Systematics and palaeobiogeography of the Sub-Saharan *Neoperla* Needham stoneflies (Plecoptera, Perlidae)

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Abstract

Neoperla is a widespread and diverse genus of Plecoptera (stoneflies), with 378 species recognized from Asia, America, and Africa. For most of the last 70 years, the Afrotropical *Neoperla* were treated as a single, widespread and heterogeneous species, *Neoperla spio* Newman. This gordian knot persisted until 2023, when 82 valid species were recognized across the Afrotropical region. As a result of this taxonomic uncertainty, remarkably little is known about the diversity, ecology and biogeographical history of *Neoperla* in Africa. In this thesis, I used newly collected specimens, museum collections, and published literature to address some of these gaps. Ten novel species of *Neoperla* were identified and described from Central and Southern Africa based on morphological and molecular evidence. A further five putative species were described, but are not recognized as species due to the unavailability of adult material. The taxonomy of *Neoperla* nymphs, which currently cannot be identified morphologically, was reviewed, and six species were described based on colouration patterns. A time-calibrated phylogeny of *Neoperla* was used to investigate the mechanisms behind the disjunct distribution of *Neoperla*, and its dispersal into Africa. The results of this analysis did not support any previous hypotheses, and a novel hypothesis that *Neoperla* spread into Africa and America via the North Atlantic land bridge during the Paleogene was presented. In the Afrotropical region, I propose that *Neoperla* radiated and dispersed alongside the evolution of drainage systems and rivers on the continent. To select calibration points for this time-calibrated phylogeny, the taxonomy of >100 fossil stoneflies was reviewed. I suggested the reclassification of almost 60% of these species, and proposed a novel assemblage of ten fossil calibration points that can be used for future phylogenetic and palaeobiogeographic analyses of Plecoptera. Finally, the diversity and ecology of the South African *Neoperla* species was reviewed, and the distribution, habitat, and river biotope preferences of nine South African species were summarized. A key for the identification of South African *Neoperla* nymphs was presented to facilitate further research on the genus.

Keywords: Afrotropical, taxonomy, species description, palaeoentomology, diversity, time-calibrated phylogeny, ecology, distribution, freshwater.

Disclaimer

During this study, I have contributed to two publications that are currently in press. While neither directly overlap with the body of work presented here, I do discuss some similar topics. These include the diversity of Plecoptera and other aquatic insects in the Drakensberg mountains (discussed in Chapter 5), the diversity and taxonomy of the African Plecoptera (discussed throughout, particularly in Chapters 1, 2, & 6), and the biogeographical history of Plecoptera (discussed in Chapters 1, 3 & 4). These articles are:

1. Taylor P, Bredenhand E, Monadjem A, Armstrong A, Rakotoarivelo A, Mdluli VM, Howard A; Modise S; Motitsoe SN, Ntloko P, **Kirkaldy AP**, Kleynhans D, Jankielsohn A, Mosikidi T, Oosthuizen MK, Payne S, Munyai TC, Carbutt C, Ramoejane M, Bereng M, Stiller M, Haddad C; Steenhuisen S, Mlambo MC, Moyo S, Nyembe N, Mofokeng L, van As J. Malekana L, Daniel GM, Gwate O, van As M, Harrison J, Thabethe NF, Kheswa N, Moloi K, Sishange N, Clark VR. (In press.). Citizen science and expert bioblitzes reveal congruent elevational patterns of biodiversity in an imperiled alpine hotspot in southern Africa. *Bothalia ABC*.

2. Eichert A, de Almeida LH, Du Y, Duarte T, Fochetti R, Giersch J, Hotaling S, Huo Q, Jouault C, **Kirkaldy AP**, Letsch H, Li W, López-Rodríguez MJ, Machingura J, McCulloch G, Mo R, Pessacq P, Rippel MLS, Rivera-Pomar R, Sproul J, Sarmento FRP, Sroka P, Tierno de Figueroa JM, Ware J (In press.). Plecoptera Systematics: Past, Present, and Future. *Insect Systematics & Diversity*.

A modified version of Chapter 3 has been submitted to Zootaxa and is currently under peer review.

3. **Kirkaldy AP**, Barber-James HM, Richoux NB, Villet MH, (in rev.). Literature Review of the Fossil Record of Systellognatha (Plecoptera) and its Implications for the Biogeography of Plecoptera. *Zootaxa*.

In Chapter 2, I recognize and describe ten novel species, which are informally named to facilitate further discussions in Chapters 4 & 5. This work is explicitly disclaimed as a nomenclatural revision and all novel names and acts within are disclaimed as nomenclatural acts in terms of Article 8.1 – 8.3 of the International Code of Zoological Nomenclature (ICZN 2000).

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1. Chapter 1: Introduction

Neoperla Needham is a widespread and diverse genus in the Plecoptera (stoneflies) family Perlidae, with 378 species known from Asia, the Nearctic, and the Afrotropical region (DeWalt *et al.* 2025; Zwick 2023, 2024; Zwick & Zwick 2023). This single genus accounts for approximately 8% of all known Plecoptera species (DeWalt *et al.* 2025), and is the only genus found across most of the Afrotropical region (DeWalt *et al.* 2025; Hynes 1952; Picker 1980; Zwick & Zwick 2023). Unfortunately, relatively little is known about *Neoperla* in Africa, because it was treated as a single, widespread and generalist species, *Neoperla spio sensu lato* Newman (Hynes 1952; Picker 1980; Zwick 1976), until it was revised in 2023 (Zwick & Zwick 2023). In this dissertation, I will investigate the diversity of the genus in the Afrotropical Region and explore its biogeographical history.

1.1. Plecoptera

Plecoptera is a small order of Insecta, with approximately 4000 extant species (DeWalt *et al.* 2025; Eichert *et al.* in press) that are found on all continents except Antarctica (DeWalt *et al.* 2015, 2025; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008). They are hemimetabolous, and in most species the immature stages are aquatic, while adults are almost exclusively winged and terrestrial (DeWalt *et al.* 2015; Fochetti & Tierno de Figueroa 2008; Stewart 2009). Following Bybee *et al.* (2015), the immature stages should be referred to as *naiads*, as the adults do not resemble the young and inhabit a different ecosystem. However, *nymph* is the most widely used term in Plecoptera literature (see for example DeWalt *et al.* 2015; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008; Sivec & Yule 2004; Stewart 2009) and is used here for consistency. Morphologically, stoneflies are similar to many Polyneoptera, but can be recognized by a combination of their two pairs of folded, membranous wings (for which the order is named, *Plecto*: "folded", *pteron:* "wing", secondarily lost in some, e.g., Scopuridae Uéno), a pair of long, filamentous cerci (reduced in the adults of some families, e.g., Notonemouridae Ricker), and three-segmented tarsi with two claws; although these features are also present in other orders (Figure 1.1; Beutel *et al.* 2013; DeWalt *et al.* 2015; Gullan & Cranston 2014; Stewart 2009). Monophyly of the order is supported by internal autapomorphies, namely looped gonads that are medially fused, specialized intersegmental muscles that allow for a laterally undulating swimming motion (Beutel *et al.* 2013; Gullan & Cranston 2014; Zwick 2000), and a cercus heart (Pass 1987), which forms part of the circulatory system.

In total, extant Plecoptera is represented by 17 families (Table 1.1) that are divided into two suborders, Arctoperlaria and Antarctoperlaria (Zwick 2000). Both suborders are largely limited to a

single hemisphere, and they may have originated through vicariance associated with the separation of Pangaea around 180 Ma (Ding *et al.* 2019; García-Girón *et al.* 2024; McCulloch *et al.* 2016; Zwick 2000). However, this timing is contested and may have occurred much earlier (Letsch *et al.* 2021). Arctoperlaria is diverse, with approximately 4000 species in 12 families (DeWalt *et al.* 2025; Eichert *et al.* in press) that are mostly limited to the Northern Hemisphere (DeWalt *et al.* 2025; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008; Zwick 2000). However, two Arctoperlaria families, Notonemouridae and Perlidae Latreille, occur in the Southern Hemisphere, probably as a result of at least three independent migrations (García-Girón *et al.* 2024; Illies 1965; Letsch *et al.* 2021; Zwick 2000). *Neoperla* falls within Perlidae, and its presence in Africa is the result of one of these migrations (Illies 1965; Letsch *et al.* 2021; Zwick 2000). Comparatively, Antarctoperlaria is small, with about 400 species from four families (DeWalt *et al.* 2025; Eichert *et al.* in rev.), and is exclusively found in the Neotropical and Australasian regions (DeWalt *et al.* 2025; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008; Zwick 2000).

Table 1.1: Distribution of the 17 extant Plecoptera families. NA: Nearctic, P: Palearctic, IM: Indo-Malay, NT: Neotropical, AF: Afrotropical, A: Australasia.

Family:	Author	Distribution
Suborder: Antarctoperlaria	Zwick, 1969	
Superfamily: Eusthenioidea	Banks, 1913	
Diamphipnoidae	Ricker, 1950	NT
Eustheniidae	Banks, 1913	NT, A
Superfamily: Gripopterygoidea	Enderlein, 1909	
Austroperlidae	Tillyard, 1921	NT, A
Gripopterygidae	Enderlein, 1909	NT, A
Suborder: Arctoperlaria	Zwick, 1969	
Infraorder: Euholognatha	Zwick, 1973	
Scopuridae	Uéno, 1935	IM
Superfamily: Nemouroidea	Billberg, 1820	
Capniidae	Banks, 1900	NA, PA, IM
Leuctridae	Klapálek, 1905	NA, PA, IM
Nemouridae	Billberg, 1820	NA, PA, IM
Notonemouridae	Ricker, 1950	NT, AF, A
Taeniopterygidae	Klapálek, 1905	NA, PA, IM
Infraorder: Systellognatha	Enderlein, 1909	
Superfamily: Perloidea	Latreille, 1802	
Chloroperlidae	Okamoto, 1912	NA, PA, IM
Kathroperlidae	Banks, 1947	NA, IM
Perlidae	Latreille, 1802	NA, PA, IM, NT, AF
Perlodidae	Klapálek, 1909	NA, PA, IM
Superfamily: Pteronarcyoidea	Newman, 1853	
Peltoperlidae	Claassen, 1931	NA, PA, IM
Pteronarcyidae	Newman, 1853	NA, PA, IM
Styloperlidae	Illies, 1966	IM

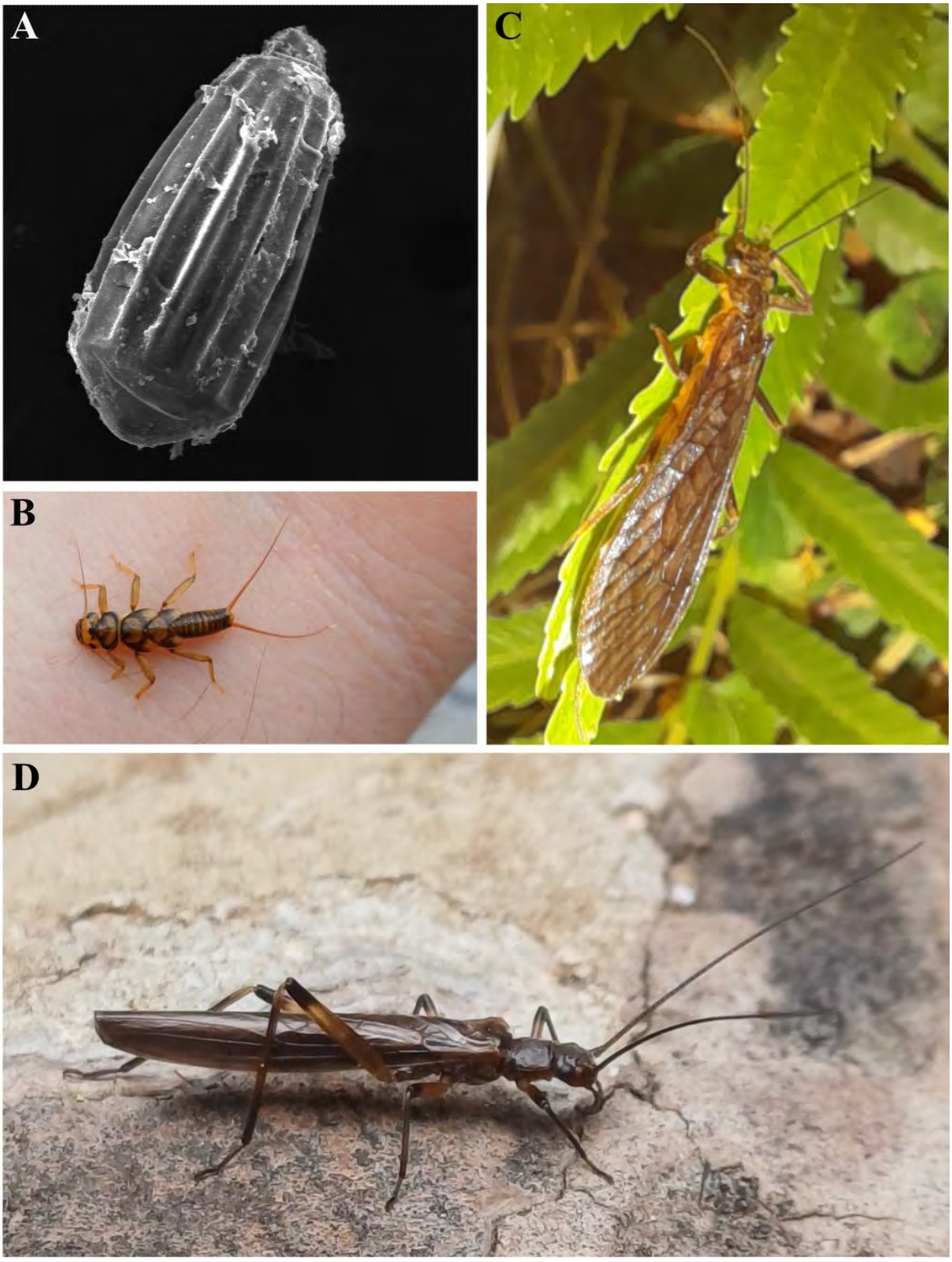

Figure 1.1: Habitus photos of African Plecoptera. **A-C:** Perlidae. **A:** Egg, *Neoperla transvaalensis* Enderlein, **B:** Nymph, *Neoperla sjostedti sjostedti* Lestage, **C:** Adult, *Neoperla sp. nov. 4*, **D:** Notonemouridae adult, *Afronemoura amatolae* Balinsky.

1.1.1. Natural history of Plecoptera, and their role in freshwater ecosystems

In most Plecoptera species, the aquatic nymphs inhabit well-oxygenated, fast-flowing rivers and springs, although some species can be found in other water bodies such as lake margins (Brittain 1974, 1990; DeWalt *et al.* 2015; Fochetti & Tierno de Figueroa 2008; Hynes 1976). In some Gripopterygidae, e.g. *Nesoperla fulvescens* Hare and *Taraperla howesi* Tillyard, the middle-to-late instar nymphs are terrestrial or amphibious (McLellan 1977; Winterbourn 2010). This characteristic is possibly an adaptation to survive in springs with unpredictable rainfall and flow patterns (McLellan 1977; Winterbourn 2010). The nymphs of *Vesicaperla* McLellan are completely terrestrial and live in cool, humid alpine vegetation, where they feed on dead plant material and spores (McLellan 1977, 1993). Nevertheless, most stoneflies are cold-water specialists, and are usually found in rivers between 5-15°C (Brittain 1990; Elliott 1988; Ross-Gillespie *et al.* 2018). Egg hatching success decreases rapidly at higher temperatures, and few species successfully reproduce or occur in waters above 25°C (Brittain 1990; Elliott 1988; Ross-Gillespie *et al.* 2018).

Stoneflies are usually univoltine, although many Perlidae and Antarctoperlaria are semivoltine (Brittain 1990; DeWalt *et al.* 2015; Fochetti & Tierno de Figueroa 2008; Hynes 1976). In semivoltine species, developmental times vary between two to three years (DeWalt *et al.* 2015; Hynes 1976). Some species switch between semivoltine and univoltine cycles, seemingly in response to unpredictable or different climatic conditions (Hynes 1976). Plurivoltinism has been inferred rarely, and is confirmed only in *Nemurella pictetii* Klapálek (Nemouridae Billberg), which is also occasionally uni- or semivoltine at higher altitudes (Lieske & Zwick 2007; Wolf & Zwick 1989). The number of instars differs between species and even between populations of the same species (Hynes 1976). However, most have between 10-24 instars (Brittain 1990; Fochetti & Tierno de Figueroa 2008; Hynes 1976), although rarely as many as 50 have been observed (Hynes 1976). Diapause is common in stonefly nymphs and eggs, and is generally used to avoid adverse conditions of low flow and particularly high or low temperatures (Brittain 1990; DeWalt *et al.* 2015; Hynes 1976; Pritchard *et al.* 1996; Stewart 2009)

Nymph growth and development are affected by environmental factors such as flow rates, micro-habitat availability, and temperature (Brittain 1990; Hynes 1976). Temperature seems to play a particularly important role, and the nymphs of most species show faster developmental times and growth rates with increased temperatures (Brittain 1990; Hynes 1976; Stewart 2009). However, these growth rates drop above optimal temperatures, which are usually low (Brittain 1990; Hynes 1976; Stewart 2009). Most species prefer clean, unpolluted rivers and are sensitive to changes in water chemistry or flow, showing significant population declines in response to anthropogenic factors such as damming or eutrophication (Brittain 1991; Dallas & Day 2004; DeWalt *et al.* 2005; DeWalt & Ower 2019; Edia *et al.* 2016; Fochetti & Tierno de Figueroa 2008). This sensitivity means they are

among the first groups that disappear from a river after disturbance, and makes them valuable biological indicators of clean water systems (Barbour 1999; Dallas & Day 2004; DeWalt & Ower 2019). Stoneflies are included in most freshwater biomonitoring systems because of their sensitivity to disturbance (Barbour 1999; Dickens & Graham 2002; Fikri *et al.* 2016; Lenat 1993; Pandiarajan *et al.* 2019). Unfortunately, when coupled with their preference for cold waters and limited dispersal ability, their sensitivity to environmental changes also makes them vulnerable to climate change (Bojková *et al.* 2014; DeWalt *et al.* 2005; Fochetti & Tierno de Figueroa 2008; Tierno De Figueroa *et al.* 2010). For example, 63% of European Plecoptera are expected to be negatively impacted by climate change, with 8% highly vulnerable to predicted changes (Tierno De Figueroa *et al.* 2010).

Plecopteran nymphs fill a variety of niches in rivers, as some species are predators (e.g., Perloidea and Eustheniidae), while others are detritivorous or phytophagous, acting as shredders (e.g., Notonemouridae), collector-gatherers (e.g., Leuctridae), or scrapers (e.g., Taeniopterygidae) (Tierno De Figueroa & López-Rodríguez 2019). Some species are omnivorous, or change feeding strategy during their development (Fochetti & Tierno de Figueroa 2008; Tierno De Figueroa & López-Rodríguez 2019). Stoneflies form an important component of freshwater trophic webs, as many species are primary consumers and important food sources for other freshwater animals such as fish (DeWalt *et al.* 2015; Tierno De Figueroa & López-Rodríguez 2019). Eleven stonefly families are shredders and break down coarse particulate organic matter (CPOM), which many other macroinvertebrate species rely on for food and the flow of fine particulate organic matter (FPOM) downstream (Vannote *et al.* 1980). Even predatory stoneflies serve as an important food source for other groups, regularly being eaten by invertebrates such as Odonata and Megaloptera (Benke *et al.* 2001; Tierno De Figueroa & López-Rodríguez 2019).

Once mature, nymphs migrate to rocks or marginal vegetation along the river and moult into the terrestrial adult stage (Hynes 1976; McCulloch & Waters 2018). Only one species, *Capnia lacustra* Jewett (Capniidae Banks), remains fully aquatic as an adult, living at depth (30 – 90 m) in Lake Tahoe (Caires *et al.* 2016; Jewett 1965). The females of another species, *Zapada cinctipes* Banks, dive 15-20 cm underwater for 20-60 minutes to thermoregulate (Tozer 1979).

The emergence period varies between stonefly species, and often occurs earlier at lower altitudes, especially in species where emergence is triggered by temperature (Brittain 1990; Hynes 1976; McCulloch & Waters 2018). In Northern Hemisphere temperate environments adult emergence is often synchronous and short, lasting under 90 days (Brittain 1990; Hynes 1976; McCulloch & Waters 2018). Comparatively, many species in the Southern Hemisphere emerge across several months or seasons (McCulloch & Waters 2018; McLellan 1991, 1993), while emergence in the tropics can occur throughout the year (Hynes 1976; Tjønneland 1961; Zwick 1976; Zwick & Zwick 2023). Some species mature in cohorts, with two sympatric populations developing, emerging and mating in

different seasons, without overlapping (Caires *et al.* 2016). Up to 30 different species can co-occur in the same river, and these staggered emergence times can reduce competition between species, and lead to an almost constant emergence of stonefly adults from the same site (DeWalt *et al.* 2015). Because of this, Plecoptera are a valuable and reliable food source for some terrestrial animals, particularly in winter (DeWalt *et al.* 2015; DeWalt & Ower 2019). Aquatic insects emerging from rivers, including stoneflies, are important sources of essential fatty acids in terrestrial ecosystems (Moyo *et al.* 2017).

Stonefly adults are short-lived, usually surviving a few days to weeks (Brittain 1990; Hynes 1976), although some species live longer (Hynes 1976), such as the females of *C. lacustra* that survive for three months (Caires *et al.* 2016). Most Euholognatha and Antarctoperlaria feed as adults, and in some species, it is necessary for the maturation of their eggs (Hynes 1976). Euholognatha have been recorded feeding on algae, pollen, lichens, nectar, and soft fruits (Fenoglio & Tierno de Figueroa 2003; Hynes 1976; Stewart 2009), while Antarctoperlaria eat rotten wood, possibly for fungal growths in the wood (Hynes 1976). Comparatively, the predatory Perloidea do not feed as adults and have reduced mouthparts (Brittain 1990; Fenoglio & Tierno de Figueroa 2003; Hynes 1976), although they may drink nectar or water (Hynes 1976). All of the energy for their egg production, dispersal, and mating, therefore, comes from their rich diet as nymphs (Fenoglio & Tierno de Figueroa 2003).

Most stoneflies fly poorly and remain close to the rivers they emerge from, often hidden in vegetation, under stones, or in crevices when they are not active (Bowman & Smith 2021; Brittain 1990; DeWalt *et al.* 2015; Hynes 1941, 1976). This behaviour is particularly common in males, which usually emerge a few days earlier than the females, and are occasionally shorter-lived (Brittain 1990; DeWalt *et al.* 2015; Petersen *et al.* 1999; Stewart 2009). Sexual dimorphism occurs in a few species, with males either apterous or brachypterous, probably to allow for greater energy investment into sperm production (Brittain 1990; Hynes 1976). However, wing development can vary even within the same species, as wing loss in both sexes can be driven by strong winds and patchy habitats in alpine regions (Hynes 1941; McCulloch *et al.* 2019, 2022). Females act as dispersal agents for stoneflies, occasionally travelling far from their natal river before ovipositing (Hynes 1941, 1976; Petersen *et al.* 1999; Zwick & Zwick 2023). Even this dispersal is limited, and in some *Leuctra* Stephens species, less than 10% travelled more than 50 m from the riverbank (Petersen *et al.* 1999). A limited dispersal ability means that stoneflies often struggle to cross even relatively small natural barriers, and commonly diversify due to vicariance. This characteristic makes them valuable tools for investigating biogeography (e.g., McCulloch *et al.* 2017; Stevens *et al.* 2018; Weiss *et al.* 2012), although the palaeobiogeographic history of the Plecoptera globally remains unresolved and heavily debated (Ding *et al.* 2019; García-Girón *et al.* 2024; Illies 1965; Letsch *et al.* 2021; McCulloch *et al.* 2016; Zwick 2000).

Males and females usually locate one another via direct searching (Tierno De Figueroa *et al.* 2019). The Arctoperlaria are united by an autapomorphy of drumming, or substrate vibrational communication, which facilitates this (Fochetti & Tierno de Figueroa 2008; Tierno De Figueroa *et al.* 2019; Zwick 2000). Mating occurs within a few days of emergence, once the eggs have hardened and are fully developed, and oviposition follows a few days later (Hynes 1976). In most species, the females form an egg packet, which is extruded onto Sternite 9 and deposited directly onto water surfaces, either by dropping them while in flight, or by flying into the current and freeing themselves once the eggs have been released (DeWalt *et al.* 2015; Hynes 1976). In Euholognatha, the eggs are clad in a gelatinous matrix, while Antarctoperlaria and Systellognatha have a filamentous or membranous cap, both of which allow the egg to adhere to substrates within the current (DeWalt *et al.* 2015). Egg development and hatching is controlled by temperature, and can occur in a few weeks, several months later, or occasionally in cohorts (Brittain 1990; DeWalt *et al.* 2015; Ross-Gillespie *et al.* 2018). Live birth, or vivipary, has been observed in Capniidae, such as *C. lacustra* and *Capnia khingana* Teslenko (Caires *et al.* 2016; Teslenko & Yavorskaya 2020).

1.2. Plecoptera in Africa

North Africa is diverse, with 38 species of Plectoptera in seven families found in three countries, Algeria, Morocco and Tunisia (Belahcen *et al.* 2023; DeWalt *et al.* 2025; Errochdi *et al.* 2014; Vinçon *et al.* 2014). One species, *Perla aegyptica* Pictet has been recorded from Egypt and is known from a single specimen (Pictet 1841). Affinities of this species are uncertain, and it may belong to *Neoperla* (Hynes 1952). The centre of this diversity is the Atlas Mountains, which have several endemic species (Belahcen *et al.* 2023; Errochdi *et al.* 2014; Vinçon *et al.* 2014). All of the North African species fall into Arctoperlaria and are closely related to European taxa (Errochdi *et al.* 2014).

Comparatively, the diversity of Plecoptera south of the Sahara desert is low, with only two families, Notonemouridae and Perlidae, found in the Afrotropical region (DeWalt *et al.* 2025; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008; Illies 1965; Zwick 2000; Zwick & Zwick 2023). Both families belong to the otherwise Northern Hemisphere-limited Arctoperlaria, and no Antarctoperlaria have been recorded from the continent (DeWalt *et al.* 2025; Zwick 2000). Notonemouridae occurs only in the Southern Hemisphere, and is found in the cold, southern reaches of South Africa, Madagascar, Australasia and the Neotropics (Avelino-Capistrano *et al.* 2018; Fochetti & Tierno de Figueroa 2008; McLellan 1991; Stevens *et al.* 2018; Zwick 2000). Perlidae is widespread in the Northern Hemisphere, but also occurs in the Neotropics (Avelino-Capistrano *et al.* 2018; Zwick 2000), while only a single genus, *Neoperla*, is found across most of Africa (Zwick 2000; Zwick & Zwick 2023). The Neotropical Perlids and *Neoperla* are unrelated, and their presence in the Southern

Hemisphere appears to be the result of two independent dispersal events (Letsch *et al.* 2021; Zwick 2000).

1.2.1. Notonemouridae

Notonemouridae occur across the Southern Hemisphere, with 124 species known from 24 genera (DeWalt *et al.* 2025; Fochetti & Tierno de Figueroa 2008; Illies 1975; McLellan 1991; Stevens *et al.* 2018). As adults, they can easily be distinguished from *Neoperla* by their rolled wings (Figure 1.1), and short cerci, with a single cercomere (Stevens & Picker 2003; Zwick 2000; Zwick & Zwick 2023). While the nymphs do have long cerci, they are smaller and thinner than *Neoperla* nymphs, lack gills and swimming hairs, and have a transverse suture dividing the frons and clypeus (Stevens & Picker 2003; Zwick 2004).

Notonemouridae lacks autapomorphies and are instead recognized by a combination of plesiomorphic characters, synapomorphies with other Euholognatha, and the absence of autapomorphies of their closest relatives (McLellan 1991, 2000; Zwick 2000). The distinctive rolled wings are also present in Leuctridae (Zwick 2000). They share several similarities with their putative sister family Nemouridae, including shortened cerci (Cui *et al.* 2019; Sroka & Prokop 2023), the lack of a sclerotized aedeagus and retractor muscles, and the presence of an unpaired, long ejaculatory duct (Zwick 2000). However, they can be separated from this family by the absence of laterally expanded coxae (Zwick 2006). While Zwick (2000) noted a shortened abdominal nerve cord with no more than six free ganglia as a similarity between the families, this is inconsistent in Notonemouridae, as at least *Aphanicercella* Tillyard, *Balinskycercella* Stevens & Picker, *Aphanicercopsis* Barnard, *Neonemura* Navás and all of the New Zealand taxa have seven (Stevens *et al.* 2018).

The monophyly of the Notonemouridae has been questioned. Originally described as a subfamily of Nemouridae (Ricker 1950), they were later assigned to the Capniidae (Illies 1961), before eventually being elevated to family rank (McLellan 1972; Zwick 1973b). Even then, they were suspected to be a paraphyletic assemblage of independent stem-Nemouridae lineages that dispersed into the Southern Hemisphere (McLellan 1991, 2000; Zwick 2000). However, molecular analyses of the family have since repeatedly supported their monophyly (García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016; Terry 2004), and most authors now accept Notonemouridae as a single valid family. However, its relationships with the remaining Euholognatha are poorly resolved (Cao *et al.* 2024; Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016; Terry 2004).

Thirty-nine species of Notonemouridae from seven genera occur in South Africa (31 spp., 29 endemic), Lesotho (2 spp., shared with South Africa) and Madagascar (8 spp., all endemic) (DeWalt *et al.* 2005; Fochetti & Tierno de Figueroa 2008; Stevens *et al.* 2018; Zwick 2015). The majority of

these species are highly localized and endemic to the Western Cape Province in South Africa (Stevens *et al.* 2018). The family is relatively well studied in Africa, and the taxonomy of adults and nymphs from all seven genera has been reviewed in the last 30 years (Picker & Stevens 1997, 1999; Stevens & Picker 1995, 1999; Zwick 2015). Despite this work, significant gaps remain. One of the most widespread species, *Aphanicerca capensis sensu lato* Tillyard, seems to be a species complex, and 15 molecularly and morphologically distinct forms have been recognized (Barnard 1934; Bellingan 2010; Stevens 2008). Additionally, the ecological and life history characteristics of most species are unknown, and many nymphs are undescribed (Picker & Stevens 1997; Zwick 2015).

In Africa, Notonemouridae are generally limited to fast-flowing and cold montane or forest streams at mid-high altitudes (Barnard 1934; Stevens *et al.* 2018; Zwick 2015). Nymphs are usually collected among stones in these small streams, but the same species are also present on marginal vegetation (Dallas 2007, pers. obs.). Some species also occur in intermittent streams, springs and seeps (Balinsky 1956; Zwick 2015, pers. obs.). While the ecological requirements of the nymphs have not been investigated for most species, *Aphanicercella scutata* Barnard and *A. capensis (s.l.)* prefer cold temperatures (Dallas 2016; Ketley 2009; Ross-Gillespie *et al.* 2018). Both species have optimal growth and survivability at around 15°C, with egg hatching and survival all decreasing rapidly at temperatures exceeding 20°C (Dallas 2016; Ketley 2009; Ross-Gillespie *et al.* 2018). The nymphs of at least *Afronemoura* Illies and *Madenemura* Paulian are shredders that feed on CPOM, fungi, wood, algae, and microorganisms (Tierno de Figueroa *et al.* 2007; Tierno De Figueroa & López-Rodríguez 2019; Zwick 2015). Most species are univoltine, but emergence periods vary between species, with some emerging for a few months in one or two seasons (Barnard 1934; Picker & Stevens 1999; Stevens & Picker 1995, 1999; Tillyard 1931). Most species emerge in autumn, winter or spring, but both *Balinskycercella* Stevens and Picker and *Desmonemoura* Tillyard are found only in summer (Balinsky 1956, 1967; Harrison 1958; Picker & Stevens 1999; Stevens & Picker 1995). Females of most species have an ovipositor and deposit their eggs directly into wet substrates near rivers (Hynes 1976; Zwick 2015).

The mechanisms behind the presence of Notonemouridae in Africa and their absence from the Northern Hemisphere remain subjects of debate (Cui *et al.* 2019; García-Girón *et al.* 2024; Illies 1965; Letsch *et al.* 2021; McCulloch *et al.* 2016; Sroka & Prokop 2023; Zwick 2000). Some authors suggest the family formed in the Northern Hemisphere, before dispersing south during the Jurassic or Early Cretaceous (Cui *et al.* 2019; Letsch *et al.* 2021; Sroka & Prokop 2023; Yushuang *et al.* 2011). However, most dated phylogenies suggested that Notonemouridae dispersed across the Southern Hemisphere later, possibly via winds circulating Antarctica (Ding *et al.* 2019; García-Girón *et al.* 2024; McCulloch *et al.* 2016).

1.2.2. Perlidae, and African Neoperla

Perlidae is the most diverse and widespread family of Plecoptera, with 1380 valid species in 55 genera found across every biogeographical region except for Australasia and Antarctica (DeWalt *et al.* 2025). Monophyly of the family is supported by several autapomorphies, the most distinctive of which is the branched thoracic gills that form clusters on either side of the nymphs' coxae (Zwick 2000). The nymphs also have an expanded genae covering the bases of the mandibles, inflated paraglossae, a wide mentum, and a complex, folded proventriculus that forms an extra valve in the alimentary canal (Zwick 2000). The family has been well supported in all molecular analyses (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; Terry 2004). The taxonomy and phylogeny of Perlidae have been uncertain at times, with many similar species described inconsistently from related genera (Sivec *et al.* 1988). However, generic diagnoses and relationships were clarified by several morphological reviews and have remained stable for the last 37 years, with only minor changes (Sivec *et al.* 1988; Stark 2001; Stark & Gaufin 1976; Stark & Kondratieff 2004; Zwick 2023).

While most stoneflies are adapted to cold waters, Perlidae have undergone significant radiation into warmer rivers (DeWalt *et al.* 2015, 2025). This radiation was probably facilitated by the branched thoracic gills, which increase the surface area available for gas exchange and oxygen absorption (DeWalt *et al.* 2015). Perlid species also show a behavioural modification to high temperatures and low oxygen, as they can repeatedly pump their body up and down in a "push-up" motion, increasing water circulation and oxygen supply across the gills (Genkai-Kato *et al.* 2000; Hynes 1976). This characteristic has allowed Perlidae to dominate tropical regions, and they are common in Central America (DeWalt *et al.* 2015; Stark 2001; Stark & Kondratieff 2004), tropical Asia, e.g,. Malaysia and India (Amiruddin & Suhaila 2020; Hamid & Rawi 2011; Muranyi & Li 2013), and Africa (Barnard 1934; Hynes 1952; Picker 1980; Zwick 2023; Zwick & Zwick 2023). In Africa, *Neoperla* can be differentiated from Notonemouridae by the presence of filamentous gills on the thorax and tenth abdominal segment, swimming hairs on the tibia, and a wide head with large mouthparts (Figure 1.2; Stevens & Picker 2003). The adults have long, filamentous cerci, and are usually light amber-brown or green (Stevens & Picker 2003; Zwick & Zwick 2023).

Neoperla is placed in the subfamily Perlinae, in the tribe Neoperlini (Sivec *et al.* 1988; Zwick 2023, 2024). Both groups are well supported morphologically. Perlinae as a whole are united by having the hemitergites on the 10th abdominal segment modified into anteriorly upcurved hooks in adult males, a transverse ridge or setal row across the occiput, and three gill shields in nymphs (Sivec *et al.* 1988; Zwick 2000). Neoperlini consists of four genera, *Neoperla*, *Chinoperla* Zwick, *Furcaperla* Sivec, and *Phanoperla* Banks (DeWalt *et al.* 2025; Zwick 2023). They are supported by a sclerotized aedeagus base with a constricted basal opening (Sivec *et al.* 1988; Zwick 2023). Perlinae has been recovered as monophyletic by four molecular analyses, but only *Neoperla* has been included in any molecular

analyses among Neoperlini (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021). The relationships of *Neoperla* to the remaining Perlinae genera have been poorly resolved, and differ between analyses (Chen *et al.* 2019; Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021). The monophyly of *Neoperla* is supported by three autapomorphic characters, namely a slender, spike like process on the hemitergites, a modified process on tergite 7 and paired, hard knobs which originate at the constricted, basal opening of the aedeagus (Sivec *et al.* 1988; Zwick 2023).

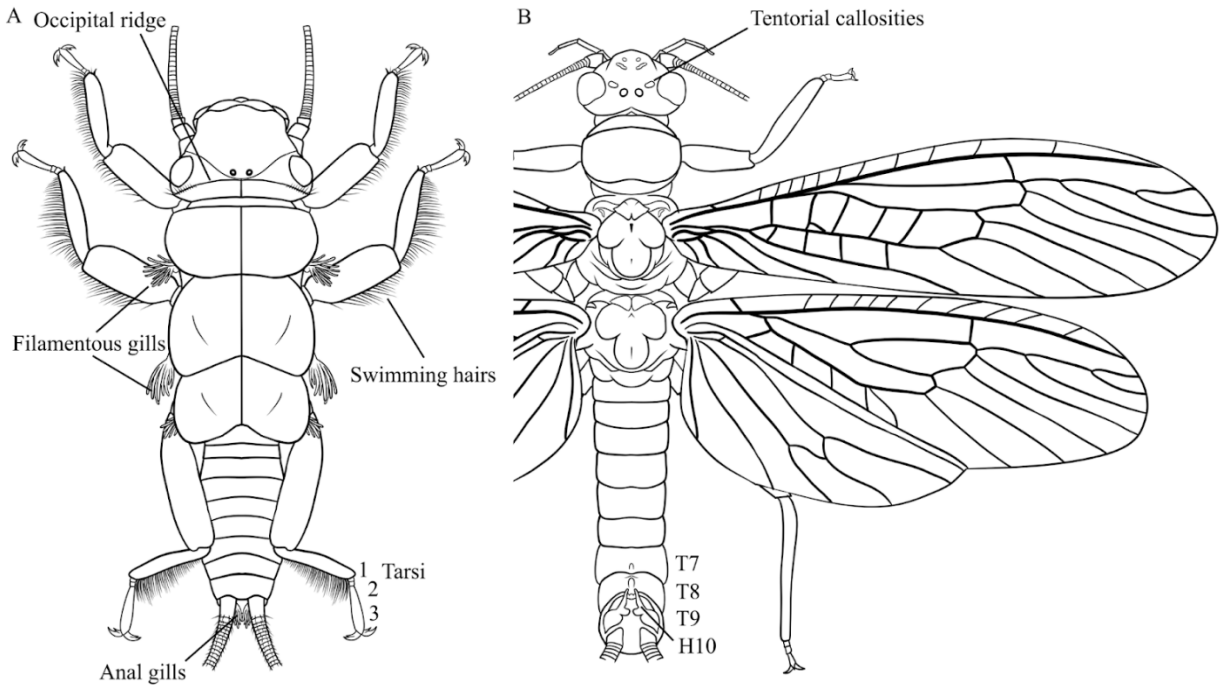

Figure 1.2: Generalized structure of *Neoperla* nymphs and adults. **A:** Dorsal view of *Neoperla transvaalensis* as a nymph, **B:** Dorsal view of a male *Neoperla bugeoni* Navás, showing external genital characters, and long process on H10.

The taxonomy of *Neoperla* was recently reviewed, with 14 species groups recognized in four subgenera (Zwick 2023, 2024). The genus has a disjunct distribution, with almost three-quarters of the known species and all four subgenera occurring in South Asia, while a single subgenus, *Neoperla* (*Neoperla*) also occurs in the east Nearctic (15 species, in two endemic species groups) and Africa (82 species, in at least four species groups) (DeWalt *et al.* 2015; Zwick 2023, 2024; Zwick & Zwick 2023). A single species of *Neoperla* (*Formosita*) Klapálek is described from the Nearctic, but its locality record is doubtful, and the species is generally excluded from species lists and keys (Stark 1994). *Neoperla* (*Neoperla*) can be differentiated from the remaining subgenera by the presence of a spermathecal stalk, which is an elongated, often folded and scale-covered region between the vagina

and spermathecal duct (Zwick 2023). As the African taxa all fall into this subgenus, further specification is unnecessary, and they will henceforth be referred to as *Neoperla.*

Neoperla is absent from Europe and the west Nearctic, and the biogeographical processes that led to its disjunct distribution are poorly understood (Hynes 1988; Illies 1965; Zwick 2000; Zwick & Zwick 2023). The genus is thought to have originated in Asia, as this is where its greatest diversity and closest relatives are located (Zwick 2000; Zwick & Zwick 2023). Its spread into the Nearctic was attributed to either vicariance or long-distance dispersal (Hynes 1988; Zwick 2000). The African taxa may have arrived recently, during the Miocene or Pliocene (Illies 1965; Letsch *et al.* 2021; Zwick 2000). However, this estimate relied on the assumed low diversity [i.e., a single species, *Neoperla spio (s.l.)*] of the African taxa, until just two years ago (Hynes 1952; Picker 1980; Zwick & Zwick 2023).

By 1936, 29 species of Perlidae in four genera had been described from the Afrotropical region (Barnard 1934; Enderlein 1909; Klapálek 1909, 1911, 1923; Navás 1919; Newman 1839; Zwick 1973a). However, many of these descriptions relied on highly variable habitus characters, which are unreliable for identification, and species were regularly misidentified, even by their own authors (Hynes 1952; Zwick & Zwick 2023). Recognising this, Hynes (1952) reviewed the Perlidae in Africa, and argued that they were all members of a single, widespread and highly variable species, *Neoperla spio*. However, Hynes largely ignored or dismissed the genitalia (Hynes 1952; Picker 1980; Zwick 1973a, 1976; Zwick & Zwick 2023), which are taxonomically informative (Picker 1980; Sivec *et al.* 1988; Stark & Baumann 1978; Zwick 1973a, 1976; Zwick & Zwick 2023). He therefore tied the African taxa into a Gordian knot instead of resolving this uncertain taxonomy, which remained until Zwick and Zwick (2023) completed a monumental review of the types and newly collected material, recognizing 82 valid species of *Neoperla* across the continent. These species are found in almost every country south of the Sahara Desert (Figure 1.3), and even occur on the volcanic Comoro islands, although they are absent from Madagascar (Zwick & Zwick 2023).

Unfortunately, this means very little is known about the individual species of African *Neoperla.* While some studies and reviews have commented on the ecological and physiological requirements of the genus (Arimoro *et al.* 2011; Chutter 1968; Dallas 2007; Edia *et al.* 2016; Fenoglio & Tierno de Figueroa 2003; Hynes 1976; Niba & Sakwe 2018; Zwick 1976), and its value as a biological indicator of pollution and other anthropogenic impacts in Africa (Edia *et al.* 2016; Niba & Sakwe 2018), all of them have focused on the genus as a whole instead of individual species. Therefore, the preferred habitat, water chemistry requirements, and physiological limits of all these species are unpublished. Similarly, while Zwick and Zwick (2023) provided detailed distribution records for all of the material examined for their review, this was based on sporadic collecting and museum records, and was not a comprehensive representation of the distribution of each of these species (Zwick & Zwick 2023).

Many of the records are old, and it is unclear if *Neoperla* ranges and diversity are shrinking, following the trend seen in stoneflies globally (Bojková *et al.* 2014; DeWalt *et al.* 2005, 2015; Macadam *et al.* 2022; Tierno De Figueroa *et al.* 2010). Investigating the ecology and distribution of *Neoperla* is further hampered by the difficulty in identifying nymphs. In Africa, six morphologically distinct nymphs have been described based on colouration patterns, but not assigned to any known species (Klapálek 1912; Lestage 1917). Barnard (1934) described the nymphs of *Neoperla transvaalensis* Enderlein, but misassociation is probable, as at least some specimens were collected from the same site as his presumed *N. transvaalensis* male (Barnard 1934), which is in fact *Neoperla burgeoni* Navás (Zwick & Zwick 2023). Hynes (1953) described the nymph of *Neoperla spio*, but already considered all African *Neoperla* to belong to this species, and dismissed different colour patterns as variation between individuals. It is therefore unclear what species these different colour patterns belong to. However, *Neoperla spio* has a relatively small range in West Africa (Zwick & Zwick 2023), and Hynes' (1953) nymphs were only collected in Central Africa. It is therefore highly unlikely that any of these forms belong to *N. spio*. *Neoperla* nymphs can, therefore, only be differentiated using DNA (Zwick & Zwick 2023).

Figure 1.3: Known distribution of *Neoperla* in the Afrotropical region; all countries with at least one record are marked in blue. Data from Hynes (1952), Zwick & Zwick (2023) and DeWalt *et al.* (2024).

In Africa, all *Neoperla* nymphs are predatory (Fenoglio & Tierno de Figueroa 2003; Hynes 1953; Stevens & Picker 2003), and are usually found in fast-flowing, cold, forested rivers (Chutter 1968; Hynes 1976; Picker 1980; Stevens & Picker 2003). They are most commonly collected from stones in and out of the current (Chutter 1968; Dallas 2007), but occur in lesser numbers on marginal vegetation and in sandy or gravel-bottomed rivers (Dallas 2007). Some species seem capable of surviving in a range of habitats, and the genus is also known from lake margins (Hynes 1976), and larger lowland rivers (pers. obs.). Whether this flexibility applies only to some species is unknown. *Neoperla* nymphs are apparently capable of surviving in warmer waters than Notonemouridae, but most species seem to prefer cool water (Dallas 2009; Edia *et al.* 2016) and are collected at an average water temperature of 14.9°C (Dallas 2009). Temperature tolerance probably varies between species because *Neoperla* has been collected from rivers with water temperatures ranging between 3.9 and 28.0°C (Dallas 2009). The species are assumed to be univoltine, like the Nearctic *Neoperla clymene* Newman (Hynes 1976; Vaught & Stewart 1974). However, nymphs from different size classes are regularly collected together (pers. obs.), suggesting that they may be semivoltine, multiple species with different emergence times are sympatric, or that nymphs develop in cohorts (Vaught & Stewart 1974). Most species are probably sensitive to changes in water chemistry and flow, making them valuable biological indicators (Dallas & Day 2004; Edia *et al.* 2016; Niba & Sakwe 2018), although some are occasionally collected from heavily impacted streams, albeit in low numbers (Niba & Sakwe 2018).

Time of emergence varies between species, with adults of some species present throughout the year, while others are present only for a few months (Zwick 1976; Zwick & Zwick 2023). Emergence occurs at night, and the adults are generally night-flying (Hynes 1976; Zwick & Zwick 2023). The adults' mouthparts are reduced, and they seem to not feed (Fenoglio & Tierno de Figueroa 2003). Males do not fly far from the rivers they emerged from, while females disperse and oviposit a few days after mating (Zwick & Zwick 2023).

Species-level taxonomy and ecology form the basis of resource management, conservation and efficient biological monitoring (Dolédec *et al.* 2000; IUCN 2024; McGeoch 1998; Melville *et al.* 2021; Menezes *et al.* 2010; Ripple *et al.* 2017; Rowntree *et al.* 2000). Without these data, it is difficult and expensive to interpret population fluctuations, range shifts, and the biological significance of a species, making management or research inefficient and impractical (Haig *et al.* 2006; Melville *et al.* 2021). Current knowledge of South African *Neoperla* species is extremely poor, and it is likely that management protocols are insufficient to conserve them. This means it is difficult to detect changes in their distribution and abundance, which limits their utility as biological indicators. It is unclear what ecological effects the loss of any of these species may have, but such losses can indicate the degradation of freshwater catchments and rivers. Additionally, *Neoperla* species are important food sources for fish and other aquatic animals (Stevens & Picker 2003).

1.3. Aims

Despite recent advances, questions about the diversity, distribution, biogeographical history and ecological needs of *Neoperla* species persist worldwide. Very little is known about the Afrotropical taxa in particular. In this dissertation, I aim to build on the baseline presented by Zwick and Zwick (2023) to address some of these knowledge gaps, and to contextualize my findings in the biogeographical history of Plecoptera worldwide. To do this, I will:

1. Describe novel species of *Neoperla* from across the Afrotropical region (Chapter 2). These descriptions will help to unravel the true diversity of this genus in the region, and allow for these species to be identified in the future.

2. Examine nymphs of *Neoperla*, and evaluate morphological characters that can be used to identify species (Chapter 2). The nymphs of several known and novel species will be described, providing the first resource that can be used for the identification of *Neoperla* nymphs in Africa.

3. Use a combination of mitochondrial DNA (COX1), nuclear DNA (H3), and RNA (28S) to investigate the molecular taxonomy of African *Neoperla* species to (1) link different life stages and sexes; (2) determine the relationships between taxa; (3) create a molecular database of South African Plecoptera barcodes to allow for future monitoring efforts using novel techniques, such as environmental DNA (Chapter 2).

3. Examine the fossil history of Systellognatha (Chapter 3). This is necessary to select fossil calibration points for a biogeographical analysis of *Neoperla* and will provide additional context for investigating the biogeography of Plecoptera globally, including the formation of the two suborders and the dispersal of Notonemouridae into South Africa.

4. Recommend a set of fossil calibration points for Systellognatha as a whole, which can be used in future phylogenetic and palaeobiogeographical analyses of Plecoptera (Chapter 3).

5. Construct two time-calibrated phylogenetic trees, utilizing publicly available mitochondrial genomes and the three genes from Chapter 2, to investigate the relationships of African *Neoperla* species (Chapter 4). These results will be used to construct novel hypotheses on the biogeography and evolutionary history of the genus in Africa (Chapter 4).

6. Present baseline ecological notes and distribution maps for the South African taxa (Chapter 5). A review of the ecology, distribution and physiological requirements of all *Neoperla* species in Africa is beyond the scope of this study. However, this baseline information will provide a valuable framework for future ecological and physiological investigations of the genus and its individual species.

2. Chapter 2: Description of Novel Species of Afrotropical *Neoperla* Needham 1905 (Plecoptera, Perlidae)

2.1. Introduction

The first *Neoperla* from Africa, *Neoperla spio* Newman, was described as a member of the Chloroperlidae in the 19th century (Newman 1839). A further 28 species were identified over the next 100 years, with 29 species and four genera recognized by 1936 (Hynes 1952; Zwick 1976; Zwick & Zwick 2023). Many of these species were described on the basis of habitus characters, such as wing venation, colouration, the position of the ocelli, and body size (Hynes 1952; Klapálek 1923; Navás 1919; Zwick 1976; Zwick & Zwick 2023). These characters are all generally unreliable for identification, as they can vary significantly within a species, and even occasionally on the same specimen (Hynes 1952; Zwick 1976). The external genitalia of males were occasionally described, but for few species, and the aedeagus was overlooked (Barnard 1934; Enderlein 1909; Hynes 1952; Klapálek 1923). This led to difficulties in identifying described species, and they were regularly misidentified, even by their original authors (Zwick & Zwick 2023).

Adding to this confusion, disagreements on the generic placement of the African *Neoperla* species were common. While some authors placed them within *Neoperla* (Klapálek 1923; Needham *et al.* 1920), Enderlein (1909) erected the genus *Ochthopetina*, arguing simply that *Neoperla* was an exclusively American genus. The genera were initially separated on the basis of wing venation, and later ocellus position, but the diagnoses of each genus underwent several revisions, with characters such as “ocelli close together” being attributed to both genera at separate times (Hynes 1952; Klapálek 1923).

Hynes (1952) recognized that the taxonomy of the group was poorly resolved and reviewed the described African species and the reliability of the characters that had been used to diagnose them. Unfortunately, while he recognised several different forms in colouration, female subgenital plate shape, and male external genitalia, he interpreted these heterogeneous characters as a developmental series, rather than distinct adult forms co-existing (Hynes 1952, 1953; Zwick 1976). For example, in males he presented nine different forms of the processes on tergite 7 (T7) and tergite 8 (T8) as a continuous series, starting from the *“kunenensis* type” (i.e., flat lappet on T7, no process on T8) and ending in the “*excisa-leroiana* type” (i.e., no process on T7, a large process on T8). No explanation was offered why these different forms should be considered a developmental series instead of distinct forms, with Hynes simply stating: “The only alternative is to postulate a large number of more or less co-extensive species which overlap one another in all characters which have so far been studied. As there is no indication of this, it is proposed that all these forms be referred to one species” (Hynes

1952, 104). On this basis, Hynes combined the Afrotropical species into a single, highly variable species, *Neoperla spio* (Hynes 1952, 1953).

The idea that the Afrotropical *Neoperla* were all a single species was challenged by Zwick (1973a, 1976) and Picker (1980). In males, they recognised multiple distinct forms of the aedeagus, which differed in length, sclerotization, and spine patterns on the endophallus, and in the shape of the processes on Tergite 7 – 10 (Picker 1980; Zwick 1973a). In females, the spermathecal stalk differed in shape and spine pattern, while the eggs differed in shape and striation patterns (Picker 1980; Zwick 1973a, 1976). As these forms were consistent between specimens, and intermediate forms were not present, this provided strong evidence that *Neoperla spio* was likely a species complex (Picker 1980; Zwick 1973a, 1976). Hynes (1952) had noted many of these different forms in the male genitalia (discussed above) and the eggs during his review. However, he dismissed variation in the eggs as possibly being caused by wrinkling during preservation. He did not consider the internal genitalia structures, dismissing them as only varying in width (Hynes 1952).

Despite the early recognition that *Neoperla spio* was possibly a species complex, no species were formally recognized or redescribed for the next 50 years. The delay was due largely to challenges in linking males, females and nymphs, which typically required rearing and mating experiments (Picker 1980; Zwick 1976). Difficulty in associating sexes ran the risk of males and females of the same species being described as heterospecifics, which occurred in the early descriptions of African *Neoperla* (see for example *"Neoperla kunenensis"* Barnard and *Neoperla transvaalensis* Enderlein; Barnard 1934; Zwick & Zwick 2023). Additionally, as many of the initial descriptions were based on variable external characters, a re-examination of the type material was needed to re-establish previously described species with the incorporation of internal morphology.

Modern molecular methods allow the sexes and life stages within a species to be linked, and provide additional evidence to differentiate cryptic species of *Neoperla.* Recently, Zwick & Zwick (2023) completed a monumental review of the Afrotropical *Neoperla* and recognized 82 valid species from the continent based on both morphological and molecular evidence. These were the first species of *Neoperla* described from Africa since they were synonymized by Hynes (1952) 71 years earlier. Additionally, Zwick (2023, 2024) reviewed the morphological relationships of *Neoperla* worldwide, recognizing four subgenera. The 82 species of African *Neoperla* all fall within the subgenus *Neoperla* (*Neoperla*), divided between four species groups, although Zwick & Zwick noted a further ten species assemblages within these groups [referred to as "complexes" in Zwick & Zwick (2023) and Zwick (2023), the term *assemblage* is used here to avoid confusion with traditional species complexes].

Despite these taxonomic advancements, nymphs were not described, as no reliable morphological characters were found to differentiate them (Zwick & Zwick 2023). Some *Neoperla* nymphs in Asia have been described using colouration patterns (Cruz *et al.* 2018; Pelingen & Freitag 2020), and the

proventriculus may allow for differentiation of species (Ogbogu 2006; Stark & Gaufin 1976). As nymphs are the most common life stage collected, descriptions of them are particularly needed.

In this chapter, I aim to further the taxonomy of *Neoperla* in Africa. To do this, I describe novel species of *Neoperla*, which will help to uncover the true diversity of the genus in Africa, and provide identification resources for these species. As nymphs cannot yet be identified, I examine the morphological features present and describe the nymphs of any species that can be identified using DNA. These are some of the first nymphs of *Neoperla* described from Africa, and they provide a valuable baseline for identifying and describing this life stage. Finally, I use three genes to investigate the relationships of *Neoperla* to link different life stages, better describe the relationships between these taxa, and improve the molecular database of South African Plecoptera barcodes.

2.2. Methods

2.2.1. Sampling

Nymphs and adults of *Neoperla* were collected from South Africa and Rwanda, starting in August 2022 and finishing in April 2024 (Figure 2.1). Nymphs were collected from rivers and streams by sweeping a standardized aquatic insect net (30 cm x 30 cm frame with mesh size 1mm) over disturbed substrates (e.g., leaf litter packs, submerged stones and gravel, marginal vegetation) in all available biotopes. Night-flying adults were collected using light traps (Zwick & Zwick 2023). Two light trap designs were used: a white tray, illuminated by a Draper Tools 12V UV fluorescent light strip and partially filled with water and a few drops of detergent to break surface tension, was left on the river bank overnight and drowned insects collected from the trap after dawn; and a white sheet illuminated by an LED lantern was hung on the banks of the river and flying insects were collected from the trap by hand after dusk. Some specimens were collected using a sweep net in terrestrial vegetation near the banks of rivers and streams during the day, or when attracted to light sources near rivers. *In situ* rearing cages, consisting of a submerged compartment with mesh walls and a floating, exposed compartment kept above the water surface with a foam float, were used at several sites. These traps were loaded with substrate (such as small stones and sticks) and a single mature nymph before being left on site for 1-4 days to capture emerging adults. Several additional specimens were collected in Malaise traps left on site next to or directly over rivers for 3-4 days.

All specimens were killed and preserved in 80% ethanol. Specimens were frozen for long-term preservation and DNA extraction once returned to the laboratory.

This collection was supplemented with specimens stored at the Albany Museum, Makhanda, South Africa, including representatives from South Africa, Angola and Rwanda. Finally, a collection of

specimens from the Democratic Republic of Congo was donated for study by François Ngera, Department of Biology, Centre de Recherche en Sciences Naturelles (CRSN)/Lwiro, Democratic Republic of Congo, Lwiro.

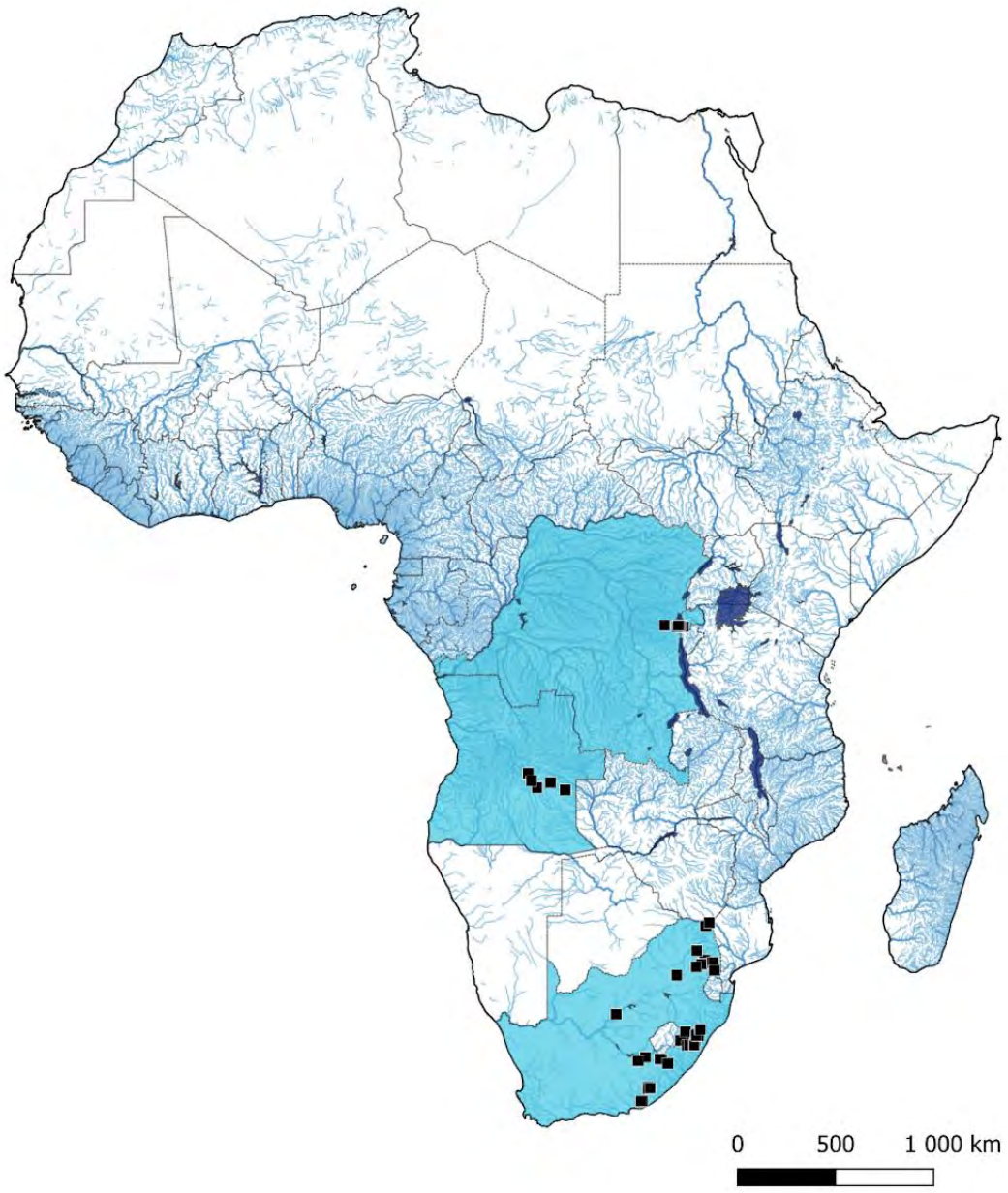

Figure 2.1: Sampling locations and countries where *Neoperla* specimens were collected. GPS co-ordinates are currently unavailable for specimens donated from the Democratic Republic of Congo.

2.2.2. Preliminary Identification

All specimens were examined under a ZEISS Stemi 508 stereo microscope and photographed using a ZEISS Aciocam ERc 5S camera attachment. Images were stacked using CombineZP 1.0 (https://combinezp.software.informer.com/). Adults were identified using the key from Zwick & Zwick (2023). As *Neoperla* nymphs currently cannot be identified without DNA, they were identified to genus using the key to South African Plecoptera (Stevens & Picker 2003).

In total, more than 700 Plecoptera specimens were examined and identified from the Albany Museum and new collections. Of these, 112 *Neoperla* were selected for molecular and morphological analysis. This selection included a combination of nymphs, adults identified to known species, and specimens that could not be assigned to any known species during the preliminary identifications.

2.2.3. Molecular Methods

2.2.3.1. DNA Extraction

A single leg was removed from each specimen for sequencing to limit damage to voucher specimens and prevent contamination from the gut contents. In nymphs, the dissection of the alimentary canal was necessary to extract the proventriculus (see below). To prevent contamination, these dissections were completed only after these specimens had already been sequenced. Total genomic DNA was extracted using an Invitrogen Purelink Genomic DNA kit, following the manufacturer's recommendations for Mammalian Tissue (available at https://tools.thermofisher.com/content/sfs/manuals/purelink_genomic_man.pdf), with an overnight lysis/digestion step. The final elution volume for all specimens was 100 µl.

2.2.3.2. Polymerase Chain Reaction (PCR):

Portions of three genes, the barcoding region of Cytochrome C Oxidase Subunit 1 (Mitochondrial, COI/COX1: 648BP), Histone 3 (Nuclear, H3: 318BP) and 28S Ribosomal RNA (Nuclear RNA: 640-756BP), were sequenced from 112 specimens. All PCRs were performed using the protocols and primer pairs summarized in Table 2.1. In cases where the COX1 gene from the South African specimens could not be sequenced using the LCO1940 / HCO2198 primer pair (Folmer *et al.* 1994), the gene was instead amplified using two overlapping internal primer pairs, LCO1490 / Ill_C_R_Perl and ILL_B_F_Perl / HCO2198 (Table 2.1), which overlapped by 82 BP and were modified from ILL_C_R and ILL_B_F (Shokralla *et al.* 2015). Successful PCR products were verified on a 1% Agarose Gel (Lee *et al.* 2012), and sent to Macrogen Europe (Amsterdam, Netherlands) for purification and sequencing.

Table 2.1: Primers used for Polymerase Chain Reactions of COX1, H3 and 28S

Gene	Primer Code	Sequence (5'-3')	Reference
COX1	LCO 1490[1]	GGTCAACAAATCATAAAGATATTGG	(Folmer *et al.* 1994)
	HCO 2198[1]	TAAACTTCAGGGTGACCAAAAAATCA	(Folmer *et al.* 1994)
	Fragment 1		
	ILL_C_R_Perl*[2]	GGVGGGTAAACBGTTCAHCC	
	Fragment 2		
	ILL_B_F_Perl*[2]	CCDGAYATRGCYTTYCCYCG	
H3	H3AF[3]	ATGGCTCGTACCAAGCAGACVGC	(Colgan *et al.* 1998)
	H3AR[3]	ATATCCTTRGGCATRATRGTGAC	(Colgan *et al.* 1998)
28S	ROAD 1A[4]	CCCSCGTAAYTTAGGCATAT	(Crandal *et al.* 2000; Whiting 2002)
	ROAD 4B[4]	CCTTGGTCCGTGTTTCAAGAC	(Crandal *et al.* 2000; Whiting 2002)

*Newly designed primers.

[1]25µl PCR reactions (12.5µl iTaqTM Universal SYBR® Green Supermix, Bio-Rad; 2.0 µl forward & reverse primer @ 10 µM concentration; 5.5 µL ddH20; 3µl DNA extract) were carried out under the following thermocycling conditions: 1. Initial denaturing: 94°C for 5 min, 2. 38 cycles of: 94°C for 40s / 50°C for 35s / 72°C for 45s, 3. Final extension: 72°C for 10 min.

[2]25µl PCR reactions (12.5µl iTaqTM Universal SYBR® Green Supermix, Bio-Rad; 2.0 µl forward & reverse primer @ 10 µM concentration; 5.5 µL ddH20; 3µl DNA extract) were carried out under the following thermocycling conditions: 1. Initial denaturing: 94°C for 4 min, 2. 40 cycles of: 94°C for 40s / 50°C for 35s / 72°C for 45s, 3. Final extension: 72°C for 8 min.

[3]25µl PCR reactions (12.5µl PowerUp™ SYBR™ Green Master Mix, Thermo-Fisher; 2.0 µl forward & reverse primer @ 10 µM concentration; 5.5 µL ddH20; 3µl DNA extract) were carried out under the following thermocycling conditions: 1. Initial denaturing: 94°C for 2 min, 2. 40 cycles of: 94°C for 30s / 50°C for 30s / 72°C for 30s, 3. Final extension: 72°C for 4 min

[4]25µl PCR reactions (12.5µl iTaqTM Universal SYBR® Green Supermix, Bio-Rad; 2.0 µl forward & reverse primer @ 10 µM concentration; 6.5 µL ddH20; 2µl DNA extract) were carried out under the following thermocycling conditions: 1. Initial denaturing: 94°C for 4 min, 2. 40 cycles of: 94°C for 40s / 56°C for 35s / 72°C for 45s, 3. Final extension: 72°C for 8 min.

2.2.3.3. Analysis

As reference 28S and H3 sequences of *Neoperla* species were unavailable, a combined phylogenetic analysis of the genus was not possible. Instead, two separate analyses were conducted, a COX1-based approach to identify novel operational taxonomic units (OTUs, estimated "species" based on tree topology), and a second to estimate a phylogeny of all newly-collected Afrotropical *Neoperla*.

Newly sequenced material was trimmed and checked manually for base-calling errors in Chromas v2.6.6 (Technelysium Pty Ltd, South Brisbane, Queensland, Australia) and AliView 1.27 (Larsson 2014). Poorly-resolved nucleotide positions were replaced with ambiguity codes.

2.2.3.4. Identification of Novel Operational Taxonomic Units (OTUs)

To confirm identifications and identify novel OTUs, the newly sequenced COX1 genes were combined with *Neoperla* reference sequences from Zwick & Zwick (2023), GenBank (Sayers *et al.*

2021) and the Barcode of Life Database (BOLD) (Ratnasingham *et al.* 2024). This dataset consisted of 284 sequences from 82 species, including 58 of the 82 described African species (Appendix 2.1). A further three species of *Neoperlops* Banks were selected as outgroups for this analysis, as this genus has been recovered as sister to *Neoperla* in recent phylogenetic analysis (Zwick & Zwick 2023; Chapter 4). The dataset was 664 homologous nucleotide positions long.

This dataset was aligned using an online version of MAFFT v7 with default parameters (Katoh *et al.* 2019), after which the alignment was translated into amino acids and checked by eye for frame shifts and the presence of stop codons (Buhay 2009). This counteracts the inadvertent inclusion of "COI-like" or nuclear copies of the mitochondrial gene ("numt").

A maximum likelihood (ML) approach was used to infer phylogenetic relationships using W-IQ-Tree (Trifinopoulos *et al.* 2016), with 1000 bootstrap replicates using the Ultrafast algorithm (Hoang *et al.* 2018). The dataset was partitioned by codon, and models (Appendix 2.2) were selected by ModelFinder (Kalyaanamoorthy *et al.* 2017). The recovered tree was visualized in FigTree V1.4.4 (Rambaut 2018) and edited in Inkscape V1.2.1 (Inkscape Project 2022).

2.2.3.5. Species Delimitation

Four molecular species delimitation tests were conducted on the COX1 dataset: a multi-rate Poisson Tree Process (mPTP: Kapli *et al.* 2017), a Bayesian Poisson Tree Process (bPTP: Zhang *et al.* 2013), Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.* 2012) and Assemble Species by Automatic Partitioning (ASAP, Puillandre *et al.* 2021). Both the mPTP and bPTP models use nucleotide substitution rates to estimate speciation events based on the branching of an imputed phylogenetic tree (Kapli *et al.* 2017; Zhang *et al.* 2013). The mPTP method is an updated version of the original Poisson Tree Process (Zhang *et al.* 2013) that improves on some of the original shortfalls, for example, by allowing intraspecific coalescent rates to vary between species instead of assuming them constant (Kapli *et al.* 2017). The analysis was run on the web server (https://mptp.h-its.org/#/tree) using the ML tree generated in W-IQ-Tree as the starting tree, with the outgroups cropped from the analysis. The bPTP method is a Bayesian implementation of the original PTP method, which uses two coalescent Markov Chain Monte Carlo (MCMC) simulations to estimate OTUs. This analysis was run on the web server (https://species.h-its.org/ptp/), with 200000 MCMC generations and a 25% burnin, and the outgroups excluded from the analysis. The ABGD method groups sequences based on pairwise genetic distances before using a range of prior intraspecific divergences to estimate species-level partitions (Puillandre *et al.* 2012). ASAP similarly groups sequences based on pairwise distances, but ranks the likelihood of the partition being correct based on the probability that the group represents a species, and the barcode gaps between partitions (Puillandre *et al.* 2021). Both analyses were conducted using the Spart explorer web server

(https://spartexplorer.mnhn.fr/), using the K2P model. The relative gap width was set to 1.0 for ABGD, as this tends to provide results closer to known taxonomy (Puillandre *et al.* 2012).

2.2.3.6. Phylogenetic Analysis

To infer phylogenetic relationships between the Afrotropical *Neoperla*, all three genes from the newly sequenced material were combined with reference genes (Appendix 2.3) obtained from GenBank (Sayers *et al.* 2021). Originally, all available Perlidae 28S sequences were used, with four outgroup species from Chloroperlidae and Perlodidae selected. However, relationships within Acroneuriinae were poorly resolved and consistently had very poor support. For this reason, these more distantly related sequences were excluded, and the clade of Acroneuriinae closest to the monophyletic Perlinae was selected as an outgroup. The final dataset contained 112 sequences from 27 *Neoperla* species, four Perlini species, and two outgroups. The dataset was 1712 homologous nucleotide positions long.

To concatenate the three data sets, each protein-coding gene was aligned separately using an online version of MAFFT v7, with default parameters (Katoh *et al.* 2019). The 28S region was aligned by ClustalW (Thompson *et al.* 2003) in MEGA v11.0.13 (Tamura *et al.* 2021). Final alignments were checked for substitution saturation in DAMBE 7.3.32 (Xia & Xie 2001) using Xia's nucleotide substitution test (Xia *et al.* 2003; Xia & Lemey 2009). Final alignments of both protein-coding genes were translated into amino acids and checked by eye for frame shifts and the presence of stop codons (Buhay 2009).

Both ML and Bayesian inference (BI) methods were used to infer phylogenetic relationships. The ML analyses were conducted using W-IQ-Tree (Trifinopoulos *et al.* 2016), using the same methods presented above. Datasets were partitioned by gene, and all protein-coding genes were further partitioned by codon position. The BI analysis was conducted via the CIPRES science gateway (Miller *et al.* 2010) using Mr Bayes 3.2.7a (Ronquist *et al.* 2012) and the models identified by ModelFinder (Kalyaanamoorthy *et al.* 2017). All BI methods were run for 20 million generations, with sampling every 1000 generations and the first 25% of the run discarded as burn-in. Convergence of the Markov chains was checked in Tracer 1.7.2 (Rambaut *et al.* 2018) to ensure the effective sample size of all parameters was >200. Final phylogenetic trees were visualized in FigTree V1.4.4 (Rambaut 2018) and edited in Inkscape V1.2.1 (Inkscape Project 2022).

2.2.4. Morphological Methods

2.2.4.1. Preparation of Adult Males

Adult males were prepared following the methods of Zwick & Zwick (2023).

As all specimens were preserved in 80% alcohol, relaxation of the specimens was not required. A median longitudinal slit was cut into sternite 9, through which fine forceps and insect pins were used to carefully extract the aedeagus. Once extracted, the aedeagus was placed into 30% potassium hydroxide (KOH, modified from Zwick & Zwick 2023), and the basal, bulb-like opening closed with soft forceps. A bent insect pin was used to wipe across the aedeagus, using the "jelly-like" internal tissue to evert the endophallus, turning it inside out and revealing the spine patterns. Once everted, the aedeagus was slide-mounted using Euparal.

External genital features from tergite 7 (T7) to the hemitergites (H10) were examined using a ZEISS Stemi 508 stereo microscope and photographed using a ZEISS Axiocam ERc 5S camera attachment. Images were stacked using CombineZP 1.0 (https://combinezp.software.informer.com/). All diagrams were made in Clip Studio Paint V2.0.0 (https://www.clipstudio.net/en/).

Slide mounts were examined under a ZEISS Primo Star microscope and photographed using the same camera. Photographs and diagrams were prepared as above.

2.2.4.2. Preparation of Adult Females

Adult females were prepared following the methods of Zwick & Zwick (2023).

To extract the vagina and spermathecal stalk, a scalpel was used to cut sternite 7 (S7) and sternite 8 (S8) free from the body. This was done by slicing through the membrane between the sternites and pleural plates, and the intersegmental membrane between Sternite 6 and S7. To prevent damage to the vagina, the intersegmental membrane in front of S8 was not cut, and instead a pair of soft forceps was used to lift and carefully pull the sternite free.

The vagina and spermathecal stalk were soaked in 30% KOH for a few minutes to clear them, after which they were thoroughly rinsed in distilled water, dehydrated in 100% Ethanol, and slide mounted in Euparal.

2.2.4.3. Preparation of Eggs

Eggs were collected from the body cavity of the dissected females. Eggs were abundant in cases where the female had been close to ovipositing, but in younger or older females, eggs would be sought in the body cavity, as there were usually a few embedded near the cerci. In young females, the follicular membrane was manually peeled from the mature oocytes with forceps and a scalpel. Eggs were then dehydrated in alcohol before being slide-mounted in Euparal, where they became transparent. As Euparal cannot penetrate the chorion, small cracks and fissures in the eggs were introduced by pressing on the slide cover with an insect pin.

The structure of a few species' eggs was examined using a TESCAN Vega scanning electron microscope, fitted with an Oxford Instruments EDS detector at the Electron Microscopy Unit hosted by Rhodes University. Due to a prolonged maintenance period, the Electron Microscope was not available to examine all species. All images and diagrams were made using the methods described above.

2.2.4.4. Preparation of Nymphs

Hynes (1953) assessed the nymphs of Afrotropical *Neoperla* to provide a generalised description of the nymph of the *Neoperla spio* species complex. He found little variation in the morphological features of the nymphs, including in the mouthparts, body shape, ocellus size and position, and gill structure. Zwick & Zwick (2023) similarly found no morphological characters that allowed for the taxonomic separation and identification of the nymphs. These same characters were checked here and did not vary systematically between genetically delimited OTUs.

However, Hynes (1953) described several distinct colouration patterns. The morphological assessment of the nymphs, therefore, focuses on habitus characters. This aligns with recent descriptions of *Neoperla* nymphs from Asia (Cruz *et al.* 2018; Pelingen & Freitag 2020). As colouration patterns appear only once the nymphs are at least 7 mm long (Hynes 1953), descriptions were limited to specimens of at least 10 mm in length (excluding antennae and cerci).

The inner lining of the proventriculus has been mentioned as a possible character for delimiting the nymphs of Perlidae (Ogbogu 2006; Stark & Gaufin 1976). The proventriculus from each species was removed by cutting a longitudinal slit through the sternal plate of the meso- and metathorax, and the first two abdominal segments. The alimentary canal was pulled through this slit using forceps, and the entire proventriculus cut away from the rest of the alimentary canal using scissors. The proventriculus was sliced open and flattened, cleared in KOH, washed, dehydrated in ethanol and slide-mounted in Euparal.

All images and diagrams were made using the methods described above.

2.3. Results

2.3.1. Identification of Novel OTUs

Relationships between the species groups established by Zwick (2023) and Zwick & Zwick (2023) were unresolved in this analysis, with low support values recovered for all basal branches (Figure 2.2). This lack of resolution was possibly due to saturation of the COX1 gene (DAMBE recovered

significant saturation in the third codon position: Iss > Iss.cSym & Iss.cAsym, p-value: >0.23; little saturation in codon position 1 & 2, and when not partitioned), as a combination of Asian, North American and African species from several subgenera and many species groups of *Neoperla* were included. Despite this, most species groups were recovered as monophyletic clades, although poorly supported (Bootstrap support < 75%). Both of Zwick's (2023) *N. transvaalensis* and *N. excisa-sjostedti* species groups were polyphyletic. Within species groups, only the *N. transvaalensis* species assemblage was polyphyletic, as four species were recovered as sister to the rest of the African and North American *Neoperla*. Nevertheless, due to poor support for basal clades, phylogenetic interpretations were not made from the results of this analysis.

However, except for a paraphyletic *Neoperla socia* Zwick & Zwick, all of the included species formed strongly supported (Bootstrap support >91%) monophyletic clades. Many of these clades included newly sequenced specimens, which served to confirm the identifications of nymphs and adults and ensure that any novel OTUs recovered were not artifacts of different extraction and sequencing protocols. Relationships between close species were generally well resolved, with strong support (Bootstrap support > 90%), although basal branches within the species groups were usually poorly supported.

Ten putative, strongly-supported (Bootstrap support >94%) monophyletic clades, that did not correspond to any known species, were identified by this analysis (Figure 2.2-2.6). In some cases, these clades were sister to one another, or had a single, long basal branch consisting of a single specimen (e.g., Figure 2.5). In these cases, delineation between the clades was resolved using a combination of species delineation tests and morphological evidence. All these clades are considered novel species, and are henceforth referred to by the informal names assigned to them in the Taxonomy section below.

The results of the four species delineation tests were mutually incongruent. The mPTP test recovered the fewest OTUs, but failed to successfully identify all the reference species, returning a total of only 73 partitions. This failure was because multiple species were occasionally grouped into a single OTU, despite long internal branch lengths. Grouping of multiple species was particularly common in sister species represented by single sequences. Comparatively, the bPTP process recovered a total of 135 OTUs. Most reference species were successfully identified by this analysis, including species with only a single sequence. However, a few species with multiple specimens were split into separate OTUs, despite short internal branch lengths.

Comparatively, ASAP and ABGD returned significantly more OTUs, with 145 and 189 partitions recovered, respectively. ASAP provides ten ranked partition files, and usually the highest-ranked partition should be selected for analysis. However, several distantly-related species from disparate species groups and subgenera were grouped into a single OTU (e.g., *Neoperla transvaalensis*,

Neoperla beta Zwick & Zwick, *Neoperla catharae* Stark & Baumann and *Neoperla* (*F.*) *dao* Stark & Sivec). As these species are morphologically distinct, and were clearly polyphyletic in all phylogenetic analyses, including the phylogenetic tree constructed by ASAP, these results were discarded. Instead, the highest-ranking partition that successfully identified the most reference species was selected. Comparatively, the ABGD results are not ranked, and the result that successfully identified the most species was selected for further analysis. Both the initial clustering of distant species and the oversplitting of individual species were probably caused by saturation of the COX1 gene and incomplete sampling of the genetic diversity within a species due to small sample sizes (Puillandre *et al.* 2012, 2021).

Despite this variation, all four species delineation tests placed most of the ten clades identified above into separate OTUs, providing additional evidence that these are different species (Figure 2.3-2.6). In some cases, these OTUs were further split by one or more tests (e.g., Figure 2.3, *Neoperla sp. nov.* 3). In these situations, the more conservative divisions were used to prevent over-splitting of single species (Carstens *et al.* 2013). The mPTP analysis failed to recover two species, which were instead placed in a single OTU (Figure 2.6). However, as this grouping included four other morphologically distinct, known species, this was probably too conservative a grouping, and the combined results of the other three tests were used instead.

2.3.2. Phylogeny

Except for the placement of *Neoperla amoena* Zwick & Zwick and *Neoperla sp. nov. 2*, all three analyses returned consistent and generally strongly supported relationships across the phylogeny (Figure 2.7, Bootstrap support: >80, Posterior probabilities: >0.90).

Both Perlini and *Neoperla* formed strongly supported monophyletic clades (ML: 100, BI: 1, BEAST: 1), with the African taxa additionally forming a monophyletic group (ML: 80, BI: 1, BEAST: 1) separate from the North American *Neoperla clymene* Newman. The African taxa were further split into two clades, with the strongly supported *N. transvaalensis* species group (ML: 98, BI: 0.99, BEAST: 0.99) forming a monophyletic clade, while the second (ML: 78, BI: 0.95, BEAST: 0.82) consisted of the *N. spio, N. duodeviginti* and *N. excisa sjostedti* species groups. The placement of *N. amoena* was unresolved, as it formed a polytomy in both the maximum likelihood and Bayesian inference methods, and a very weakly supported sister to the second clade (BEAST: 0.18) in the BEAST analysis. The *N. spio, N. excisa-sjostedti* and *N. duodeviginti* species groups were polyphyletic, but all of the Afrotropical species assemblages (except for *duodeviginti)* were monophyletic and well-supported (ML: >84, BI: >0.96, BEAST: >0.97).

All included species formed well-supported monophyletic clusters (ML: >89, BI: >0.98, BEAST: >0.99). In addition to the ten OTUs identified above, five additional monophyletic clades were recovered that did not correspond to any known species. The sequencing of the COX1 gene for these species all failed, so their taxonomy was assessed using morphological characters alone.

2.3.3. Morphology

In total, nine morphologically novel species were identified and described. Descriptions used previously identified characters, focusing on the adult genital characters (Picker 1980; Zwick 1976, 2023; Zwick & Zwick 2023). Specifically, in males the shapes and processes on abdominal tergites from T7 – H10 and the spine patterns of the aedeagus were used, while in females the shape and spinulation of the vagina, the spermathecal stalk, spermathecal duct, and the eggs were used. Habitus features were described only to provide supplementary, secondary identifying characteristics. A previously described morphologically distinct form of the *Neoperla sjostedti needhami* Lestage subspecies (Zwick & Zwick 2023) was elevated to species rank, which also necessitated the elevation of both *Neoperla sjostedti* Klapálek subspecies. Novel species are not named here and have been denoted as *sp. nov. 1-10,* until they can be formally described and published.

As it is impractical to develop a new key to the adult *Neoperla*, amendments to the key developed by Zwick & Zwick (2023) are presented here. These changes are denoted by the letter i and should be inserted immediately before the given number in the original key (e.g. 3i should be inserted just before 3, one choice in the couplet will then proceed to 3). In some cases, the previous couplet needed to be modified. In these cases, the new form of that couplet is also presented.

Nymphs of several species are described. Habitus characters provided the only sources of morphological variation between species. As these characters are unreliable for species identification, these species will not be formally named until adults can be collected. Descriptions are therefore provided only as supplementary characters. To prevent informal homonyms, each species has been named using the same format that Zwick & Zwick (2023) used for unnamed species, continuing numbering from that same paper. Therefore, these species are denoted as *Neoperla* sp. Afr_C-F.

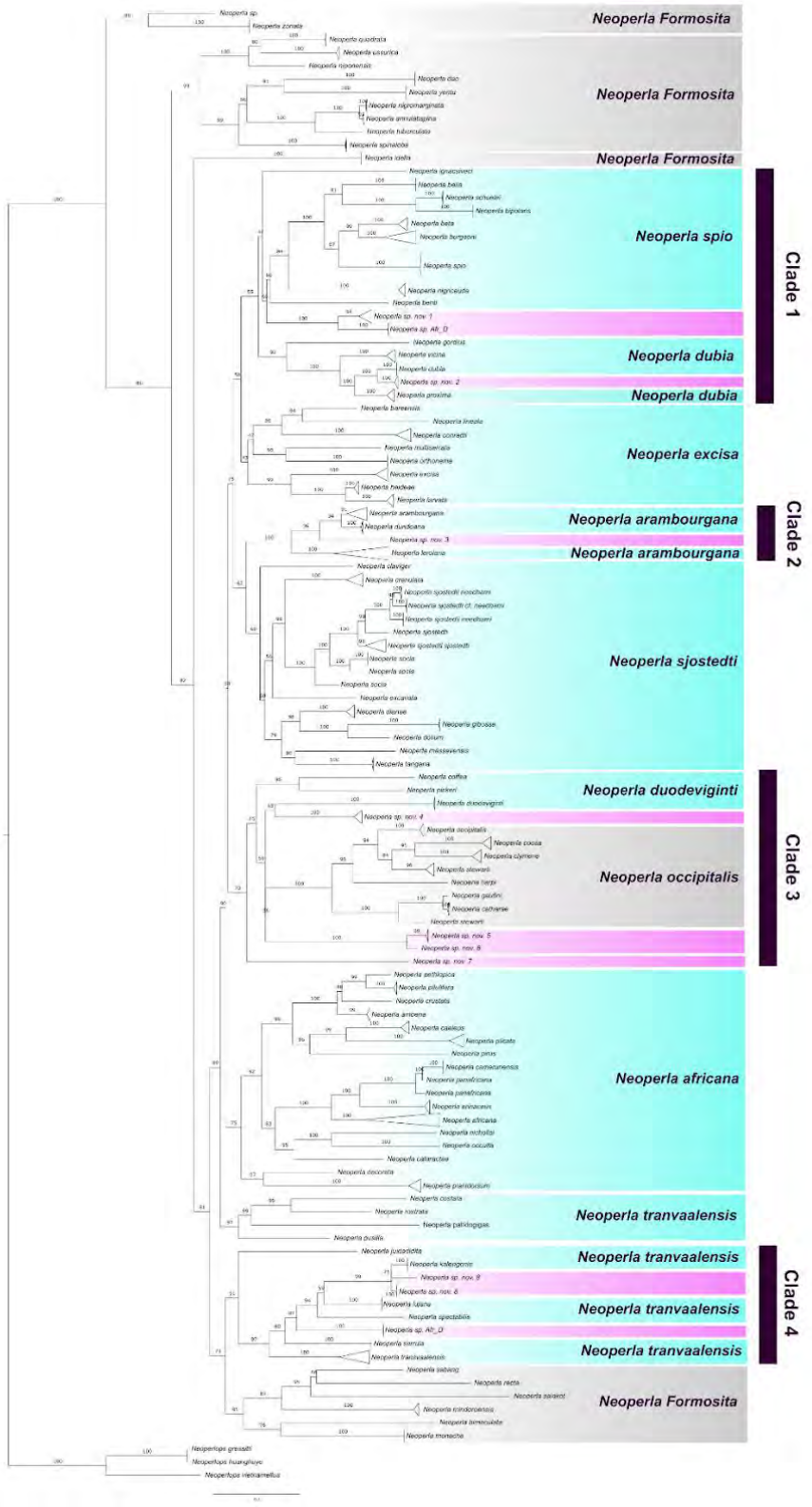

Figure 2.2: Phylogenetic tree constructed using the barcoding region of the COX1 gene of 284 *Neoperla* specimens from 92 species (including novel OTUs). Blue blocks on the right of the tree show species assemblages, following Zwick & Zwick, 2023, while novel species are highlighted in pink. Members of *Neoperla* (*Formosita*) and *Neoperla occipitalis* are highlighted in grey. Values on each node represent bootstrap support values from UFBoot (% of 1000 bootstrap analyses).

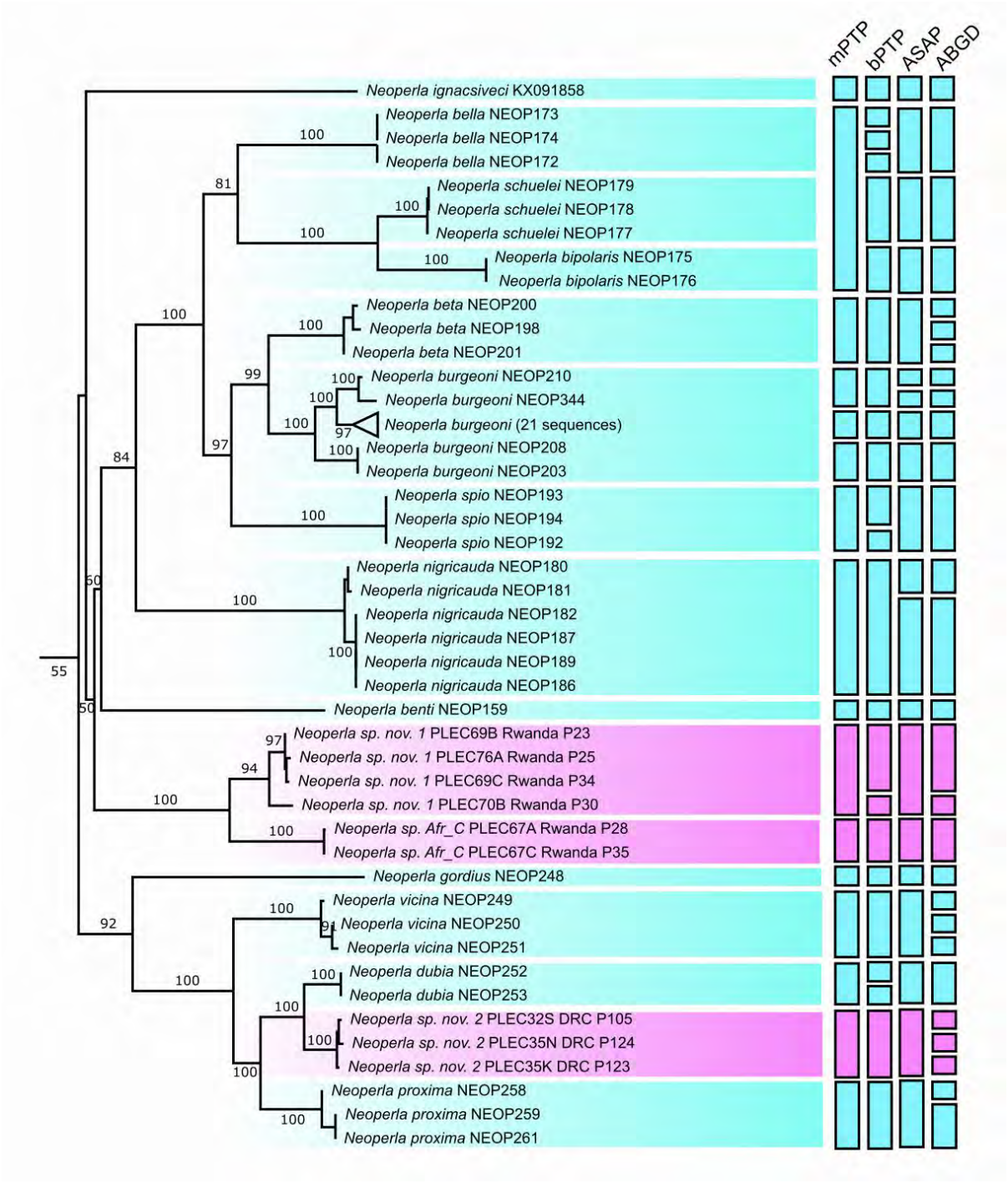

Figure 2.3: Phylogenetic relationships of the *Neoperla spio* and *Neoperla dubia* species assemblages based on the barcoding region of the COX1 gene, Clade 1 from Figure 2.2. Blue blocks on the right of the tree show described species, while novel species are highlighted in pink. Boxes on the right represent each OTU recovered by the four species delineation tests, mPTP, bPTP, ASAP, and ABGD; each box represents a single OTU. Values on each node represent bootstrap support values from UFBoot (% of 1000 bootstrap analyses).

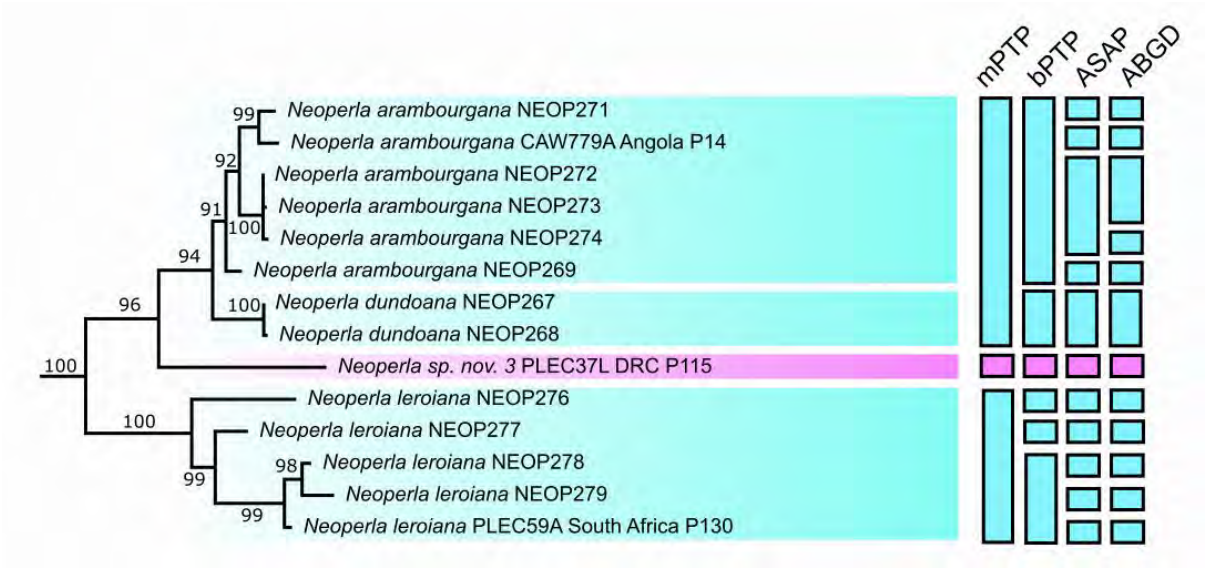

Figure 2.4: Phylogenetic relationships of the *Neoperla arambourgana* species assemblage based on the barcoding region of the COX1 gene, Clade 2 from Figure 2.2. Known species are highlighted in blue, while novel species are highlighted in pink. Format follows Figure 2.3.

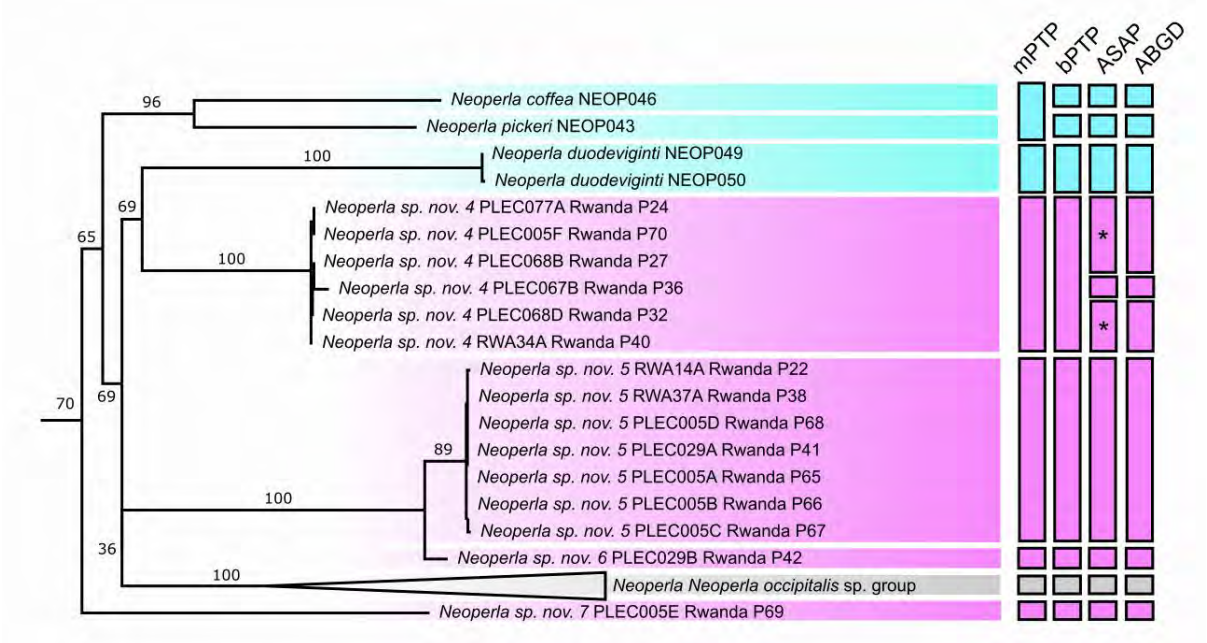

Figure 2.5: Phylogenetic relationships of the *Neoperla duodeviginti* and *Neoperla occipitalis* species assemblages based on the barcoding region of the COX1 gene, Clade 3 from Figure 2.2. Format follows Figure 2.3, with the addition of a grey highlight to denote the *Neoperla occipitalis* species group. *recovered as a single OTU.

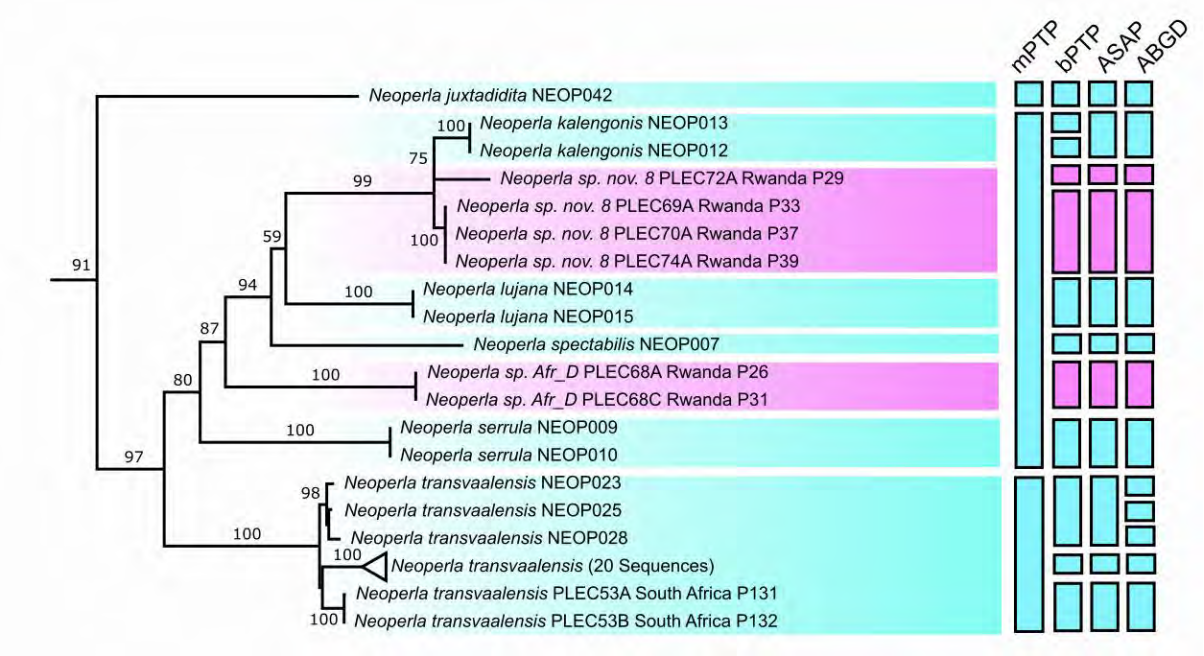

Figure 2.6: Phylogenetic relationships of the *Neoperla transvaalensis* species assemblages based on the barcoding region of the COX1 gene, Clade 4 from Figure 2.2. Format follows Figure 2.3.

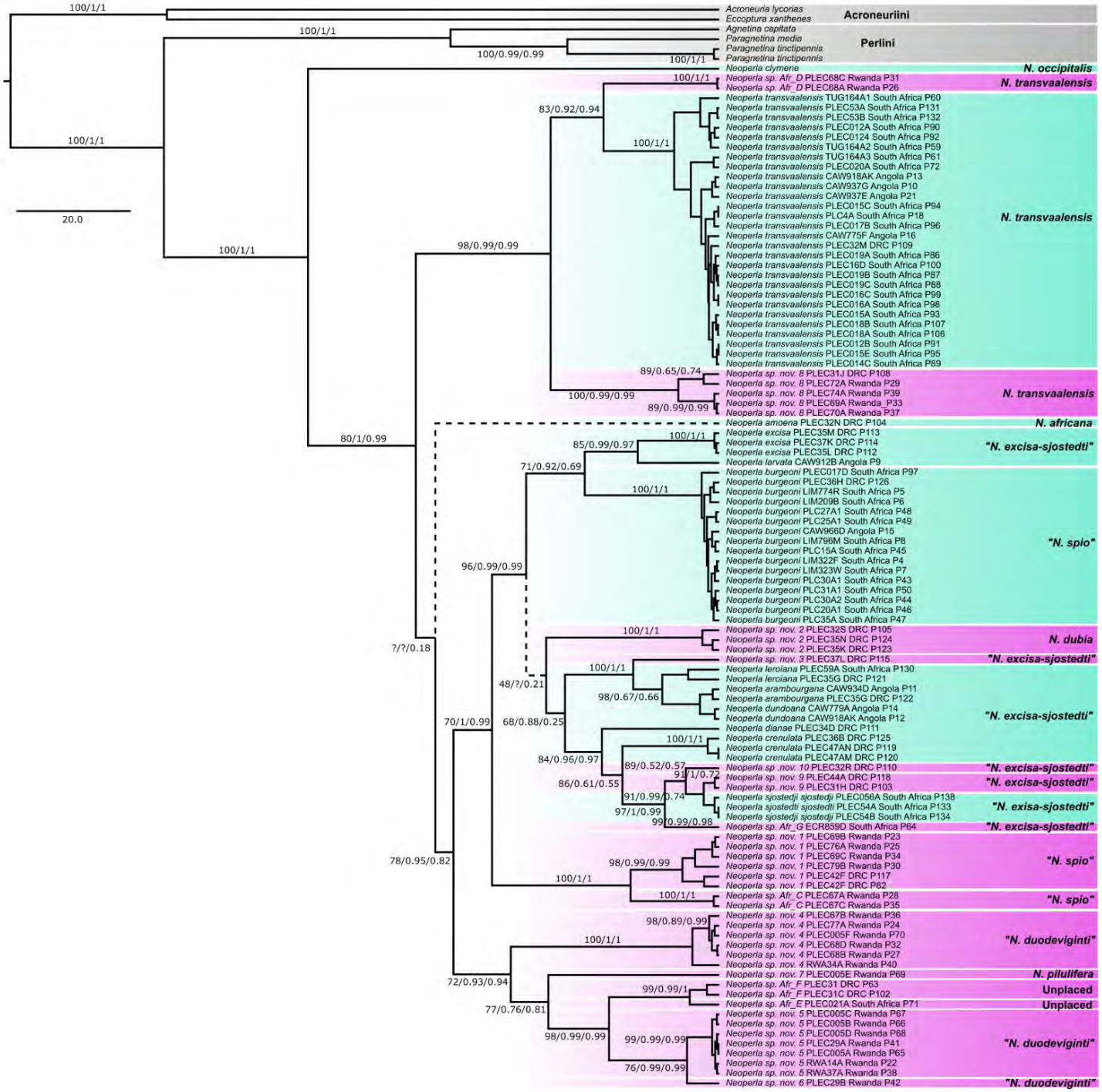

Figure 2.7: Phylogenetic tree showing the relationships of African *Neoperla*. Values on each node represent bootstrap support values from UFBoot (% of 1000 bootstrap analyses), and posterior probabilities recovered from MrBayes and BEAST. Blocks on the right of the tree show species assemblages, following Zwick & Zwick, 2023, Novel species are highlighted in pink.

2.4. Taxonomy

Genus *Neoperla* Needham, 1905

Subgenus *Neoperla* (*Neoperla*) Needham, 1905

Species group *Neoperla spio* Zwick & Zwick, 2023

Neoperla sp. nov. 1

Images: Figure 2.8

Material Examined: *Prospective Holotype*: PLEC 42F: 1♂ adult || Democratic Republic of Congo | Npungwe River | PNKB | 03.vi.2016 | Coll.: F. Ngera. ***Other Material*: PLEC 69B**: 1♀ nymph || Rwanda | Mujabagiro River | Nyungwe Forest | 2°26'51.9"S, 29°06'27.8"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Currenti || **PLEC 69C**: 1♀ nymph || Rwanda | Mujabagiro River | Nyungwe Forest |2°26'51.9"S, 29°06'27.8"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 70A**: 1♂ nymph || Rwanda | uWasenkoko Swamp | Nyungwe Forest | 2°31'44.5"S, 29°21'12.4"E | 10.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 76A**: 1♀ nymph || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°28'08.7"S, 29°04'28.7"E | 14.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current.

Nymph: Dark brown. Head dark brown, without distinctive patterning; vertex and region around eye sometimes paler or faded. Ocelli separate, approximately one ocellus-width apart. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae light brown; with very pale, fine setae distally.

Prothorax wide, dark brown, without obvious markings, but darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax dark brown, but medially pale in an indistinct circle, with a very pale "w" marking; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs brown; tibia slightly lighter; tarsomeres brown; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

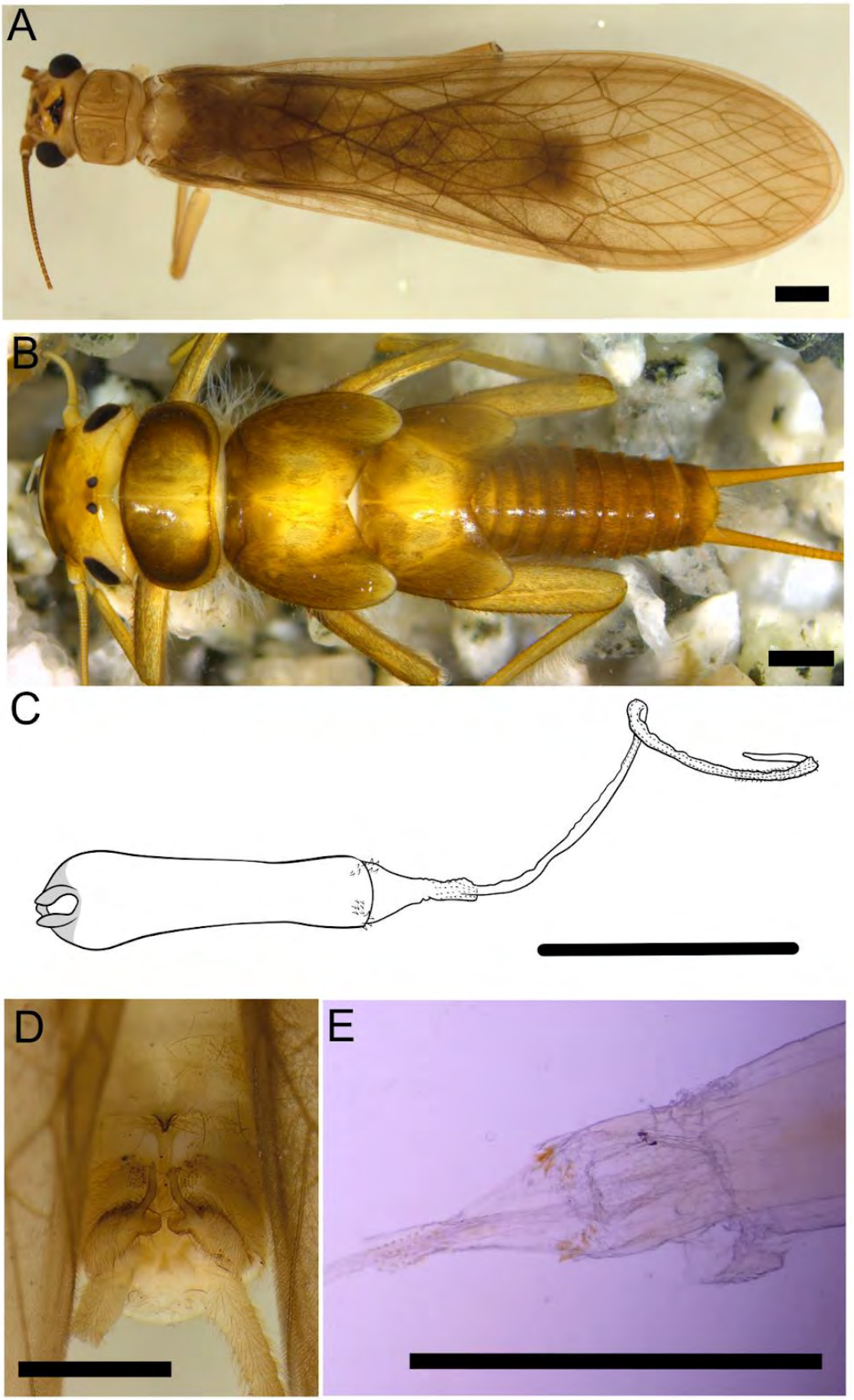

Figure 2.8: *Neoperla sp. nov. 1*. **A:** Adult male habitus. **B:** Nymph habitus. **C:** Diagram of the everted aedeagus. **D:** Tergite 7 – Hemitergites in the adult male. **E.** Spine patterns on tip of aedeagus. Unless otherwise stated, all scale bars represent 1mm.

Adult Habitus: ♂ FW length: 14.1 mm. Pale yellow brown. Head pale brown, with brown "spade" pattern over ocelli, edges drawn out into thin ridges; a small brown square antero-medially. Tentorial callosities pale. Antennae dark, amber brown. Ocelli separate, approximately one ocellus-width apart. Thorax amber, with margin and midline brown. Legs amber; fore- and mid-femora with dark brown stripe caudally; tarsi pale; tip of tarsomere III and claws black. Wings pale brown, veins uniform brown. Abdomen pale, darkened distally. Cerci yellow, densely covered with fine setae.

Males: T7 without forefield; with an unpaired, three-dimensional caudal process. The process is a pyramidal cone, with sensilla basiconica on tip and underside. T8 distinctly sclerotized in midline, T-shaped, caudally wide and almost straight; with a large, raised hump that is skewed towards T7 and bearing some sensilla basiconica on face; forming a narrow band from bump to posterior margin. T9 with antecosta divided in front of pale median field; rest of T9 is sclerotized; lateral humps flat and widely spaced, with many sensilla basiconica and some pilosity. H10 mediabasal callus large, dorsally flat, tongue-shaped. Process sinuous, with tip rounded. Epiproct triangular.

Aedeagus: Aedeagus short, distally curved, moderately sclerotized, distally with a long patch of small spicules dorsomedially, that spread into two large lateral spine bands along margin.

Everted endophallus approximately 1.5 times longer than aedeagus; base tapering rapidly, remainder thin, rope-like. Spines straight and sharp-tipped, pointing slightly caudally; base of endophallus with short bare section, followed by four rows of spinules, that converge as endophallus becomes rope-like; rope-like section with strikingly long bare section, after which two ordered rows of fine spines appear, becoming four ordered spines rows distally, spines grow larger; distally tip bare.

DNA: *Neoperla sp. nov. 1* forms a strongly supported (Bootstrap support: 94%), monophyletic cluster at the base of the *Neoperla spio* species assemblage in COX1 analysis (Figure 2.3). However, its relationship to the rest of the *N. spio* species group was poorly supported (Bootstrap support: 50%), and the phylogeny (Figure 2.7) placed it as sister to both the *N. spio* and *N. excisa-sjostedti* species groups, in a strongly supported monophyletic clade (Bootstrap support: 100%, Posterior probability: 1, BEAST: 1). All four species delimitation tests identified this species as an OTU, although the bPTP and ABGD analyses each separated a single specimen (PLEC 70B). As there are no morphological differences to support this, the more conservative grouping of mPTP and ASAP was followed.

Notes: The species group placement of *Neoperla sp. nov. 1* is unclear, but for now it is placed within the *N. spio* species group. This is supported by the long, “rope-like” endophallus with ordered rows of spines (Zwick 2023; Zwick & Zwick 2023). Males of many specimens within the *N. spio* group are similar, and share the moderately sclerotized aedeagus, and external genital characters of *Neoperla sp. nov. 1* (Zwick & Zwick 2023). However, it is most similar to *Neoperla funiculata* Zwick & Zwick and *Neoperla biserrata* Zwick & Zwick, due to the unmodified, bluntly pointed tips of the hemitergites,

the lack of external spine patches on the aedeagus except along the tip, and the lack of a process on T8 (Zwick & Zwick 2023).

Neither species has been sequenced, preventing diagnosis by molecular methods. However, *Neoperla sp. nov. 1* can be differentiated from both species by the dorsodistal patch of small spicules on the aedeagus that spread into two large lateral spine bands, the four ordered rows of spines dorsally on the base, and the long void section of the endophallus (Zwick & Zwick 2023).

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. spio-complex, Males, pg. 88

19i. Aedeagus tip dorsodistally with microscopic spines, develops into lateral patches of large, pointed spines; endophallus base with four rows of spines, followed by a long void section ……. ……. *Neoperla. sp. nov. 1*

19i'. Aedeagus tip with some scattered spines, but lacking dorsodistal patch of spines; no long void section on endophallus…….……. 19ii

Neoperla burgeoni Navás, 1926

Neoperla burgeoni – Navás, 1926
Neoperla bredoana – Navás, 1932

Images: Figure 2.9

Material Examined: *Other Material*: PLEC 17D: 1 nymph || South Africa | Trout River | -29.756778, 29.434139 | 04.iv.2024 | Coll.: A. Kirkaldy, M. Villet || Stones in Current | Temp: 18.9 C | EC: 36 us/cm | TDS: 18mg/l || **PLC 27:** 1 nymph || South Africa | Blyde River Cabins | -24.297028, 30.851361 | 24.xi.2022 | Coll.: A. Kirkaldy || Stones in Current || **PLC 31:** 1 nymph || South Africa | Blyde River Cabins | -24.297028, 30.851361 | 24.xi.2022 | Coll.: A. Kirkaldy || Stones in Current || **LIM 209:** 1 nymph || South Africa | Sabie River, at Tinga | -24.94067, 31.50496 | 24.ix.2015.

Nymph: Light brown, amber. Head pale brown; frons brown; vertex with a transverse yellow band, stretching from occipital ridge to front of eyes; no markings between occipital ridge and ecdysial line, or with very faint, indistinct brown medially; slight, dusty brown between ocelli. Ocelli small or large, approximately one ocellus-width apart when small, if large almost touching. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae pale yellow; with fine dark setae, more along internal margin basally, distally very few.

Prothorax wide, amber brown, no markings, but sometimes pale medially; a dark line around margin. Mesothorax amber brown, medially pale; sometimes with very indistinct, light markings on either side

of ecdysial line; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale yellow; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small, curved hook emerging near base of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: The identification of the nymphs was supported by molecular evidence. All of the specimens formed a strongly supported (COX1 Bootstrap: 100%) monophyletic clade with sequences taken from Zwick & Zwick (2023). While many of the nymphs described here have a transverse yellow stripe on the head, only *N. burgeoni* does not have brown markings between the occipital ridge and the ecdysial line.

Figure 2.9: *Neoperla burgeoni* Navás, nymph habitus. Scale bar represents 0.1mm.

Species group *Neoperla africana* Zwick & Zwick, 2023

Neoperla sp. nov. 2

Images: Figure 2.10

Material Examined: *Prospective Holotype*: PLEC 35N: 1♀ adult || Democratic Republic of Congo | Ongayi River | Nkuba | 03.x.2020 | Coll.: F. Ngera. ***Prospective Paratypes*: PLEC 32S**: 1♀ adult || Democratic Republic of Congo | Obonje River | Nkuba | 13.iii.2020 | Coll.: F. Ngera || **PLEC 35K**: 1♀ adult | Democratic Republic of Congo | Ongayi River | Nkuba | 03.x.2020 | Coll.: F. Ngera.

Adult Habitus: ♀ FW length: 13.3–13.7 mm. Amber brown. Head amber, with small indistinct brown oval over ocelli. Tentorial callosities pale. Antennae pale brown. Ocelli separate, approximately one ocellus-width apart. Thorax amber, with margin and midline brown. Legs pale; femur yellow, darker distally; tibia brown; tarsi pale brown. Wings pale brown, veins uniform brown. Abdomen pale, darkened distally. Cerci yellow, densely covered with fine setae.

Females: Caudal edge of S7 unmodified. S8 with two squarish brown marks in a median row, separated by a pale line, sometimes indistinct. Posterior margin of S8 with a small sclerotized nail, length variable, in some specimens barely projecting. Vagina calyx shaped; spermathecal stalk inserted towards top of vagina. Spermathecal stalk coiled, 1 ring(s), longer than vagina, wide at base, narrowing distally; base with elongate folds on concave side. Scales internal; armature consists of sharp, slightly scattered and irregular scales; at base scale pattern is thick, but does not cover folded region; distally covers entire diameter, except for a narrow band on convex side. Spermatheca densely coiled.

Egg: Egg approximately 408 µm long, 217 µm wide; ovoid. Collar constricted at base, widening distally, rim slightly flared; thin base with one row of elongated cells formed by costae, wider section with single row of large, deep cells with high walls. Anchor cap with a truncate stalk, mushroom shaped. Approximately 20 striae, straight, with very broad sulci and narrow costae. Costae very high crests, drawn into lamellae; lamellae are rough and scalloped along edge, with a wave like pattern caused by striations; sulci with relatively coarse punctures in indistinct transverse rows; striae extend onto collar, creating a row of high, elongated cells, ridge like lamellae continue to top of operculum, lamellae interrupted with a calyx shaped incision along eclosion line. Microphyles are located in sulci, large, exposed holes. Operculum punctate, with a high parabolic top.

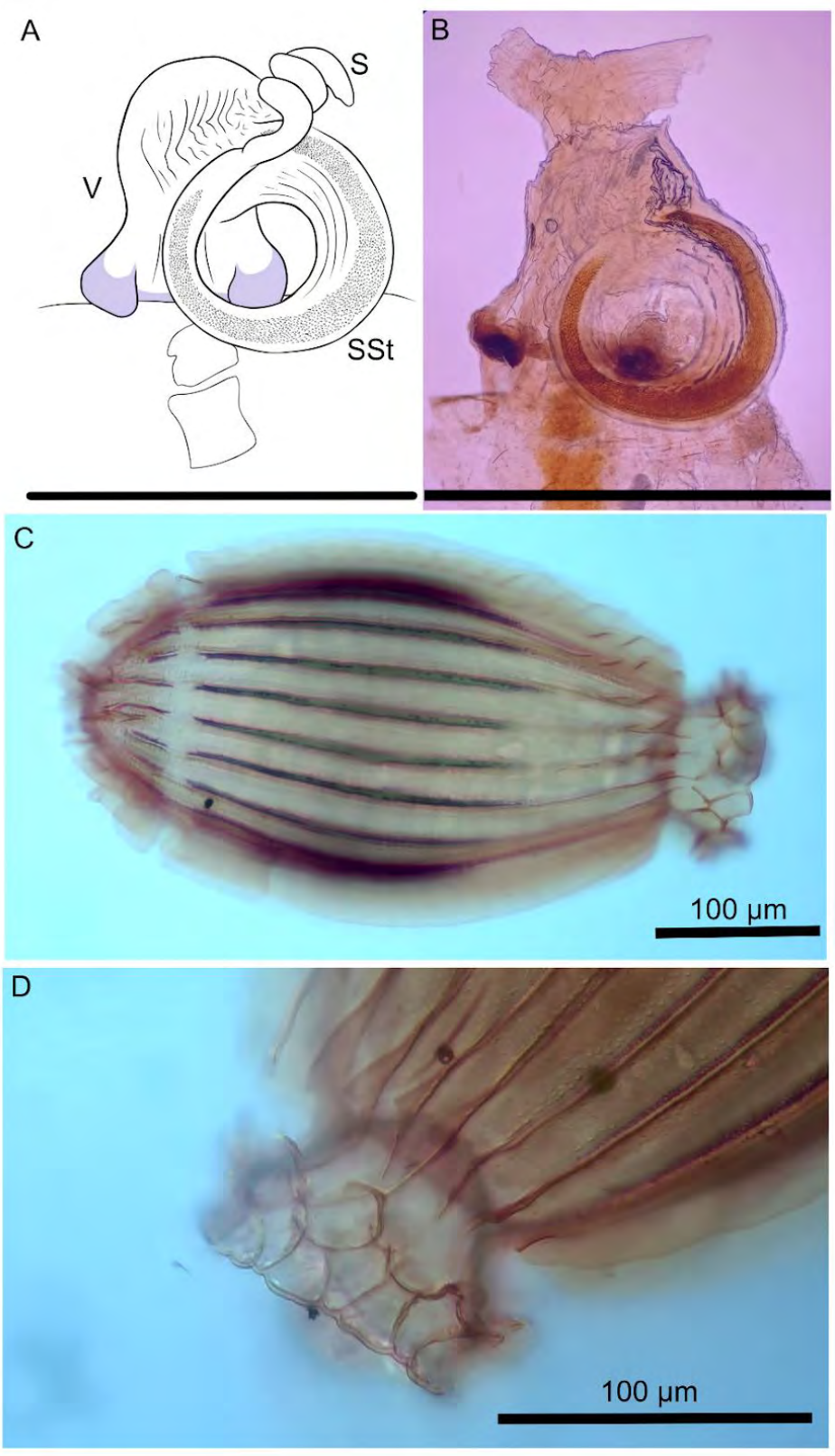

Figure 2.10: *Neoperla sp. nov. 2*. **A:** Diagram of vagina and spermathecal stalk, V: Vagina, Sst: Spermathecal stalk, S: Spermatheca. **B:** Photograph of vagina and spermathecal stalk, X40 **C:** Egg structure, (X200) **D:** Egg collar, showing constricted base (X200). Unless otherwise stated, all scale bars represent 1mm.

DNA: *Neoperla sp. nov. 2* forms a strongly supported (Bootstrap support: 100), monophyletic sister to *Neoperla dubia* Klapálek in the COX1 analysis (Figure 2.3). In the phylogeny, it formed a monophyletic clade, and was sister to the *Neoperla excisa-sjostedti* species group (Figure 2.7), However, this relationship was unresolved, as the *N.spio* species group formed a polytomy with these two branches in some analyses. All four species delineation tests separated this species from *N. dubia*, although ABGD further split the species into three OTUs.

Notes: *Neoperla sp. nov. 2* falls in the *N. africana* species group. This is supported by the coiled spermathecal stalk, which has a dense coat of internal scales arranged like "fish scales", except for the bare, folded concave side on the base (Zwick 2023). Additionally, *Neoperla sp. nov. 2* is placed within the *N. dubia* species assemblage by the two squarish brown marks on S8, the spermathecal coil forming only one ring, and the costae being drawn out into high flanges (Zwick & Zwick 2023).

Neoperla sp. nov. 2 is extremely similar to *N. dubia* and was originally misidentified as such. The vagina and spermathecal stalk share the generalized structure of the *N. dubia* assemblage, although the spermathecal stalk is slightly narrower in *Neoperla sp. nov. 2*. The eggs also share the high flanged costae, which continue from the collar to the top of the operculum, with a small incision delineating the base of the operculum (Zwick & Zwick 2023). However, all species delimitation tests repeatedly separated the two species. Morphologically, the species can be separated by the egg collar, which is constricted at its base, with elongate cells extending from the costae in *Neoperla sp. nov. 2*. While the collar in *N. dubia* is variable, it is never markedly constricted at the base (Zwick & Zwick 2023).

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. spio-complex, Females, pg. 89

5. Costae with ridges or with high flanges which are bent sideways ……. 5i

5'. No ridges or flanges on costae ……. 56 *Neoperla proxima* Zwick & Zwick

5i. Egg collar constricted at base, with elongate cells ……. *Neoperla sp. nov. 2*

5i'. Egg collar not constricted at base ……. 55 *Neoperla dubia* Klapálek

Species group *Neoperla exisa-sjostedti* Zwick & Zwick, 2023

Neoperla sp. nov. 3

Images: Figure 2.11

Material Examined: *Prospective Holotype:* PLEC 37L: 1♀ adult || Democratic Republic of Congo | Maiga River | Nkuba | 24.ix.2020 | Coll.: F. Ngera.

Adult Habitus: ♀ FW length: 12.1 mm. Pale yellow brown. Head pale yellow, slightly darker along midline; with dark marking around ocelli; pale "W" mark on the frons. Tentorial callosities pale. Antennae dark, amber brown. Ocelli separate, approximately one ocellus-width apart. Thorax pale, without prominent markings. Legs pale; mid- and fore-tibia slightly darker brown; tarsi pale brown. Wings pale brown, veins brown, subcosta paler. Abdomen pale, darkened distally. Cerci yellow, densely covered with strong setae.

Females: Caudal edge of S7 with a fringe of strong setae. S8 pale yellow, without markings. Posterior margin of S8 straight to very slightly convex. Vagina short, wide; spermathecal stalk inserted towards top of vagina. Spermathecal stalk coiled, 2 ring(s), longer than vagina, slightly wider at base, constricts rapidly and then similar width over entire length; without folds. Scales internal; armature consists of sharp, slightly scattered and irregular scales; at base scales are only on convex side; distally widening to cover entire diameter. Spermatheca densely coiled.

Egg: Egg approximately 312 µm long, 172 µm wide; ovoid, with a slight angle at base of operculum. Collar narrow, rim flanged and scalloped; appears smooth, but costae extend onto collar, creating a few cells with smooth walls. Anchor cap with a truncate stalk, mushroom shaped. Approximately 38 striae, levogyrous, costae narrow, but still approximately double the width of deep, narrow sulci. Costae smooth and flat; sulci narrow and deep, with two rows of micropunctures; striae extend onto collar, creating a few low cells, and to near top of operculum, with pale ring at base. Microphyles were not found. Operculum appears smooth, without cells, conical.

DNA: *Neoperla sp. nov. 3* formed a monotypic branch in all analyses, and was placed as sister to *Neoperla arambourgana* Navás and *Neoperla dundoana* Zwick & Zwick. This was strongly supported in both the COX1 analysis (Bootstrap support: 94, Figure 2.4) and the phylogeny (Bootstrap support: 100, Posterior probability: 1, BEAST: 1; Figure 2.7). Both *N. arambourgana* and *N. leroiana* had multiple, deeply split branches within their clades, suggesting possible species complexes. *Neoperla sp. nov. 3* was identified as a novel OTU by all four of the species delineation tests.

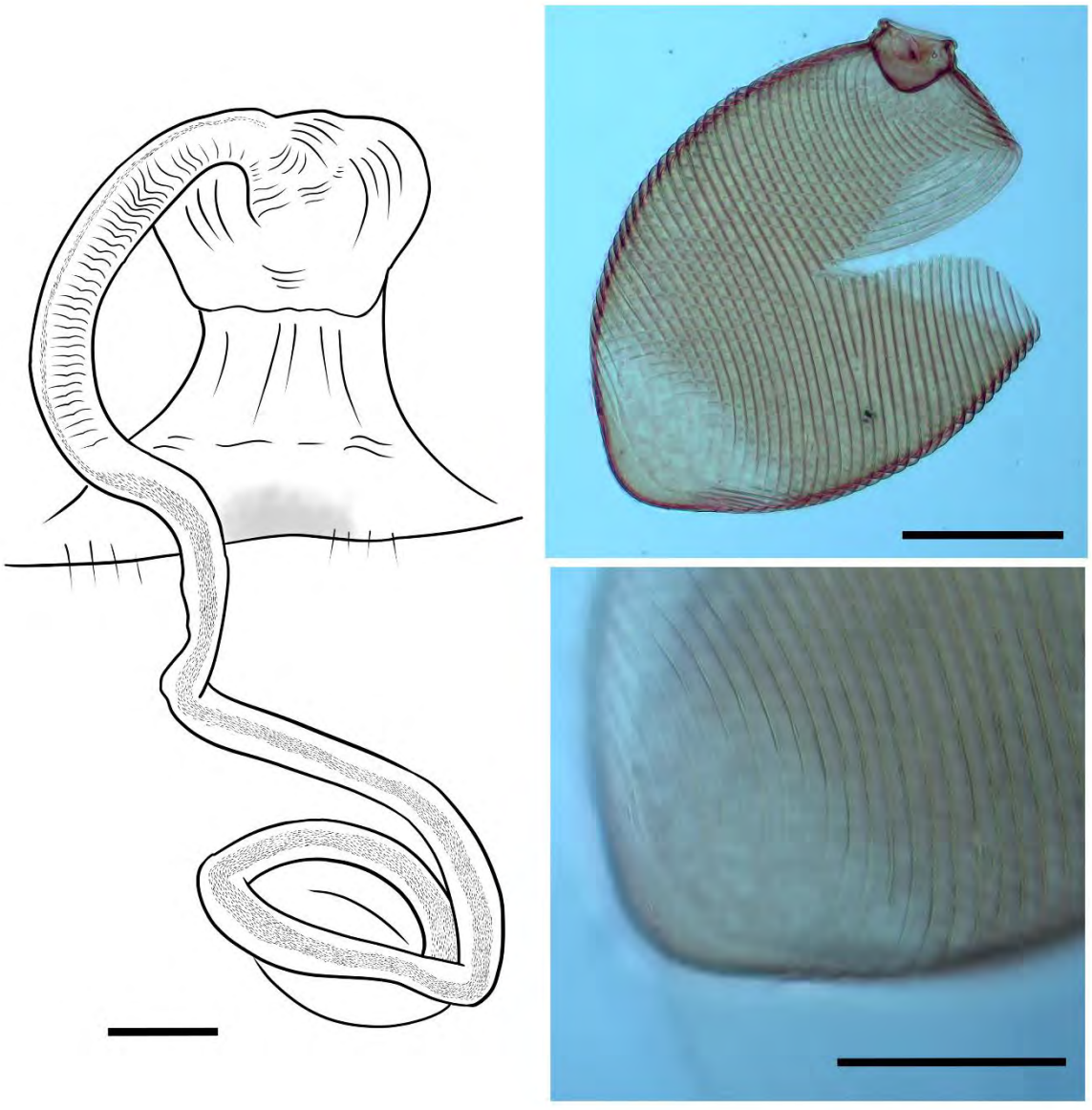

Figure 2.11: *Neoperla sp. nov. 3*. **A:** Diagram of vagina and spermathecal stalk, scale bar represents 1mm. **B:** Egg structure (X200), scale bar represents 0.1mm **C:** Operculum (X200), scale bar represents 0.1mm.

Notes: *Neoperla sp. nov. 3* is placed in the *N. excisa-sjostedti* species group. This is supported by the coiled spermatheca with a dense coat of scales, eggs with flat costae, and sulci with two-ordered rows of micropunctures (Zwick 2023; Zwick & Zwick 2023). Within this species group, *Neoperla sp. nov. 3* can be placed in the *Neoperla arambourgana* species assemblage based on its molecular relationships.

Despite emerging as sister to *N. arambourgana* and *N. dundoana*, *Neoperla sp. nov. 3* is most similar to *Neoperla leroiana* Klapálek. It can be separated from both *N. arambourgana* and *N. dundoana* by the levogyrous striae, instead of oblique and straight respectively, and the narrower collar (Zwick &

Zwick 2023). The striae are almost identical to those of *N. leroiana,* but the two can be distinguished by the lack of cells on the operculum in *Neoperla. sp. nov. 3*, and the narrow collar (Zwick & Zwick 2023). Furthermore, it can be separated from all three other species in the *N. arambourgana* species assemblage by the lack of a sclerotized nail or notch on S8.

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. excisa and N. sjostedti-operational complex, Females, pg. 132

24i. Egg with a collar, operculum punctate …… *Neoperla. sp. nov. 3*

24i'. Egg either without a collar, or if collar is present, striae extend to top of operculum …… 24

Neoperla sp. nov. 9

Images: Figure 2.12

Material Examined: ***Prospective Holotype:*** **PLEC 31H**: 1 ♀ adult || Democratic Republic of Congo | Lwiro River | Kakeza | 25.viii.2017 | Coll.: F. Ngera. ***Prospective Paratypes*****: PLEC 32R**: 1 ♀ adult || Democratic Republic of Congo | Obonje River | Nkuba | 13.iii.2017 | Coll.: F. Ngera || **PLEC 44A**: 1 ♀ adult || Democratic Republic of Congo | Kankere River | PNKB | 04.ix.2017 | Coll.: F. Ngera.

Adult Habitus: ♀ FW length: 17.9–19.1 mm. Amber brown. Head amber; with brown "spade" pattern over ocelli. Tentorial callosities distinct, small knobs. Antennae pale near base, darken rapidly. Ocelli separate, approximately one ocellus-width apart. Thorax amber, with margin and midline brown. Legs amber; tarsi slightly darker. Wings cloudy, pale, veins uniform brown. Abdomen pale, darkened distally. Cerci yellow, densely covered with fine setae.

Females: Caudal edge of S7 unmodified. S8 pale yellow, without markings. Posterior margin of S8 with a small sclerotized nail, usually sinuous or slightly excavate; without crests; a few long, strong setae laterally. Vagina slender, elongate; with concentric folds at top; spermathecal stalk inserted towards top of vagina. Spermathecal stalk coiled, 0.5 ring(s), slightly longer than vagina, similar width over entire length; without folds. Scales internal; cover entire diameter from base, fade away near the tip. Spermatheca very long, almost as long as spermathecal stalk.

Egg: Egg approximately 343 µm long, 172 µm wide; ovoid. Collar fused into egg contour, parallel sided; with two rows of cells with raised walls. Anchor cap with a truncate stalk, mushroom shaped. Approximately 35 striae, straight, with very broad sulci and narrow costae. Costae smooth and flat; sulci with two rows of micropunctures in the walls of the costae, not clearly visible, diverge around the microphyles; striae originate from smooth collar, terminate at base of operculum. Microphyles are

located in sulci, large, exposed holes. Operculum without cells, but with punctures forming polygonal shapes, rounded.

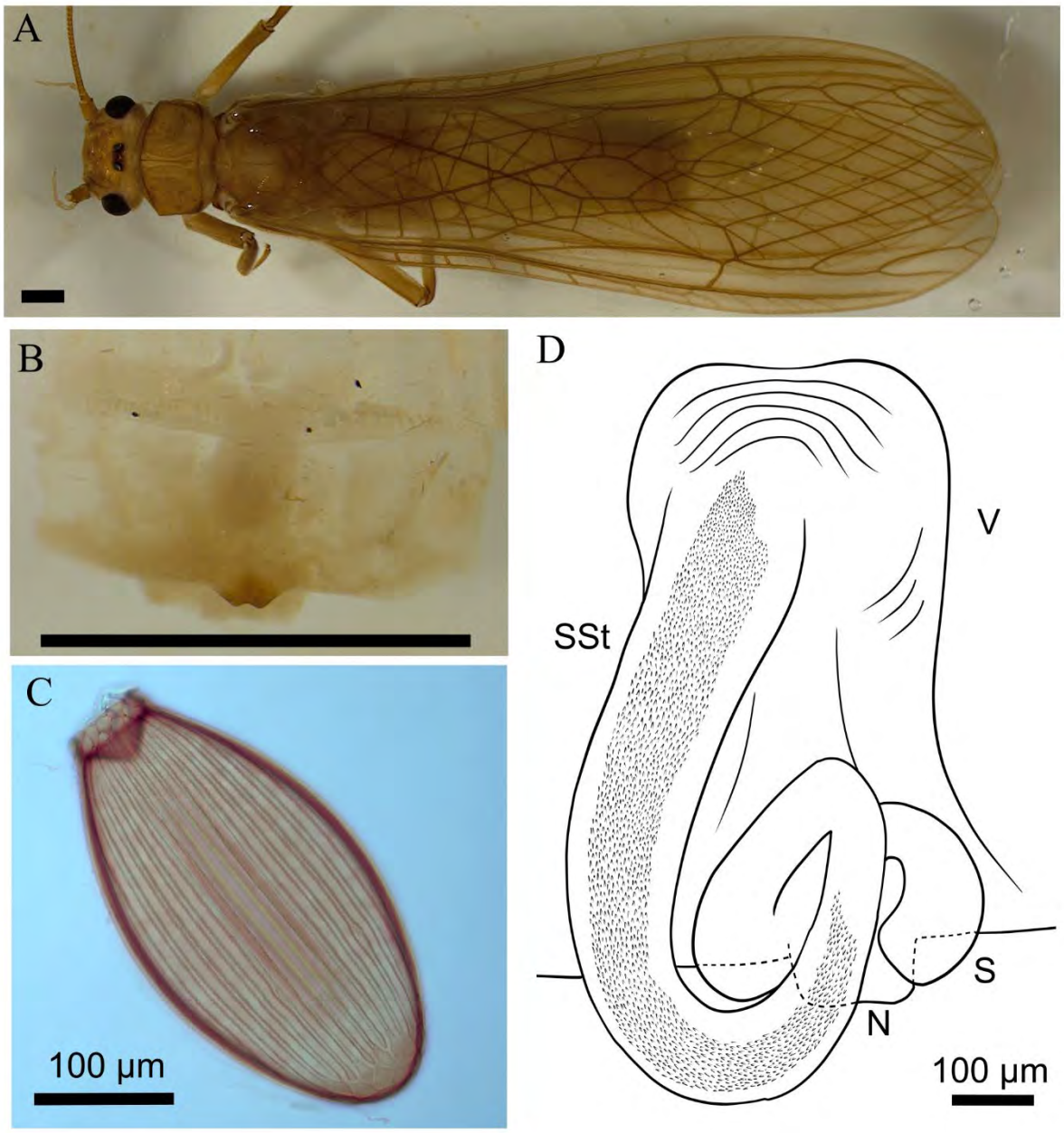

Figure 2.12: *Neoperla sp. nov. 9*. **A:** Adult female habitus. **B:** Sclerotized nail on S8 of the female subgenital plate. **C:** Egg structure (X200). **D:** Diagram of vagina and spermathecal stalk, V: Vagina, SSt: Spermathecal stalk, S: Spermatheca, N: Nail. Unless otherwise stated, all scale bars represent 1mm.

DNA: The COX1 sequencing of this species failed for all specimens. However, in the multi-gene phylogeny, *Neoperla sp. nov. 9* was placed as a moderately-to-strongly supported sister of *Neoperla*

sjostedti sjostedti Klapálek (Bootstrap support: 91%, Posterior probability: 1, BEAST: 0.72, Figure 2.7).

Notes: *Neoperla sp. nov. 9* falls in the *N. excisa-sjostedti* species group. This is supported by the coiled spermathecal stalk, with a dense coat of "fish-scale" like scales, the sclerotized nail on S8, and the eggs with flat, impunctate costae.

The spermathecal stalk and short, sclerotized nail are most similar to *Neoperla socia* Zwick & Zwick; however, the spermathecal stalk is not as wide at the base, nor does it taper distally in *Neoperla sp. nov. 9.* Additionally, it lacks the drawn-out crests on the egg costae which are present in *N. socia.* This spermathecal stalk pattern, along with the concentric folds on top of the vagina, are also present in *Neoperla tangana* Zwick & Zwick; however, the two species can be separated by the lack of a nail in *N. tangana,* and the lack of spines on the vagina next to the spermathecal stalk in *N. sp. nov. 9*. The egg of *N. sp. nov. 9* also lacks the coriaceous surface of the costae seen in *N. tangana.*

The eggs are the same as the *N. sjostedti* complex, with polygonal cells on the parabolic operculum, smooth sulci, and a collar fused into the egg contour, with two rows of cells (Zwick & Zwick 2023). However, *Neoperla sp. nov. 9* can be separated from this species complex by the short spermathecal stalk, with only half a coil, and the lack of a long basal section that appears void of scales.

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. excisa and N. sjostedti-operational complex, Females, pg. 132

20i. Spermathecal stalk short and wide, only slightly longer than vagina. At most, a half coil ……. ……..…… *Neoperla. sp. nov. 9*

20i'. Spermathecal stalk long and coiled, at least one complete coil …….…….. 24

Neoperla sp. nov. 10

Neoperla sjostedti cf. needhami – Zwick & Zwick 2023

Material Examined: ***Prospective Holotype:*** **PLEC 32R**: 1 ♀ adult || Democratic Republic of Congo | Obonje River | Nkuba | 13.iii.2020 | Coll.: F. Ngera.

Notes: Zwick & Zwick (2023) identified and described *Neoperla sp. nov. 10* as a morphologically distinct form of *N. sjostedti needhami* Lestage, but did not formally name it. They avoided naming this species as it would have made *N. sjostedti needhami* polyphyletic according to their COX1 data. However, as this species is clearly morphologically distinct, and the *N. sjostedti* species complex has been made polyphyletic by the addition of *Neoperla sp. 9* (Figure 2.7), it should be elevated to species rank. As this form was described by Zwick & Zwick (2023), it is not redescribed here.

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. excisa and N. sjostedti-operational complex, Females, pg. 132

20’. 79 *N. sjostedti cf. needhami* (by DNA) is replaced with *Neoperla sp. nov. 10*

Neoperla sjostedti Klapálek, 1909 **Stat. nov.**

Neoperla sjöstedti – Klapálek 1909
Neoperla sjostedti sjostedti – Zwick & Zwick 2023

Images: Figure 2.13

Material Examined: ***Additional Material:*** **PLEC 54A:** 1 nymph || South Africa | Upstream from Lisbon Falls | 24°52'00.3"S, 30°51'02.9"E | 07.iv.2020 | Coll.: A. Kirkaldy, M. Villet, G. Diedericks & R. Palmer || Stones in Current | River in heavy flow after rain || **PLEC 54B:** 1 nymph || South Africa | Upstream from Lisbon Falls | 24°52'00.3"S, 30°51'02.9"E | 07.iv.2020 | Coll.: A. Kirkaldy, M. Villet, G. Diedericks & R. Palmer || Stones in Current | River in heavy flow after rain || **PLEC 56A:** 1 nymph || South Africa | Upper Blyde River | Hartebeestvlaagte | 25°03'13.2"S, 30°40'07.3"E | 08.iv.2020 | Coll.: A. Kirkaldy, M. Villet, & G. Diedericks || Stones in Current | River in heavy flow after rain.

Nymph: Dark brown. Head dark brown; with medial dark brown markings between occipital ridge and ecdysial line, extending to halfway between ocelli and eye; vertex with a transverse pale band, from ecdysial line to front of eyes; frons brown, fading posteriorly, not sharply delineated. Ocelli separate, approximately one ocellus-width apart. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae pale yellow; with fine dark setae, more along internal margin basally, distally very few.

Prothorax wide, dark brown, with a clearly delineated, pale, three-lobed "clover" marking; a dark line around margin; ecdysial line pale. Mesothorax pale brown; anteromedially dark brown, but with a very thick pale band that covers almost the entire segment; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs brown; tibia slightly lighter; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen pale yellow, banded; dark brown bands on posterior margin of each segment. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: The *N. sjostedti* species complex is made polyphyletic by the splitting of the *N. sjostedti sjostedti* and *N. sjostedti needhami* sub-species around *Neoperla sp. nov. 9* (Figure 2.7). While the females of these two species cannot be identified using morphological methods, the males can be reliably differentiated, and they are clearly genetically distinct. For this reason, the subspecies should be raised to species rank.

The nymphs are distinctive and can easily be recognized by the three-lobed marking on the prothorax and the banded abdomen.

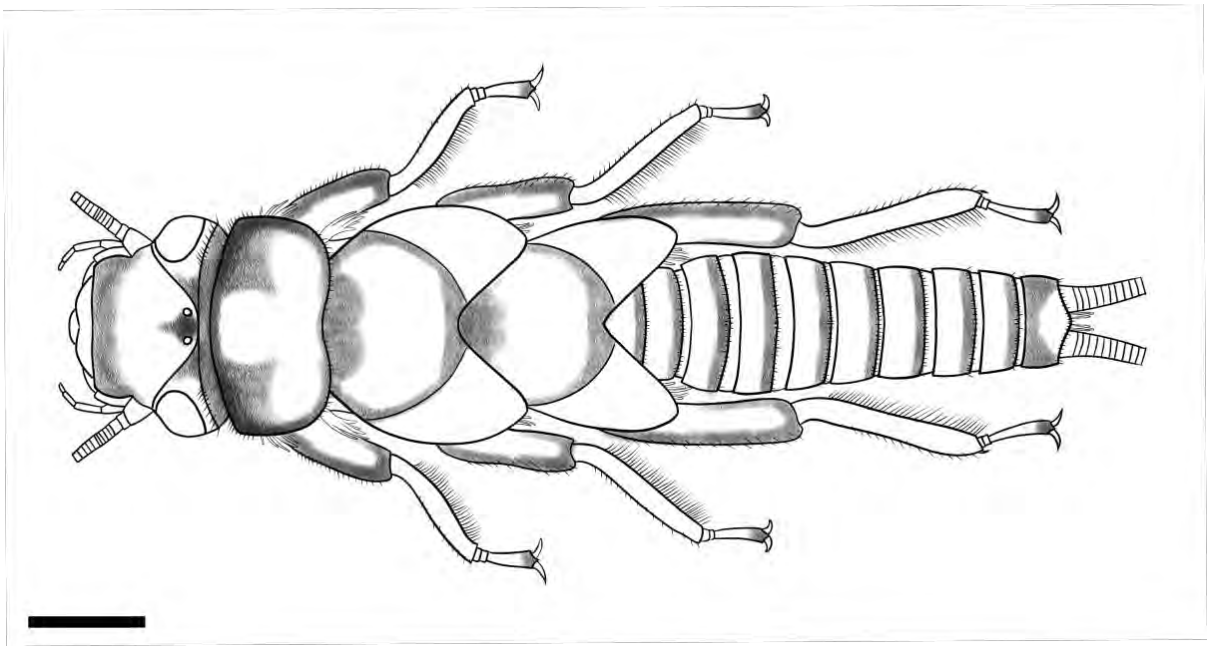

Figure 2.13: Diagram of *Neoperla sjostedti* Klapálek, nymph habitus. Scale bar represents 0.1mm.

Neoperla needhami Lestage, 1921 **Stat. nov.**

Neoperla excisa – Needham, 1920 (nec Klapálek, 1909)
Neoperla needhami – Lestage, 1921
Neoperla bottegoana – Navás, 1933
Neoperla sp. 33 – Zwick, 1976a
Neoperla species C – Picker, 1980 (male)
Neoperla sjostedti needhami – Zwick & Zwick, 2023

Notes: The *N. sjostedti* species complex is made polyphyletic by the splitting of the *N. sjostedti sjostedti* and *N. sjostedti needhami* sub-species around *Neoperla sp. nov. 9* (Figure 2.7). While the females of these two species cannot be identified using morphological methods, the males can be reliably differentiated, and they are clearly genetically distinct. For this reason, the subspecies should be raised to species rank.

Species group *Neoperla duodeviginti* Zwick & Zwick, 2023

Neoperla sp. nov. 4

Neoperla sp. Afr_B – Zwick & Zwick, 2023

Images: Figure 2.14 - 2.16

Material Examined: *Prospective Holotype*: RWA 34A: 1♀ adult || Rwanda | Kamiranzovu Waterfall | Nyungwe Forest | 2°26'48.7"S, 28°06'53.0"E | 14.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Malaise Trap | downstream of the waterfall. ***Prospective Paratypes*: PLEC 005F**: 1♂ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°26'48.7"S, 28°06'53.0"E | 28.viii.2023 | Coll.: A. Kirkaldy, E. Twagirayezu || Light Trap | downstream of the waterfall. ***Other Material*: PLEC 67B:** 1 nymph || Rwanda | uWinka Stream | Nyungwe Forest | 2°28'31.5"S, 29°11'45.2"E | 10.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones and marginal vegetation in current || **PLEC 68B**: 1 nymph || Rwanda | Kamiranzovu Waterfall | Nyungwe Forest | 2°26'48.7"S, 29°06'53.0"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 68D**: 1 nymph || Rwanda | Kamiranzovu Waterfall | Nyungwe Forest | 2°26'48.7"S, 29°06'53.0"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 77A**: 1 nymph || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°28'08.7"S, 29°04'28.7"E | 14.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current.

Nymph: Light brown, amber. Head amber brown, with a small, slightly darker patch over ocelli and white "W" marking on frons. Ocelli separate, approximately one ocellus-width apart. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae light brown; with very pale, fine setae distally.

Prothorax wide, dark brown, without obvious markings, but darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax pale brown, with some faint pale markings on either side of ecdysial line; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale brown; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs; a long fringe of swimming hairs along inner margin of the cerci.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

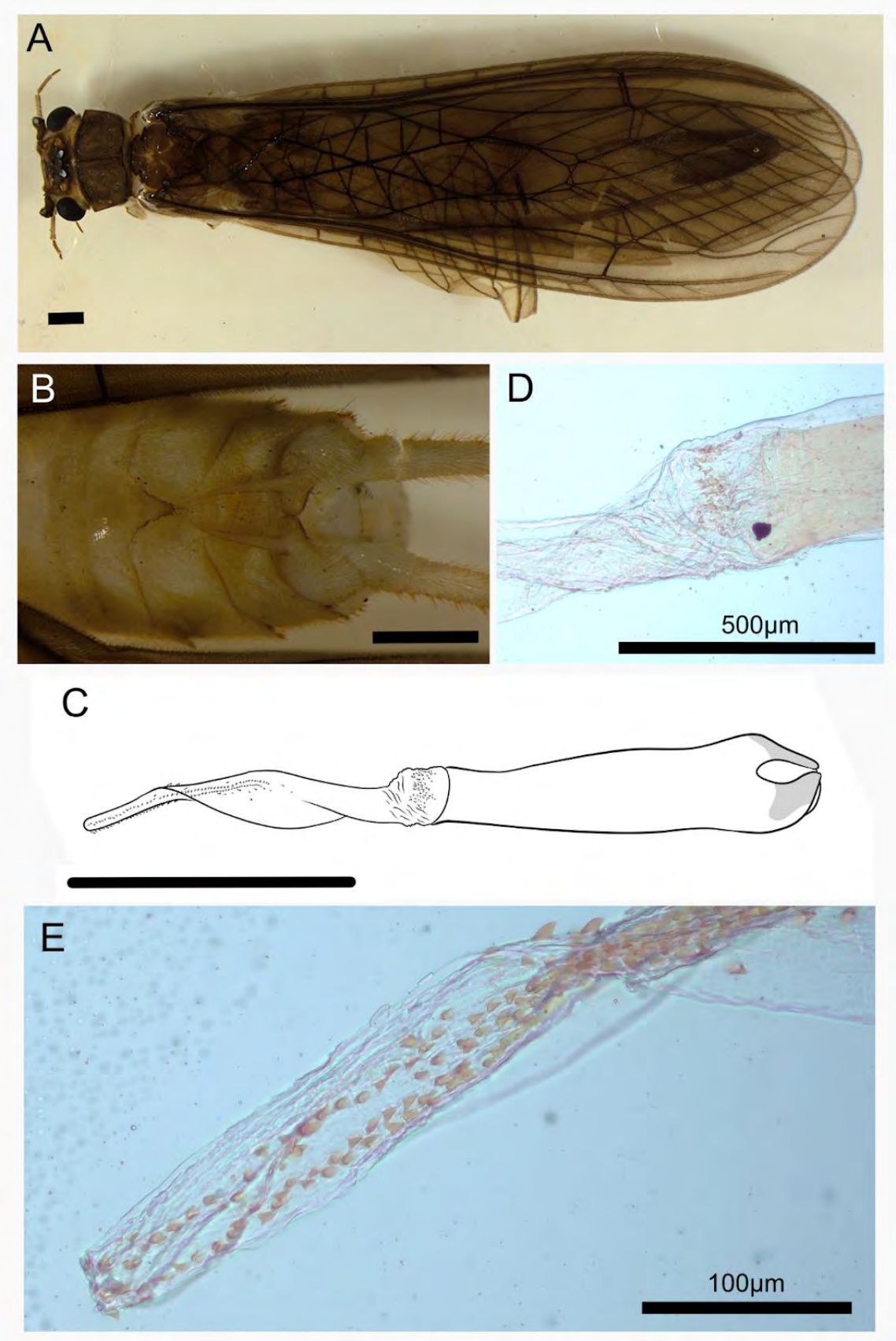

Figure 2.14: *Neoperla sp. nov. 4*. **A:** Adult male habitus. **B:** Tergite 7 – Hemitergites in the adult male. **C:** Diagram of the everted aedeagus. **D:** Spine patterns on tip of aedeagus. **E**: Distal spine pattern on the endophallus. Unless otherwise stated, all scale bars represent 1mm.

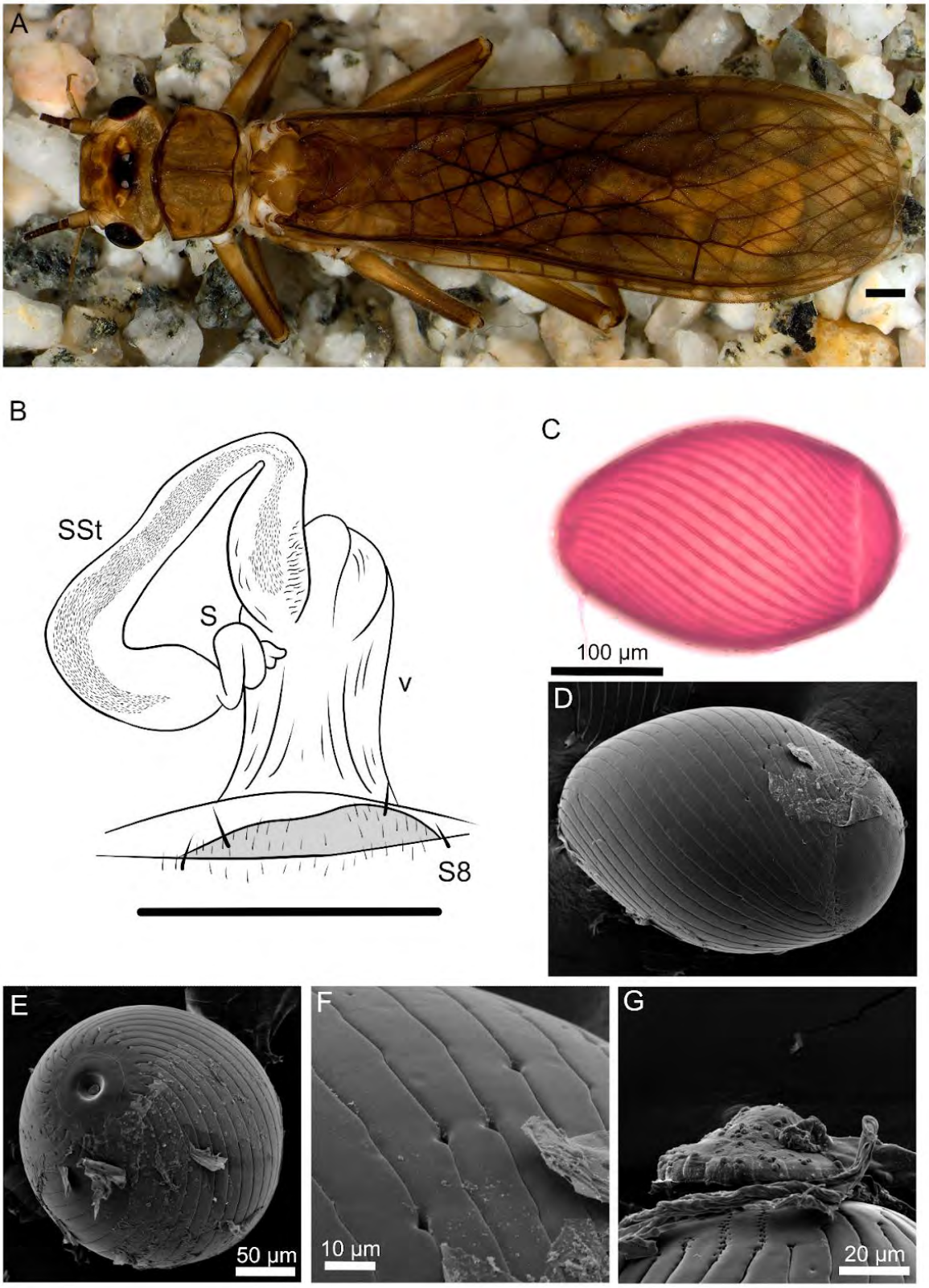

Figure 2.15: *Neoperla sp. nov. 4*. **A:** Adult female habitus. **B:** Diagram of vagina and spermathecal stalk, V: Vagina, Sst: Spermathecal stalk, S: Spermatheca. **C:** Egg (X200). **D:** SEM photograph of the egg. **E**: SEM photograph of the anchor pole. **F:** SEM photograph of the microphyles. **G:** SEM photograph of the anchor cap. Unless otherwise stated, all scale bars represent 1mm.

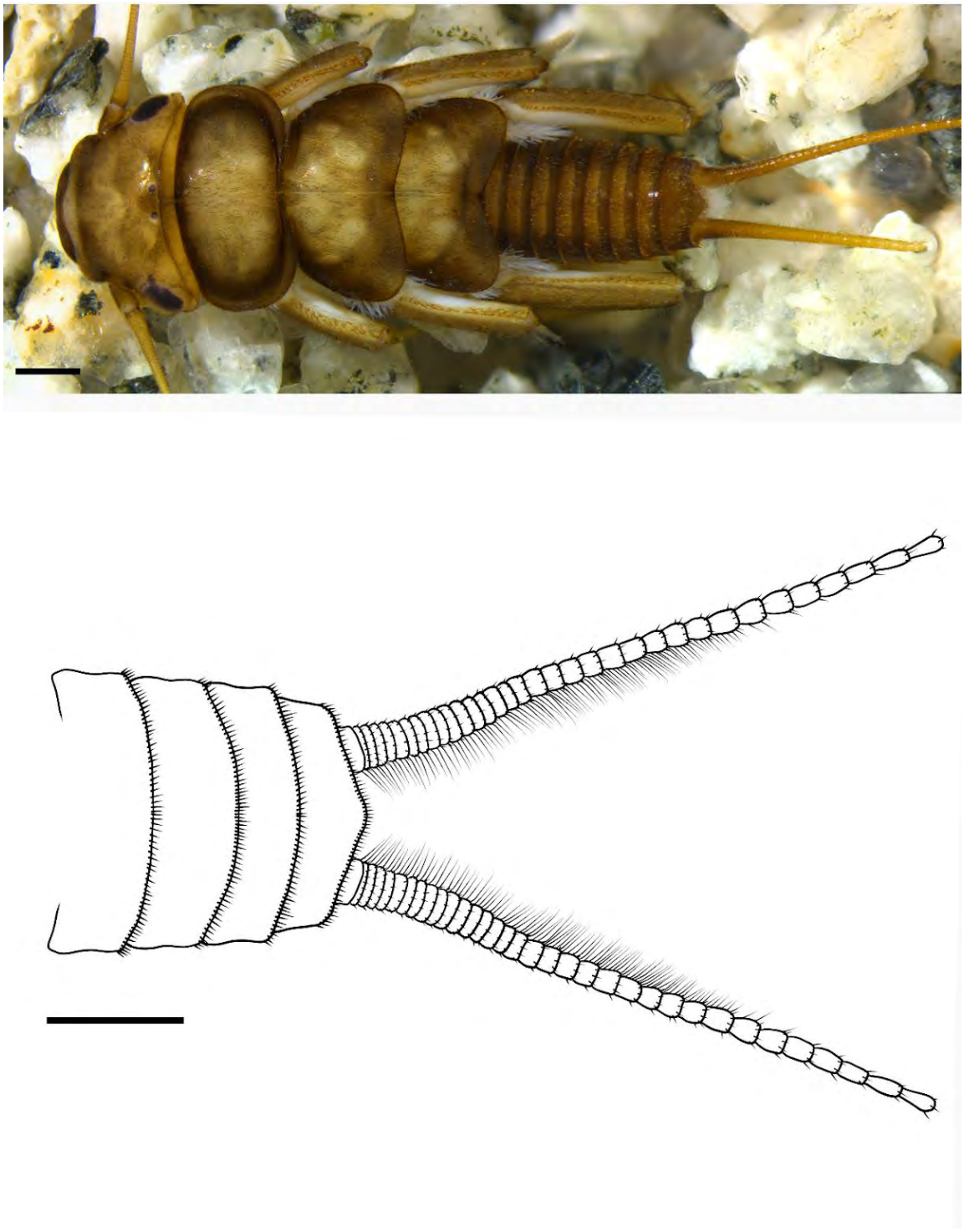

Figure 2.16: *Neoperla sp. nov. 4*. **A:** Nymph habitus. **B:** Diagram of swimming hairs on the cerci. All scale bars represent 0.1mm.

Adult Habitus: ♂ FW length: 17.6 mm. ♀ FW length: 15.6 mm. Dark amber brown. Head dark; with strong, three-lobed marking over ocelli, anteriorly narrows to a band in male; female paler, with "spade" pattern over ocelli and pale M marking medially. Tentorial callosities distinct, small knobs. Antennae dark, amber brown. Ocelli separate, approximately one ocellus-width apart. Thorax dark

brown, with black line along margin and midline. Legs pale; femur yellow, darker distally; tibia brown; tarsi pale; tip of tarsomere III and claws black. Wings forewings dark brown, hind wings pale, veins uniform brown. Abdomen pale, darkened distally. Cerci yellow, densely covered with fine setae.

Males: T7 divided at midpoint by raised ridge; a rectangular concave region leads to posterior margin; with triangular sclerite medially; with an unpaired caudal process. The process is a long triangular cone, almost parallel to T8, with sensilla basiconica on tip, along margins and covering underside. T8 very weakly sclerotized in the midline; with a small forward-skewed hump, that forms a narrow ridge with sensilla basiconica, which align with the parallel, pyramidal T7 process, laterally with low, raised humps, without sensilla basiconica. T9 with antecosta divided in front of median membranous area; lateral humps widely spaced, densely pilose and with almost no sensilla basiconica; median furrow weakly sclerotized, with a patch of sensilla basiconica medially. H10 mediabasal callus tongue shaped. Process slender, slightly curved inwards, with tip slightly hooked. Epiproct flat and wide, Y-shaped.

Aedeagus: Aedeagus long, slender, distally sinuous, moderately sclerotized, except for a soft bulge at tip, with a patch of backwards facing spines laterally, on either side of membranous bulge.

Everted endophallus approximately 1.5 times longer than aedeagus; curves ventrad from bulb like base; initially same width as aedeagus, but narrows to thin tube distally. Spines sharp-tipped, backwards curved hooks; base of endophallus with long bare section; rope-like section with some scattered and unordered spines dorsally; ventrally with hooks arrayed in two irregular rows (in the holotype, the tip is not fully everted and the internal spines give the illusion of four rows); distally distal spines smaller.

Females: Caudal edge of S7 with a fringe of strong setae. S8 pale yellow, without markings; some scattered, long setae. Posterior margin of S8 with a slightly projecting, somewhat bilobed, sclerotized brown lip; a few strong setae along margin. Vagina slender, elongate; spermathecal stalk inserted near base of wide section of vagina. Spermathecal stalk initially directed upwards, but rapidly turns back on itself, extending towards posterior margin of S7, ends in a half coil at base of vagina, slightly longer than vagina, wider at base, constricts at first bend into a equal tube, that widens again distally; cuticle thick on convex side, without spines, slightly folded, wide at tip. Scale band wide at base, narrows in turn of spermathecal stalk, then widens to a consistent width for rest of stalk; covers most of stalk diameter, except for thick cuticle along concave edge. Spermatheca densely coiled.

Egg: Egg approximately 348 µm long, 240 µm wide; drop-shaped. Collar absent. Anchor cap with a truncate stalk, mushroom shaped. Approximately 38 striae, dextrogyrous, with broad costae and narrow sulci. Costae smooth and flat; sulci narrow and deep, with two rows of micropunctures; striae end close to anchor pole, creating a small, low ring around anchor cavity, stretch to a pale ring at base

of operculum. Microphyles are located in sulci, somewhat indistinct, sulci margin barely enlarged around them. Operculum punctate, low or convex.

DNA: *Neoperla sp. nov. 4* formed a strongly supported, monophyletic clade in both the COX1 (Bootstrap support: 100%, Figure 2.5) and three-gene phylogeny (Bootstrap support: 100%, Posterior probability: 1, BEAST: 1, Figure 2.7). It was recovered as sister to *Neoperla duodevinginti* Zwick & Zwick in the COX1 analysis, but this relationship was poorly supported (Bootstrap support: 60%). *Neoperla sp. nov. 4* was separated from all known species by the species delineation tests, although ASAP and ABGD further split the species into multiple OTUs. As the branches of this clade were short, and these further splits were inconsistent, it has been treated as a single species.

Notes: *Neoperla sp. nov. 4* is placed in the *N. duodeviginti* species group, as *N. duodeviginti* was its closest relative in the COX1 analysis (Figure 2.4). Morphological species group affinities are contradictory. The eggs are similar to those of the *N. excisa-sjostedti* species group, with flat, impunctate, spiral costae, and very narrow sulci with two ordered rows of micropunctures that diverge around the microphyles (Zwick 2023; Zwick & Zwick 2023). However, while the spermathecal stalk shares the dense coat of internal, "fish-scale" like spines, it is divided into two sections, that are initially directed caudad, before turning back on itself. This is instead shared with the *N. transvaalensis* species group (Zwick 2023; Zwick & Zwick 2023). A similar spermathecal stalk is also present in *N. duodeviginti*, which nevertheless differs in the structure of the egg and the lack of the folded cuticle along the concave side of the spermathecal stalk. The male genitalia resemble the *N. spio* species group, with a thin, "rope-like" endophallus, and the process on T7, rising parallel to the slightly humped T8 (Zwick & Zwick 2023). The latter of these characters also prevents assignment to the *N. excisa-sjostedi* species group, which lacks a process on T7 (Zwick 2023; Zwick & Zwick 2023). These external genital features are also shared with the *Neoperla occipitalis* species group, but differ in the absence of very large spines on the endophallus, and the coiled spermatheca (Zwick 2023).

Females of *Neoperla sp. nov. 4* can be differentiated from all other females in the *N. duodevinginti* species group by the structure of the egg, which are otherwise unstriated or have straight, punctate costae in this group. Similarly, they can be differentiated from the species within the *N. excisa-sjostedti* group by the spermathecal stalk, which is not coiled or spiral, and the very distinctive egg structure.

The males differ from all other species in the *N. duodeviginti* species group by having a long, "rope-like" endophallus. *Neoperla sp. nov. 4* can be differentiated from *Neoperla sp. nov. 6* by the lack of small spines along the distal 1/3 of the aedeagus, and the curved hemitergal spikes. The males can also be separated from all the males in the *N. spio* species assemblage by the lack of external spine patches on the aedeagus, and the simple pointed hemitergites.

Neoperla sp. nov. 4 is the same species as the informally designated *Neoperla sp. Afr_B* (Zwick & Zwick 2023). Zwick & Zwick (2023) noted similarities in the eggs to *Neoperla conradti* Enderlein. However, the two species can be differentiated by the drop-shaped egg, with a narrow ring around the anchor pole, and simple spermathecal stalk in the females. In males, the absence of a hook-like process on T8 in *Neoperla sp. nov. 6* separates these two species.

Finally, *Neoperla. sp. nov. 4* can be identified reliably as a nymph, as it has a long fringe of swimming hairs on the inner margin of the cerci. While this is known for some *Neoperla* (Sivec *et al.* 1988), it has not been observed in any of the other African species (Barnard 1934; Hynes 1952; Klapálek 1912; Lestage 1917; Ogbogu 2006).

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. spio-complex, Males, pg. 88

19ii. Endophallus with a thick band of spines dorsally, laterally extends into two narrow lateral bands ……...……..……. *Neoperla. sp. nov. 4*

19ii’. Endophallus base without spine band, either unordered or smooth ……...……...……. 19

Keys to species groups and species of African Neoperla, Females, Pg. 18

4i. Egg with dextrogyrous striae, costae smooth. Spermathecal stalk a tube with internal, fish-scale like spines ….……..……...… *Neoperla. sp. nov. 4*

4i’. Egg with straight striae, costae punctate. Spermathecal stalk bag shaped, or narrow at base ……... ……..……..... 4

Neoperla sp. nov. 5

Images: Figure 2.17

Material Examined: ***Prospective Holotype*****: PLEC 005A**: 1 ♀ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°26'48.7"S, 28°06'53.0"E | 28.viii.2023 | Coll.: A. Kirkaldy, E. Twagirayezu || Light Trap | Downstream from Waterfall. ***Prospective Paratypes*****: PLEC 005B**: 1♀ adult | Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°26'48.7"S, 28°06'53.0"E | 28.viii.2023 | Coll.: A. Kirkaldy, E. Twagirayezu || Light Trap | Downstream of the Waterfall || **PLEC 005D**: 1 ♀ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°26'48.7"S, 28°06'53.0"E | 28.viii.2023 | Coll.: A. Kirkaldy, E. Twagirayezu || Light Trap, Downstream of the Waterfall || **PLEC 29A**: 1 ♀ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°32'05.6"S, 29°25'00.2"E | 15.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Light Trap | Set next to waterfall. ***Other Material*****: PLEC 005C**: 1 ♀ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°26'48.7"S,

28°06'53.0"E | 28.viii.2023 | Coll.: A. Kirkaldy, E. Twagirayezu || Light trap | Downstream from Waterfall || **RWA37A**: 1 ♀ adult || Rwanda | Kamiranzovu Swamp | Nyungwe Forest | 2°32'05.6"S, 29°25'00.2"E | 15.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Malaise Trap | Downstream of Waterfall || **RWA14A**: 1♀ adult || Kamiranzovu Swamp | Nyungwe Forest | 2°29'20.2"S, 29°09'14.7"E | 31.viii.2021 | Coll.: H. Barber-James, F. Ngera, L. Ngirinshuti & O. Mugisha || Nymph collected, reared overnight in emergence trap.

Adult Habitus: ♀ FW length: 15.7–19.6 mm. Pale yellow. Head pale yellow; with small brown marking over the ocelli. Tentorial callosities pale. Antennae pale brown, lighter distally. Ocelli separate, approximately one ocellus-width apart. Thorax pale, slightly darker than head, without prominent markings. Legs pale; mid- and fore-tibia slightly darker brown; tarsi with small brown patch on final tarsomere, and between claws. Wings cloudy, pale, veins brown, subcosta darker. Abdomen pale white-yellow. Cerci yellow, densely covered with fine setae.

Females: Caudal edge of S7 with a medial patch of long, strong setae, continuing onto S8. S8 pale yellow, without markings; with median patch of long setae. Posterior margin of S8 with a distinct, rounded notch; in some specimens reduced, a barely visible, small indent, which is slightly sclerotized. Vagina long, with parallel sides; with some transverse folds near base; spermathecal stalk inserted near base of wide section of vagina. Spermathecal stalk coiled, 1–1.5 ring(s), approximately 1.5 times as long as vagina, wide at base, narrowing to a tube at approximately 1/3 the length; base with elongate folds on concave side. Scales internal; armature consists of two-tipped and sharply pointed scales; forms a narrow band along convex side of base, widening distally to cover entire diameter of tube-like spermathecal stalk. Spermatheca densely coiled.

Egg: Egg approximately 384 µm long, 226 µm wide; drum-shaped. Collar fused into egg contour, a low ring; without cells. Anchor cap with a truncate stalk, mushroom shaped. Approximately 34 striae, straight, with broad costae and narrow sulci. Costae smooth and flat; sulci with two lines of micropunctures, which diverge around microphyles; costae narrow towards poles, near collar form a connected network with small ridges across sulci, terminate with small indents into collar, form deep cells at base of operculum. Microphyles are located in sulci, large, exposed holes. Operculum with deep cells, rounded or low.

DNA: *Neoperla sp. 5* formed a strongly supported, monophyletic clade in both the COX1 analysis (Bootstrap support: 89%, Figure 2.5) and phylogeny (Bootstrap support: 99%, Posterior probability: 0.99, BEAST: 0.99, Figure 2.7). In both analyses, it was a close sister to *Neoperla sp. nov*. 6, but was consistently separated by all of the species delineation tests, which returned identical partitions.

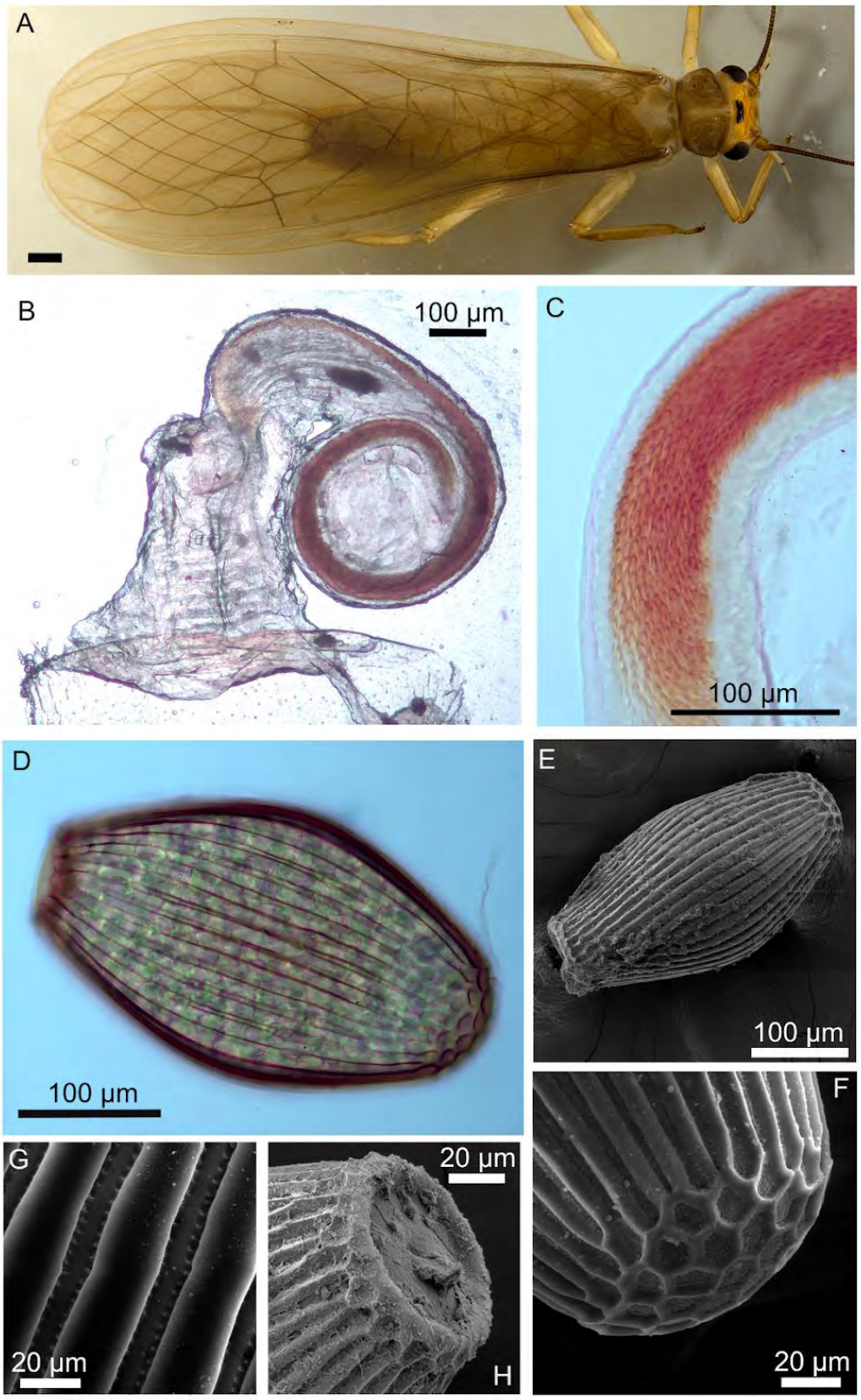

Figure 2.17: *Neoperla sp. nov. 5*. **A:** Adult female habitus. **B:** Vagina and spermathecal stalk. **C:** Internal "Fish scales" in the spermathecal stalk (X200). **D:** Egg (X200). **E**: SEM photograph of the egg. **F:** SEM photograph of the operculum. **G:** SEM photograph of the two rows of micropunctures. **H:** SEM photograph of the anchor pole. Unless otherwise stated, all scale bars represent 1mm.

Notes: Morphologically, *Neoperla sp. nov. 5* should be placed in the *N. excisa-sjostedti* species group. This is supported by the flat, impunctate costae, with narrow sulci that have two rows of micropunctures, and the coiled spermathecal stalk with a dense coating of "fish-scales" internally (Zwick 2023; Zwick & Zwick 2023). However, this was not supported by the molecular evidence: as *Neoperla sp. nov. 5* formed a polytomy between the *N. occipitalis* and paraphyletic *N. duodevinginti* species groups.

The eggs of *Neoperla sp. nov. 5* are strikingly similar to those of the *N. occipitalis* species group, with >25 flat costae, an operculum densely covered in raised polygonal cells, and the rim like collar without any cells (Stark & Baumann 1978; Zwick 2023). However, it differs from the *N. occipitalis* group as the micropunctures in the sulci are arranged in ordered rows instead of in a dense and unordered pattern, the spermathecal stalk is coiled, rather than conical and large, and the spermatheca is not cup shaped (Zwick 2023). The *N. duodevinginti* species group is heterogeneous, and at least *Neoperla sp. nov. 4* suggests that the females can have similar eggs to the *N. excisa-sjostedti* species group. However, in the three-gene phylogeny, the two species were distantly related, although on sister clades.

Neoperla sp. nov. 5 is therefore putatively assigned to the *N. duodevinginti* species group because of molecular affinities. It is possible that the males of this species will allow for this to be altered, or further resolved. *Neoperla sp. nov. 6* may represent the males of this species.

Neoperla sp. nov. 5 can be differentiated from all of the other species in the *N. duodevinginti* species complex by a combination of the coiled spermathecal stalk, and smooth, straight costae (Zwick 2023; Zwick & Zwick 2023).

Within the *N. excisa-sjostedti* group, the distinct notch on S8 is similar to females of *Neoperla excavata* Zwick & Zwick, *Neoperla arambourgana* Zwick & Zwick, and *Neoperla gibbosa* Zwick & Zwick. However, the eggs allow for *Neoperla sp. 5* to be separated from all three of these species. The costae of *N. sp. nov. 5* are straight, instead of levogyrous in *N. gibossa* and *N. arambourgana*, and smooth, instead of humped in *N. gibossa* (Zwick & Zwick 2023). The collar is rim-like, and does not have cells, unlike *N. gibossa* and *N. arambourgana* (Zwick & Zwick 2023). While the costae, egg shape and collar are similar to *N. excavata*, *N. sp. nov. 5* has deep polygonal cells on the low, flat operculum, compared to unordered punctures in *N. excavata*. The two species can be further differentiated by the spermathecal stalk, as *N. excavata* lacks the folded region of the cuticle.

In specimens where the notch on S8 is small or indistinct, the females appear most similar to *N. sp. nov. 10*. However, *Neoperla sp. nov. 5* differs from all species in the *Neoperla sjostedti* Klapálek complex, as it lacks a sclerotized nail, does not have a strikingly void section of the spermathecal stalk, and does have cells on the collar (Zwick & Zwick 2023).

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. excisa and N. sjostedti-operational complex, Females, pg. 132

6. Egg drum-shaped, striae straight ……..……...……. 6i

6'. Egg striae levogyrous ……..……..… 7

6i. Base of spermathecal stalk wide, with large folds, scales only on convex side; S8 not strongly sclerotized ……..…….…… *Neoperla. sp. nov. 5*

6i'. Base of spermathecal stalk entirely paved with scales; S8 with a strong sclerite with convergent sides and oblique crests ……. 73 *N. excavata* Zwick & Zwick

Neoperla sp. nov. 6

Images: Figure 2.18

Material Examined: ***Prospective Holotype:*** **PLEC 29B**: 1♂ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°32'05.6"S, 29°25'00.2"E | 15.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Light Trap | Next to Waterfall.

Adult Habitus: ♂ FW length: 14.7 mm. Pale yellow. Head pale yellow; very faintly darkened in an oval around ocelli. Tentorial callosities pale. Ocelli separate, approximately one ocellus-width apart. Thorax pale, without prominent markings. Legs pale; mid- and fore-tibia slightly darker brown; tarsi pale brown. Wings cloudy, pale, veins yellow, with subcosta brown. Abdomen pale white-yellow. Cerci yellow, densely covered with fine setae.

Males: T7 with a triangular sclerite; with an unpaired, three-dimensional caudal process. The process is conical, curved downwards, parallel to raised platform on T8, with sensilla basiconica on lower face, opposite patch of sensilla basiconica on T8. T8 unsclerotized; with a strong, forward-curved hump that bears many sensilla basiconica medially; forms a mouth-like structure with the parallel T7 process. T9 soft; lateral humps widely spaced, pilose and with many sensilla basiconica. H10 mediabasal callus parabolic. Process straight, with tip sharply pointed. Epiproct not present.

Aedeagus: Aedeagus long, slender, straight, soft, distally covered with regular, long, straight spines directed distad.

Everted endophallus approximately 1.5 times longer than aedeagus; base annulated, remainder straight. Spines sharp-tipped; arrayed in ordered rows; base of endophallus with four irregularly spaced rows of spines; bands continue along length of endophallus, become more regular; distally spines smaller and denser distally, forming 8 rows; tip is bare.

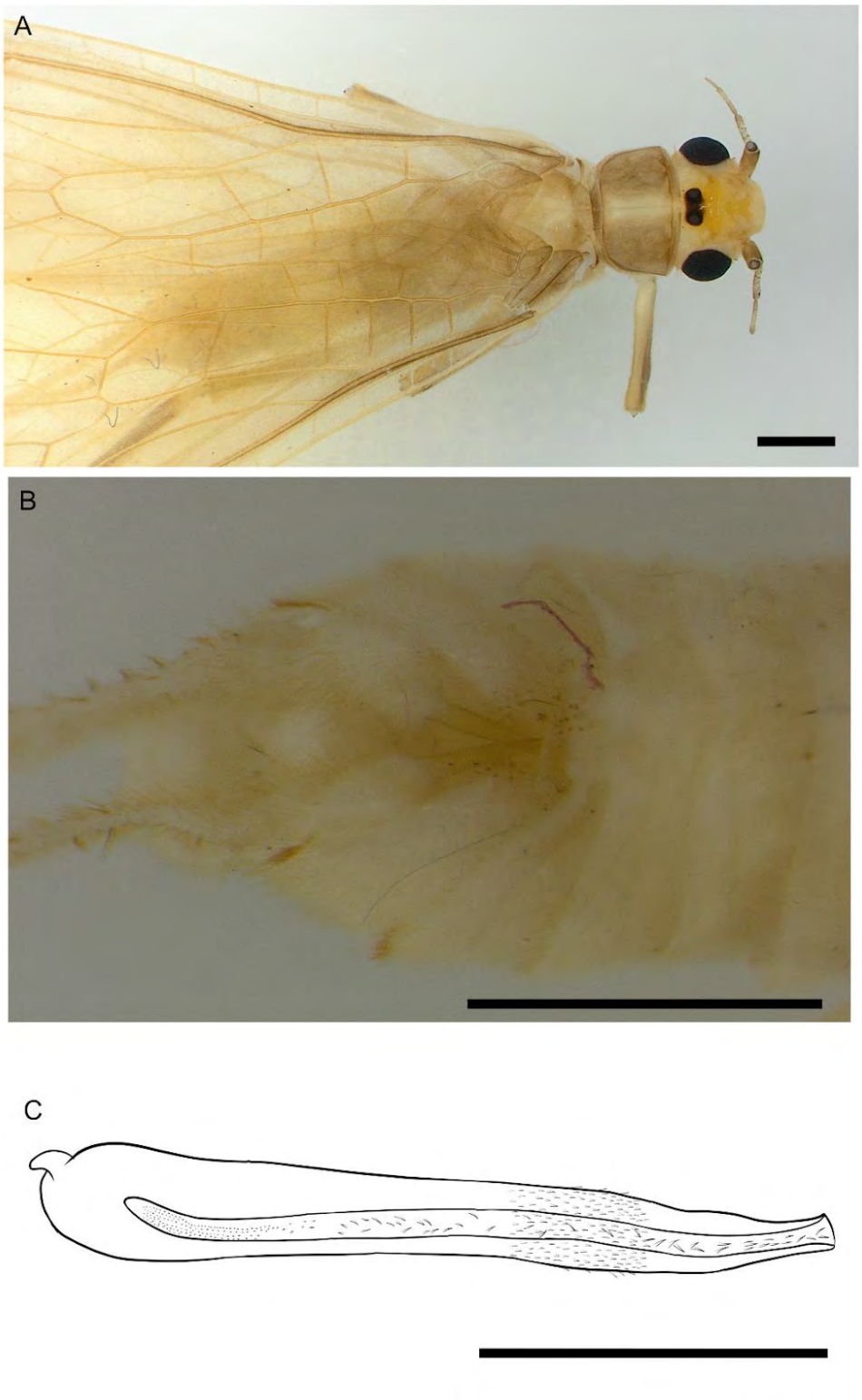

Figure 2.18: *Neoperla sp. nov. 6*. **A:** Adult male habitus. **B:** Tergite 7 – Hemitergites in the adult male. **C:** Diagram of the everted aedeagus. Unless otherwise stated, all scale bars represent 1mm.

DNA: *Neoperla sp. nov. 6* was recovered as a close sister to *Neoperla sp. nov. 5* in both the COX1 analysis (Bootstrap support: 100%, Figure 2.5) and the phylogeny (Bootstrap support: 76%, Posterior

probability: 0.99, BEAST: 0.99; Figure 2.7). As the males of *Neoperla sp. nov. 5* have not been described, it is possible this is the male of that species. However, the branches between the two species are long, and all four species delineation tests separated the two into different OTUs.

Notes: As with *Neoperla sp. nov.* 5, *Neoperla sp. nov. 6* is putatively placed in the *N. duodevinginti* species group because of its molecular affinities, as morphology is unclear. The processes on T7 and T8, and the long "rope-like" endophallus are both similar to genital characters of the *Neoperla spio* species assemblage. However, *Neoperla sp. nov. 6* is not sclerotized externally, and the hemitergites are straight spikes. The *N. occipitalis* species group has similar processes on T7 and T8, i.e. a conical pyramidal process on T7 and a raised hump on T8, but differ in having large spines on the endophallus (Zwick 2023).

Neoperla sp. nov. 6 can be differentiated from all species within the *N. spio* assemblage by the coating of fine spines that coat the distal two-thirds of the aedeagus (Zwick & Zwick 2023). *N. funiculata* does have some small spines distally on the aedeagus, but this coating extends further back in *Neoperla sp. nov. 6.* The two species can also be separated by the spines on the endophallus, which are scattered and sparse basally in *Neoperla sp. nov. 6* instead of forming long, regular rows (Zwick & Zwick 2023).

The external genital features of *N. duodeviginti*, which falls in the same clade, are also similar to those of *Neoperla sp. nov 6.* The two can be separated by the straight hemitergites, the long "rope-like" endophallus with sparse spines, and the coating of fine spines on the aedeagus of *Neoperla sp. nov. 6*, compared to angularly bent hemitergites, a short, densely spined endophallus, and a smooth aedeagus in *N. duodeviginti* (Zwick & Zwick 2023).

Neoperla sp. nov. 6 forms a relatively close sister to *Neoperla sp. nov. 5*, and may be the male of the species. However, as it was consistently separated by all of the species delineation tests, they are treated as two separate species here.

Amendments to Key, Zwick & Zwick 2023:

Keys to species groups and species of African Neoperla, Males, pg. 17

4. Process of T7 downcurved, parallel to the caudally rising surface of T8 …… 4i

4'. Process of T7 not parallel to the surface of T8, more or less raised ……...……. 5

4i. H10 straight; distal third of aedeagus coated with basad-pointing spines, endophallus thin and tube-like ….…….. *Neoperla sp. nov. 6*

4i'. H10 sinuous, endophallus wide …….. *Neoperla planidorsum* Zwick & Zwick

Species group *Neoperla africana* Zwick & Zwick 2023

Neoperla sp. nov. 7

Images: Figure 2.19

Material Examined: ***Prospective Holotype:*** **PLEC 005E**: 1 ♂ adult || Rwanda | Ndambarare Waterfall | Nyungwe Forest | 2°26'48.7"S, 28°06'53.0"E | 28.viii.2023 | Coll.: A. Kirkaldy, E. Twagirayezu || Light Trap | Downstream from Waterfall.

Adult Habitus: ♂ FW length: 14.3 mm. Amber brown. Head amber, with indistinct, dark brown mark over ocelli; brown stripe near the anterior margin. Tentorial callosities pale. Antennae dark, amber brown. Ocelli separate, approximately half an ocellus-width apart. Thorax brown, without prominent markings. Legs amber; fore- and mid-femora with dark brown stripe caudally; tarsi pale; tip of tarsomere III and claws black. Wings pale brown, veins brown, subcosta paler. Abdomen pale white-yellow. Cerci yellow, densely covered with fine setae.

Males: T7 without forefield; with an unpaired, three-dimensional caudal process. The process is a conical, rounded lobe; underside three-dimensional, with a large hump, with sensilla basiconica on tip and underside. T8 flat; distinctly sclerotized in the midline, caudally wide, narrows to a band before widening slightly at margin of T9; few sensilla basiconica medially, laterally sclerotized. T9 with antecosta divided in front of pale median field; rest of T9 is sclerotized; lateral humps very low, with some setae and sensilla basiconica; membranous furrow short, ends in a strong, downward-curved sclerotized plate with some sensilla basiconica. H10 mediabasal callus large, dorsally flat, tongue-shaped. Process short, slightly curved outwards, with tip pointed. Epiproct heart shaped.

Aedeagus: Aedeagus long, slender, straight, strongly sclerotized, with a dense coat of spines, starting laterally at end of bulb, and spreading to cover entire diameter of aedeagus for distal third; towards tip with dorsal, conical bare patch, fringed with longer spines.

Everted endophallus approximately 1.5 times longer than aedeagus; base inflated, bulb-like; curves ventrad; approximately the same width as aedeagus, but tip a thin tube. Spines conical, almost vertical, and sharp-tipped; arrayed in ordered rows; base of endophallus inflated and appears smooth, but under high magnification (>200x) tiny spicules surround tube-like portion; rest of the endophallus with 14 rows of dense, regular, saw like spines dorsally; laterally with two bare strips; a single row of spines ventrally; distally distal spines smaller.

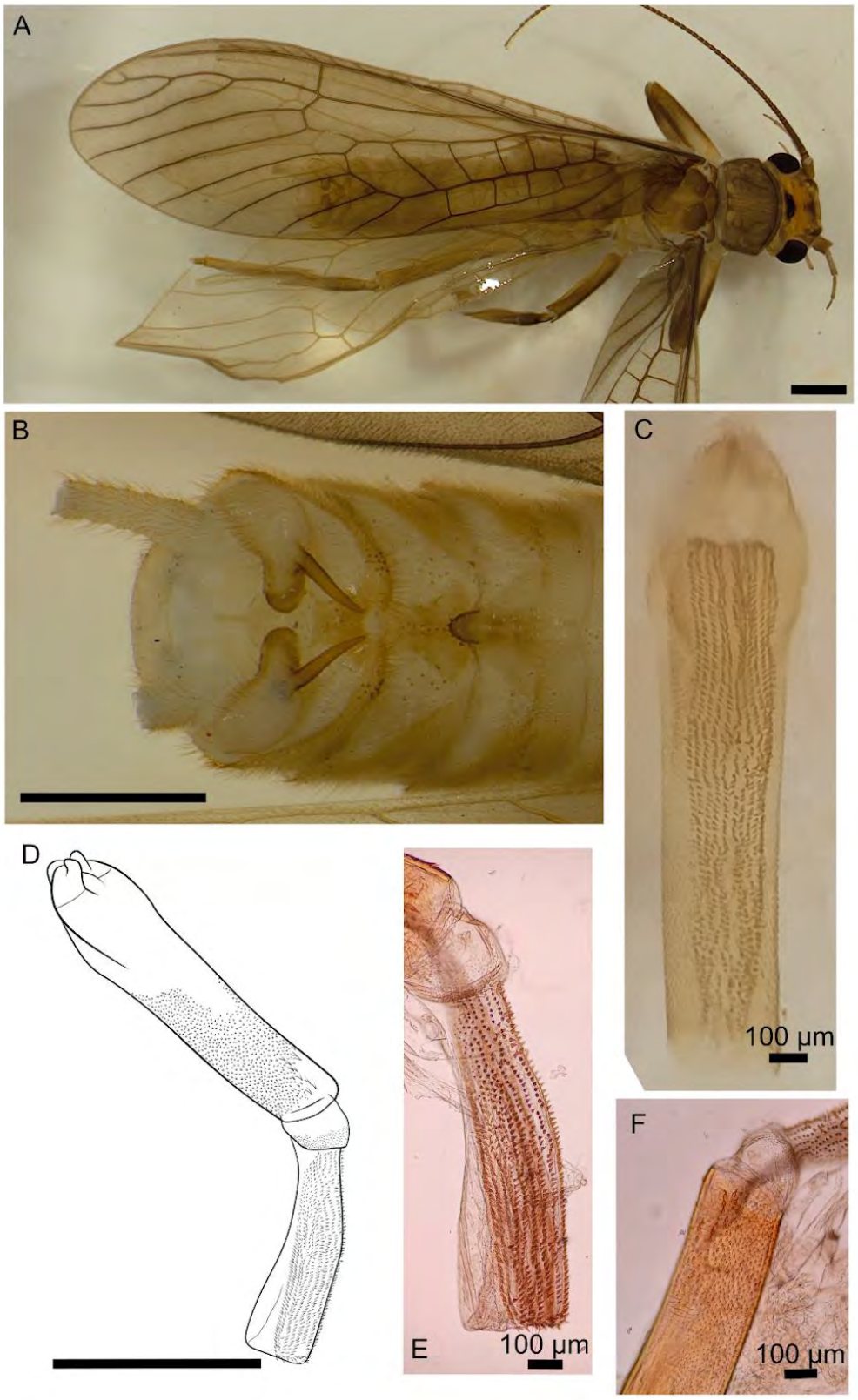

Figure 2.19: *Neoperla sp. nov. 7*. **A:** Adult male habitus. **B:** Tergite 7 – Hemitergites in the adult male. **C:** Aedeagus, not everted. **D:** Diagram of the everted aedeagus and spine patterns. **E:** Spine pattern on the endophallus. **F:** Spine patterns on tip of aedeagus. Unless otherwise stated, all scale bars represent 1mm.

DNA: In both analyses, *Neoperla sp. nov. 7* formed a monotypic branch, distant from any other species included in the analysis. In the COX1 analysis it was sister to the *N. duodevinginti* and *N. occipitalis* polytomy, but this relationship was only slightly supported (Figure 2.5, Bootstrap support: 70%). In the phylogeny, it was placed between the polyphyletic *N. duodevinginti* species group, but these branches were only moderately supported (Figure 2.7, Bootstrap support >72, Posterior probability > 0.76, BEAST >0.81).

Notes: *Neoperla sp. nov. 7* can be placed in the *N. africana* species group by the three-dimensional process on T7, the sclerotized aedeagus, and the endophallus with distinctly different spine patterns on lateral and ventral faces (Zwick 2023; Zwick & Zwick 2023). Furthermore, *Neoperla sp. nov. 7* can be placed in the *Neoperla pilulifera* species assemblage by slightly convex T8, and the bare, bulb-like base of the endophallus (Zwick 2023; Zwick & Zwick 2023).

Morphologically, *Neoperla sp. nov. 7* differs from all described males within the *N. pilulifera* assemblage. The endophallus is thick and tubular, approximately the same width as the aedeagus, and is covered in 14 rows of fine spines. Both *Neoperla pusilla* Zwick & Zwick and *Neoperla multiserrata* Zwick & Zwick have a similar spine pattern on the endophallus, but with fewer and smaller spine rows (Zwick 2023; Zwick & Zwick 2023). Both species additionally lack the membranous bulb at the base of the endophallus, and the spines covering the distal 1/3 of the aedeagus (Zwick & Zwick 2023).

Amendments to Key, Zwick & Zwick 2023:

Keys to species groups and species of African Neoperla, Males, pg. 17

10i. Process of T7 short and blunt, rounded. Endophallus thick, about as wide as aedeagus, with 14 ordered rows of saw-like teeth ……..…….... *Neoperla sp. nov. 7*

10i'. Process differs, either long, or a pyramidal cone …….…… 10

Species group *Neoperla transvaalensis* Zwick & Zwick 2023

Neoperla sp. nov. 8

Images: Figures 2.20 – 2.21

Material Examined: *Prospective Holotype*: PLEC 74A: 1♀ adult || Rwanda | Kamiranzovu Swamp | Nyungwe Forest | 2°29'20.2"S, 29°09'14.7"E | 12.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Light Trap | Over some small streams near the edge of the swamp and forest. ***Prospective Paratypes*: PLEC 31J**: 1♂ adult || Democratic Republic of Congo | Lwiro River | Kakeza

| 25.viii.2017 | Coll.: F. Ngera. ***Other Material*: PLEC 69A**: 1♀ nymph || Rwanda | Mujabagiro River | Nyungwe Forest | 2°26'51.9"S, 29°06'27.8"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 70A**: 1♂ nymph || Rwanda | uWasenkoko Swamp | Nyungwe Forest | 2°31'44.5"S, 29°21'12.4"E | 10.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 72A**: 1♂ nymph || Rwanda | Kitabi Tea Estate | Nyungwe Forest | 2°32'03.6"S, 29°25'00.4"E | 11.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current.

Nymph: Dark brown. Head dark brown, without distinctive patterning, but slightly pale over ocelli; "W" marking on frons. Ocelli small, separate, approximately one ocellus-width apart. Eyes small, slightly sunken. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae light brown; with fine dark setae, more along internal margin basally, distally very few.

Prothorax wide, dark brown, without obvious markings, but darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax dark brown, with a pale indistinct circle medially; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale brown; tibia pale yellow; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen dark brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Adult Habitus: ♂ FW length: 16.6 mm. ♀ FW length: 18.9 mm. Amber brown in females, pale yellow in males. Head uniform brown; with rounded, darker triangle over ocelli. Tentorial callosities pale. Antennae pale brown. Ocelli separate, approximately half an ocellus-width apart. Thorax pale, without prominent markings. Legs pale, without markings; tarsi pale; tip of tarsomere III and claws black. Wings clear to slightly cloudy, veins brown, subcosta paler. Abdomen pale, darkened distally. Cerci yellow, densely covered with fine setae.

Males (presumed): T7 without forefield; with an unpaired caudal process. The process is a large, flat, rectangular lobe, with sensilla basiconica along edges. T8 unsclerotized in midline; with few unordered sensilla basiconica, laterally with low, raised humps, with few sensilla basiconica along lateral margins. T9 soft; lateral humps low, with unordered sensilla basiconica and setae; median furrow soft, with some sensilla basiconica. H10 mediabasal callus large, dorsally flat, tongue-shaped. Process straight, with tip blunt. Epiproct trident shaped.

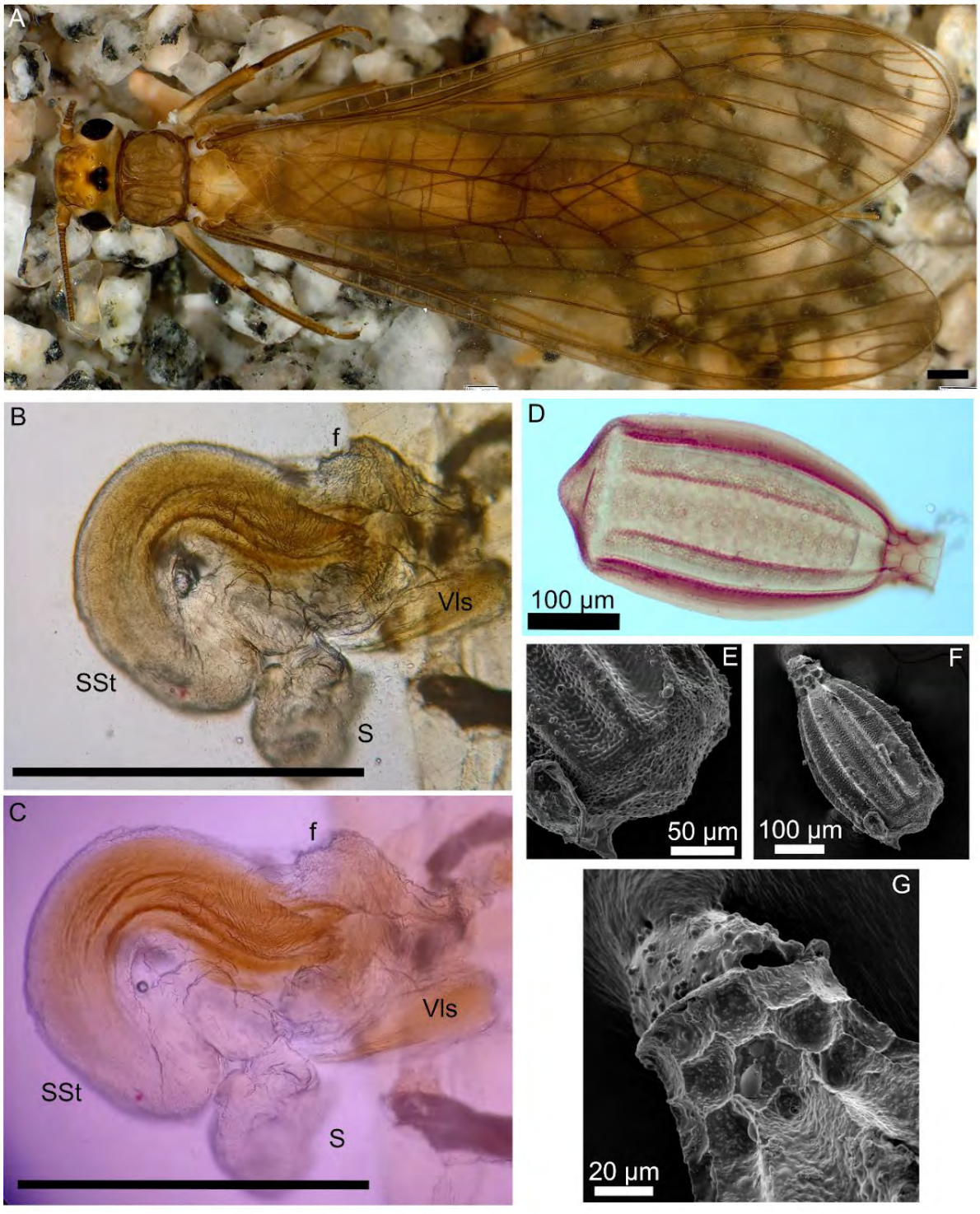

Figure 2.20: *Neoperla sp. nov. 8*. **A:** Adult female habitus. **B-C:** Vagina and spermathecal stalk, Vls: Lateral vaginal sclerites, f: folds, Sst: Spermathecal stalk, S: Spermatheca. **D:** Egg (X200). **E:** SEM photograph of operculum. **F**: SEM photograph of the egg. **F:** SEM photograph of the egg collar and anchor cap. Unless otherwise stated, all scale bars represent 1mm.

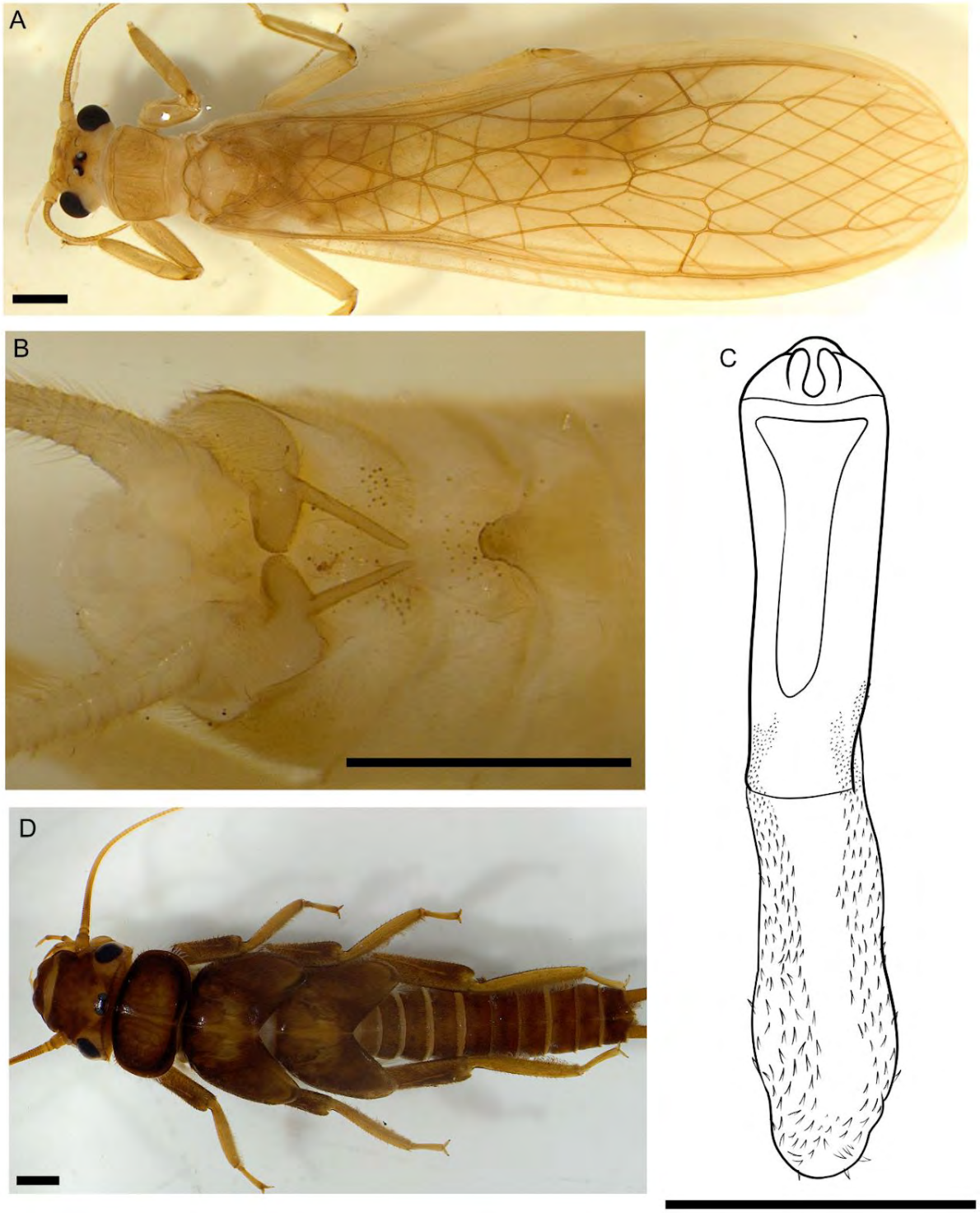

Figure 2.21: *Neoperla sp. nov. 8*. **A:** Adult male habitus. **B:** Tergite 7 – Hemitergites in the adult male. **C:** Diagram of the everted aedeagus and spine patterns. **D:** Nymph habitus. Unless otherwise stated, all scale bars represent 1mm.

Aedeagus: Aedeagus large, straight, moderately sclerotized, with lateral rows of small spines that continue onto the everted endophallus.

Everted endophallus slightly longer than aedeagus; straight. Spines sharp-tipped, pointing caudally; base of endophallus with a patch of small spinules ventrally, that extend into lateral bands along the distal tip of the aedeagus; no spinules dorsally; rest of endophallus densely covered with spines, except for a clear long strip dorsally, that stretches from base to tip and is approximately 1/3 the width of endophallus; distally tip bare, but surrounded by small spinules.

Females: Caudal edge of S7 with strong setae. S8 pale yellow, without markings. Posterior margin of S8 with a wide, slightly sclerotized, projecting lappet. Vagina short, wide; with lateral sclerite rods; a stack of folds on each side, laterally from vagina; spermathecal stalk widely connected to vagina. Spermathecal stalk strongly curved, divided into two sections, initially directed forward, then turned caudad; flat, slightly longer than vagina, similar width over entire length; cuticle thick on convex side; most of stalk with many longitudinal folds. Scales internal; armature consists of dense, tiny red spicules; dense spines aggregate in folds of spermathecal stalk, forming strong lines; spines fade away after vagina curves. Spermatheca coiled, with a long duct.

Egg: Egg approximately 481 µm long, 240 µm wide; with convex sides, base of operculum wider than base of collar. Collar large, inflated, rim slightly flanged; with three rows of cells with very high walls. Anchor cap with a long stalk, mushroom shaped. Approximately 12 striae, straight, with very broad sulci and narrow costae. Costae high, densely punctate ridges, narrowest at midpoint and wider towards poles; sulci with relatively coarse punctures in indistinct transverse rows; striae start from cells of collar, terminate at base of operculum. Microphyles are located in sulci, large, exposed holes, form a ring approximately 1/4 of the length of the egg from operculum. Operculum punctate, wide, flat, conical.

DNA: *Neoperla sp. nov. 8* is sister to *Neoperla kalengonis* Zwick & Zwick, as both species formed a strongly supported clade in the COX1 analysis (Figure 2.6, Bootstrap support: 99%). The species was not monophyletic, as one specimen (PLEC72A) formed a polytomy between the two clusters, which were otherwise clearly separated and strongly supported (Bootstrap support: 100%). This divide was echoed in the species delineation results, with bPTP, ASAP and ABGD all splitting these into two OTUs. mPTP comparatively grouped both species with *Neoperla lujana* Navás, *Neoperla spectabalis* Zwick & Zwick, and *Neoperla serrula* Zwick & Zwick. As these species can be reliably delineated by both morphological and molecular methods, this was clearly too conservative a grouping. As no morphological differences were present in the nymphs, PLEC72A (and PLEC31J, where the COX1 gene could not be sequenced) are included in this species.

Notes: *Neoperla sp. nov. 8* belongs to the *N. transvaalensis* species group. In females, this is supported by the U-shaped spermathecal stalk, while in the males it is supported by the flat, squarish

process on T7 with a soft and flat T8, and the soft endophallus with spines similar on both sides (Zwick 2023; Zwick & Zwick 2023).

The male of *Neoperla sp. nov. 8* is most similar to those of *N. kalengonis*, as they share the straight, tube-like endophallus with a bare strip dorsally (Zwick & Zwick 2023). However, the two can be differentiated by the trident-shaped epiproct in *Neoperla sp. nov. 8*, and the bare dorsal strip, which is much wider in this species, covering approximately 1/3 of the width of the endophallus. The male is presumed to belong to this species, as COX1 could not be sequenced from it, and it clustered with the PLEC72A nymph in the three-gene phylogeny.

The females are also similar to *N. kalengonis*, particularly in the structure of the spermathecal stalk, which shares the dense red spines, forming undulating rows when concentrated in folds (Zwick & Zwick 2023). However, the spermathecal stalk in *N. sp. nov. 8* is shorter, and does not have spines after the bend. Additionally, *N. kalengonis* lacks the lateral sclerites and folds on the vagina. While the eggs are similar, they can easily be differentiated by the collar, which is smooth and inflated in *N. kalengonis*, while *N. sp. nov. 8's* collar is long, with three clear rows of cells.

The females are also similar to *N. lujana*, which has a shorter spermathecal stalk with spines until the bend, lateral sclerites on the vagina, and eggs with a long collar (Zwick & Zwick 2023). However, the two can be separated by the stacks of folds on the lateral edges of the vagina in *N. sp. nov. 8*, which are absent in *N. lujana*, and the collar not distally widening.

Amendments to Key, Zwick & Zwick 2023:

Keys to species in the N. transvaalensis-clade, Males, pg. 21

10. Apex of penis tube unmodified. Endophallus covered by short conical spines ……...……. ……...……..…….…… *Neoperla. planidorsum* Zwick & Zwick

10'. A bare membranous nipple dorsally on penis apex, endophallus long and straight, with armature of long needles ……...……...…….……. 10i

10i. Epiproct trident shaped; dorsal bare strip covering ⅓ of the endophallus ...…. *Neoperla. sp. nov. 8*

10i'. Dorsal bare strip narrow……...……...……. *Neoperla kalengonis* Zwick & Zwick

Keys to species in the N. transvaalensis-clade, Females, pg. 21

2. SSt long and parallel, U-shaped, the two sections of similar length, full of elongate reddish spinules ……. 2i

2'. Vagina and/or base of SSt with many large folds. SSt bent, the forward-directed section much shorter than the sideward section, armature colourless and hard to see. Egg collar narrow …… ……..……...……. 3

2i. Vagina and base of SSt without large stacks of folds; Egg collar large, inflated and cup-like …… ……..……. *Neoperla kalengonis* Zwick & Zwick

2i'. Vagina with lateral sclerite rods, and stacks of folds laterally; Egg collar long, with three rows of cells …… *Neoperla sp. nov. 8*

Neoperla transvaalensis Enderlein, 1909

Images: Figure 2.22

Material Examined: *Other Material*: PLEC 12B: 1 nymph || South Africa | Bokspruit River | 30°53'5.5"S, 27°53'2.7"E | 09.xi.2023 | Coll.: A. Kirkaldy, N. Balmer || Stones in Current || **PLEC 15A, B, E:** 3 nymphs || South Africa | Bell River | 30°50'42.2"S, 27°48'23.2"E | 10.xi.2023 | Coll.: A. Kirkaldy, N. Balmer || Stones in Current || **PLEC 16A, C:** 2 nymph || South Africa | Nculwane River | 29°22'26.3"S, 30°22'23.8"E | 13.xi.2024 | Coll.: A. Kirkaldy, N. Balmer || Stones in Current || **PLEC 19A, B, C:** 3 nymphs || South Africa | Mzimkhulu River | 29°45'24.4"S, 29°26'2.9"E | 15.xi.2024 | Coll.: A. Kirkaldy, N. Balmer || Stones in Current || **PLEC 53A, B:** 2 nymphs || South Africa | Maria Shires Waterfall | 24°59'06.7"S , 30°48'39.7"E | 07.iv.2024 | Coll.: A. Kirkaldy, M. Villet, G. Diedericks, R. Palmer || Stones in Current ||

Nymph: Dark brown, chocolate to almost black. Head dark brown; with dark brown, sharply delineated markings between occipital ridge and ecdysial line, medial and extending half way between ocellus and eye, or covering eye; a transverse yellow band crosses head from ecdysial line, fades away in front of eyes, if region around eyes brown, instead forms a sharply delimited semi-circle; anterior margin of head pale yellow; a small brown oval covers ocelli. Ocelli separate, approximately one ocellus-width apart. Eyes large. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae pale yellow; with sparse setae basally, tiny setae distally.

Prothorax wide, dark brown, usually with a strongly delineated marking medially, either a rounded band or a clear patch on either side of ecdysial line; ecdysial line pale. Mesothorax brown, with a long, curved band medially to base of wing pads, in typical morph band with a sharp angle, almost square, but sometimes rounded or incomplete medially; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale brown; tibia pale yellow; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen dark brown, ventrally pale yellow and brown distally. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: The nymphs of *Neoperla transvaalensis* were fairly variable, but could usually be recognized by their very dark, almost black colouration. The head was strongly patterned, and always had clearly delineated, brown markings between the occipital ridge and ecdysial line, although the extent varied. The most distinctive marking was the clearly delineated, almost square band on the meso- and metathorax, although in some specimens this was curved, and sometimes medially interrupted. However, it was always clearly delineated and pale.

Figure 2.22: *Neoperla transvaalensis* Enderlein, nymph habitus. Scale bar represents 0.1mm.

2.5. Unnamed species

Neoperla sp. Afr_C

Images: Figure 2.23

Material Examined: ***Other Material:*** **PLEC 67A**: 1♀ nymph || Rwanda | uWinka Stream | Nyungwe Forest | 2°28'31.5"S, 29°11'45.2"E | 10.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current and Marginal Vegetation || **PLEC 67C**: 1♀ nymph || Rwanda | uWinka Stream | Nyungwe Forest | 2°28'31.5"S, 29°11'45.2"E | 10.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current and Marginal Vegetation.

Nymph: Light brown, amber. Head yellow, except for a transverse brown band along the anterior margin; without markings over ocelli. Ocelli separate, approximately one ocellus-width apart. Labrum

trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae pale yellow; with sparse setae basally, tiny setae distally.

Prothorax wide, dark brown, without obvious markings, but darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax pale brown, with a large white band curving from medial line to base of the wing pads; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale yellow; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with small hook near claw base heavily reduced, almost fused into the contour of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: *Neoperla* sp. Afr_C can be recognized by the almost completely yellow head, except for a brown, transverse band across the frons. While other species share the pale vertex, it can be separated from all of these species by the Occiput also being yellow, as this is usually pale brown. Molecular evidence strongly supports the monophyly of this species (Figure 2.3, COX 1: Bootstrap support: 100%, recognized by all species delineation tests; Figure 2.7, Phylogeny: Bootstrap support: 100%, Posterior probability: 1, BEAST: 1). However, its phylogenetic placement differs between analyses, so it is not placed in a species group.

Neoperla sp. Afr_D

Images: Figure 2.23

Material Examined: ***Other Material*: PLEC 68A**: 1♀ nymph || Rwanda | Kamiranzovu Waterfall | Nyungwe Forest | 2°26'48.7"S, 29°06'53.0"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current || **PLEC 68C**: 1♀ nymph || Rwanda | Kamiranzovu Waterfall | Nyungwe Forest | 2°26'48.7"S, 29°06'53.0"E | 09.viii.2022 | Coll.: A. Kirkaldy, F. Ngera & L. Ngirinshuti || Stones in Current.

Nymph: Dark brown. Head dark brown, with a strong V shaped white mark medially, reaching ocelli. Ocelli small, separate, approximately one ocellus-width apart. Eyes large. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae pale yellow; with fine dark setae, more along internal margin basally, distally very few.

Prothorax wide, dark brown with two small pale marks medially; posterior margin concave; median ecdysial line pale. Mesothorax dark brown, medially a little paler, with two small white marks on either side of pale median ecdysial line. Metathorax with same colouration as mesothorax. Legs pale brown; tibia pale yellow; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen dark brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: *Neoperla* sp. Afr_D can be recognized from the strong, white triangle marking on the head. Molecular evidence strongly supports the monophyly of this species (Figure 2.6, COX 1: Bootstrap support: 100%, recognized by all species delineation tests except mPTP; Figure 2.7, Phylogeny: Bootstrap support: 100%, Posterior probability: 1, BEAST: 1), and places it in the *Neoperla transvaalensis* species group.

Neoperla sp. Afr_E

Images: Figure 2.24

Material Examined: ***Other Material:*** **PLEC 021A**: 1 nymph || South Africa | Fairy Vale Farm | Grahamstown [SIC, now called Makhanda] | 33°19'21.8"S, 26°35'25.3"E | 15.ii.2015 | Coll.: V. Dames.

Nymph: Light brown, amber. Head amber brown, a transverse yellow band separates amber vertex from amber anterior half of the head. Ocelli separate, approximately one ocellus-width apart. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae pale yellow; with very pale, fine setae distally.

Prothorax wide, light brown, with two faint pale patches medially, darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax pale brown, with a pale curved band from medial line to base of the wing pads; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale yellow; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: The colouration of *Neoperla* sp Afr_E is somewhat indistinct, and it is similar to *Neoperla transvaalensis*. However, it is much paler than *N. transvaalensis*. Molecular evidence for this species is unclear; it forms a branch with singletons (Figure 2.7), making relationships difficult to resolve. COX1 sequencing was unsuccessful, preventing identification using this gene.

Figure 2.23: A: *Neoperla sp. Afr_C*, nymph habitus. **B:** *Neoperla sp. Afr_D*, nymph habitus. Scale bar represents 0.1mm.

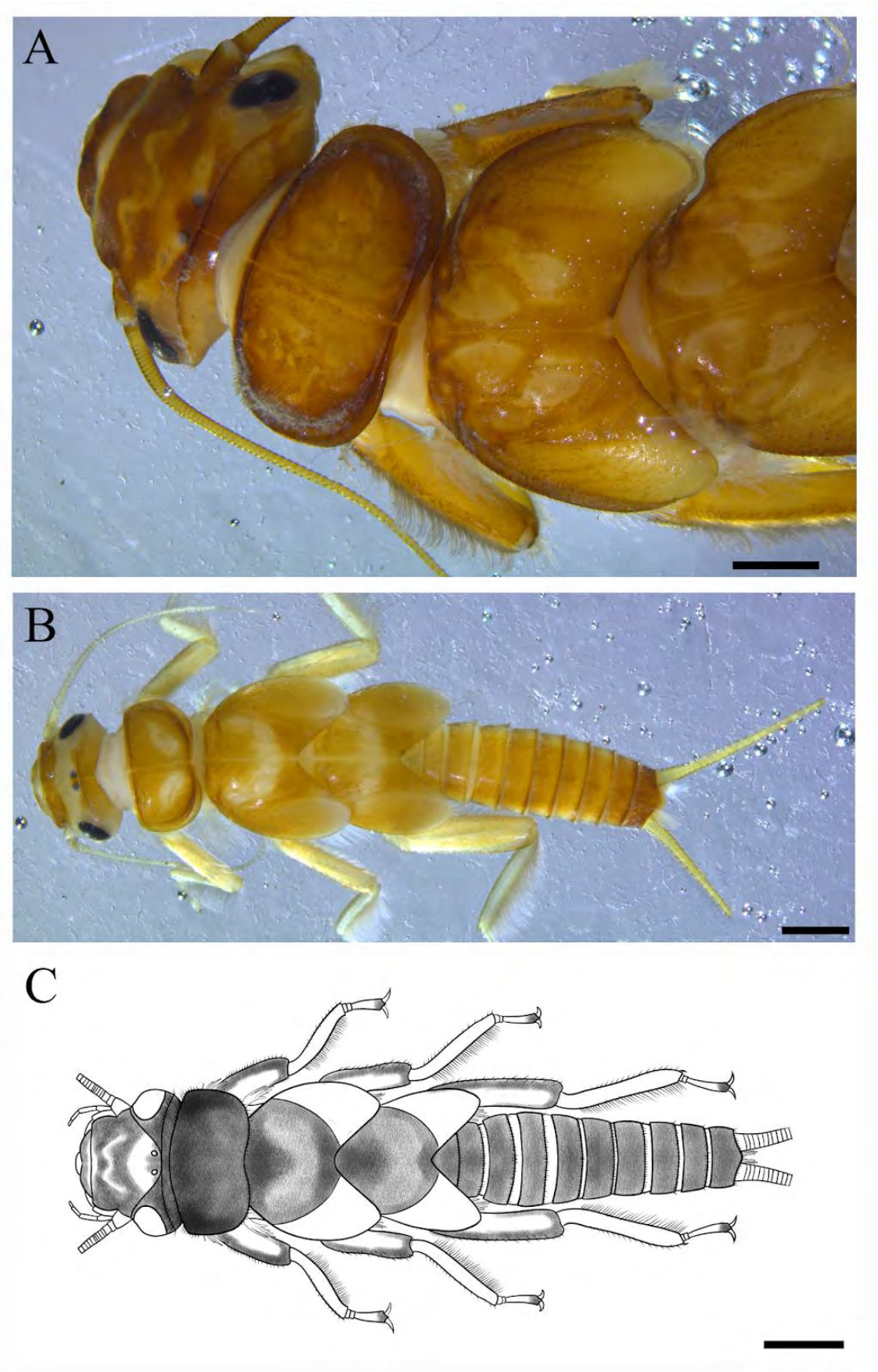

Figure 2.24: A: *Neoperla sp. Afr_F*, nymph habitus. **B:** *Neoperla sp. Afr_E*, nymph habitus. **A:** *Neoperla sp. Afr_G*, nymph habitus. Scale bar represents 0.1mm.

Neoperla sp. Afr_F

Images: Figure 2.24

Material Examined: ***Other Material:*** **PLEC 31C**: 1 nymph || Democratic Republic of Congo | Lwiro River | Kakeza | 25.viii.2017 | Coll.: F. Ngera.

Nymph: Light brown, amber. Head amber brown, with a small, slightly darker patch over ocelli and white "W" marking on frons. Ocelli small, separate, approximately two ocellus-widths apart. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along anterior margin. Antennae light brown; with very pale, fine setae distally.

Prothorax wide, dark brown, without obvious markings, but darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax pale brown, with some faint pale markings on either side of ecdysial line; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale brown; tibia fades to yellow distally; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: Molecular evidence for this species is unclear, as it forms a branch with singletons (Figure 2.7), making relationships difficult to resolve. COX1 sequencing was unsuccessful, preventing identification using this gene. It can be differentiated from the other *Neoperla* nymphs by its completely brown head, with a very bright white W-marking.

Neoperla sp. Afr_G

Images: Figure 2.24

Material Examined: ***Other Material:*** **ECR 859**: 1 nymph || South Africa | Hofani River | -31.456528, 29.718472 || In a forest patch: riparian trees on both sides of the stream, dense forest on one side of the river.

Nymph: Dark brown. Head dark brown, without distinctive patterning, but slightly pale over ocelli; "W" marking on frons. Ocelli small, separate, approximately one ocellus-width apart. Eyes large. Labrum trilobed; outer lobes brown; inner lobe pale white; with a fringe of long, strong setae along

anterior margin. Antennae light brown; with fine dark setae, more along internal margin basally, distally very few.

Prothorax wide, dark brown, without obvious markings, but darker along margin; posterior margin concave; median ecdysial line pale. Mesothorax pale brown, with a pale curved band from medial line to base of the wing pads; ecdysial line pale. Metathorax with same colouration as mesothorax. Legs pale brown; tibia fades to yellow distally; tarsomeres pale yellow; tarsal claw pale yellow, hooks tipped in brown, with a small curved hook emerging near base of claw.

Abdomen amber brown. Cerci amber brown; each cercomere with regular rings of stiff, spike-like setae distally, and fine, flat clothing hairs.

Proventriculus with straight, sharp-tipped spines, forming a spine band, wide at base and rapidly constricting to a narrow band of approximately 3–4 rows of spines; terminating in a small bulb with many large spines.

Notes: The colouration of *Neoperla sp. Afr_G* is similar to many of the other nymphs, making it hard to differentiate. It can be separated from most other species by the brown head, with a pale patch over the ocelli and a W mark on the frons. The COX1 gene from this species failed during sequencing, but it forms a strongly separated singleton branch (Figure 2.7) as sister to the *Neoperla sjostedti* complex.

2.6. Discussion

2.6.1. Diversity of Neoperla in Africa

In total, nine novel species are recognized and described here, while the two subspecies in the *Neoperla sjostedti* complex, and an informal, morphologically distinct form, are elevated to species rank. In total, this brings the number of *Neoperla* in the Afrotropical region to 93.

This total is likely still an underestimate. A further five informal, genetically distinct species are known as nymphs, but not named due to their lack of diagnostic features. It is highly likely that continued sampling efforts will allow more species to be identified across the Afrotropical region, especially as endemism is common in Plecoptera (Stevens *et al.* 2018). Endemism may also be common in the Afrotropical *Neoperla*, as 31 species (33%) are only known from a single locality. However, this might be a sampling artifact due to inconsistent collections across the continent (Zwick & Zwick 2023). Additionally, all four of the species delineation tests identified deep divides within some known species such as *Neoperla transvaalensis* and *Neoperla burgeoni*, suggesting some of the widespread African species may in fact be morphologically similar species complexes. This echoes the recent discovery of Plecopteran species complexes in Europe (Hlebec *et al.* 2022; Kermek *et al.*

2024; Vitecek *et al.* 2017; Vuataz *et al.* 2024; Weiss *et al.* 2012), and was similarly anticipated by Zwick & Zwick (2023).

2.6.2. Identification of Nymphs

In this preliminary survey of the nymphs of some Afrotropical species, I found that species differed in colouration patterns, and that these patterns were fairly consistent in specimens longer than 1 cm, excluding cerci and antennae. Therefore, habitus descriptions of nymphs are valuable, and may allow for the identification of different species, or at least species groups.

The taxonomy of *Neoperla* nymphs in Asia similarly focuses on colouration patterns (Cruz *et al.* 2018; Pelingen & Freitag 2020). However, while general colouration patterns were fairly consistent in the specimens studied here, relatively few nymphs of each species were examined, and some variation in the size and position of the ocelli, pigmentation, and patterning of the thorax and head was observed between specimens and sexes. Additionally, besides colouration, no other morphological features were observed that would allow for the differentiation of the nymphs. This included the proventriculus (Ogbogu 2006; Stark & Gaufin 1976), which had the same generalized form and spine patterns in all species examined. While *Neoperla sp. nov. 4* can be differentiated from all other Afrotropical *Neoperla* nymphs examined here by the fringe of swimming hairs on the cerci, this character occurs rarely in other *Neoperla* species (Sivec *et al.* 1988). Considering this limitation, identification will likely still be unreliable across the entire continent. However, region specific keys, especially in areas with a low diversity, will likely allow for relatively consistent identification of this life stage.

Until more descriptions of known species are available, and the variation within individual species has been assessed, the habitus characters of the nymphs should be treated as auxiliary characters rather than to diagnose species. Molecular methods remain the most reliable way to identify nymphs.

2.6.3. Subgenus Neoperla (Neoperla), the Neoperla duodeviginti species group, and implications for biogeography

All of the African species clearly belong to the subgenus *Neoperla* (*Neoperla*) because of the heavily modified spermathecal stalk, which is simple in the other subgenera (Zwick 2023). African species can be differentiated from the North American representatives of the subgenus by the delicate, curled or coiled spermatheca, which is cuplike in the Nearctic (Zwick 2023).

Most of the novel species described here can be placed in the species groups established by Zwick & Zwick (2023) and Zwick (2023, 2024) by a combination of morphological characteristics and

molecular evidence. While some of these species groups were poly- or paraphyletic in the three-gene phylogeny, most of the African species assemblages were well supported and resolved (Zwick & Zwick 2023). The exception to this was the *Neoperla duodeviginti* species group, which was morphologically heterogeneous, and polyphyletic in both the COX1 and three-gene phylogeny. Zwick & Zwick (2023) noted that the *N. duodeviginti* species group did not fit well into any other species group, and the novel species described here have further confounded this problem by exhibiting more variation in the spermathecal stalk and egg structures. The males were generally similar to those of the *N. spio* and *N. occipitalis* species groups, while females shared characters of multiple other species groups including *N. excisa-sjostedti*, *N. transvaalensis*, *N. spio* and *N. occipitalis.*

In both the phylogeny constructed here, and the phylogeny presented by Zwick & Zwick (2023), the African *Neoperla* are split into two clades. The *N. duodeviginti* species group forms some of the basal-most branches in one of these clades, suggesting that they may represent some of the earliest divergences of the African species. This highly varied morphology may therefore be due to rapid divergences as *Neoperla* spread across the Afrotropics and into new environments. Unfortunately, the position of the *N. duodeviginti* species group differs in both phylogenies, and affinities to the other species groups are poorly supported in both. Intriguingly, in the COX1 analysis, *N. duodeviginti* formed a clade with the Neotropical *N. occipitalis* species group, although the relationships had low support values. As mentioned above, there are also several pronounced morphological similarities between these species groups, particularly in some of the eggs and the endophallus.

This finding differed in Zwick & Zwick's (2023) phylogeny, where the *N. occipitalis* group (which were also only represented by COX1 genes) was sister to all the remaining *Neoperla* (*Neoperla*) species. *Neoperla* (*N.*) then split into two clades, with the *N. duodeviginti* and *N. spio* (including an Asian species, *Neoperla ignacsiveci* Li & Li) species groups forming the basal branches of each of these separate clades. The *N. spio* species group is the only group within *Neoperla* (*N.*) that occurs in Asia, where *Neoperla* are thought to have first appeared (DeWalt *et al.* 2025; Hynes 1976; Zwick & Zwick 2023), with all of the other species found in Africa and America.

The uncertain placement of the *N. occipitalis* species group and the relatively derived position of the *N. spio* group in both analyses point to several possibilities. Firstly, if the basal position of the *N. occipitalis* species group is validated, it suggests that *Neoperla* (*N.*) originated in the Nearctic, not Asia, as was previously accepted (Fochetti & Tierno de Figueroa 2008; Letsch *et al.* 2021; Zwick 2000). As the earliest branches of Zwick & Zwick's (2023) *Neoperla* phylogeny are made up of paraphyletic Asian *Neoperla* (*Formosita*), this divergence likely would have been due to the isolation of a common ancestor, possibly via vicariance (Hynes 1976; Zwick 2000). In this scenario, *Neoperla* (*N.*) would then have migrated into Africa and Asia, perhaps simultaneously.

Alternatively, if the close relationship between the *N. occipitalis* and *N. duodeviginti* species groups is validated, two other explanations present themselves. Firstly, many *Neoperla* in Asia are not currently placed into species groups (DeWalt *et al.* 2025; Zwick 2023). If any of these form more basal clades in *Neoperla* (*N.*), it remains possible that they originated in Asia and migrated into North America and Africa simultaneously, perhaps via humid, tropical corridors in the Northern Hemisphere (Denk *et al.* 2011; Graham 2018; Tiffney 1985; Wen *et al.* 2016). Alternatively, *Neoperla* may have spread into North America earlier, giving rise to the *N. occipitalis* and *N. choctaw* species groups after becoming isolated from Asia due to vicariance (Hynes 1988; Zwick 2000). In this case, Africa would have likely been colonized by *Neoperla* twice, with one clade dispersing southwards from Asia, possibly via Miocene land bridges (Fochetti & Tierno de Figueroa 2008; Letsch *et al.* 2021; Zwick 2000), and the second from America, leading to the two distinct clades seen in the phylogenies discussed here. Unfortunately, until these relationships can be investigated with more species and genes represented, this picture remains unclear.

The biogeography of the Afrotropical *Neoperla* is discussed further in Chapter 4.

3. Chapter 3: Literature Review of the Fossil Record of Systellognatha (Plecoptera) and its Implications for the Biogeography of Plecoptera

Note: A version of this chapter has been submitted to Zootaxa for peer review, and potential publication. It is still currently under review.

3.1. Introduction

Illies (1965) proposed the first relatively complete hypothesis of Plecoptera biogeographical history (Figure 3.1a), with Plecoptera originating in the austral region of Pangea during the Permian. They crossed the equator by at least the late Permian, and subsequently gave rise to the "Setipalpia" (= Pteronarcyidae + Perloidea) and "Filipalpia" (= Euholognatha + (Gripopterygoidea + Scopuridae + Peltoperlidae)) in the Northern Hemisphere. Those that remained in the Southern Hemisphere founded the Eusthenioidea. Later, the initially boreal Notonemouridae migrated over the equator prior to the rifting of Madagascar and became extinct in the north. During the Cenozoic, the Perlidae followed into post-Gondwanan South America and Africa in two independent migrations.

Alternative explanations or variations have since been presented (Banarescu 1990; Hynes 1988). Zwick (2000) summarized these in two possible scenarios: either the Antarctoperlaria and Arctoperlaria had a pan-Pangean distribution and countervailing extinctions then restricted each to a single hemisphere (Figure 3.1b); or stem stoneflies were present across Pangea, and the suborders diverged because of vicariance after the rifting of Pangea (Figure 3.1c). Zwick (2000) considered the latter explanation more likely. The disjunct distribution of Antarctoperlaria across the Southern Hemisphere was explained by the suborder evolving on a continuous landmass that subsequently split. Zwick was equivocal about whether this occurred on Gondwana (in which case Antarctoperlaria later became extinct in Africa) or after Africa split from the rest of Gondwana (circa 165–162 Ma: McLoughlin 2001), but New Zealand, Australia, Antarctica, and South America remained connected via Antarctica (McLoughlin 2001). Relationships in the Northern Hemisphere are more complex, and the modern distributions appear to reflect a combination of vicariance, faunal exchange, extinction and displacement throughout the Jurassic – Pleistocene (Hynes 1988; Zwick 2000). Estimates for the dispersal times of Perlidae and Notonemouridae were left uncertain, but *Neoperla* probably migrated into Africa during the Miocene or Pliocene, 23–2.6 Ma (Zwick 2000).

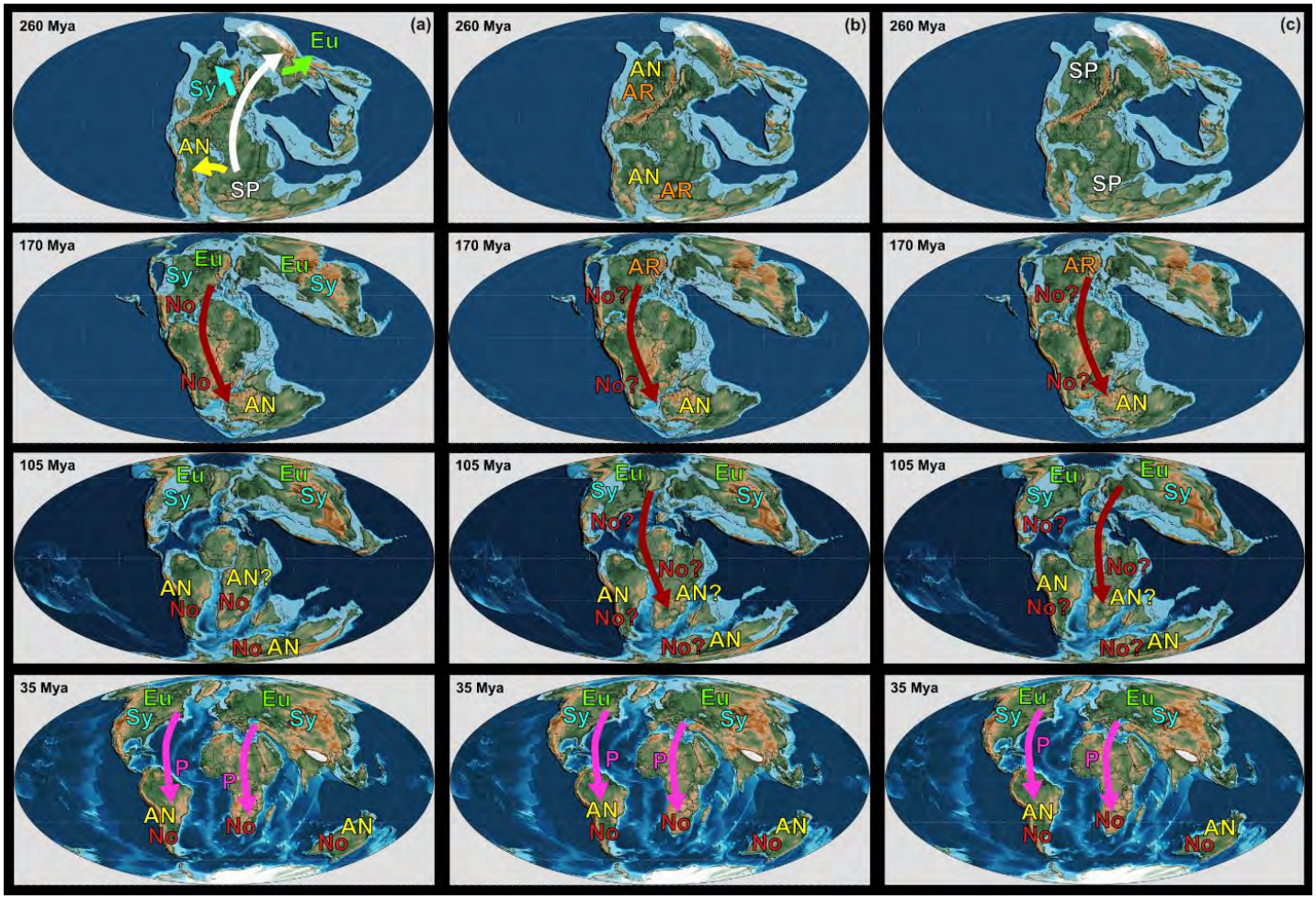

Figure 3.1: Hypotheses of the biogeography of Plecoptera, from the Permian (270 Ma) – Palaeogene (35 Ma), based on interpretations of extant and fossil distributions. **A:** Origination and migration from the Southern Hemisphere (Illies 1965). **B:** Both suborders were present across Pangea, but reciprocal extinctions limited each to a single hemisphere (Zwick 2000). **C:** Vicariance caused by the rifting of Pangea (Zwick 2000). Abbreviations are as follows: SP: Stem-Plecoptera, AN: Antarctoperlaria, AR: Arctoperlaria, Eu: Euholognatha, Sy: Systellognatha, No: Notonemouridae, P: Perlidae. Palaeomaps used in Figure are from Scotese *et al.* (2025) and are utilized under the Creative Commons Attribution 4.0 International License, available from https://doi.org/10.5281/zenodo.10659112 (Accessed 24 February 2025).

Interest has persisted into the molecular era, and four time-calibrated phylogenies have been used to investigate these questions (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016). While some consistent patterns have emerged from these studies, most dates have varied significantly. In particular, explanations for the origin of each suborder have differed, with Letsch *et al.* (2021) recovering a much older divergence in the Permian (265 Ma) than the remaining studies (< 181 Ma). In this scenario (Figure 3.2a), the suborders were interpreted as evolving in the high latitudes of Pangea, separated from the globally-distributed stem-Plecoptera by arid bands across the midlatitudes of the supercontinent (Chaboureau *et al.* 2014) and the equatorial Variscan mountain range (Kroner & Romer 2013). Antarctoperlaria and Notonemouridae both had a Northern Hemisphere origin and dispersed into the south approximately 180 Ma as the Variscan mountain

range subsided with the rifting of Pangea to form Gondwana and Laurasia (Hasterok *et al.* 2022; Letsch *et al.* 2021)

In this interpretation, Antarctoperlaria and Notonemouridae were present across Gondwana prior to its rifting, and their modern distributions are likely the result of vicariance, followed by extinction of the former in Africa. Faunal exchanges between South America, Australia, and New Zealand persisted over Antarctica until those continents separated. This separation started with New Zealand, approximately 84 Ma (Mayes *et al.* 1990; McLoughlin 2001; Storey 1996), and was followed by Australia and South America with the opening of the Drake passage 41 Ma (McLoughlin 2001; Scher & Martin 2006). Euholognatha and Systellognatha were present in the Northern Hemisphere by the Jurassic, and their modern distribution follows the model presented by Zwick (2000). Acroneuriinae dispersed from North to South America by "island-hopping" via a chain of meso-American islands some 67 Ma. *Neoperla* first appeared in Asia approximately 92 Ma, suggesting a possible earlier expansion into the Afrotropics, but the lack of Afrotropical taxa in the analysis (at the time only a single African species was regarded as valid and was not included) prevented a test of this hypothesis.

McCulloch *et al.* (2016), Ding *et al*. (2019), and García-Girón *et al.* (2024) all instead placed the divergence of the two suborders after the splitting of Pangea (121, 181, or 170 Ma respectively), supporting Zwick's hypothesis of vicariance. In this interpretation, most families and genera are younger, first appearing in the Cretaceous (Figure 3.2b). Antarctoperlaria arose on Gondwana, after the isolation of Africa, and its extant distribution is explained, in part, by the same vicariance patterns between New Zealand, Australia, South America and Antarctica discussed above. However, as New Zealand shares more recent taxa with other continents, long-distance dispersal between continents must have occurred. This dispersal was attributed to wind, with stoneflies carried as aerial plankton by the Antarctic Circumpolar Current, or West Wind Drift, circulating Antarctica (García-Girón *et al.* 2024; McCulloch *et al.* 2016). However, while stoneflies can disperse relatively far from rivers as adults (Hynes 1976; Zwick & Zwick 2023), they are generally poor flyers, even in ideal conditions (Fochetti & Tierno de Figueroa 2008; McCulloch *et al.* 2009, 2019; Zwick 2000). Most stoneflies remain at ground level rather than migrating vertically into the canopy (Bowman & Smith 2021), and winds above the treeline have been correlated to the loss of wings in Plecoptera (McCulloch *et al.* 2019, 201). This suggests dispersal as aerial plankton is unlikely. Long-distance dispersal between continents may instead have been facilitated by rafting, with stoneflies carried across the ocean by wind-driven currents. This route seems more likely than flight for the Plecoptera, and has been proposed for the 800 km marine dispersal of flightless insects from New Zealand to the Chatham Islands (Trewick 2000). Either way, Notonemouridae were estimated to have arisen recently (110, 76, or 71 Ma), suggesting that their distribution across the Southern Hemisphere is attributed to some form of long-distance dispersal. The dates recovered for the Northern Hemisphere taxa are largely

congruent with those of Letsch *et al.* (2021) and the summary presented by Zwick (2000). The dispersal of Perlidae into South America and Africa was not investigated.

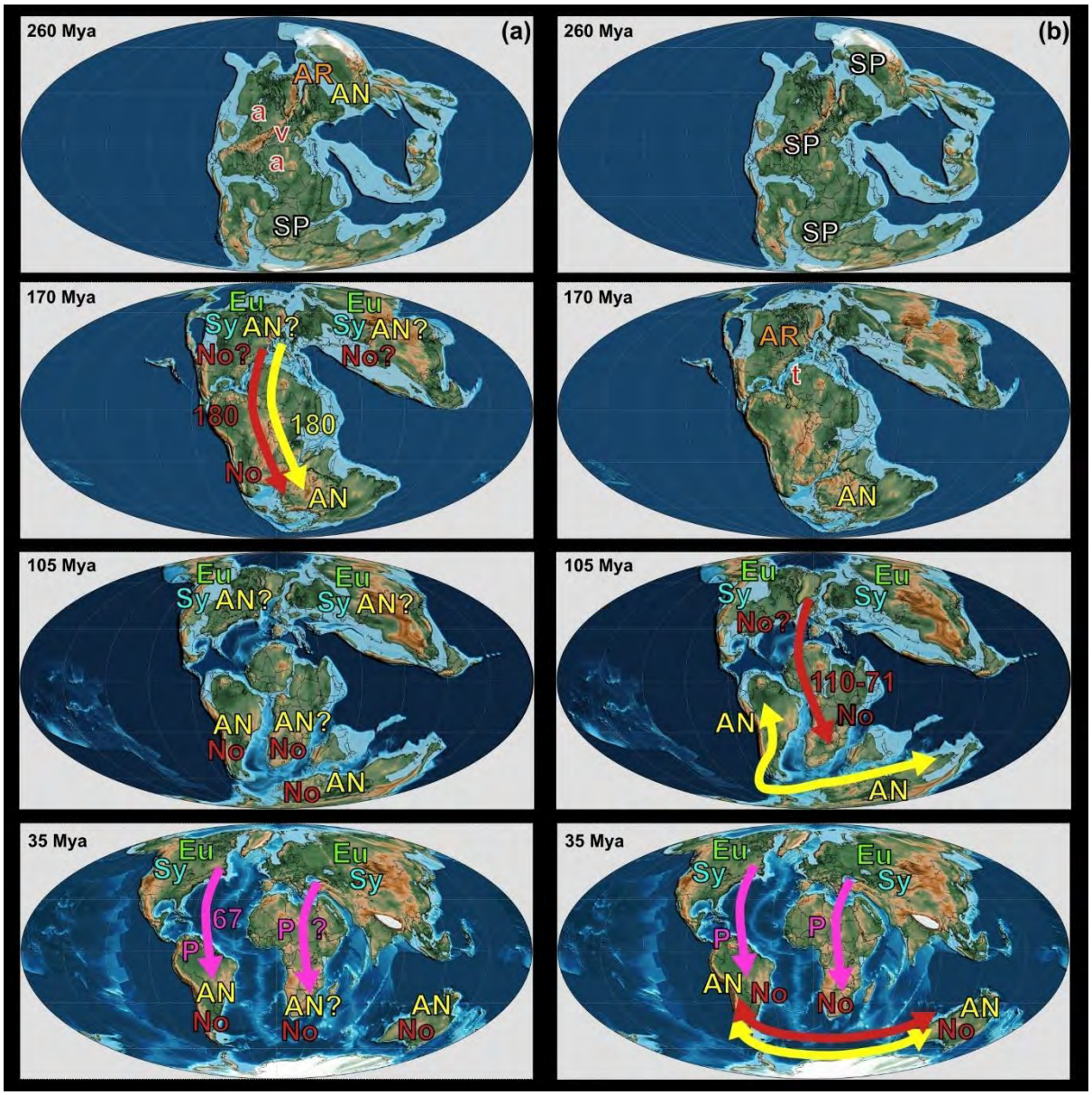

Figure 3.2: Hypotheses of the biogeography of Plecoptera, from the Permian (270 Ma) – Palaeogene (35 Ma), based on time-calibrated phylogenies. **A:** Origination and migration from the Northern Hemisphere, followed by extinction of Antarctoperlaria in the Northern Hemisphere (Letsch *et al.* 2021). **B:** Vicariance, followed by long-distance dispersal (Ding *et al.* 2019; García-Girón *et al.* 2024; McCulloch *et al.* 2016). Abbreviations are as follows: SP: Stem-Plecoptera, AN: Antarctoperlaria, AR: Arctoperlaria, Eu: Euholognatha, Sy: Systellognatha, No: Notonemouridae, P: Perlidae, a: Arid region, v: Variscan orogeny. Palaeomaps used in Figure are from Scotese *et al.* (2025), and are utilized under the Creative Commons Attribution 4.0 International License, available from https://doi.org/10.5281/zenodo.10659112 (Accessed 24 February 2025).

The pronounced differences in these interpretations indicate that the biogeographic history of the Plecoptera remains unclear. While patterns in the Northern Hemisphere after the rifting of Pangea are generally congruent between studies, the processes that led to the divergence of the suborders, the modern distribution patterns of Antarctoperlaria, and the dispersal of Notonemouridae and Perlidae into the Southern Hemisphere remain unresolved.

The fossil record of Plecoptera represents the best source of evidence to resolve and improve the dating estimates of some of these processes. Fossils provide the only primary source of evidence of ancient distributions, evolutionary times and changes in phenotypes including transitionary forms and extinct groups (Mongiardino Koch *et al.* 2021; Prevec *et al.* 2022; Raff 2007). Fossil data significantly improves phylogenetic and biogeographical analyses, even when they are marred by large gaps or incomplete sampling (Crisp *et al.* 2011; Louca & Pennell 2020; Mongiardino Koch *et al.* 2021; Puttick 2016; Raff 2007; Silvestro *et al.* 2016; Slater *et al.* 2012). Within time-calibrated phylogenies, fossil data can constrain divergence times (Cai *et al.* 2022; Mongiardino Koch *et al.* 2021; Raff 2007), ground-truth widely different biogeographical interpretations (Louca & Pennell 2020; Prevec *et al.* 2022; Raff 2007), and significantly improve the resolution and biogeographical understanding of ancient and diverse groups (Cai *et al.* 2022). Despite these advantages, fossil data must be carefully selected and constrained, as inaccurate taxonomy, age estimates or expected prior probabilities of calibration points can all alter results, leading to inaccurate or inconsistent dating estimates (Heads 2005a; Klopfstein 2021; Parham *et al.* 2012; Wolfe *et al.* 2016). Crucially, fossils only provide a minimum age for divergence events, and may represent clades that are much older (Klopfstein 2021; Mongiardino Koch *et al.* 2021).

At first glance, applying the fossil record of the Plecoptera to these biogeographical questions should be straightforward. The fossil record is rich, with 322 species and 1742 specimens (Jouault *et al.* 2022b) described from the Carboniferous (Béthoux *et al.* 2011; Schubnel *et al.* 2019) to Holocene (Sinitshenkova 1987). Additionally, at least one fossil species is attributed to 13 of the 17 extant families (DeWalt *et al.* 2025; Uhen *et al.* 2023). Unfortunately, the assignment of many Plecopteran fossils, and their relationships to extant taxa, are unreliable (Cui *et al.* 2019; Jouault *et al.* 2021; Zwick 2000). Many established apomorphic characters of extant stonefly clades and families are internal, present only in a single life stage, or seldom visible in fossils (Jouault *et al.* 2021; Zwick 2000). Fossil stoneflies are therefore often assigned based on wing venation, which can vary significantly, even in a single species or individual specimen (Béthoux 2005; Cui *et al.* 2015; Jouault *et al.* 2021). Furthermore, many Plecopteran fossils are assigned to a different phylogenetic system than the extant representatives (Sinitshenkova 1987, 2002). While Sinitshenkova's proposed phylogeny broadly matches the extant groupings of Zwick (2000), which have been corroborated by molecular studies with only a few disparities (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.*

2021; McCulloch *et al.* 2016; South *et al.* 2021; Terry 2004), it differs significantly in topology (Figure 3.3).

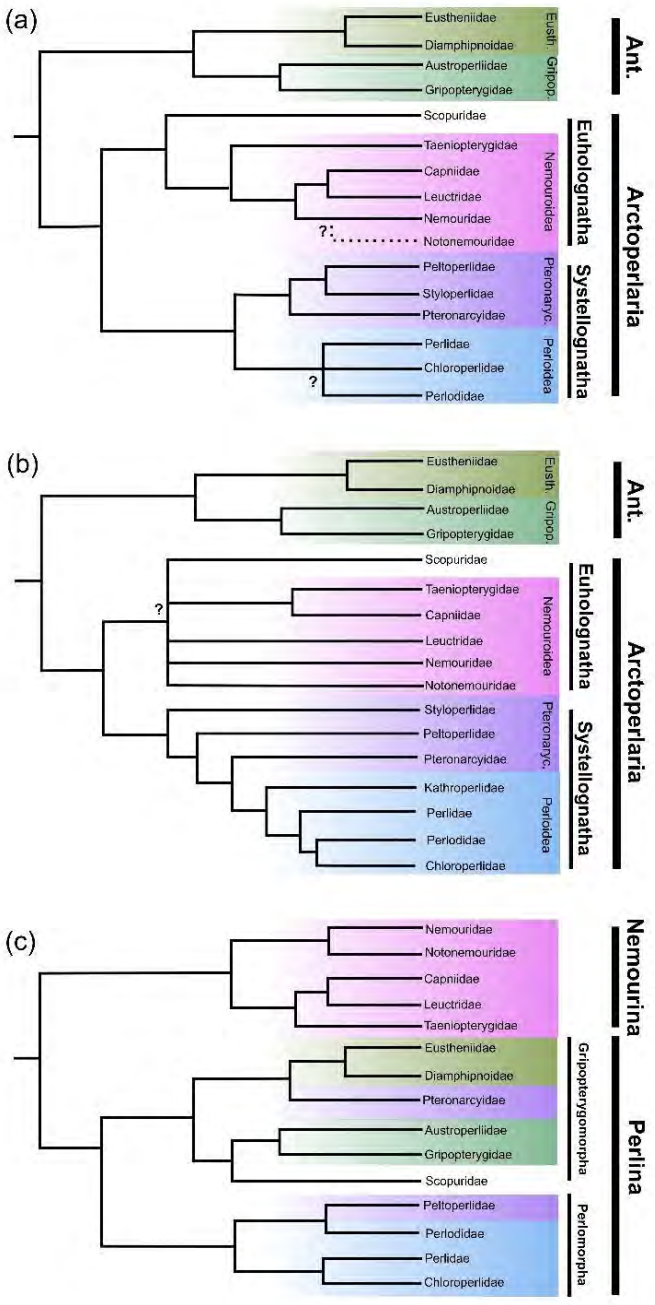

Figure 3.3: Cladograms showing hypothesized phylogenetic relationships in Plecoptera. **A:** Based on the morphological review of Plecoptera by Zwick (2000). Supported by a phylogeny constructed by Ding *et al.* (2019) using Mitochondrial genomes. **B:** Consensus cladogram based on phylogenies from Letsch *et al.* (2021), South *et al.* (2021) and Garcia-Giron *et al.* (2024). **C:** Phylogeny of Plecoptera proposed by Sinitshenkova (1987, 2002) to which most fossil species are assigned. Sub-orders and infra-orders are represented by bars on the right of each phylogeny. Each coloured block represents the currently accepted superfamily assignment of each family. Eusth.: green, Eusthenioidea; Gripop.: sea green, Gripopterygoidea; Nemouroidea: pink, Nemouroidea; Pteronarcy.: purple, Pteronarcyoidea; Perloidea: blue, Perloidea.

This uncertainty confounds any biogeographic interpretation of the fossil record and prevents the selection of reliable or consistent fossil calibration points for phylogenetic analyses. Indeed, calibration points have varied significantly between molecular studies. For example, the root of Plecoptera was constrained to 319.9 Ma by Letsch *et al.* (2021) and 167.41 Ma by García-Girón *et al.* (2024), an almost twofold difference. Differences in these calibration points can probably explain much of the variation recovered in the results and interpretations of these analyses. A review of the fossil record of Plecoptera is, therefore, urgently required, and calibration points for genetic analyses must be chosen conservatively from fossils with genuine (syn/aut)apomorphic characters. A complete review of the Plecopteran fossil record is beyond the scope of this dissertation, and instead all Systellognatha and austral fossils are reviewed here to provide calibration points for a time-calibrated phylogeny of *Neoperla* in the Afrotropical region, which is presented in Chapter 4.

3.2. Methods

I examined possible relationships and affinities of individual Systellognathan and austral fossil species and critically assessed their relationships to extant Plecoptera. Likely polyphylies in families and genera are discussed only when species are preserved with incongruent (syn/aut)apomorphic characters. These results provide a set of reliable calibration points for future molecular and fossil phylogenetic analyses (Bell & Lloyd 2015; Parham *et al.* 2012) and highlight fossil families and genera whose systematic placement should be reassessed.

A complete species list of fossil Plecoptera was obtained from the Palaeobiology Database (Uhen *et al.* 2023) and the Plecoptera Species File (DeWalt *et al.* 2025). Original descriptions and published figures were examined to assess the presence and absence (and unavailability) of diagnostic (syn/aut)apomorphic characters. Nineteen austral fossil species and 81 Systellognatha, or "Perlomorpha", from the Northern Hemisphere were reviewed. Some related fossil taxa from Euholognatha and "Gripopterygomorpha" were considered when affinities were relevant. Assignments to suborder, superfamily, and extant families proposed here are based on previously published phylogenetic reviews (Cui *et al.* 2015; Sivec *et al.* 1988; Uchida & Isobe 1989; Zwick 2000). All date ranges for species were obtained from the Palaeobiology Database (Uhen *et al.* 2023), which follow the 2023 estimates provided by the International Commission on Stratigraphy (www.stratigraphy.org).

Recommended calibration points were selected only from specimens that clearly had multiple, reliable, derived character states supporting their assignment. Taxonomic terminology follows Klopfstein (2021), and wing vein definitions and abbreviations (explained in Figure 3.5) follow Béthoux (2005).

A comprehensive taxonomic review of fossilised Plecoptera is beyond the scope of this study. Many extinct families and genera are supported by purely morphometric conditions such as body proportions (Sinitshenkova 1987; Zwick 2000) and may be para- or polyphyletic. With few exceptions, I did not assess the monophyly of extinct families and genera, nor are the diagnoses of these families and genera reviewed here. For this reason, no formal systematic changes are proposed.

3.3. Results

The recommended re-classification of all reviewed fossils is summarized in Tables 3.1–3.3, Appendix 3.1 and Figure 3.4.

3.3.1. Austral fossils

Although only 19 species of fossil Plecoptera are reported from the Southern Hemisphere, they are currently assigned to at least eight families and 11 genera (Table 3.1). Four of these families are extinct: †Euxenoperlidae Sinitshenkova, †Perlapsocidae Pinto and Piñeiro, †Palaeonemouridae Sinitshenkova and †Platyperlidae Sinitshenkova. Four more are extant: Eustheniidae, Notonemouridae, Gripopterygidae and Austroperlidae. A single genus, †*Afroperla* van Dijk & Geertsema, is unplaced.

3.3.1.1. Incertae sedis

†*Afroperla* is known from only a single Permian species, †*A. permiana* van Dijk & Geertseema. This genus was not assigned to a family, although similarities to †Palaeonemouridae, a widespread family from Russia, Kazakhstan and Antarctica (Sinitshenkova 1987) were noted (Van Dijk & Geertsema 2004). Possible affiliations of †Palaeonemouridae are discussed below. This species is known from only a single forewing that has no preserved apomorphies to support its assignment anywhere within crown-Plecoptera. Assignment to stem-Plecoptera is supported by an anterior Cubitus (CuA) with three distal branches, an anterior Radius (RA) – posterior Radius (RP) crossvein, and a Media (M) with two branches (Béthoux 2005; Béthoux *et al.* 2011; Van Dijk & Geertsema 2004).

Several specimens of likely Plecoptera have been collected from the Middle Permian, dated 272–265 Ma (Prevec *et al.* 2022). These species are currently undescribed, but an assignment to stem-Plecoptera was proposed (Prevec *et al.* 2022).

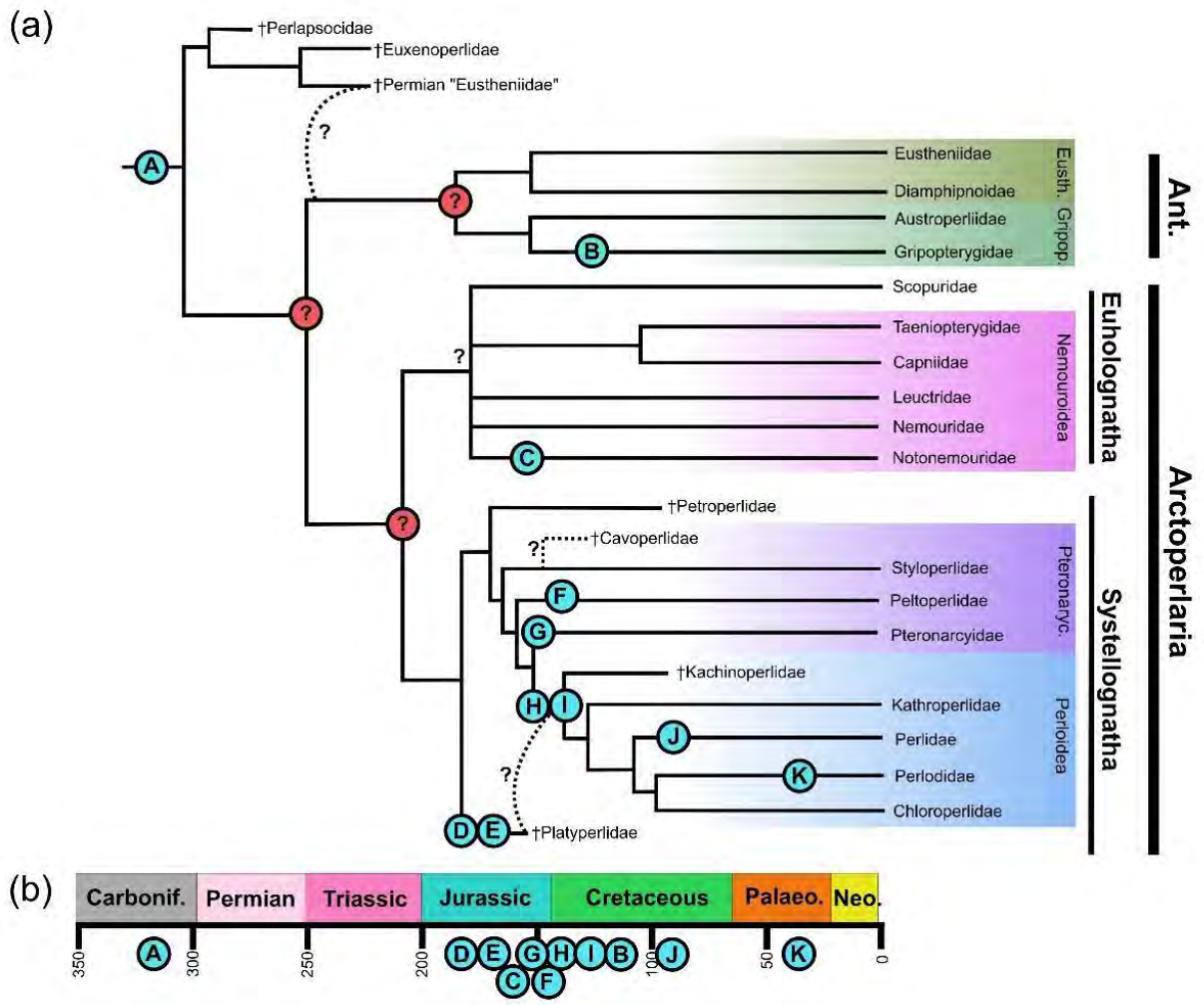

Figure 3.4: (a) Cladogram showing likely phylogenetic relationships of the fossil families reviewed here, mapped to the consensus phylogeny based on Letsch *et al.* (2021), South *et al.* (2021) and Garcia-Giron *et al.* (2024). Dotted lines represent the uncertain affinities of Southern Hemisphere Permian "Eustheniidae" to Antarctoperlaria, †Cavoperlidae to Pternonarcyioidea, and Platyperlidae to Perloidea respectively. Key fossil species that are recommended as calibration points for future phylogenetic studies are shown by blue circles. (b) Time scale, showing relative ages of key calibration points for the phylogeny of Systellognatha. Calibration points are as follows: **A:** First stem Plecoptera: †*Gulou carpenteri* Béthoux et al. (314.6–311.45 Ma) & †*Gulou oudardi* Schubnel et al. (314.6–306.95 Ma). **?**: Lack of clear fossil representatives of the earliest Antarctoperlaria and Arctoperlaria. **B:** First Gripopterygidae: †*Eodinotoperla duncanae* Jell & Duncan (122.46–112.6 Ma). **C**: First stem-Notonemouridae: †*Talbragaria australis* Sroka & Prokop (157.3–145.0 Ma). **D-E**: First stem-Systellognatha: **D**: †*Platyperla platypoda* Brauer *et al.* (183.0–155.7 Ma), **E**: †*Platyperla conferta* Sinitshenkova (171.6–164.7 Ma). **F**: First Peltoperlidae: †*Ecdyoperla fairlightensis* Sinitshenkova (145.5–140.2 Ma). **G**: First Pteronarcyidae: †*Pteroliriope sinitshenkovae* Cui et al. (164.7–155.7 Ma). **H**: †*Bestioperlisca inulta* Sinitshenkova, affinity to stem-Systellognatha or stem-Perloidea uncertain (150.8–145.5 Ma). **I:** First stem-Perloidea: †*Trianguliperla quassa* Sinitshenkova (140.2–125.45 Ma). J: First Perlidae (Subfamily Acroneuriinae): Several species from Myanmar, e.g. †*Lagusoperla acus* Chen *et al.* & †*Electroneuria ronwoodi* Sroka *et al.* (99.7–94.3 Ma) **K:** First Perlodidae (& *Isoperla*): *Isoperla †baltica* Jouault *et al.* (37.2–33.9 Ma).

Table 3.1: Recommended assignment of Plecoptera fossils from the Southern Hemisphere, and some related boreal fossils. New classifications denoted with *

Plecoptera

Species	Authority	Age (Ma)	G. Period
incertae sedis			
†*Boreoperlidium* Sinitshenkova, 2013*			
†*Boreoperlidium callopterus**	Sinitshenkova, 2018	268-265	Permian
†*Boreoperlidium cercus**	Sinitshenkova, 2018	268-265	Permian
†*Boreoperlidium ramificans**	Sinitshenkova, 2018	268-265	Permian
†*Boreoperlidium borealis**	Sinitshenkova, 2013	259-254	Permian
†*Afroperla* van Dijk & Geertsema, 2004			
†*Afroperla permiana*	van Dijk & Geertsema, 2004	254-252.3	Permian
†*Stenoperlidium* Tillyard, 1935 ?Stem Antarctoperlaria*			
†*Stenoperlidium anomala**	Tillyard, 1935	254-252.3	Permian
†*Stenoperlidium permianum**	Tillyard, 1935	254-252.3	Permian
†*Mesonotoperla* Riek, 1954 ?Stem Antarctoperlaria*			
†*Mesonotoperla sinuata**	Riek, 1954	241.45-232.85	Triassic
†Perlapsocidae Pinto and Piñeiro, 2000			
†*Perlapsocus* Pinto and Piñeiro, 2000			
†*Perlapsocus formosoi*	Pinto and Piñeiro, 2000	290.1-279.5	Permian
†Palaeonemouridae Sinitshenkova, 1987 (part)			
†*Ohionympha* Sinitshenkova, 1987*			
†*Ohionympha schopfi**	Carpenter, 1969	259-252.3	Permian
†Euxenoperlidae Sinitshenkova, 1987*			
†*Euxenoperla* Riek, 1973*			
†*Euxenoperla oliveri**	Riek, 1976b	254 to 252.3	Permian
†*Euxenoperla similis**	Riek, 1973	254 to 252.3	Permian
†*Euxenoperla simplex**	Riek, 1973	254 to 252.3	Permian
†*Euxenoperla clara**	Riek, 1976a	235 to 221.5	Triassic
†*Euxenoperlella* Riek 1976*			
†*Euxenoperlella jacquesi**	Riek, 1976b	254 to 252.3 Ma	Permian
†*Argentinoperlidium* Martins-Neto & Gallego, 2003*			
†*Argentinoperlidium rogersi**	Martins-Neto & Gallego, 2003	235-221.5	Triassic
†*Gondwanoperlidium* Pinto & Purper, 1978*			
†*Gondwanoperlidium argentina**	Pinto & Purper, 1978	235-221.5	Triassic
†*Gondwanoperlidium mendozensis**	Pinto & Purper, 1978	235-221.5	Triassic
†*Gondwanoperlidium triassicum**	Riek, 1956	221.5-205.6	Triassic
†Platyperlidae Sinitshenkova, 1982 (part) - ?Stem Antarctoperlaria			
†*Platyperla* Brauer *et al.*, 1889			
†*Platyperla marquati**	Gallego *et al.*, 2011	235-221.5	Triassic

Antarctoperlaria

Gripopterygidae Enderlein, 1909			
	†*Eodinotoperla* Jell & Duncan, 1986		
†*Eodinotoperla duncanae*	Jell & Duncan, 1986	122.46-112.6	Cretaceous

Arctoperlaria

Euholognatha

†**Paranotonemouridae Cui & Béthoux, 2019**[*]			
	†*Paranotonemoura* Cui & Béthoux, 2019[*]		
†*Paranotonemoura fidelis*[*]	Sinitshenkova, 1987	171.6-164.7	Jurassic
†*Paranotonemoura zwicki*[*]	Cui & Béthoux, 2019	164.7-155.7	Jurassic
Notonemouridae Ricker 1950 (part, stem-)			
†*Talbragaria australis*	Sroka & Prokop, 2023	157.3-145	Jurassic

3.3.1.2. †Euxenoperlidae

†Euxenoperlidae is represented in South Africa by five species from two genera, †*Euxenoperla* Riek and †*Euxenoperlella* Riek, described from the Upper Permian—Upper Triassic (Riek 1973, 1976a; b). Some additional †*Euxenoperla* are not assigned to any species owing to poor preservation of the main veins (Riek 1976b). Two additional genera, †*Argentinoperlidium* Martins Neto & Gallego (1 sp.) and †*Gondwanoperlidium* Pinto & Purper (4 spp.), are known from Argentina and Australia during the same period (Martins-Neto *et al.* 2003; Pinto & Purper 1978; Riek 1956). †Euxenoperlidae was originally placed, with some uncertainty, as a subfamily of the extant Antarctoperlarian Gripopterygidae based on similarities in wing venation (Riek 1973) but later shifted to its own family within Gripopterygoidea in "Gripopterygomorpha" (a polyphyletic assemblage roughly equivalent to Antarctoperlaria, but including Pteronarcyidae and Scopuridae) by Sinitshenkova (1987). This shift was made as the wing venation characters used by Riek (1976b) to diagnose the subfamily, such as a distinctly upturned R, a reduction in crossveins and widely separated posterior (CuP) and anterior (CuA) Cubitus, were not shared with any other "Gripopterygomorpha" families or genera (Sinitshenkova 1987).

Monophyly of Antarctoperlaria is supported by a modified sternal depressor muscle in the fore-trochanter, while Gripopterygoidea is supported by internal genital characters, namely a large accessory gland in males and the loss of the seminal vesicle in females (Zwick 2000). †Euxenoperlidae has only been described from wings (Martins-Neto *et al.* 2003; Pinto & Purper 1978; Riek 1973, 1976a; b), so none of these characters can be assessed in any Euxenoperlid specimens.

The wing venation characters that Riek (1973) used to place the family, namely widely separated CuP and CuA veins, and a reduction in crossveins, are unconvincing. While the CuP and CuA veins are widely spread, it is not to the degree commonly seen in extant Antarctoperlaria, and the distance between the two is comparable to that of the stem-Plecopteran †*Gulou carpenteri* Bethoux *et al.* Similarly, a reduction in crossvenation is common to many Plecoptera (Béthoux 2005; Béthoux *et al.* 2011), and may be a homoplasy correlated with body size and wing loading (Combes & Daniel 2003; Wootton 1981, 1990). Carpenter (1992) suggested there was insufficient evidence to support the familial placement of any of the included species, and Grimaldi & Engel (2005) placed the family in stem-Plecoptera as sister to Antarctoperlaria. Considering the lack of substantive evidence to resolve the relationships of †Euxenoperlidae, the family should be considered stem-Plecoptera (Table 3.1, Figure 3.4).

3.3.1.3. †Perlapsocidae

The placement of the †Perlapsocidae even within Plecoptera is uncertain (Pinto *et al.* 2000). Wing venation (M with two branches, CuA with more than three branches) provides evidence for its placement in the stem-Plecoptera.

3.3.1.4. †Palaeonemouridae

†Palaeonemouridae, a large Permian family, is represented in the Southern Hemisphere by a single nymph from Antarctica, †*Ohionympha schopfi* Carpenter. Nymphs are placed in this family based on characters such as morphometric proportions in the wing pads, legs, abdominal segments, and pronotum (Sinitshenkova 1987).

Sinitshenkova places this family within “Nemourina” (equivalent to Nemouroidea Billberg), based on the lack of crossveins in the distal region of the wing (Figure 3.5) and long first and third tarsomeres, compared to subequal, moderate or shortened first two tarsomeres in Antarctoperlaria and Systellognatha (Nelson 2009; Sinitshenkova 1987). Both characters are derived, as they differ from the stem-group species †*G. carpenteri* (Béthoux *et al.* 2011), and are regularly used in keys [see for example: (DeWalt & Vincent 2015; Fenoglio *et al.* 2021; Zwick 2004)]. A long first tarsomere is a synapomorphy of most Nemouroidea (Nelson 2009), but can be inconsistent for identification; Gripopterygidae also regularly have long first tarsomeres (McLellan & Zwick 2007; Nelson 2009; Sinitshenkova 1987), and all three are the same size in Taeniopterygidae Klapalek (Fenoglio *et al.* 2021).

The combination of both characters together provides at least circumstantial evidence that †Palaeonemouridae are stem-Euholognatha. However, the assignment of †*O. schopfi* to this family, or

even stem Euholognatha, is unsupported as the fossilised nymph lacks wing venation and preserved tarsi.

3.3.1.5. †Platyperlidae (part)

Sinitshenkova (1987, 2002) and Grimali & Engel (2005) both consider †Platyperlidae, a predominantly Jurassic family (ages between 201.6–130.0 Ma), as a stem representative of the Systellognatha. This family is known only from nymphs that inhabited montane lakes (Gallego *et al.* 2011; Sinitshenkova 1985, 1987, 2002). Their placement is well supported; some species have a short first tarsomere (Brauer *et al.* 1889; Gallego *et al.* 2011; Sinitshenkova 1985, 1987), and at least the nymphs of †*Platyperla platypoda* Brauer *et al*. show adaptations to carnivory (Sinitshenkova 1985, 2002). As mentioned above, proportions of tarsomeres can be unreliable, but a short basal tarsomere is likely a homoplasious apomorphy of Systellognatha and some Antarctoperlaria, and was present in some of the earliest representatives of the infraorder (Nelson 2009; Sroka *et al.* 2018). A short-moderate basal tarsomere was independently derived in Antarctoperlaria (Avelino-Capistrano *et al.* 2018; McLellan & Zwick 2007; Nelson 2009). However, as Antarctoperlaria (or at least its stem relatives) is speculated to have become limited to the Southern Hemisphere by the early–mid Jurassic (Ding *et al.* 2019; García-Girón *et al.* 2024; Illies 1965; Letsch *et al.* 2021; McCulloch *et al.* 2016; Zwick 2000), boreal †Platyperlidae are almost certainly Systellognathan. Carnivory is an autapomorphy of the Perloidea (Perlodidae + Perlidae + Chloroperlidae + Kathroperlidae) (Zwick 2000), which may suggest an affinity with this superfamily. However, the very different habitat preferences and omnivorous diet of some †Platyperlid species (Sinitshenkova 1985) raise some doubt, as they may represent an extinct sister or transitionary form to the Perloidea rather than a crown group member.

The placement of the only representative in the Southern Hemisphere, †*Platyperla marquati* Gallego et al. (235–221.5 Ma), within this lineage is doubtful because it is assigned to †Platyperlidae on the basis of expanded, flat femora, and its discovery in a fluvial–to–lacustrine deposit (Gallego *et al.* 2011). The tarsomeres were not preserved, and while a carnivorous lifestyle is suggested by the gut contents and mouthpart structure, the head differs from the Perloidea, as the frons and clypeus are clearly divided by a suture in †*P. marquati*, instead of being fused (Zwick 2004). Carnivory is not limited to Systellognatha and occurs in some Antarctoperlaria such as Eustheniidae (Zwick 2000). In addition, †*Platyperla marquati* shows a significant reduction in the size of its wing pads, a state shared with †*Platypoda caudiculata* Sinitshenkova. However, wing reductions are common across Plecoptera, including Antarctoperlaria, and have evolved independently multiple times (McCulloch *et al.* 2019, 2022). This suggests a greater affinity of this species with the stem group of Antarctoperlaria than with Systellognatha. However, in the absence of any autapomorphies of this suborder or its

component superfamilies, this cannot be confidently concluded. It is also possible that this species instead belongs to †Euxenoperlidae, which was present across the Southern Hemisphere concurrently, but the nymphal stage of this family has not yet been described.

3.3.1.6. Eustheniidae

Eustheniidae fossils are known from the Southern Hemisphere by two genera, †*Stenoperlidium* Tillyard and †*Mesontoperla* Riek. This placement was supported by similarities in the wing and nymph of †*Stenoperlidium* to the extant *Stenoperla* McLachlan (Tillyard 1935), and the presence of many crossveins throughout the wing in †*Mesontoperla* (Riek 1954). The assignment of both ancient genera (Permian and Triassic, respectively) to an extant family was considered uncertain by the authors, and is still questioned (Aristov *et al.* 2013; Carpenter 1992; Grimaldi & Engel 2005; Riek 1954; Tillyard 1935). Interestingly, Eustheniidae is one of the few families with an autapomorphy in the wing: the posterior margin of the hind wing is smoothly rounded, without a notch separating the anal fan from the remigium (Zwick 1979, 2000). Unfortunately, the hind wing is not preserved, or this character is not visible, in all fossil representatives of this family including the Northern Hemisphere †*Boreoperlidium* Sinitshenkova (Aristov *et al.* 2013; Riek 1954; Sinitshenkova 2018; Tillyard 1935). Extensive crossvenation is plesiomorphic (Figure 3.5) to the order (Béthoux *et al.* 2011) and the character state "CuA reaching wing margin in three branches", suggested by Aristov et al. (2013), cannot be accepted as synapomorphic because it differs in many extant representatives of the family (Tillyard 1935). Furthermore, all †*Boreoperlidium* fossils have very few or no crossveins in the (ScP+) RA – RP field (Aristov *et al.* 2013; Sinitshenkova 2018). The loss of crossveins in this field is an autapomorphy of Arctoperlaria (Cui *et al.* 2015), making the assignment to Eustheniidae highly unlikely. Instead, a narrow space between the ScP and anterior margin of the wing, and many crossveins in the basal region of the costal field both support an assignment to stem-Systellognatha (Cui *et al.* 2015). Therefore, the placement of none of these genera in Eustheniidae can be supported.

Nevertheless, the wings of †*Stenoperlidium* and †*Mesontoperla* are similar to those of extant Eustheniidae. The distal half of the wing has extensive crossvenation, including in the posterior Radius (RP) field. In †*Mesontoperla*, the poorly preserved CuA appears to join the wing margin distally. These are both potentially homoplasious characters, but nevertheless provide some evidence for the placement of these genera among stem-Antarctoperlaria. The same cannot be said for †*Boreoperlidium*, in which neither is present. Tillyard (1935) described the nymph of †*Stenoperlidium* with simple abdominal gills. These gills are not clear in the published photograph, and the validity of this character cannot be assessed. If these gills are indeed present, they are an autapomorphy of Eusthenioidea (Avelino-Capistrano *et al.* 2018; Zwick 2000).

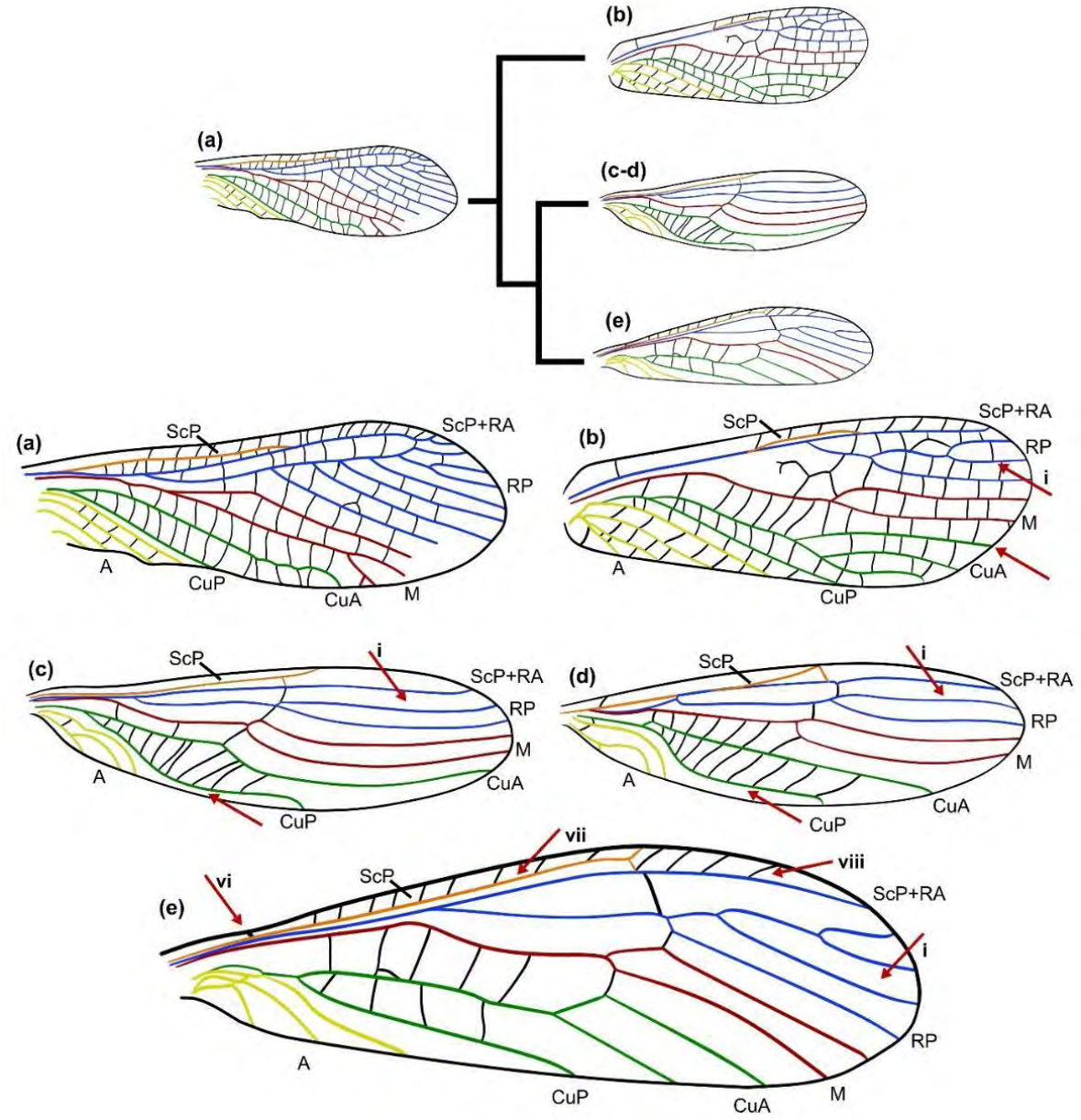

Figure 3.5: Differences in the forewing venation pattern of Plecoptera. Wing veins are as follows: Posterior Subcosta (ScP): orange, Anterior (RA) and Posterior (RP) Radius: blue, Media (M): red, Anterior (CuA) and Posterior (CuP) Cubitus: green, Analis (A): yellow. (a): Stem Plecoptera: Gulouidae, *Gulou Carpenteri* Béthoux *et al.* Diagram of wing modified from Figure 2H in Béthoux *et al.* (2011). (b) Antarctoperlaria: Eustheniidae, *Eusthenia costalis* Banks, showing the presence of crossveins in the (ScP +) RA - RP field (Lost in Arctoperlaria), and a distally terminating CuA (arrowed). (c) Arctoperlaria, Euholognatha: Notonemouridae, *Afronemoura amatolae* Balinsky, showing the loss of crossveins in the (ScP +) RA - RP field (arrowed), and the CuP approaching the hind margin of the wing, before turning away and rejoining distally (arrowed). (d) Arctoperlaria, Euholognatha: Leuctidae, *Leuctra despaxi* Mosely, showing the same venation characters as Notonemouridae. (e) Arctoperlaria, Systellognatha: Perlidae, *Neoperla burgeoni* Navás, showing the short, obliquely opposed first crossvein between ScP and anterior margin (arrowed), the narrow area between ScP and the anterior margin (arrowed), numerous crossveins in the costal field (arrowed), and the absence of crossveins in the (ScP +) Ra - RP field. Labels on arrows correspond to relevant characters in Appendix 3.1.

3.3.1.7. Notonemouridae

The first fossil of a putative Notonemouridae in the Southern Hemisphere is †*Talbragaria australis* Sroka & Prokop. This family lacks accepted apomorphies, and until recently, its monophyly was considered uncertain (Cui *et al.* 2019; McLellan 1972, 1991; Sroka & Prokop 2023; Zwick 2000, 2006). Cui et al. (2019) erected the Northern Hemisphere family †Paranotonemouridae Cui & Béthoux as a stem sister to Notonemouridae based on wing venation. The characteristic "Nemourid X" wing vein pattern, which is present in some Notonemouridae and in its putative sister, Nemouridae, is also shared with this family. This is plesiomorphic; it is the remains of an ancient crossvein condition (Zwick 2000), and is present in other Euholognatha. Cui et al. (2019) separated the Notonemouridae lineage from Nemouridae by the shape of the CuP vein, which approaches the wing margin, diverges, and rejoins distally in Notonemouridae. While this shape does differ between the families, a similar pattern is present in at least some Leuctridae (e.g. Figure 3.5; pers. obs.; Fig. 7 *in* Béthoux 2005). As the sister relationship of Notonemouridae and Nemouridae is uncertain (García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016), this vein pattern may be the remnants of a plesiomorphic condition, an independently derived homoplasy, or a synapomorphy of these two families. Therefore, the assignment of †Paranotonemouridae to stem-Notonemouridae must remain uncertain. Sroka & Prokop (2023) used the same characters to assign †*T. australis* to Notonemouridae. In this case, the above characters are synapomorphies of members within Euholognatha. As Notonemouridae is the only Euholognathan family in the Southern Hemisphere, it is most likely this fossil represents at least a stem relative of Notonemouridae.

3.3.1.8. Gripopterygidae and Austroperlidae

†*Eodinotoperla duncanae* Jell & Duncan clearly belongs to Gripopterygidae (Jell 2004; Jell & Duncan 1986), as evidenced by the autapomorphic anal rosette of fine gills (Zwick 2000).

A possible Austroperlid fossil species from the Early Miocene was recently discovered, but remains undescribed (Kaulfuss *et al.* 2015; Lee *et al.* 2016).

3.3.2. Stem-Systellognatha

Systellognatha, or "Perlomorpha", fossils are relatively common, with 87 species from 47 genera and 10 families found predominantly in the Northern Hemisphere (Table 3.2 & 3.3). Currently, fossilised species are assigned to five of the seven extant families in Systellognatha, with only Styloperlidae and Kathroperlidae not yet represented.

Table 3.2: Recommended assignment of Plecoptera fossils with no clear affinities to any Crown Plecoptera Sub-Orders. Descriptions of species marked with [#] were not available for this review. New classifications denoted with [*]

Species	Authority	Age (Ma)	G. Period
Ephemeroptera			
†Platyperlidae Sinitshenkova 1982 (part)			
	†*Platyperla* Brauer *et al.*, 1889		
†*Platyperla kingi*[*]	Ping, 1935	161.2-145.5	Jurassic
Plecoptera			
incertae sedis			
	†*Tungussonympha* Sinitshenkova, 1987[*]		
†*Tungussonympha meyeni*[*]	Sinitshenkova, 1987	254-252.3	Triassic
	†*Berekia* Sinitshenkova, 1987[*]		
†*Berekia neglecta*[*]	Sinitshenkova, 1987	221.5-205.6	Triassic
	†*Trianguliperla* Sinitshenkova, 1985 (part)[*]		
†*Trianguliperla aequalis*[*]	Sinitshenkova, 1987	221.5-205.6	Triassic
†*Trianguliperla innoxia*[*]	Sinitshenkova, 1987	221.5-205.6	Triassic
†*Trianguliperla orbiculata*[*]	Sinitshenkova, 1985	167.7-164.7	Jurassic
†*Trianguliperla attenuata*[*]	Sinitshenkova, 1990	161.2-155.7	Jurassic
†*Trianguliperla optanda*[#, *]	Sinitshenkova, 1995	150.8-145.5	Jurassic
†*Trianguliperla limosa*[#, *]	Sinitshenkova, 1998	130.0-125.45	Cretaceous
†*Trianguliperla volucris*[#, *]	Sinitshenkova, 1998	130.0-125.45	Cretaceous
	†*Triassoperla* Lin, 1977[*]		
†*Triassoperla yongrenensis*[#, *]	Lin, 1977	221.5-205.6	Jurassic
	†*Perlomimus* Sinitshenkova, 1985[*]		
†*Perlomimus multus*[*]	Sinitshenkova, 1985	161.2-155.7	Jurassic
	†*Pectinoperla* Sinitshenkova, 1987[*]		
†*Pectinoperla notabilis*[*]	Sinitshenkova, 1987	150.8-145.5	Jurassic
†Tshekardoperlidae Sinitshenkova 1987[*]			
	†*Sylvoperlodes* Sinitshenkova, 1987[*]		
†*Sylvoperlodes zhiltzovae*[*]	Sinitshenkova, 1987	279.5-272.5	Permian
	†*Tshekardoperla* Sinitshenkova, 1987[*]		
†*Tshekardoperla depicta*[*]	Sinitshenkova, 1987	279.5-272.5	Permian
†*Tshekardoperla expulsa*[*]	Sinitshenkova, 1987	279.5-272.5	Permian
†*Tshekardoperla squarrosa*[*]	Sinitshenkova, 1987	279.5-272.5	Permian
	†*Issadoperla* Sinitshenkova, 2013[*]		
†*Issadoperla permiana*[*]	Sinitshenkova, 2013	259-254	Permian
†Palaeoperlidae Sharov 1961			
	†*Palaeoperla* Sharov, 1961		
†*Palaeoperla exacta*	Sharov, 1961	272.5-268	Permian
†*Palaeoperla prisca*	Sharov, 1961	272.5-268	Permian
†*Palaeoperla perfracta*	Sinitshenkova, 1987	265-259	Permian
	†*Kargaloperla* Sinitshenkova, 1987		

†*Kargaloperla fibrosa*	Sinitshenkova, 2018	268-265	Permian
†*Kargaloperla grata*	Sinitshenkova, 2018	268-265	Permian
†*Kargaloperla postica*	Sinitshenkova, 2018	268-265	Permian
†*Kargaloperla queata*	Sinitshenkova, 2018	268-265	Permian
†*Kargaloperla exuperata*	Sinitshenkova, 1987	265-259	Permian
†*Kargaloperla avulsa*	Sinitshenkova, 1987	259-254	Permian
†*Kargaloperla decipiens*	Sinitshenkova, 2013	259-254	Permian
†*Kargaloperla furcata*	Sinitshenkova, 2013	259-254	Permian
	†*Properla* Sharov, 1961		
†*Properla incrassata*	Sinitshenkova, 2018	268-265	Permian
†*Properla umbrosa*	Sinitshenkova, 2018	268-265	Permian
†*Properla issadensis*	Sinitshenkova, 2013	259-254	Permian
†*Properla tungussica*	Sharov, 1961	254-252.3	Permian
	†*Permoleuctropsis* Martynov, 1937		
†*Permoleuctropsis cracilis*	Martynov, 1937	265-259	Permian
†Platyperlidae Sinitshenkova 1982 (part)[*]			
	†*Platyperla* Brauer *et al*., 1889		
†*Platyperla rigida*[*]	Sinitshenkova, 1987	201.6-161.2	Jurassic
†*Platyperla caudiculata*[*]	Sinitshenkova, 1985	183-175.6	Jurassic
†*Platyperla admissa*[*]	Sinitshenkova, 1987	175.6-171.6	Jurassic
†*Platyperla propera*[*]	Sinitshenkova, 1987	171.6-164.7	Jurassic
†*Platyperla mendosa*[#, *]	Sinitshenkova, 1995	150.8-145.5	Jurassic
†*Platyperla parricidalis*[*]	Sinitshenkova, 1990	136.4-130.0	Cretaceous

Table 3.3: Recommended assignment of Plecoptera fossils within Systellognatha. New classifications denoted with [*]

Plecoptera Burmeister, 1839

Systellognatha Enderlein, 1909

incertae sedis

Species	**Authority**	**Age (Ma)**	**G.Period**
	†*Perlisca* Sinitshenkova, 1985		
†*Perlisca aufuga*	Sinitshenkova, 1985	183-175.6	Jurassic
	†*Chloroperloides* Sinitshenkova, 1985		
†*Chloroperloides fusiformis*	Sinitshenkova, 1985	161.2-150.8	Jurassic
	†*Bestioperlisca* Sinitshenkova, 1990		
†*Bestioperlisca inulta*	Sinitshenkova, 1990	150.8-145.5	Jurassic
	†*Dipsoperla* Sinitshenkova, 1987[*]		
†*Dipsoperla kunikanensis*[*]	Sinitshenkova, 1990	150.8-145.5	Jurassic
†*Dipsoperla serpentis*[*]	Sinitshenkova, 1987	150.8-145.5	Jurassic
	†*Isoperlodes* Sinitshenkova, 1992[*]		
†*Isoperlodes perstrictus*[*]	Sinitshenkova, 1992	150.8-145.5	Jurassic
	†*Perlitodes* Sinitshenkova, 1987		
†*Perlitodes aenigmaticus*	Sinitshenkova, 1987	150.8-145.5	Jurassic

†*Perlitodes borealis*	Sinitshenkova, 1992	150.8-145.5	Jurassic
	†*Savina* Sinitshenkova, 1987		
†*Savina laeta*	Sinitshenkova, 1987	150.8-145.5	Jurassic
	†*Sinosharaperla, Liu et al.* 2007		
†*Sinosharaperla zhaoi*	Liu *et al*., 2007	125.45-122.46	Cretaceous
†Platyperlidae Sinitshenkova 1982 (part)			
	†*Platyperla* Brauer *et al*., 1889		
†*Platyperla platypoda*	Brauer *et al*., 1889	183-155.7	Jurassic
†*Platyperla conferta*	Sinitshenkova, 1985	171.6-164.7	Jurassic
†Petroperlidae Sroka et al. 2018			
	†*Branchioperla* Sroka & Staniczek, 2019		
†*Branchioperla ianstewarti*	Stroka & Staniczek, 2019	99.7-94.3	Cretaceous
	†*Lapisperla* Sroka *et al*., 2018		
†*Lapisperla keithrichardsi*	Stroka *et al*., 2018	99.7-94.3	Cretaceous
	†*Petroperla* Sroka *et al*., 2018		
†*Petroperla mickjaggeri*	Stroka *et al*., 2018	99.7-94.3	Cretaceous
†Crossoperlidae Chen, 2025			
	†*Crossoperla* Chen, 2025		
†*Crossoperla teslenkoae*	Chen, 2025	100.5 - 93.9	Cretaceous
†Cavoperlidae Chen, 2023			
	†*Cavoperla* Chen, 2023		
†*Cavoperla excavata*	Chen, 2023	164.7 to 155.7	Jurassic
Pteronarcyidae Newman, 1853 (part)			
	†*Pteroliriope* Cui *et al*., 2016		
†*Pteroliriope sinitshenkovae*	Cui *et al*., 2016	164.7 to 155.7	Jurassic
Peltoperlidae Claassen 1931 (Part)			
	†*Ecdyoperla* Sinitshenkova, 1998*		
†*Ecdyoperla fairlightensis**	Sinitshenkova, 1998	145.5-140.2	Cretaceous
	†*Siberiopelta* Sinitshenkova & Yan, 2024		
†*Siberiopelta bashkuevi*	Sinitshenkova & Yan, 2024	121.4 - 113.2	Cretaceous
	†*Borisoperla* Chen & Xu, 2020		
†*Borisoperla kondratieffi*	Chen & Xu, 2020	99.7-94.3	Cretaceous
	†*Dewaltoperla* Chen, 2023		
†*Dewaltoperla edwardi*	Chen, 2023	99.7-94.3	Cretaceous
	†*Zwickoperla* Chen & Wang, 2020		
†*Zwickoperla brevicauda*	Chen & Wang, 2020	99.7-94.3	Cretaceous

Perloidea Latreille, 1802

incertae sedis

†*Trianguliperla* Sinitshenkova, 1985 (part)*

†*Trianguliperla quassa*[*]	Sinitshenkova, 1987	140.2-125.45	Cretaceous
	†*Derancheperla* Sinitshenkova, 1990[*]		
†*Derancheperla collaris*[*]	Sinitshenkova, 1990	125.45-122.46	Cretaceous
	†*Burmacroneuria* Chen, 2019[*]		
†*Burmacroneuria projecta*[*]	Chen, 2019	99.7-94.3	Cretaceous
	†*Pinguisoperla* Chen, 2018[*]		
†*Pinguisoperla yangzhouensis*[*]	Chen, 2018	99.7-94.3	Cretaceous
	†*Starkoperla* Chen & Wang, 2020[*]		
†*Starkoperla longusocollum*[*]	Chen & Wang, 2020	99.7-94.3	Cretaceous
†Kachinoperlidae Chen 2022			
	†*Kachinoperla* Chen, 2022		
†*Kachinoperla zwicki*	Chen, 2022	99.7-94.3	Cretaceous
Perlodidae Klapálek, 1909 (Part)			
	Isoperla Banks, 1906		
Isoperla †*baltica*	Jouault *et al*., 2021	37.2-33.9	Palaeogene
Perlidae Latreille 1802 (Part)			
	†*Euperlida* Cifuentes-Ruiz, 2007		
†*Euperlida parvicercifera*	Cifuentes-Ruiz, 2007	33.9-23.03	Palaeogene
Acroneuriinae Klapálek 1914			
	†*Burmaperla* Jouault & Nel, 2021		
†*Burmaperla pouilloni*	Jouault & Nel, 2021	99.7-94.3	Cretaceous
	†*Burmesoperla* Chen, 2019		
†*Burmesoperla expansa*	Chen, 2019	99.7-94.3	Cretaceous
	†*Cretacroneuria* Chen, 2020		
†*Cretacroneuria angularis*	Chen, 2020	99.7-94.3	Cretaceous
	†*Electroneuria* Sroka *et al.*, 2018		
†*Electroneuria ronwoodi*	Sroka *et al*., 2018	99.7-94.3	Cretaceous
	†*Largusoperla* Chen, 2018		
†*Largusoperla acus*	Chen *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla arcus*	Chen *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla billwymani*	Sroka *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla borisi*	Chen 2018	99.7-94.3	Cretaceous
†*Largusoperla brianjonesi*	Sroka *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla charliewattsi*	Sroka *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla crassus*	Chen, 2018	99.7-94.3	Cretaceous
†*Largusoperla dewalti*	Chen, 2018	99.7-94.3	Cretaceous
†*Largusoperla difformitatem*	Chen, 2018	99.7-94.3	Cretaceous
†*Largusoperla flata*	Chen, *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla micktaylori*	Sroka *et al*., 2018	99.7-94.3	Cretaceous
†*Largusoperla reni*	Chen & Wang, 2019	99.7-94.3	Cretaceous
	†*Dominoperla* Stark & Lentz, 1992		
†*Dominiperla antigua*	Stark & Lentz, 1992	20.4-13.7	Neogene
Perlinae Latreille, 1802			
	Perla Geoffroy, 1792 (part)		

"Perla" †prisca	Pictet, 1856	37.2.9-33.9	Palaeogene

3.3.2.1. Incertae sedis

†*Sinosharaperla zhaoi* Liu *et al*. was originally placed within "Gripopterygomorpha", but was since moved to Systellognatha due to the lack of crossveins in the distal half of the posterior Subcosta (ScP) + R – RP area (autapomorphy: Arctoperlaria), an opposed, strong first vein between the anterior margin of the wing and ScP (autapomorphy: Systellognatha), and extensive branching of the CuA (autapomorphy: Systellognatha) (Figure 3.5; Cui *et al.* 2015). Further affinities within Systellognatha are uncertain (Cui *et al.* 2015; Sroka *et al.* 2018). "†A*rchaeoperla rarissimus"* Liu *et al.*, which was synonymized with †*S. zhaoi* (Cui *et al.* 2015; Sroka *et al.* 2018), was originally assigned to the tribe Neoperlini as it had hemitergal lobes and a process on tergite 7 (Liu *et al.* 2008). This reason is insufficient. Neoperlini is diagnosed by a sclerotized aedeagus base (Sivec *et al.* 1988; Zwick 2023; Zwick & Zwick 2023), which cannot be assessed in the fossil. While a process on T7 is an autapomorphy of *Neoperla*, this structure is unclear in †*S. zhaoi*. No other apomorphy of *Neoperla*, or its sister genera, is present. Modified hemitergal lobes with anteriorly upturned hooks are an autapomorphy of the subfamily Perlinae (Sivec *et al.* 1988; Zwick 2000, 2023). The hemitergal lobes in †*S. zhaoi* are not noticeably modified into upwardly turned hooks, precluding its inclusion in crown group Perlidae (Cui *et al.* 2015; Sroka *et al.* 2018).

A large number of fossil families and genera are currently placed in Sinitshenkova's "Perlomorpha" (Sinitshenkova 1987, 2002; Zwick 2000). While this roughly corresponds to Systellognatha, it is paraphyletic; Pteronarcyidae is excluded, and the inclusion of Peltoperlidae makes the Perloidea polyphyletic (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; Zwick 2000). Sinitshenkova (1987) diagnosed this group using variable or plesiomorphic wing venation characters: rich wing venation in large forms, reduced wing venation in small forms, and a multibranched CuA. Wing venation is strongly linked to insect flight patterns and wing loading, as changes to the structure of main veins and the complexity of crossveins affect the support and flexibility of wings during flight (Wootton 1981). Larger insects tend to have stiffer wings, regardless of their phylogenetic position (Combes & Daniel 2003). This stiffness is often provided by increased crossvenation (Wootton 1990), suggesting that the richness of veins in "Perlomorpha" is likely a homoplasy related to body size, rather than an independently derived autapomorphy.

With few exceptions, recognized apomorphies of the Perloidea, Pteronarcyoidea and extant families are not preserved in "Perlomorpha" species, but most species do have a short basal tarsomere. For this reason, most of these fossils should be placed within stem-Systellognatha, as their relationships with the crown group are unresolved. This applies to †*Bestioperlisca* Sinitshenkova, †*Chloroperloides*

Sinitshenkova, †*Perlisca* Sinitshenkova, †*Perlitodes* Sinitshenkova, and †*Savina* Sinitshenkova. †*Bestioperlisca* shows clear adaptations to carnivory, such as narrow, sharp-toothed mandibles, and sharpened galea and lacinia, supporting a possible assignment to the Perloidea (Sinitshenkova 1990). However, the galea and lacinia are "lance-like", which is usually seen in herbivorous or detritivorous Plecoptera (Sinitshenkova 1990). This combination of mouthpart types suggest that †*Bestioperlisca* was omnivorous (Sinitshenkova 1990), and makes its possible relationship to Perloidea ambiguous. The following genera do not have sufficient evidence to safely place them even within Systellognatha: †*Berekia* Sinitshenkova, †*Perlomimus* Sinitshenkova, †*Pectinoperla* Sinitshenkova, †*Trianguliperla* Sinitshenkova (in part; exception below), and †*Tungussonympha* Sinitshenkova. Unfortunately, I was unable to obtain the original descriptions of †*Trianguliperla optanda* Sinitshenkova, †*Trianguliperla limosa* Sinitshenkova, †*Trianguliperla volucris* Sinitshenkova and †*Triassoperla yongrenensis* Lin for this review. As the character states could not be assessed, they are treated as Plecoptera *incertae sedis* here.

3.3.2.2. †Tshekardoperlidae and †Palaeoperlidae

The placements of †Tshekardoperlidae Sinitshenkova and †Palaeoperlidae Sharov are uncertain. While many of the component species are figured with a short basal tarsomere (Aristov *et al.* 2013; Sinitshenkova 1987, 2018), this is the only apomorphy present in both families. Wing venation in †Palaeoperlidae retained the plesiomorphic condition. As these families are much older than the other stem-Systellognatha discussed here (limited to the Permian vs. Jurassic and Cretaceous), it is safer to not assign these families to the crown group, although it is possible that they represent a very early stem-ancestor of Systellognatha.

3.3.2.3. †Platyperlidae (part)

The position of †Platyperlidae as stem-Systellognatha is discussed above. As several characteristics, such as tarsal proportions, vary between species, it is likely that this family is polyphyletic.

†*Platyperla kingi* Ping is unlikely to be a Plecoptera at all. It is drawn with at least four tarsomeres instead of three and has tracheated, oval gills on the first eight abdominal segments (Ping 1935). This gill structure is not seen in any Plecoptera, and rather suggests that this species is an Ephemeropteran.

Only †*P. platypoda* and †*P. conferta* Sinitshenkova can confidently be assigned to stem-Systellognatha due to their sharply-toothed mandibles providing evidence of carnivory and a short basal tarsomere (Sinitshenkova 1985).

3.3.2.4. †Petroperlidae

†Petroperlidae Sroka *et al.* is an extinct, independent line of stem-Systellognatha (Sroka *et al.* 2018; Sroka & Staniczek 2020). This classification is supported by several apomorphies; the wing venation characters discussed above and a short basal tarsomere are both present, and there are setae on the arolium (Nelson 2009; Sroka *et al.* 2018; Sroka & Staniczek 2020). However, autapomorphies of both superfamilies are absent (Sroka *et al.* 2018; Sroka & Staniczek 2020).

3.3.2.5. †Crossoperlidae

†Crossoperlidae Chen was only described this year, and is known from a single species, *Crossoperla teslenkoae* Chen (Chen 2025). Its placement within stem-Systellognatha is supported by a short first tarsomere, the presence of euplantulae, and a multibranched CuA (Chen 2025; Cui *et al.* 2016; Nelson 2009). However, the family lacks apomorphies of any of the seven extant families, preventing its assignment to the crown group (Chen 2025).

3.3.3. Pteronarcyoidea

3.3.3.1. †Cavoperlidae

†*Cavoperla excavata* Chen forms the monotypic family †Cavoperlidae Chen and is considered sister to Styloperlidae, due to similarities in wing venation, the loss of giant tibial spurs, and elongated basal cercomeres in the males (Chen 2023b). However, no autapomorphies of Styloperlidae are preserved, such as a dense setal brush on sternite nine in males, an X-shaped sclerotization of sternite ten, or modified tibial setae (Chen 2023b; Uchida & Isobe 1989; Zwick 2000). Contrarily, the laterally expanded arolium is an autapomorphy of Pteronarcyidae (Uchida & Isobe 1989; Zwick 2000). However, as other apomorphies of all families within Pteronarcyoidea are not present in this species, its relationship to any of the extant families cannot be confidently resolved (Chen 2023b).

3.3.3.2. Pteronarcyidae

The monophyly of the superfamily Pteronarcyoidea has recently been contradicted by molecular data that place Pteronarcyidae as sister to Perloidea (Figure 3.3c; García-Girón *et al.* 2024; Letsch *et al.* 2021). Pteronarcyidae is represented by a single fossil species, †*Pteroliriope sinitshenkovae* Cui *et al.* This species clearly belongs to Systellognatha as it shares most of the wing venation characters of †*S. zhaoi*, but differs in having extensive crossvenation throughout all wings (Cui *et al.* 2016). As discussed above, this character is present in the ground-plan of Plecoptera and Antarctoperlaria (Béthoux *et al.* 2011; Cui *et al.* 2015, 2016) and seems to have reappeared independently in several

lines within Systellognatha, including Pteronarcyidae and Perlidae (Cui *et al.* 2016), possibly because it is correlated with body size and wing loading (Wootton 1981, 1990). †*Pteroliriope sinitshenkovae* is a large species (forewing length >30mm), and it is possible that the increased wing venation is associated with this, rather than phylogenetic affinities. Although no clear autapomorphies of Pteronarcyidae are preserved, close morphometric similarities with extant *Pteronarcys* Newman, and the presence of wing venation characters [M occasionally with more than two branches, numerous crossveins between M and CuA, crossveins between branches of Anterior Analis 2 (AA2)] seen only in Pteronarcyidae amongst Systellognatha provide convincing evidence for this placement (Cui *et al.* 2016).

3.3.3.3. Peltoperlidae

Peltoperlidae is known from five genera, most of which are preserved in Burmese amber from the same locality in Myanmar (Chen 2023a; Chen & Wang 2020; Chen & Xu 2020). The placement of the three Burmese genera is supported by a shortened head inserted into a strongly angular prothorax, which is an autapomorphy of the family (Uchida & Isobe 1989; Zwick 2000). In addition, the "Perlomorpha" †*Ecdyoperla fairlightensis* Sinitshenkova clearly belongs to this family because of the autapomorphic "cockroach-like" body shape of the nymph (Uchida & Isobe 1989; Zwick 2000). Uncertainties regarding the number of ocelli and fusion of the basal cercomeres (Sinitshenkova 1998) do not necessarily contradict this assignment, as these are autapomorphies of the subfamily Peltoperlinae rather than the family as a whole (Zwick 2000). Finally, a new species, †*Siberiopelta bashkuevi* Sinitshenkova & Yan, was described last year, and can also be placed into this family due to the autapomorphic "cockroach-like" body shape (Sinitshenkova & Yan 2024).

3.3.4. Perloidea

3.3.4.1. Incertae sedis

†*Trianguliperla* is almost certainly polyphyletic. The genus is described from the Triassic—Late Cretaceous and varies significantly in morphology throughout that time (Sinitshenkova 1985, 1987, 1990). Apomorphies of Systellognatha are not preserved, except in the nymph of †*Trianguliperla quassa* Sinitshenkova, which likely belongs to the Perloidea. This species has a small basal tarsomere, sharply toothed lacinias, and long, slender palpi (Sinitshenkova 1987). The latter two states are established autapomorphies of Perloidea (Zwick 2000).

3.3.4.2. †Kachinoperlidae

The monotypic family †Kachinoperlidae Chen should be assigned to the stem-Perloidea. This assignment is strongly supported by slender mandibles lacking a mola, long sharply-pointed lacinias, and slender palps (Chen 2022), which all suggest a predatory lifestyle. Subequal glossae and paraglossae prevent the inclusion of †Kachinoperlidae among any extant Perloidea families, but the lack of these synapomorphies does not preclude inclusion of the family amongst stem-Perloidea.

3.3.4.3. Chloroperlidae

Chloroperlidae fossils are known from a single genus, †*Dipsoperla* Sinitshenkova. This genus was assigned to Chloroperlidae based on short wing pads (Sinitshenkova 1987); no other characteristic features of the family or its subfamilies (e.g., minute terminal palpomere, oval prothorax; Zwick 2000, 2006) were preserved. Mouthparts are not figured or described, and it is unclear whether the nymphs were carnivorous. Some morphometric similarity supports a close affiliation to Chloroperlidae, including cerci shorter than the abdomen and a slender body shape, but without more conclusive evidence, this genus must be placed in Systellognatha *incertae sedis*.

3.3.4.4. Perlodidae

Perlodidae fossils are known from two extinct Jurassic genera and two extant Palaeogene genera. Unfortunately, this family lacks established apomorphies and is generally supported by variable characters, such as colouration, that are not visible in many fossils (Zwick 2000). The nymph of †*Isoperlodes perstrictus* Sinitshenkova is incomplete, without the head or tip of the abdomen preserved. It was assigned to Perlodidae as the wingpads are elongated near the apex, which is similar to the condition of extant *Isoperla* Banks (Sinitshenkova 1992). This is not an autapomorpy of the family or genus. Nevertheless, †*I. perstrictus* may be considered Systellognatha, as it has a short first tarsomere.

†*Derancheperla* Sinitshenkova clearly falls within Perloidea, supported by the presence of slender mandibles without a mola suggesting a carnivorous lifestyle, and the loss of the frontoclypeal suture (Sinitshenkova 1990). The nymph shows strong similarities to the nymphs of both Perlidae and Perlodidae, but lacks any clear autapomorphies of either. This absence of apomorphies does not preclude a possible assignment to stem-Perlidae instead of stem-Perlodidae, and therefore this species can be placed confidently only within Perloidea. The presence of a notal contour between the wing pads, present in †*Derancheperla*, may be derived in Perlodidae. The wing pads are instead rounded and meet medially in Perlidae and Chloroperlidae (Zwick 2000), a trait that appears to have been secondarily lost in the Perlodidae.

The type specimens of *Isoperla* †*succinica* Hagen and *Perlodes* †*resinatus* Hagen have been lost (Caruso & Wichard 2010; Jouault *et al.* 2021). As the original descriptions are somewhat vague, we follow Jouault et al. (2021) in treating their placement as uncertain and exclude them from this review. The assignment of *Isoperla* †*baltica* Jouault et al. is relatively well supported. While it is assigned to the Perlodidae predominantly on the absence of any apomorphies of other families, it can be placed within Isoperlinae based on paraprocts modified into simple upcurved hooks, and specifically in *Isoperla* based on wing venation (e.g., lack of cubital crossveins in the hindwing) (Jouault *et al.* 2021). *Isoperla* †*baltica* also shares the longitudinal thoracic stripe commonly seen in Perlodidae (Zwick 2000).

3.3.4.5. Perlidae

Perlidae is well represented in the fossil record, with 23 species from 11 genera known. Of these, eight genera and 19 species are preserved in Baltic amber from Myanmar. The placements of †*Pinguisoperla* Chen, †*Burmacroneuria* Chen and †*Starkoperla* Chen & Wang in Perlidae are likely, but somewhat uncertain. Their placements are supported by a short first tarsomere, numerous crossveins in the basal region of the costal field (plesiomorphy: Systellognatha), tarsal euplantulae (plesiomorphy: Systellognatha), occasionally an abdominal hammer (synapomorphy within Arctoperlaria, secondarily lost in some families; homoplasious apomorphy: Acroneuriinae), slender palpi (autapomorphy: Perloidea), and similar wing venation to *S. zhaoi* (autapomorphy: Perloidea) (Chen 2018d, 2019b; Chen & Wang 2020). As most established apomorphies of Perlidae are internal, or visible only in nymphs (Zwick 2000), assignment to the family must be considered uncertain.

A modified cercomere 1 may support moving †*Pinguisoperla* to Acroneuriinae.

Comparatively, the remaining Burmese fossil genera [†*Burmaperla* Jouault & Nel (1 sp.), †*Electroneuria* Sroka et al. (1 sp.), †*Cretacroneuria* Chen (1 sp), †*Burmesoperla* Chen (1 sp.) and †*Largusoperla* Chen (12 spp.), including adults and a single nymph] can all confidently be assigned to Acroneuriinae. These assignments are supported by the presence in adults of a hammer on the abdomen (which excludes membership of Perlinae) and paraprocts altered into anteriorly curved hooks (autapomorphy: Acroneuriinae), and in nymphs of an occipital row of short spinules (autapomorphy: Perlidae), incomplete laterally (autapomorphy: Acroneuriinae) (Chen 2018c, b; a, 2019a, 2020a; Chen *et al.* 2018; Chen & Wang 2019; Jouault *et al.* 2022a; Sroka *et al.* 2018).

†*Euperlida parvicercifera* Cifuentes-Ruiz can be assigned to Perlidae due to the tufted thoracic and anal gills visible in the impression fossil (Cifuentes-Ruiz *et al.* 2007). Affinities with either subfamily are not clear because the occipital ridge is poorly preserved (Cifuentes-Ruiz *et al.* 2007).

†*Dominiperla antigua* Stark & Lentz is represented by a female specimen, thus preventing assessment of the male autapomorphies in relation to other species. However, the similarity of the specimen's eggs to extant *Acroneuria* Pictet supports the placement of the species within Perlidae, and most likely in Acroneuriinae (Stark & Lentz 1992).

Perla †*prisca* Pictet belongs to the Perlinae and can be placed within the (*Perla* + (*Eoperla* + *Dinocras*)) clade due to its interrupted T9 (Carpenter 1992; Pictet & Hagen 1856; Sivec *et al.* 1988). However, affinities with any of these genera cannot be confirmed. The description of a fossilised representative of the extant *Perla burmeisteriana* Claasen was unavailable for this review and is therefore excluded.

3.4. Discussion

These findings strongly agree with previous authors (Cui *et al.* 2015, 2019; Jouault *et al.* 2021; Zwick 2000) that the classifications and assumed phylogenetic relationships of many Plecoptera fossils are unreliable. Of the 107 fossilised species reviewed (including some Euholognatha and "Gripopterygomorpha"), I recommend the reclassification of 62 species (58%). Some of these shifts completely alter the phylogenetic significance of these species. For example, †*Boreoperlidium* is currently assigned to extant Eustheniidae (Aristov *et al.* 2013; Sinitshenkova 2018) and is reclassified here as a stem-Plecopteran, while †*P. kingi* is moved from stem-Systellognatha to Ephemeroptera. These significant shifts are not unprecedented, as †*S. zhaoi* was previously moved from "Gripopterygomorpha" to Systellognatha (Cui *et al.* 2015).

I was cautious and assigned species only when several (syn/aut)apomorphies, or multiple lines of characters, were present. The sharing of plesiomorphic character states is insufficient to place exemplars in a crown group. Some genuine representatives of extant families and clades may, therefore, have been excluded through lack of preserved evidence. This is particularly true of several of the Perloidea *incertae sedis* species, where definitive morphological similarities to extant families are present, but equivocal (Chen 2018d; Chen & Wang 2020; Sinitshenkova 1987, 1990, 1992). Because incorrectly interpreted fossils can significantly alter the results of fossil-calibrated phylogenies (Heads 2005a; Parham *et al.* 2012), I recommend that only fossil species with uncontested (syn/aut)apomorphies preserved are used for the calibration and interpretation of these analyses (Crisp *et al.* 2011; Heads 2005a; Klopfstein 2021; Parham *et al.* 2012; Wang *et al.* 2016; Wolfe *et al.* 2016).

These results are congruent with the summary of the fossil record presented by Jouault et al. (2022b). The Paleozoic Era is dominated by stem-Plecoptera or dubiously-stem representatives of the Arctoperlaria and Antarctoperlaria. Throughout the Mesozoic, the first clear representatives of the

crown-group Plecoptera began to appear. This started with stem-Systellognatha and stem-Perloidea in the mid-Jurassic, and was followed by stem representatives of extant families in the mid-Jurassic and early-Cretaceous. Finally, extant genera and families arose during the Cenozoic Era. The Systellognatha fossil record suffers from the same gaps seen across the Plecopteran fossil record in general, with very few species preserved from the Early Cretaceous or the Late Cretaceous - Oligocene (Jouault *et al.* 2022b). Both of these gaps are problematic, as they seem to coincide with the early diversification of the extant stonefly families and genera, respectively (Jouault *et al.* 2022b). In the Southern Hemisphere, only two post-Triassic fossils are known: a stem-Notonemourid from the Jurassic (Sroka & Prokop 2023), and a Gripopterygid from the Cretaceous (Jell 2004; Jell & Duncan 1986).

3.4.1. Divergence of Antarctoperlaria and Arctoperlaria

None of the fossils reviewed here can confidently be assigned to stem-Antarctoperlaria or stem-Arctoperlaria (Figure 3.4). While some early fossils from the Permian and Triassic do show some morphological affinities to these suborders (e.g., †*Stenoperlidium* and †*Mesonotoperla* to Antarctoperlaria), these relationships are tenuous. This uncertainty prevents any direct conclusions on the origin of these suborders from being drawn.

As the first definitive Systellognatha appeared during the mid-Jurassic (see below), both suborders must have originated prior to this. It is possible that this divergence was caused by the rifting of Pangea, followed by a period of relatively rapid diversification giving rise to the Euholognatha and Systellognatha (Figure 3.1c, 3.6c). This scenario was partially corroborated by a recent analysis of the drivers of diversification in Plecoptera, which found that the timing of continental fragmentation was positively correlated with lineage origination (Jouault *et al.* 2022b). However, this same analysis found stable origination, extinction and net-diversification rates throughout the Jurassic. Zwick (2000) suggested that an ancient origin of extant families so soon after the rifting of Pangea would leave insufficient time for the formation of the suborders via the breakup of Pangea.

These early Systellognatha fossils could also point to an ancient origin of the suborders on Pangea, supporting either reciprocal extinctions in each hemisphere (Figure 3.1b; Zwick 2000), or dispersal of one suborder, followed by a localized extinction, from either the Northern Hemisphere (Figure 3.2a; Letsch *et al.* 2021) or Southern Hemisphere (Figure 3.1a; Illies 1965). Both scenarios have seen some support from fossils assigned to Northern "Gripopterygomorpha" and Southern "Perlomorpha" and "Nemourina" during the Permian–Jurassic (Aristov *et al.* 2013; Cui *et al.* 2015; Gallego *et al.* 2011; Liu *et al.* 2008; Sinitshenkova 2002, 2018). If these records were correctly interpreted, they provide evidence of both suborders sharing a distribution on Pangea. However, all of the species from these

groups that we examined were instead assigned to either stem-Plecoptera or a different suborder. Although not discussed in detail here, none of the remaining "Gripopterygomorpha" in the Northern Hemisphere (Aristov *et al.* 2013; Sinitshenkova 1987, 1990, 1992, 2018) have convincing apomorphies of Antarctoperlaria, let alone the families within it. Jouault et al. (2022b) similarly did not find evidence to support either hypothesis.

Beyond the lack of palaeontological evidence, both hypotheses are unlikely. They require the complete extinction of at least one suborder from across an entire hemisphere, with neither remnant lineages in the fossil record nor extant species. In the case of long-distance dispersal of one suborder, a further extinction of all stem-Plecoptera in the receiving hemisphere is required. No mechanisms have been proposed for these asymmetrical hemisphere-wide extinctions, nor for the ability of the remaining suborder to survive such a widespread event. Competitive exclusion is unlikely, as the Arctoperlarian Notonemouridae coexist with Antarctoperlaria today. While Jouault et al. (2022b) found evidence of two major extinction events in Plecoptera during the Permian—Triassic crisis and the end of the Early Cretaceous, apparently correlated with the appearance of angiosperms, these extinctions do not align with either hypothesis. Therefore, divergence of a common stock of stem-Plecoptera after becoming isolated remains the most likely explanation for the origin of the suborders (Figure 3.6).

If this divergence occurred on Pangea, the mechanisms proposed by Letsch et al. (2021) may have resulted in the isolation of each suborder in its respective hemisphere (Figure 3.6). The earliest stoneflies are known from the equator of Pangea at the end of the Carboniferous, some 310 Ma (Béthoux *et al.* 2011; Schubnel *et al.* 2019). The transition to the Permian appears to have resulted in a diversification process for Plecoptera (Jouault *et al.* 2022b). A similar trend has occurred in many plants and animals (Condamine *et al.* 2020a; Montañez *et al.* 2007). This diversification was likely accompanied by significant range expansions, facilitated by several periods of cooling and increased rainfall throughout the transition to the Permian, despite a general warming trend (Jouault *et al.* 2022b; Montañez *et al.* 2007; Roscher & Schneider 2006). Increased orogeny and an abundance of glacial streams would have provided suitable habitats for these early stoneflies (Jouault *et al.* 2022b). This expansion is clear in the fossil record, and stem-Plecoptera species were globally distributed by at least the Middle Permian (Figure 3.6a; Illies 1965; Letsch *et al.*, 2021; Prevec *et al.* 2022; Sinitshenkova 1987; Zwick 2000).

With few exceptions, such as the tropically-adapted *Neoperla,* Plecoptera are cold-adapted, occurring in cold, fast-flowing and oxygen-rich waters such as mountain streams (Fochetti & Tierno de Figueroa 2008; Hynes 1976). Aridification, particularly in the middle reaches of Pangea (Chaboureau *et al.* 2014; Péron *et al.* 2005; Roscher & Schneider 2006) and the Variscan orogeny (López-Gómez *et al.* 2021) throughout the Permian may have limited stoneflies to mid–to–high latitudes and

prevented exchanges between the hemispheres (Figure 3.6b). It is possible the Variscan Mountains instead acted as refuges for stem Plecoptera at low latitudes, by providing colder habitats at high altitude. However, these mountains were generally hot and humid in the early Permian, becoming more arid into the late Permian and Triassic (López-Gómez *et al.* 2021; Péron *et al.* 2005), likely limiting Plecopteran diversity. Nevertheless, by at least the late Triassic, Plecopteran species were present in this region, as †*Siberioperla angulata* Sinitshenkova (221.5–205.6 Ma), †*Berekia neglecta* Sinitshenkova (221.5–205.6 Ma) and †*Trianguliperla innoxia* Sinitshenkova (221.5–205.6 Ma) were all recorded from modern-day Ukraine. It is unclear if stoneflies were present prior to this, or if these records instead represent range expansions and dispersals during the Late Triassic, possibly facilitated by changing climatic conditions (Miller & Baranyi 2019).

Unfortunately, until clear ancient representatives of either suborder can be found, the above hypotheses remain speculation. As stoneflies apparently showed increased diversification immediately following extinction events (Jouault *et al.* 2022b), both the end Permian–Triassic and end Triassic – Jurassic extinction events may have facilitated their divergence into the extant suborders while isolated on either Pangea or Gondwana and Laurasia, respectively. Hoyal Cuthill *et al.* (2020) showed that extinctions and radiations are generally decoupled, suggesting origination does not necessarily follow a mass extinction event. Nevertheless, a period of generally increased radiation was associated with the end Permian mass extinction (Hoyal Cuthill *et al.* 2020), correlating with the period of increased diversification detected in the fossil record of Plecoptera at the same time (Jouault *et al.* 2022b).

3.4.2. Diversification of Antarctoperlaria

Only a single species, †*Eodinotoperla duncanae*, can confidently be assigned to the Antarctoperlaria (Figures 3.4, 3.6). As this species is known from the Early Cretaceous (122.46 to 112.6 Ma), it provides evidence that the extant families first appeared on Gondwana while at least Australia, New Zealand and South America were intermittently connected via Antarctica (Figure 3.6c–d; (Mayes *et al.* 1990; McLoughlin 2001; Storey *et al.* 1999). As fossils only provide a minimum age for their associated clades (Klopfstein 2021), the appearance and dispersal of Antarctoperlaria may have occurred prior to this. However, the Weddellian Isthmus land bridge between Antarctica and South America likely only formed in the Late Cretaceous, and may not have been consistently aerial (Reguero *et al.* 2014). This may point to a Late Cretaceous dispersal of Antarctoperlaria across the Southern Hemisphere, perhaps in stages and over millions of years.

It is unclear if Antarctoperlaria were ever present in Africa, which separated from Eastern Gondwana during the Jurassic (approx. 165 Ma) (McLoughlin 2001). As Antarctoperlaria may have first

appeared in the austral realm of Pangea or later on Gondwana, dispersal into Africa seems possible (Figure 3.6c–d). Even after its separation from Eastern Gondwana, Western Africa remained connected to South America until the end of the Lower Cretaceous (119–105 Ma) (McLoughlin 2001). This land bridge may have provided a route for the suborder to reach the continent, although absences of otherwise pan-Gondwanan groups are common (Gheerbrant & Rage 2006). If the suborder were ever present in Africa, it is likely that hot and dry periods during the Late Cretaceous and Palaeogene (Jacobs 2004; Myers *et al.* 2011; Rees *et al.* 2004; Sellwood & Valdes 2008) led to its localized extinction (Figure 3.6d–e).

As many Plecopteran taxa and clades are not geographically isolated across the rest of the Southern Hemisphere, at least some movement across the austral region must have continued after these families first appeared (García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016). Migrations likely occurred predominantly while these landmasses were connected via Antarctica, which was generally cool and covered in temperate rainforests during the Cretaceous (Bowman *et al.* 2014; Francis & Poole 2002). However, migrations may have persisted after the separation of South America, Australia and New Zealand, possibly via the West Wind Drift, which could have dispersed stoneflies as aerial plankton (postulated by Ding *et al.* 2019; García-Girón *et al.* 2024; McCulloch *et al.* 2016), or via ocean currents (Figure 3.6e; discussed above).

3.4.3. Diversification of Systellognatha, and selection of calibration points for node-dating in phylogenetic analyses

The earliest stem-Systellognatha appeared in the fossil record of the mid-Jurassic, shortly after the separation of Gondwana and Laurasia some 180 Ma (McLoughlin 2001; Schettino & Scotese 2005; Schettino & Turco 2009; Scotese *et al.* 2025). As a short first tarsomere can be unreliable for classification, this estimate is based on †*Platyperla platypoda* (183–155.7 Ma), †*Platyperla conferta* (171.6–164.7) and †*Bestioperlisca inulta* (150.8–145.5), as the sharply toothed mandibles support a possibly carnivorous feeding strategy, which supports their assignment (Zwick 2000). Carnivory is an autapomorphy of Perloidea (Zwick 2000). However, while these species all have predatory mandibles, they maintain herbivorous maxillae, suggesting that they were detritivores or omnivores (Sinitshenkova 1985, 1987). These differences prevent their assignment to stem-Perloidea, but do not preclude the possibility that †*Bestioperlisca* and †Platyperlidae may be the remnants of a slow shift to carnivory that eventuated in extant Perloidea. These early fossils are all from Russia and Mongolia, suggesting that the initial diversification of Systellognatha occurred in the eastern high latitudes of the Northern Hemisphere (Figure 3.6). This was likely caused by a combination of isolation with the cold and wet climate associated with the movement of Laurasia (Jouault *et al.* 2022b; Schettino & Turco 2009).

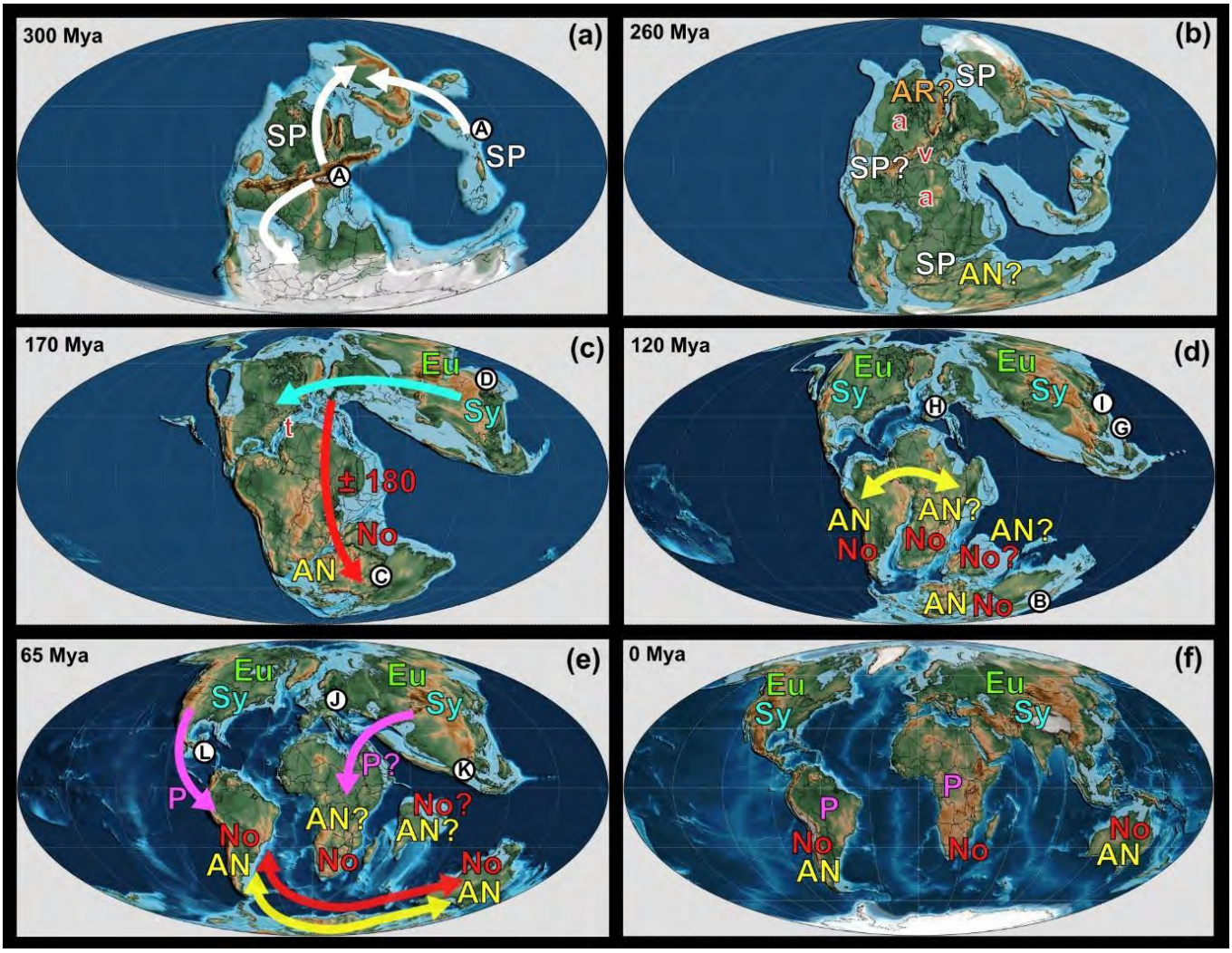

Figure 3.6: Hypothesized biogeographical history of Plecoptera, from the Carboniferous (300 Ma) – Present Day, based on the results of this review of the fossil record. Abbreviations: SP: Stem-Plecoptera, a: arid zone, v: Variscan orogeny, t: Tethys Seaway, AN: Antarctoperlaria, AR: Arctoperlaria, Eu: Euholognatha, Sy: Systellognatha, No: Notonemouridae, P: Perlidae. Labels in white circles represent the same fossils species and labels as Figure 4, with the following addition: L: *Dominiperla antigua* Stark & Lentz (20.4–13.7 Ma). (a) Carboniferous: origin of Plecoptera, and oldest fossil stem-Plecoptera. (b) Permian: stem-Plecoptera have distributed worldwide and become isolated in the austral and boreal regions of Pangea by arid bands and the Variscan orogeny. Possible origin of Antarctoperlaria in the Southern Hemisphere, and Arctoperlaria in the Northern Hemisphere. (c) Jurassic: If not formed on Pangea, Antarctoperlaria and Arctoperlaria form due to Vicariance with the separation of Laurasia and Gondwana. Systellognatha and Euholognatha arise in the Northern Hemisphere and stem-Notonemouridae disperse into the Southern Hemisphere. (d) Cretaceous: the Northern and Southern Hemisphere are isolated by the Tethys sea, preventing faunal exchanges. The first records of extant families begin to appear, possibly correlated with Angiosperm radiation. Extant Antarctoperlaria first appear in the fossil record, and spread across the remnants of Gondwana, either via land bridges or long-distance dispersal (e) Palaeogene: extant genera first appear in the fossil record. Perlidae migrate into the Southern Hemisphere in two independent events. The timing of the migration of *Neoperla* into the Afrotopics remains unclear. (f) Modern day. Palaeomaps from Scotese et al. (2025), and are utilized under the Creative Commons Attribution 4.0 International License, available from https://doi.org/10.5281/zenodo.10659112 (Accessed 24 February 2025).

Diversification throughout the Jurassic appears to have been slow (Jouault *et al.* 2022b), and most extant families and superfamilies appeared only in the Early Cretaceous. The exception to this is a

Pteronarcyid, †*P. sinitshenkovae* (164.7–155.7 Ma) (Cui *et al.* 2016). Recent molecular phylogenetic analyses have suggested that Pteronarycyidae is a basal sister to Perloidea (García-Girón *et al.* 2024; Letsch *et al.* 2021), which is congruent with this early appearance. As †*P. sinitshenkovae* was from China, these basal Systellognathans must have spread across Laurasia by the Early Cretaceous (Figure 3.6c–d).

Perloidea and the remaining Pternonarycoidea arose during the Cretaceous. In Pteronarcyoidea, †*Ecdyoperla fairlightensis* (145.5–140.2 Ma) was the first Peltoperlid (Sinitshenkova 1998). Stem-Perloidea were simultaneously evidenced by †*Trianguliperla quassa* and †*Derancheperla collaris* (Sinitshenkova 1987, 1992). This diversification overlapped with the first appearance of angiosperms (Chaboureau *et al.* 2014; Condamine *et al.* 2020a; b) and a mass extinction of Plecoptera hypothesized to have caused a shift from "pre-Angiosperm Plecoptera" to the extant groups (Jouault *et al.* 2022b). Similar extinctions and diversifications during this period have been noted in other insect groups, such as Dictyoptera (Condamine *et al.* 2020a). These shifts were potentially precipitated by both direct and indirect challenges to Plecoptera such as eutrophication, replacement of food sources for herbivores, and shifts in the diets and behaviours of predators (Jouault *et al.* 2022a). This scenario is harmonious with our results, as representatives of Peltoperlidae (†*Zwickoperla*, †*Borisoperla* and †*Dewaltoperla*: Chen 2023a; Chen & Wang 2020; Chen & Xu 2020) and Perlidae (Acroneuriinae: †*Burmaperla*, †*Electroneuria*, †*Cretacroneuria*, †*Burmesoperla* and †*Largusoperla*: Chen 2018c, b; a; Chen *et al.* 2018; Sroka *et al.* 2018) were present by the late Cretaceous, some 99.7–94.3 Ma (Figure 3.6e).

Peltoperlidae and Perlidae continued to diversify throughout the Cretaceous, and extant genera first appeared by at least the Palaeogene (*Isoperla* †*baltica*, 37.2–33.9 Ma; Jouault et al. 2021). Many of these genera probably arose and shifted between continents following the complex network of exchanges and isolations during the Palaeogene and Miocene posited by Zwick (2000).

3.4.4. Dispersal of Notonemouridae

The discovery of †*Talbragaria australis* in Australia shows that stem-Notonemouridae was present in the Southern Hemisphere by the mid-Jurassic, 157.3–145 Ma (Sroka & Prokop 2023). This scenario matches most biogeographical interpretations, as the current presence of this family in Madagascar suggests that it dispersed prior to the island splitting from Africa (Cui *et al.* 2016; Illies 1965; Letsch *et al.* 2021; Sroka & Prokop 2023; Zwick 2000), some 165 Ma (Kusky *et al.* 2007; McLoughlin 2001). Madagascar remained connected to India until the Late Cretaceous (McLoughlin 2001), suggesting the family likely occurred in Greater India but became locally extinct, possibly with increased temperatures during the landmasses movement North. Alternatively, faunal exchanges

between Africa and Madagascar persisted after the separation of the two landmasses (Gheerbrant & Rage 2006), and a latter dispersal across the Mozambique Channel via aerial plankton or marine currents is possible (Figure 3.6d–e). One species of *Neoperla*, *N. burgeoni*, occurs on the Comoros islands between mainland Africa and Madagascar (Zwick & Zwick 2023), which only formed in the Miocene – Tertiary (Emerick & Duncan 1982). A latter dispersal is therefore feasible.

Notonemouridae probably entered the Southern Hemisphere via Africa (Letsch *et al.* 2021), shown by the Afrotropical taxa forming a basal clade in molecular phylogenies (Figure 3.6c; Letsch *et al.* 2021; McCulloch *et al.* 2016; Terry 2004). This route and timing aligns with Jurassic geographical and paleoenvironmental patterns. The opening of the Tethys seaway led to the separation of Gondwana and Eastern Laurasia, with isolation occurring in the Middle to Late Jurassic (Figure 3.6). At that point, the seaway would have formed a significant barrier to stonefly migration (Gheerbrant & Rage 2006). By the time of this final split, Africa was concurrently separating from the rest of the East Gondwanan landmasses and Western Laurasia (McLoughlin 2001), and a later date of migration likely would have prevented dispersal across the rest of the austral realm (Figure 3.6c–d). This timing coincided with a major cooling event during the Aalenian (174.7–170.9) that resulted in cold temperate climates across northern Africa and Europe, increased rainfall in northern Africa, and a significant drop in sea level during the middle Jurassic (Haq 2018; Kairouani *et al.* 2024; Korte *et al.* 2015), all providing ideal conditions for Notonemouridae to spread south. If this interpretation is correct, the family may have also occurred in Greater India, but likely went extinct as it moved north, across the equator, and conditions became hotter and dryer.

This narrative has been contradicted by some dated molecular phylogenies that place the origin of Notonemouridae later, during the Cretaceous (García-Girón *et al.* 2024; McCulloch *et al.* 2016). A Cretaceous origin and migration for Notonemouridae seem unlikely because, although trans-Tethyan migrations of mammals, reptiles and dinosaurs into Africa occurred repeatedly during the Cretaceous (Gheerbrant & Rage 2006), northern Africa had become hot and arid by the late Jurassic (Myers *et al.* 2011; Rees *et al.* 2004; Sellwood & Valdes 2008). This climate persisted throughout the Early Cretaceous, with increased humidity and rainfall eventually leading to more tropical habitats only in the late Cretaceous (Jacobs 2004). This warm climate would be a significant barrier to the spread of the cold-adapted Notonemouridae, beyond the already significant physical barrier of the Tethys. These conditions persisted into the Paleogene and Miocene, after the Tethys closed (Jacobs 2004; Morley 2000; Steinthorsdottir *et al.* 2021). If Notonemouridae was widespread in Africa, it is plausible that this period of warming led to its extinction in the northern reaches of the continent, resulting in its restricted distribution in South Africa and Madagascar today.

It is generally assumed that stem-Notonemouridae went extinct in the Northern Hemisphere after migrating south (Ding *et al.* 2019; García-Girón *et al.* 2024; Hynes 1988; Illies 1965; Letsch *et al.*

2021; McCulloch *et al.* 2016; Zwick 2000). This was obliged by its assumed derived position within Euholognatha. However, most molecular analyses have not supported the presumed sister relationship between Notonemouridae and Nemouridae, and the position of the family in Euholognatha is poorly resolved by both morphological and molecular analyses (García-Girón *et al.* 2024; Letsch *et al.* 2021; McLellan 1991; Zwick 2000). This journey may, therefore, have been made by a stem-Euholognathan, which gave rise to the Notonemouridae in the south, while its remnants in the north developed into some, or all, of the extant crown-Euholognatha.

3.4.5. Dispersal of Perlidae

None of the recovered fossils provide biological context or biogeographical evidence for the migration of *Neoperla* into the Afrotropics.

In the New World, †*Dominiperla antigua* (20.4–13.7 Ma) is likely a remnant of Acroneuriinae migrating into South America from North America. No extant Plecopterans are known from the Dominican Republic, where this fossil was collected from rich amber beds (DeWalt *et al.* 2025; Stark & Lentz 1992). It is dated to the early Neogene, suggesting that this migration occurred prior to the Miocene, perhaps during the early Palaeogene (Letsch *et al.* 2021; Zwick 2000). As a land bridge did not exist between the continents at the time, this migration could have occurred by "island hopping" via a chain of meso-American islands (Letsch et al. 2021).

4. Chapter 4: Biogeography of *Neoperla* in Africa and its links to drainage evolution

4.1. Introduction

Neoperla has a disjunct distribution in the eastern Nearctic, Indo-Malay, and Afrotropical regions (DeWalt *et al.* 2025; Fochetti & Tierno de Figueroa 2008; Zwick 2023, 2024). A single undescribed species has also been reported from the Australasian region, in New Guinea (Zwick 2023). It is one of only three Arctoperlarian groups to occur in the Southern Hemisphere, along with Notonemouridae and Acroneuriinae (Fochetti & Tierno de Figueroa 2008; Illies 1965; Letsch *et al.* 2021; McCulloch *et al.* 2016; Zwick 2000), making it very interesting for understanding the biogeography of Plecoptera. However, surprisingly little is known of its relationships, both within the genus and with the remaining Perlidae, or the historical biogeographical processes that led to its current distribution.

Some of these gaps have begun to be addressed. The taxonomic infrastructure of the genus was recently reviewed, with four sub-genera recognized and several paraphyletic and polyphyletic species groups addressed, including in the African *Neoperla* (discussed in Chapter 2; Zwick 1976, 2023, 2024; Zwick & Zwick 2023). Despite this work, the affinities of some species remain uncertain (Zwick 2023), and novel species continue to be found across the genus's range (Chapter 2; Zwick 2023; Zwick & Zwick 2023). The monophyly of the tribe Neoperlini, and its position as sister to Perlini, has similarly not been supported by recent molecular analyses, although the relationships recovered by each analysis have differed (García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021; Zwick 2023; Zwick & Zwick 2023). Additionally, the historical biogeographical processes that led to its extant distribution largely remain unresolved.

It is likely that Neoperlini, including *Neoperla*, originated in the Indo-Malay region, later dispersing into the Nearctic and Afrotropical regions (Zwick 2000, 2023). Hynes (1988) considered the genus to be old, reasoning that its absence from the western Nearctic supported its evolution on a connected landmass of the eastern Nearctic and western Palearctic, prior to the opening of the Atlantic Ocean in the Cretaceous. Zwick (2000) presented an alternative hypothesis, suggesting the genus more likely arose recently and spread into the Nearctic via the northwest of the continent, before disappearing from this region.

Illies (1965) proposed a late Cenozoic arrival of *Neoperla* in the Afrotropics due to the supposedly low diversity (at the time, one species: "*Neoperla spio*") of the region. Zwick (2000) agreed with this hypothesis, adding that this journey could have occurred in the Miocene or Pliocene via a land bridge over a warm and wet Arabian peninsula (Steinthorsdottir *et al.* 2021) alongside mammal lineages. Before the benefit of Zwick & Zwick's (2023) taxonomic review, this perspective largely persisted

(Fochetti & Tierno de Figueroa 2008; Sroka & Prokop 2023), but the high diversity of the genus in Africa may instead point to an earlier arrival.

In the absence of direct fossil evidence, time-calibrated phylogenies that estimate the age of cladogenesis from reference calibration points (usually taken from paleogeographic events, the presence of fossil species, substitution rates and/or derived node ages) have become a commonly-used tool to investigate the biogeographical history and evolution of clades (Donoghue & Yang 2016; Drummond *et al.* 2006; Hipsley & Müller 2014; Parham *et al.* 2012; Rutschmann 2006). These methods are controversial, and the results of similar analyses, even using the same molecular datasets and calibration points, can vary significantly (Guindon 2020; Heads 2005a; Hipsley & Müller 2014; Klopfstein 2021). Variation can arise from several factors, such as a fossil's uncertain age or taxonomy (Heads 2005a; Ksepka *et al.* 2015; Parham *et al.* 2012), and differing prior probability expectations applied to each calibration point (Donoghue & Yang 2016; Klopfstein 2021). These problems persist even when well-justified fossils are used, as these represent only a minimum age for clades that may be far older (Klopfstein 2021). Despite this variation, molecular clock analyses provide valuable estimates for the timing of cladogenesis, particularly in groups with a poor or incomplete fossil record, and form an important basis for exploring the biogeography and evolution of these groups (Wilke *et al.* 2009). While these time estimates can be correlated to biogeographical events (such as vicariance, the opening of land bridges, etc.) to generate hypotheses for the modern distributions of clades, interpretations based on these data are inherently limited, as these hypotheses cannot be tested or observed outside of the discovery of further fossil evidence (Crisp *et al.* 2011; Klopfstein 2021).

Four such time-calibrated phylogenies have examined the historical biogeography of Plecoptera as a whole (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016), although they have not provided any specific explanation for the extant distribution of *Neoperla*. However, Letsch et al. (2021) recovered a Cretaceous origin for the genus, suggesting that their migration out of Asia may have occurred much earlier than the Miocene or Pliocene. This would align with the recent taxonomic review of the Afrotropical *Neoperla* that found a higher diversity of the genus in the region than expected spread across five species groups, four of which are endemic (Zwick 2023; Zwick & Zwick 2023).

Recently, new genetic sequences of Afrotropical *Neoperla* have become available, both from a taxonomic review (Zwick & Zwick 2023), and from the phylogeny presented in Chapter 2. These new data provide a valuable opportunity to investigate the timing of cladogenesis and the historical biogeography of *Neoperla* across Africa. To this end, I will use a time-calibrated phylogeny to investigate the relationships and biogeographical history of the genus and test the current hypotheses explaining the distribution of *Neoperla.* First, I will estimate the origin of *Neoperla*, assessing whether

its distribution in the Nearctic and Indo-Malayan regions is more likely due to vicariance associated with the breakup of Eurasia (Hynes 1988), or a later dispersal (Zwick 2000). Secondly, I will estimate the timing of the dispersal of *Neoperla* into Africa and examine whether this dispersal occurred in the Miocene (Illies 1965; Zwick 2000), or earlier (Letsch *et al.* 2021). Finally, I will present novel hypotheses for the spread of this genus across the Afrotropical region.

4.2. Methods

4.2.1. Sampling, Morphology, DNA sampling & PCR

The methods used to collect, identify, and sequence three genes (28S, H3 and COX1) from African *Neoperla* are described in Chapter 2. In total, 26 species of *Neoperla,* including 15 novel species, were identified and sequenced from all four of the Afrotropical species groups. For each species, a single specimen was selected for further analysis in the time-calibrated phylogeny. Only a single specimen was selected as within-species divergences can be interpreted as speciation events by BEAST, altering cladogenesis estimates (Drummond *et al.* 2006).

4.2.2. Analysis

As reference 28S and H3 sequences were not available for any other African *Neoperla,* a combined phylogenetic analysis of the newly sequenced material and the species described and sequenced by Zwick & Zwick (2023) was not possible. Instead, two separate analyses were used to estimate a time-calibrated phylogeny of *Neoperla*. The first of these used mitochondrial genomes (Mitogenome approach), while the second used a combination of the three genes extracted and sequenced in Chapter 2 (Three-gene approach).

Newly sequenced material was trimmed and checked manually for base-calling errors in Chromas v2.6.6 (Technelysium Pty Ltd, South Brisbane, Queensland, Australia) and AliView 1.27 (Larsson 2014). Poorly resolved nucleotide positions were replaced with ambiguity codes. To concatenate the three datasets, each protein-coding gene was aligned separately using an online version of MAFFT v7, with default parameters (Katoh *et al.* 2019). The 28S region was aligned by ClustalW (Thompson *et al.* 2003) in MEGA v11.0.13 (Tamura *et al.* 2021). Final alignments were checked for substitution saturation in DAMBE 7.3.32 (Xia & Xie 2001) using Xia's nucleotide substitution test (Xia *et al.* 2003; Xia & Lemey 2009). Final alignments of all protein-coding genes were translated into amino acids and checked by eye for frame shifts and the presence of stop codons (Buhay 2009).

In both analyses, maximum likelihood (ML) and Bayesian inference (BI) methods were used to infer phylogenetic relationships. The ML analyses were conducted using W-IQ-Tree (Trifinopoulos *et al.* 2016) with 1000 bootstrap replicates using the Ultrafast algorithm (Hoang *et al.* 2018). Datasets were partitioned by gene, and all genes, except for 28S, were further partitioned by codon position. Models (Appendix 4.1) were selected using ModelFinder (Kalyaanamoorthy *et al.* 2017). The BI analysis was conducted via the CIPRES science gateway (Miller et al. 2010) using Mr Bayes 3.2.7a (Ronquist et al. 2012) and the models identified by ModelFinder (Kalyaanamoorthy *et al.* 2017). All BI analyses were run for 20 million generations, with sampling every 1000 generations, and the first 25% of the run discarded as burn-in. Convergence of the Markov chains was checked in Tracer 1.7.2 (Rambaut *et al.* 2018) to ensure the effective sample size of all parameters was >200. Final phylogenetic trees were visualized in FigTree V1.4.4 (Rambaut 2018) and edited in Inkscape V1.2.1 (Inkscape Project 2022).

4.2.3. Molecular Clock Calibration and Analysis: Mitogenome approach

All available Perlidae mitochondrial protein-coding genomes were obtained from Zwick & Zwick (2023) and NCBI GenBank (Sayers *et al.* 2021) and merged with four outgroup species from Chloroperlidae (*Suwallia teleckojensis* Šámal and *Sweltsa* sp.) and Perlodidae (*Isoperla eximia* Zapekina-Dulkeit and *Perlodes microcephalus* Pictet). The final dataset was 11490 bp long and included 64 species, each represented by a single mitochondrial genome (Appendix 4.2). Zwick (2023, 2024) recently proposed a new classification of *Neoperla*, recognizing four subgenera, two of which were further divided into species groups: *Neoperla* (*Neoperla*) (7 species groups), *Neoperla* (*Borneella*), *Neoperla* (*Formosita*) (5 species groups) and *Neoperla* (*Oodeia*). All African *Neoperla* are placed in four species groups within *Neoperla* (*Neoperla*): *N. transvaalensis* group, *N. africana* group, *N. sjostedti* and *N. spio* group. Except for the *N. spio* group, which also occurs in parts of Asia, these species groups are endemic to Africa. These species groups are occasionally further divided into species assemblages, following the morphological and genetic review of Zwick & Zwick (2023). All Afrotropical species groups and assemblages were included in this phylogeny.

Divergence times were estimated using a Bayesian Markov chain Monte Carlo approach conducted in BEAST V1.10.4 (Drummond *et al.* 2012; Drummond & Rambaut 2007; Suchard *et al.* 2018) via the CIPRES science gateway (Miller *et al.* 2011). Tree topology and divergence times were estimated simultaneously, but Perlidae and the outgroups were each constrained as monophyletic. The concatenated dataset was partitioned by gene and codon position, with the models identified by ModelFinder (Kalyaanamoorthy *et al.* 2017) applied to each partition, and all parameter values unlinked across partitions. A relaxed-clock model with a lognormal distribution and a birth-death speciation prior were applied. The analysis was run for 200 million generations with a sampling frequency of 1000 generations, and the first 25% of the samples discarded as burn-in. Convergence of

the Markov chains was checked in Tracer 1.7.2 (Rambaut *et al.* 2018), and a maximum clade credibility tree using median node heights was constructed in TreeAnnotator v1.10.4 (Suchard *et al.* 2018). The final tree was annotated in FigTree V1.4.4 (Rambaut 2018) and edited in Inkscape V1.2.1 (Inkscape Project 2022).

The divergence time estimate was calibrated using fossils chosen from the results presented in Chapter 3. For each calibration node, a lognormal distribution was applied, with a lower bound constrained by the minimum age of the fossil deposit, following the 2023 estimates provided by the International Commission on Statigraphy (www.stratigraphy.org), and soft upper bounds. The root of the Perloidea was calibrated using †*Trianguliperla quassa* Sinitshenkova (140.2-125.45 Ma), while the root of the Perlidae was constrained using several genera of Acroneuriinae from Burmese amber (99.7-94.3 Ma). The latter of these calibration points was applied to Perlidae as a whole, as Acroneuriinae were paraphyletic in both the ML and BI analyses (see below). This paraphyly has been recovered in several recent phylogenetic analyses (García-Girón *et al.* 2024; Letsch *et al.* 2021; Xiang *et al.* 2021).

4.2.4. Molecular Clock Calibration and Analysis: Three-gene approach

Newly-sequenced *Neoperla* genes were combined with reference genes obtained from NCBI GenBank (Sayers *et al.* 2021). Originally, all available Perlidae 28S sequences were used, with four outgroup species from Chloroperlidae and Perlodidae selected to match the sampling of the Mitogenome approach. However, relationships within Acroneuriinae were poorly resolved with consistently low support. For this reason, the closest clade of Acroneuriinae to the monophyletic Perlinae was selected as an outgroup and used to root the analysis.

Only a single specimen from each species identified in Chapter 2 was used, as divergences between populations within a species can be interpreted as speciation events, potentially altering date estimates (Drummond *et al.* 2006). The final dataset was 1712 nucleotide positions long and included 26 species (Appendix 4.3).

Analysis and divergence time estimates were conducted using the same methods described for the Mitogenome approach, with the following changes: the subfamily Perlinae was constrained as a monophyletic group instead of the entire family, 28S was not partitioned by codon position, and the analysis was run for 400 million generations to ensure convergence. As Chloroperlidae and Perlodidae were excluded as outgroups, only the Burmese Perlidae fossils were used to constrain the root of the tree. Models are summarized in Appendix 4.1

4.2.5. Biogeographical analyses

Biogeographical analyses for both the Mitogenome and Three-gene approaches were conducted in RStudio using the BioGeoBEARS V1.1 package (Matzke 2013, 2014) on the maximum clade credibility tree from each BEAST analysis. The BioGeoBEARS package uses six biogeographical models (DEC, DEC + J, DIVALIKE, DIVALIKE + J, BayAreaLIKE and BayAreaLIKE + J) (Matzke 2013, 2014) to estimate the ancestral ranges of lineages in a phylogeny. As several species of *Neoperla* are widespread in the Afrotropical region (e.g. *N. burgeoni* and *N. transvaalensis*: Zwick & Zwick 2023), the ten ichthyological provinces in Africa (Lévêque *et al.* 2008; Roberts 1975) were modified for the analysis. These regions were based on the distribution of extant *Neoperla,* with distribution records obtained from Zwick & Zwick (2023) and new records presented in Chapter 2. Regions in close proximity with similar species and a low number of endemic species were merged (Appendix 4.4; Figure 4.1). In cases where a province had a high degree of endemism, e.g., the East Coast province, it was kept separate. The Nilo-Sudan province, as defined by Roberts (1975) and Lévêque *et al.* (2008), was separated into the Western portion (i.e. the Niger River and tributaries) and the Eastern portion (i.e. the Nile and tributaries) because the *Neoperla* species recorded from each portion differed. This protocol follows the recent redefinition of the Upper Guinea province used by Arroyave *et al.* (2020) based on new findings in hydrological basin mapping. The final regions were i: Upper Guinea province and the western portion of the Nilo-Sudan province, ii: the eastern portion of the Nilo-Sudan province (i.e., the Nile and tributaries), iii: The Lower Guinea, Congo, and Cuanza provinces, iv: the East Coast province, and v: the Zambezi and southern rivers province (Figure 4.1; Appendix 4.4). All non-African taxa were attributed to a single “Holarctic” region. Each lineage was allowed a maximum range of five combined areas (based on widespread species that occur in every region). Best fit models were selected by the Akaike information criterion corrected for small sample sizes.

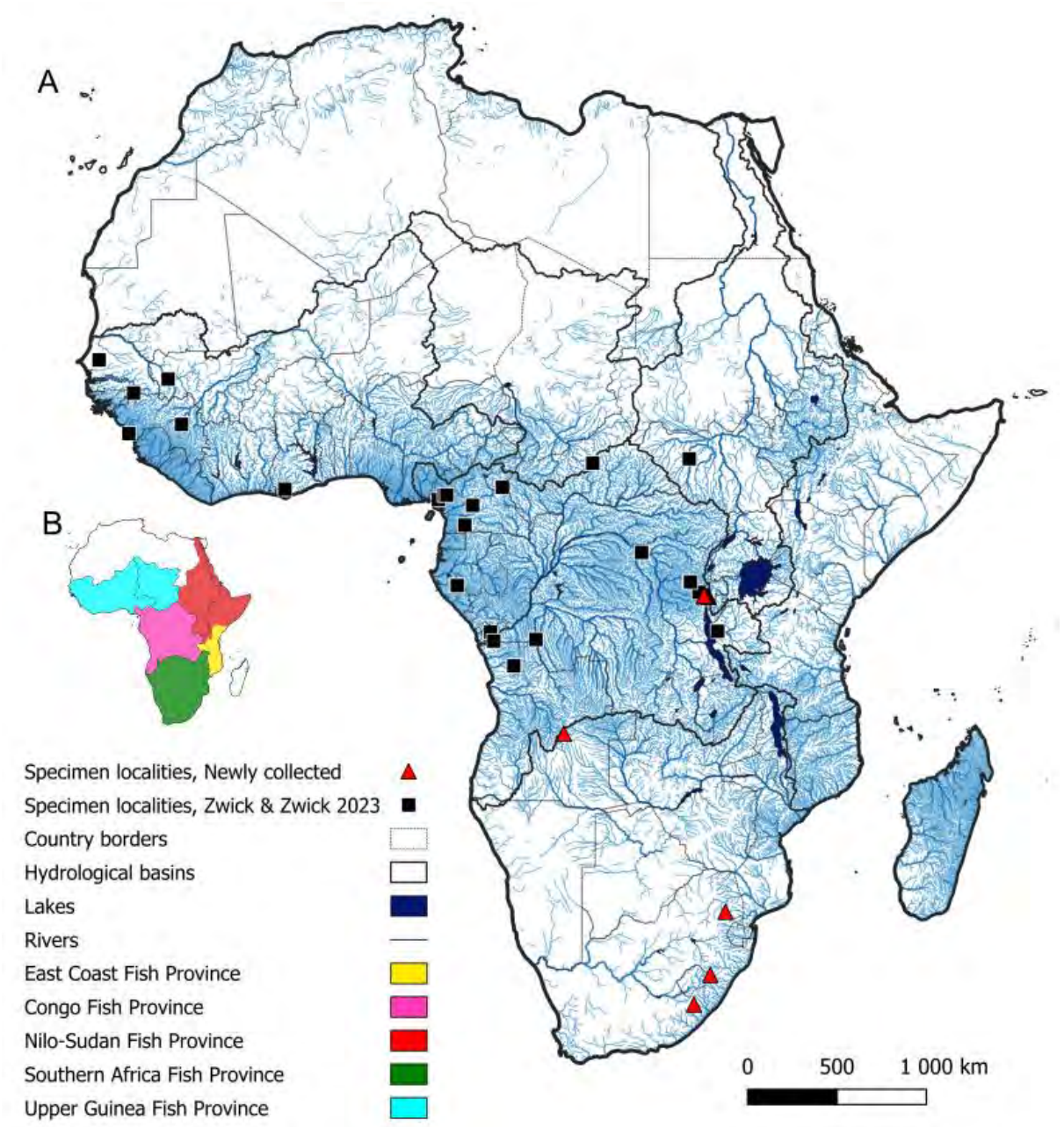

Figure 4.1: A: Map of Africa, showing specimen localities for the Mitogenome and Three-Gene approaches, and the major river systems and hydrological basins from the continent. River and hydrological basin data were obtained from HydroSHEDS (http://www.hydrosheds.org; Lehner & Grill 2013). Continent and country borders were obtained from Natural Earth (https://www.naturalearthdata.com/). **B:** Modified regions of Africa used for the Biogeographical analysis of *Neoperla* (*Neoperla*) on the continent. Fish regions are modified from Leveque *et al.* 2008. Blue: Upper Guinea, Red: Nilo-Sudan, Pink: Congo, Yellow: East Coast, Green: Southern Africa.

4.3. Results

4.3.1. Phylogeny and divergence time estimates: Mitogenome approach

All three analyses returned consistent and predominantly strongly supported (>80% bootstrap support, >0.9 Posterior Probability) phylogenetic relationships across the phylogeny, with only a few weakly supported nodes (Figure 4.2). Acroneuriinae was paraphyletic, with its tribes Kiotinini and Acroneuriini each forming strongly supported, monophyletic clades (ML: 100%, BI: 1, BEAST: 1 for all branches). Perlinae was recovered as monophyletic (ML: 77%, BI: 1, BEAST: 1), with Claasseniini forming a basal clade to the rest of the subfamily. *Neoperlops* was sister to a monophyletic *Neoperla*, making Perlini paraphyletic with strong support (ML: 97%, BI: 0.99, BEAST: 1).

Within *Neoperla*, the subgenus *Neoperla* (*Neoperla*) formed a strongly supported monophyletic clade (ML: 100%, BI: 1, BEAST: 1). Two of the species groups, *N. spio* and *N. excisa-sjostedti* were recovered as paraphyletic. As very few *Neoperla* sequences from outside the Afrotropical region were available, and this phylogeny only examines biogeographical patterns within the region, a complete analysis of the subgenera and species groups is not considered further here. Instead, species assemblages following Zwick & Zwick (2023) are discussed.

The African taxa did not form a single monophyletic clade, but were instead split between two distinct, well-supported clades (Clade 1: ML: 60%, BI: 0.99, BEAST: 0.99; Clade 2: ML: 100%, BI: 1, BEAST: 1). Clade 1 consisted only of the endemic *transvaalensis*, *duodeviginti*, *pilulifera* and *camerunensis* (Clade E in Zwick & Zwick 2023) assemblages. Clade 2 basally included an Asian species, *Neoperla ignacsiveci* Li & Li (*N. spio* group), with the Afrotropical taxa forming a derived monophyletic grouping consisting of the *spio, orthonema, dubia, excisa* (Clade J in Zwick & Zwick 2023), *sjostedti* and *arambourgana* assemblages. Except for *orthonema*, which fell within the *spio* assemblage, all assemblages were monophyletic and generally strongly supported (ML: >88 %,BI: 1, BEAST: 1; *N. excisa:* ML: 86%, BI: 0.66, BEAST: 0.77).

The divergence time estimates (Table 4.1) placed the origin of Perlidae in the Upper Cretaceous, 98.1 Ma (95% highest posterior density: HPD: 103.3 - 93.0 Ma). The origin of *Neoperla* was placed at the boundary of the Upper Cretaceous and Paleogene, 66.2 Ma (HPD: 72.9 - 59.3 Ma), while *Neoperla* (*Neoperla*) originated during the Eocene (50.2 Ma, HPD: 55.8 – 44.7 Ma). The initial separation of the two African clades occurred shortly after this, still during the Eocene (**Clade 1**: 47.3 Ma, HPD: 52.9 – 41.9 Ma; **Clade 2:** 45.3 Ma, HPD: 50.8 – 40.1 Ma). The African assemblages began to appear during the late Eocene and continued throughout the Oligocene, although some diversified later during the Pliocene (Figure 4.2; Table 4.2).

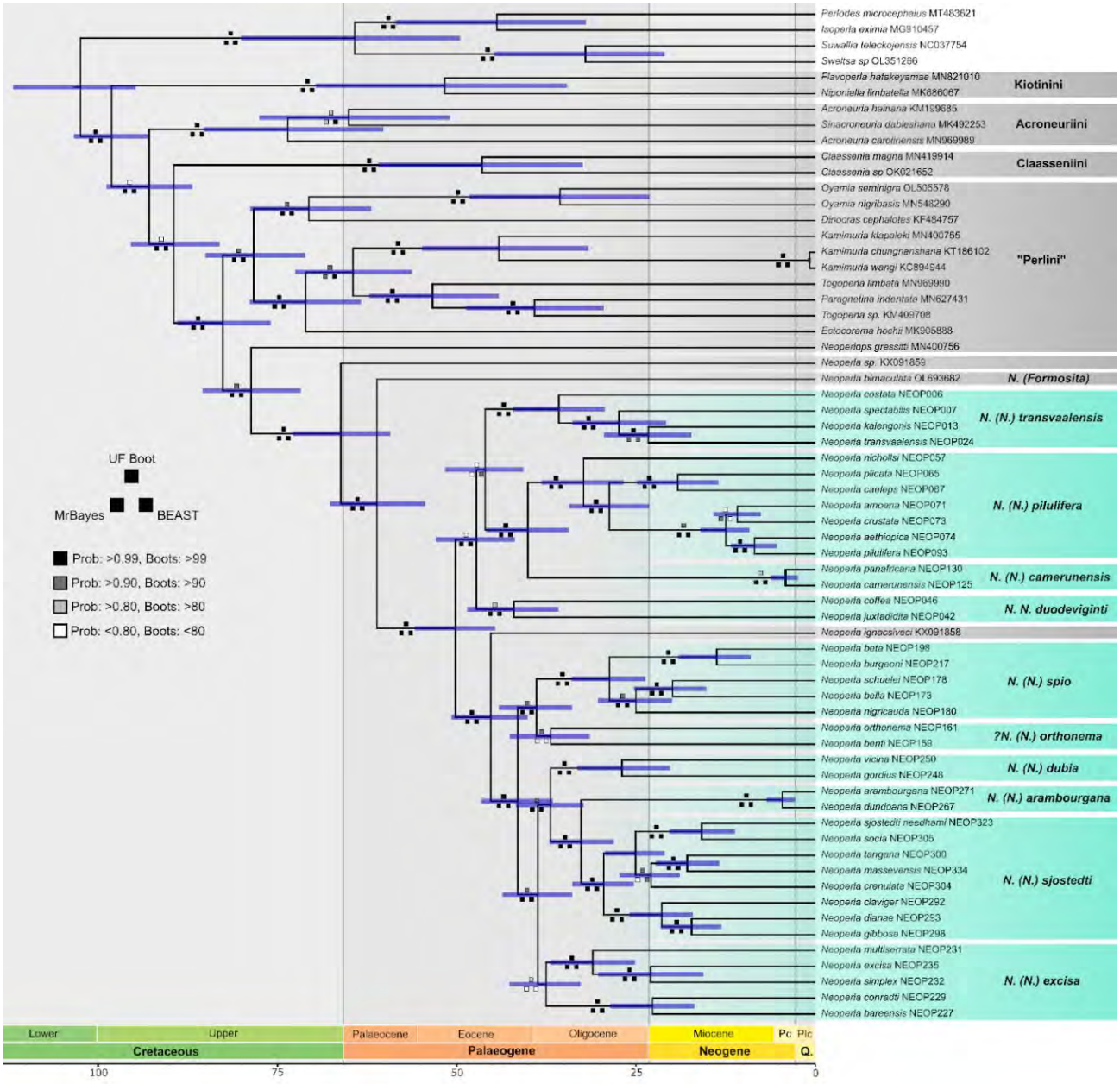

Figure 4.2: Chronogram showing the relationships and divergence times of genera in the Perlidae (Plecoptera) as recovered by the BEAST Bayesian inference analyses of the Mitochondrial Genome approach. Blocks on each node represent bootstrap support values from UFBoot (% of 1000 bootstrap analyses), and posterior probabilities recovered from MrBayes and BEAST. Blue rectangles on each node show the 95% confidence interval for recovered median ages. Blocks on the right of the tree show species assemblages, following Zwick & Zwick (2023).

Table 4.1: Median divergence time and 95% highest posterior density for all major clades estimated by the BEAST analysis using the Mitogenome approach.

Clade	**Median age (Ma, 95% HPD)**
Perloidea	102.5 (111.8-94.8)
Perlidae	98.1 (103.3-93.0)
Subfamily	
"Acroneuriinae"	-
Tribe	
Kiotinini	51.7 (69.7 - 34.7)
Acroneuriini	73.6 (85.2 - 60.2)
Subfamily	
Perlinae	89.4 (95.4 - 83.1)
Tribe	
Claasseniini	46.5 (60.9 - 32.4)
"Perlini"	78.3 (85.4 - 71.1)
Neoperlini + *Neoperlops*	78.7 (78.9 - 71.7)
Genus	
Neoperla	66.2 (72.9 - 59.3)
Subgenus	
Neoperla (*Neoperla*)	50.2 (55.8 - 44.7)
Species Groups	
Clade 1	47.3 (52.9 - 41.9)
N. transvaalensis	35.8 (42.1 - 29.4)
N. africana	42.9 (50.8 - 34.7)
pilulifera assemblage	32.3 (38.2 - 26.7)
camerunensis assemblage	4.1 (6.2 - 2.5)
N. duodeviginti	42.1 (48.6 - 35.8)
Clade 2	45.3 (50.8 - 40.1)
"*N. spio*" (African clade)	38.9 (44.1 - 33.9)
spio assemblage	28.7 (34.0 - 23.7)
orthonema + N. benti	36.9 (42.6 - 31.5)
"N. excisa-sjostedti"	38.7 (43.6 - 33.9)
excisa assemblage	37.5 (42.5 - 33.8)
dubia assemblage	27.0 (33.2 - 20.3)
arambourgana assemblage	4.6 (6.8 - 2.8)
sjostedti assemblage	32.7 (37.1 - 28.1)

4.3.2. Biogeographical analyses: Mitochondrial genome approach

The BayAreaLIKE + J model was selected as the best-fit biogeographical model (Figure 4.3; Table 4.2). An Afroptropical origin for *Neoperla* (*Neoperla*) in a combined range of Upper Guinea and Congo was recovered. Both Clade 1 and Clade 2 radiated during the Eocene, with the same ancestral range of Upper Guinea and Congo recovered for both. This ancestral range was also found for most

species assemblages, namely *pilulifera, camerunensis, duodeviginti, spio, orthonema*, and *excisa.* During the Oligocene, the *transvaalensis* assemblage originated in a range of Upper Guinea, Congo and the East Coast, while the *arambourgana* and *sjostedti* assemblages both originated in the Congo. Within the *spio* assemblage a clade consisting of *N. schuelei, N. bella* and *N. nigricauda* similarly originated in the Congo during the Oligocene. Finally, during the Oligocene–Miocene, a clade within the *sjostedti* assemblage, consisting of *N. sjostedti, N. socia, N. tangana, N. massevensis,* and *N. crenulata,* originated in a combined range of Congo and the East Coast.

Table 4.2: Results of BioGeoBears Analysis. #: number of parameters, d: rate of dispersal, e: rate of extinction, j: probability of jump dispersal, AICc: bias corrected Akaike's information criterion, AICw: Akaike weight.

Model	LnL	#	d	e	j	AICc	AICw
DEC	-153.9	2	0.01	0.01	0	311.9	0.3
DEC + J	-153.9	3	0.01	0.01	0	314.2	0.1
DIVALIKE	-165.8	2	0.01	0	0	335.8	0
DIVALIKE + J	-164.2	3	0.01	0	0.01	334.9	0
BAYAREALIKE	-156.9	2	0.01	0.01	0	318.1	0.01
BAYAREALIKE + J	-152.1	3	0.01	0.01	0.01	310.6	0.5

4.3.3. Phylogeny and divergence time estimates: Three-gene approach

The overall topology of the three-gene approach broadly agreed with the Mitogenome approach, with the African taxa forming two separate clades (Figure 4.4). Each of these clades consisted of the same species assemblages as in the Mitogenome approach (Clade 1: *duodeviginti*, *transvaalensis, pilulifera* and two species from the *spio* assemblage, *Neoperla sp. nov. 1* and *Neoperla sp. Afr_C*; Clade 2: *arambourgana, spio, sjostedti*, *excisa* and *dubia*), but differed in topology. Support values across all depths of the tree were generally low, and relationships between the *sjostedti*, *arambourgana* and *dubia* assemblages were unresolved, differing between each analysis. The divergence time estimates were consistently slightly younger than those returned in the Mitogenome approach, but had larger error margins that overlapped with the median ages returned in the earlier analysis. Although specific dates varied, a similar diversification of the African *Neoperla* during the Eocene was recovered, followed by diversification of the species groups in the Oligocene.

Disagreement in topology was due to supported incongruence between each of the three genes in the *sjostedti, dubia* and *arambourgana* clades. Adding multiple specimens of each species to improve resolution of these relationships can lead to conflicts between the coalescent population prior and speciation models in BEAST, potentially altering the time estimates of cladogenesis (Drummond *et*

al. 2006), so this analysis was disregarded in the following discussion. Similarly, as BioGeoBEARS relies on the topology of the tree to estimate ancestral ranges, its results are not presented here or considered further in the discussion.

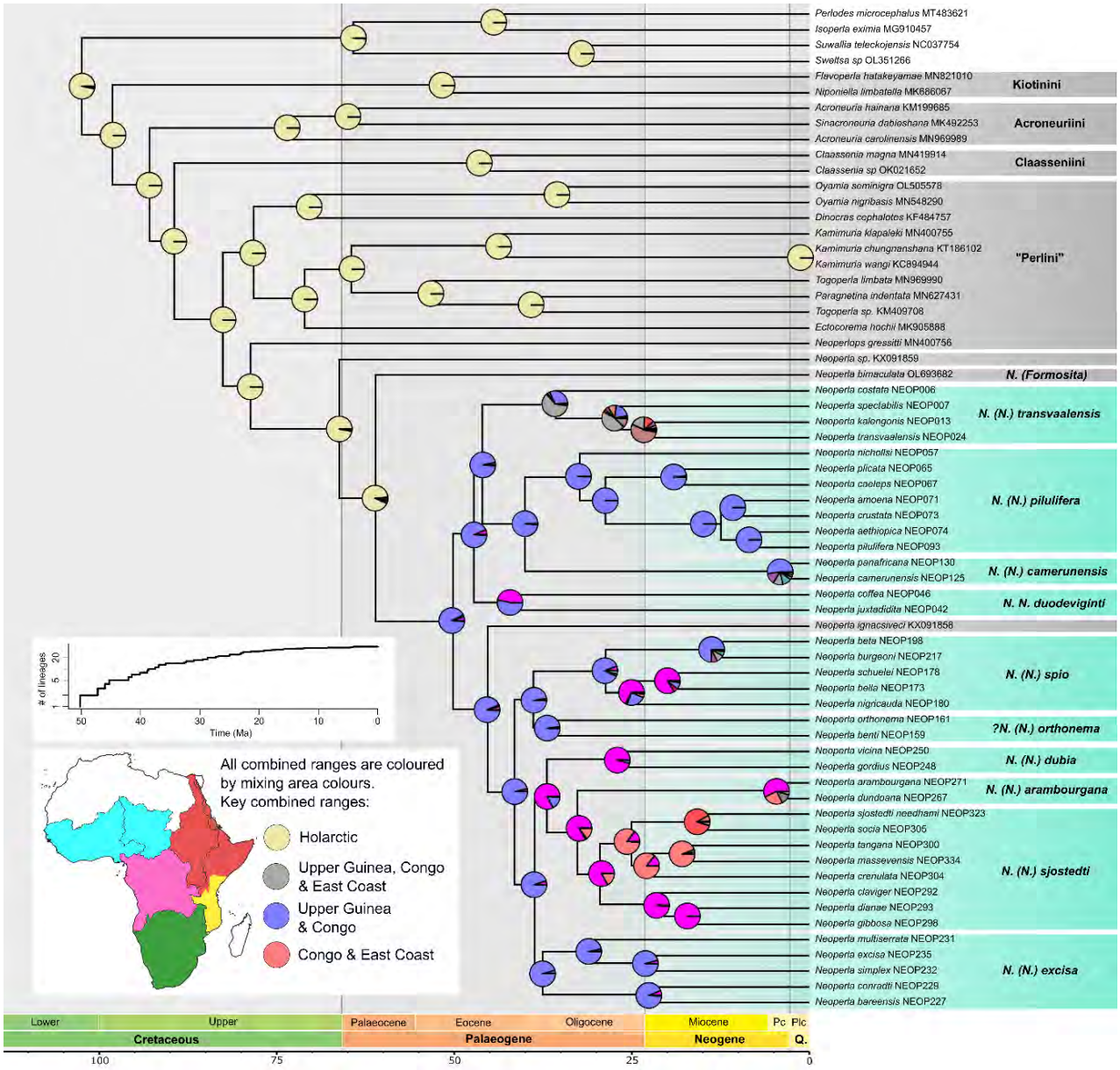

Figure 4.3: Results of the biogeographic analysis of Perlidae (Plecoptera) conducted in BioGeoBEARS for the Mitochondrial Genome approach. Pie-Charts on each node represent the relative likelihood of each area being a part of the reconstructed ancestral range. Map of Africa shows each extant range used for the BioGeoBEARS analysis, and provides a key to common combined ranges. Blocks on the right of the tree show species assemblages following Zwick & Zwick (2023).

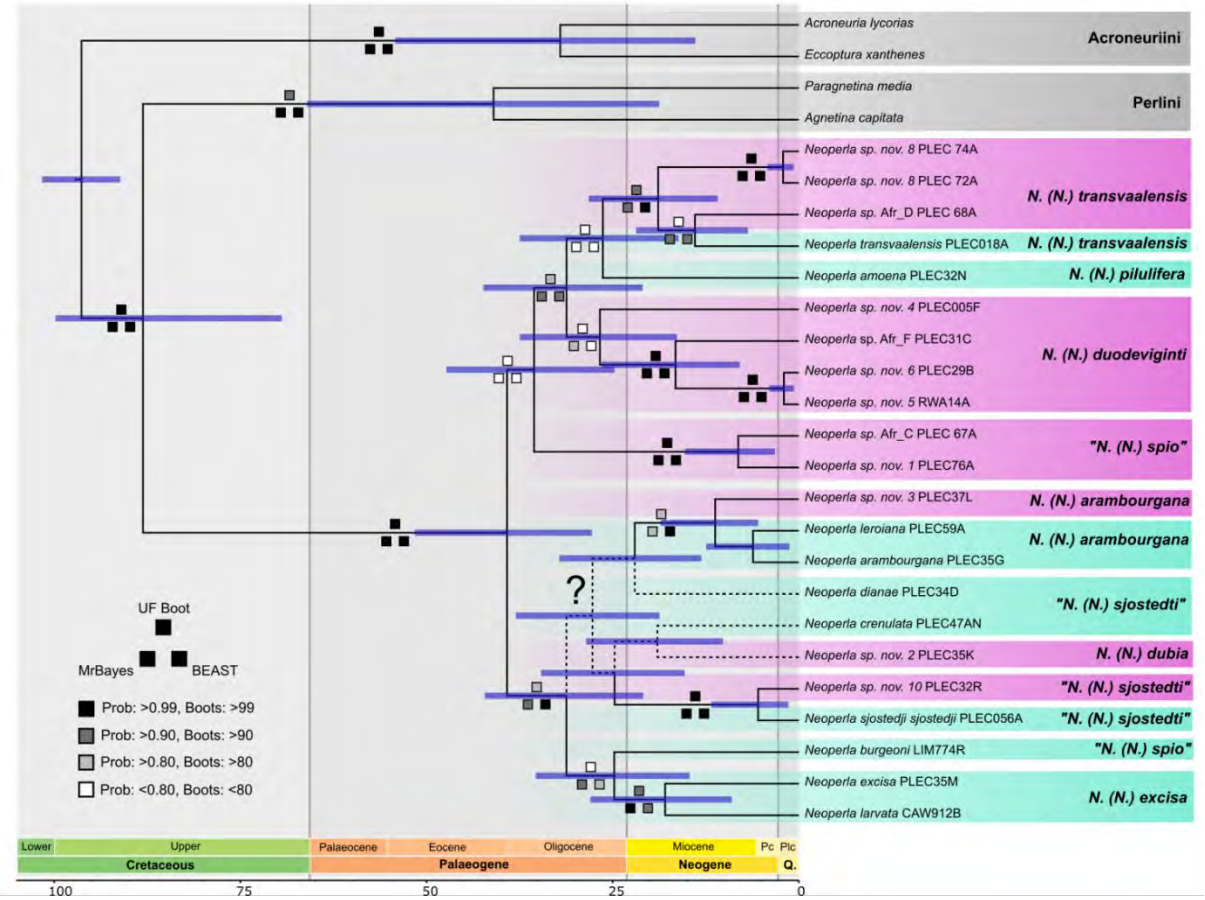

Figure 4.4: Chronogram showing the relationships and divergence times of genera in the Perlidae (Plecoptera) as recovered by the BEAST Bayesian inference analyses of the Three-gene approach. Blocks on each node represent bootstrap support values from UFBoot (% of 1000 bootstrap analyses), and posterior probabilities recovered from MrBayes and BEAST. Blue rectangles on each node show the 95% confidence interval for recovered median ages. Blocks on the right of the tree show species assemblages, following Zwick & Zwick (2023). Dotted lines between *N. arambourgana*, *N. sjostedti*, and *N. dubia* show conflicting relationships between different analyses.

4.4. Discussion

4.4.1. Phylogeny of Perlidae

Two subfamilies of Perlidae are currently recognized; Acroneuriinae and Perlinae (Zwick 2000). Acroneuriinae is united morphologically by the modification of male paraprocts into upturned hooks, a drumming hammer on sternite IX in adults, and an incomplete or irregular row of occipital setae in nymphs (Murányi & Li 2016; Zwick 2000). Although the subfamily is widely accepted, and both the Nearctic and Neotropical genera are well-defined (Froehlich 2010; Stark 2001; Stark & Gaufin 1976), most relationships within and between the Asian genera are unresolved (Murányi & Li 2016). Acroneuriinae was paraphyletic in this analysis, as its member tribes, Acroneuriini Klapálek and Kiotinini Uchida, formed successive monophyletic clades relative to Perlinae. Recent phylogenetic

reviews similarly recovered a paraphyletic Acroneuriinae (García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021), although all of the member tribes were paraphyletic or polyphyletic in Letsch *et al.* (2021), South *et al.* (2021), and García-Girón *et al.* (2024). Letsch *et al.* (2021) found that the Neoptropical representatives formed a monophyletic clade consisting of both Acroneuriini and Anacroneuriini Stark & Gaufin. It is therefore likely that this subfamily is a grade consisting of multiple independent basal clades within Perlidae. Unfortunately, sampling of the Acroneuriinae in all phylogenetic analyses is generally poor, with only a few species and genera consistently represented, often by the same sequence(s). Considering this, the complexity of these relationships and their possible links to biogeographical events remains unclear. However, the monophyly of the subfamily should be considered uncertain. All autapomorphies and synapomorphies of the Acroneuriinae are present in the earliest definitive Perlidae fossils (Chen *et al.* 2018; Chen & Wang 2020; Sroka *et al.* 2018), and may instead represent plesiomorphies of Perlidae as a whole that were secondarily lost in Perlinae.

Perlinae are united by modified hemitergites that form anteriorly curved hooks and hair-brushes on the abdominal sclerites of adults, and a transverse occipital ridge or setal fringe in nymphs (Sivec *et al.* 1988; Zwick 2000, 2023). A morphological phylogeny proposed by Sivec *et al.* (1988) has remained largely accepted, with only a few minor revisions and the addition of new genera (Zwick 2023). Perlinae was similarly monophyletic in this analysis, forming a basal clade nested within a paraphyletic Acroneuriinae. A monophyletic Perlinae has also been recovered in all recent molecular phylogenies, although topology within the subfamily has differed (Chen *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021). Perlinae is further divided into three tribes, Perlini Latreille, Neoperlini and Claasseniini Sivec *et al.*. The position of Claasseniini is uncertain as the tribe has characters of both Perlinae ("specialized hemitergites", or antecosta 10 modified into a bridge, connecting the deeply cleft tergite 10) and Acroneuriinae (a circular drumming hammer) (Sivec *et al.* 1988; Zwick 2000). Here, it was recovered as the basal-most tribe within Perlinae. This same position was recovered by Letsch *et al.* (2021), South *et al.* (2021) and Xiang *et al.* (2021), although it has also been recovered as sister to all other Perlidae (Chen *et al.* 2019) or nested within Perlini (García-Girón *et al.* 2024) in other analyses. Its position here may suggest an intermediate form between the two subfamilies, as plesiomorphic characters from Acroneuriinae are lost and the autapomorphies of Perlinae begin to appear.

Perlini was recovered as paraphyletic, with *Neoperlops* as sister to *Neoperla*. This result is difficult to evaluate; several phylogenies (Xiang *et al.* 2021; Zwick & Zwick 2023) recovered this same relationship, but they have all reused the same mitochondrial genomes, while other phylogenies have placed *Neoperla* as sister to *Oyamia, Agnetina* or *Paragnetina* (García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021). Nonetheless, most molecular phylogenies have repeatedly recovered a

paraphyletic Perlini, with *Neoperla* nested somewhere within the tribe (García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021; Zwick & Zwick 2023).

Neoperla formed a monophyletic clade in our analysis. The monophyly of both Neoperlini and *Neoperla* were discussed by Zwick (2023). Both are supported morphologically by autapomorphies; namely a sclerotized aedeagus base and an unusually slender, spike-like processes on the anterior half of hemitergite 10, respectively (Sivec *et al.* 1988; Zwick 2023). Unfortunately, as the remaining three genera of Neoperlini, *Chinoperla*, *Furcaperla* and *Phanoperla* were not available for this analysis, definitive conclusions cannot be drawn on the monophyly of either tribe. [Note: Zwick 2023 and DeWalt *et al.* 2025 include †*Archaeoperla* Liu, Ren & Sinitshenkova within Neoperlini. †*Archaeoperla* is a synonym of †*Sinosharaperla* Liu, Sinitshenkova & Ren, and has been assigned to Systellognatha *incertae sedis* (Cui *et al.* 2015; Sroka *et al.* 2018)].

4.4.2. Neoperla Biogeography

4.4.2.1. Origin of Perlidae and Neoperla

Three dated phylogenies have previously estimated the origin of the Perlidae, recovering a divergence for the family in either the Jurassic (Letsch *et al.* 2021), or Cretaceous (Ding *et al.* 2019; García-Girón *et al.* 2024), some 165.7 – 74.6 Ma. I recovered the root of Perlidae within this range, at the border between the Lower and Upper Cretaceous, approximately 98.1 Ma. This aligns with the earliest unambiguous fossil records of Perlidae from the Upper Cretaceous of Myanmar, which have been dated to 99.7 – 94.3 Ma (Chen 2020a; Chen *et al.* 2018; Chen & Wang 2019; Jouault *et al.* 2022a; Sroka *et al.* 2018), and an Early Cretaceous origin of the Perloidea, potentially precipitated by the appearance of Angiosperms (discussed in Chapter 3, *Diversification of Systellognatha*). This timing corresponds to a period of increased diversification in Plecoptera (Jouault *et al.* 2022b). This period of diversification followed increased extinction rates in the order during the Lower Cretaceous, 120 - 100 Ma (Jouault *et al.* 2022b), possibly caused by global “hot-house” temperatures and/or the diversification of Angiosperms, which may have altered the diets of insects and led to the eutrophication of freshwater systems because of increased plant biomass in these systems (Condamine *et al.* 2020b; a, 2021; Jouault *et al.* 2022b). Unfortunately, as sampling of the extant Perlidae outside of Africa is minimal here, more detailed conclusions on the biogeography and drivers of this diversification cannot be drawn.

Only a single dated phylogeny has previously estimated the age of *Neoperla*, recovering an origin in the Upper Cretaceous, approximately 92 Ma (95% HPD = 113 – 71 Ma: Letsch *et al.* 2021). A significantly younger age of about 66.2 Ma (72.9 - 59.3 Ma), at the boundary of the Cretaceous and Paleogene, was estimated here (Figure 4.5). Counter-intuitively, this period coincides with the

Cretaceous–Paleogene (K/PG) mass extinction, a global mass extinction that led to the disappearance of non-Avian dinosaurs, inter alia (Brusatte *et al.* 2015; Condamine *et al.* 2021; Hoyal Cuthill *et al.* 2020). This extinction appears to have had relatively little impact on the diversity of insects (Labandeira 2014; Labandeira & Sepkoski Jr 1993), including Plecoptera (Jouault *et al.* 2022b). Instead, increases in global temperatures, that eventually led to the Palaeocene–Eocene thermal maximum approximately 55 Ma (Jenkyns 2003; MacLeod *et al.* 2018; O'Leary *et al.* 2013; Shellito *et al.* 2003; Zachos *et al.* 1993), may have precipitated the initial diversification of tropical-adapted *Neoperla*. It is likely that the genus originated in Asia (Figure 4.5; Zwick 2000; Zwick & Zwick 2023) as an undescribed Asian species and *Neoperla* (*Formosita*) represents the earliest divergences in the genus (Figure 4.2). The same pattern was recovered by Zwick & Zwick (2023), who also placed the Nearctic *Neoperla occipitalis* species group between the Asian and African clades. Large sections of Eurasia, at least in the north and south of the continent, were tropical and humid during the Paleogene, providing a suitable climate for these early ancestors (Bondarenko & Utescher 2022, 2024; Quan *et al.* 2014). During the early Eocene, the collision of India and Asia resulted in the uplift of the Tibetan plateau and reduction of the Tethys Ocean, altering drainage, rainfall and thermal patterns across the continent (Jagoutz *et al.* 2015; Meng *et al.* 2023; Ni *et al.* 2020; Quan *et al.* 2014). These changing conditions have been linked to rapid increases in diversification and origination of mammals during the Paleogene (Ni *et al.* 2020), and may have led to further diversification in the Plecoptera, including *Neoperla*.

Unfortunately, mitochondrial genomes of the Nearctic *N. occipitalis* and *N. choctaw* species groups were not available for this analysis, preventing direct interpretation of their origin and relationships. Nevertheless, based on the relationships recovered in recent phylogenies, the Nearctic *Neoperla* either form the basal-most clade of *Neoperla* (*Neoperla*) (Chapter 2; Zwick & Zwick 2023), or a more derived group, which are related to the *Neoperla duodeviginti* species group (Chapter 2). Hynes (1988) hypothesized that the disjunct distribution of *Neoperla* in Asia and the Nearctic was a result of vicariance, associated with the opening of the Atlantic Ocean during the Cretaceous. However, my results contradict this, as they suggest *Neoperla* first appeared in the latest Cretaceous or Paleogene, long after this separation occurred (Figure 4.5). This aligns with the biogeographical patterns of several other groups. Disjunctions between East Asia and the East Nearctic are common in plants (Denk *et al.* 2011; Graham 2018; Jiang *et al.* 2019; Tiffney 1985; Wen *et al.* 2010, 2016; Xiang *et al.* 2000; Xiang & Soltis 2001), amphibians (Pyron 2014), and even other stonefly groups, such as Pteronarcyidae and Peltoperlidae (Nelson 1988; Stark & Nelson 1994; Zwick 2000). However, most of these disjunctions are estimated to have formed in the Paleogene or the Miocene (Graham 2018; Pyron 2014; Wen *et al.* 2010, 2016; Xiang *et al.* 2000; Xiang & Soltis 2001), long after the opening of the Atlantic Ocean.

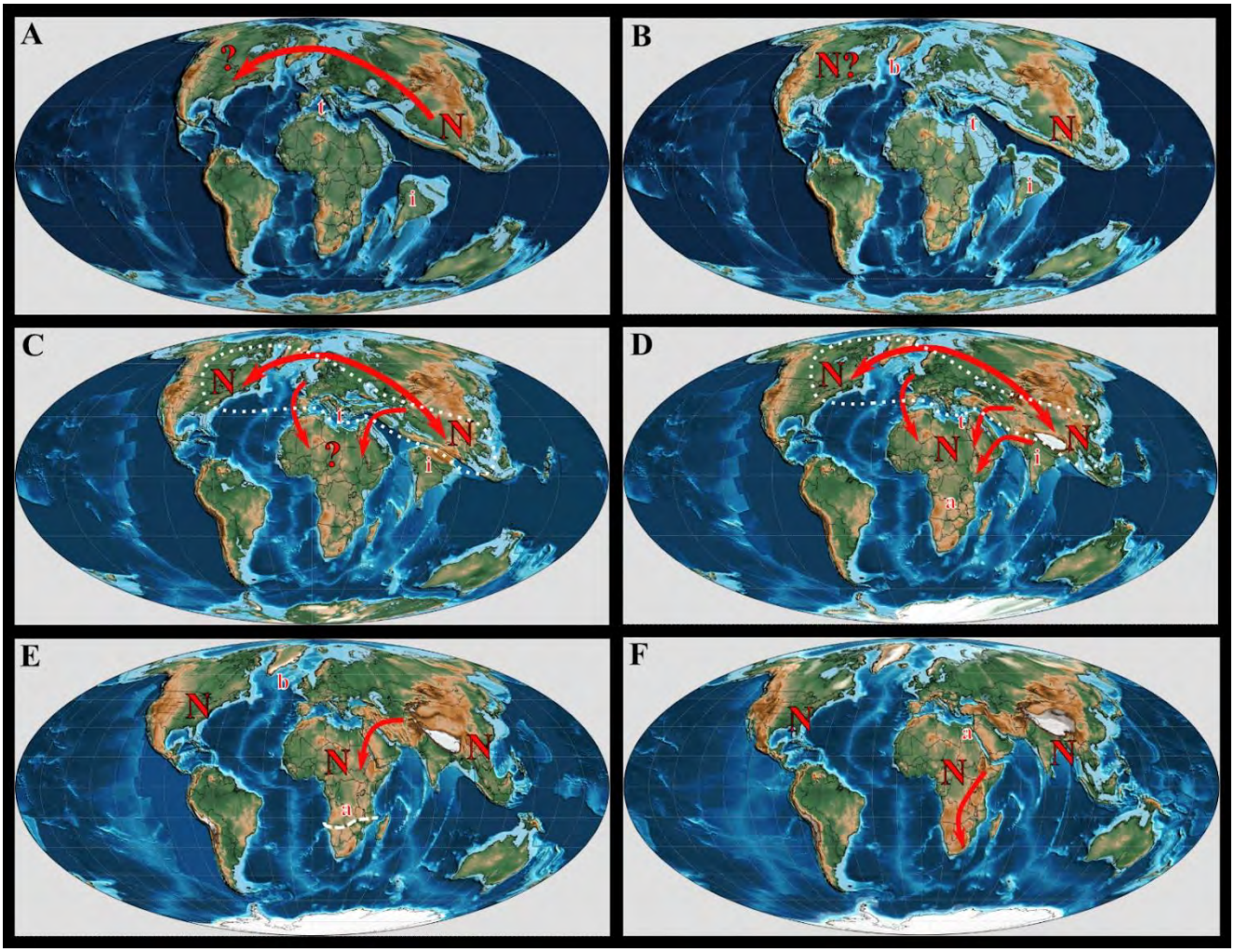

Figure 4.5: Hypotheses of the biogeographical history of *Neoperla*, from the Paleocene (65 Ma) – Pliocene (5 Ma), based on interpretations of the time-calibrated phylogeny. **A:** Paleocene, 65 Ma: Origination in southeast Asia during the Paleocene. It is possible *Neoperla* dispersed into the Nearctic via the North Atlantic land bridge. **B:** Eocene (Ypresian), 55 Ma. The Eocene Thermal Maximum. Increasing temperatures and rising sea levels widened the Tethys seaway (t), preventing migration into the Afrotropical region. If *Neoperla* had dispersed into the Nearctic, rising sea levels may have isolated them (b), giving rise to *Neoperla* (*Neoperla*). **C:** Eocene (Lutetian), 45 Ma. Boreotropical forests (white line) cover most of Europe, and allow for *Neoperla* to disperse between the Nearctic and Indo-Malayan regions. The Tethys seaway (t) is still wide, possibly preventing the spread of *Neoperla* into Africa. India (i) connects with Asia, resulting in uplift of the Himalayas. **D:** Oligocene (Rupelian), 35 Ma. Weakening of the Tethys seaway and drops in sea level due to the formation of the Atlantic ice sheets potentially allow *Neoperla* to disperse into Africa via a tropical Iberian peninsula, or the Iranian Route. Multiple mammal exchanges occur during this period (Gheerbrant & Rage 2006). **E:** Miocene, 20 Ma: Possible arrival, or continued dispersal, of *Neoperla* from Asia via the *Gomphotherium* landbridge and the Arabian plate. By the Miocene, dropping temperatures over Europe led to the mass extinction of Tropical fauna and flora in Europe, and severed the North Atlantic land bridge. In Africa, dispersal into Southern Africa was prevented by an arid band, and the separation of the Zambezi river into two distinct systems. **F:** Formation of the Saharo-Arabian desert severed any connections with Asia. *Neoperla* is left isolated in the Nearctic, Indo-Malay and Afrotropical regions. In Southern Africa, activity in EARS changes climatic conditions, and causes the Zambezi river to connect to the Limpopo system, allowing dispersal into Southern Africa. Palaeomaps used in figure are from Scotese *et al.* (2025) and are utilized under the Creative Commons Attribution 4.0 International License, available from https://doi.org/10.5281/zenodo.10659112 (Accessed 24 February 2025).

Therefore, these findings instead support Zwick's (2000) hypothesis that *Neoperla* entered the Nearctic later, probably during the Paleogene. This dispersal possibly occurred via a land bridge. Two such bridges were present intermittently throughout the Cenozoic, namely the Bering land bridge, which connected eastern Asia to the northwest of North America, and the North Atlantic land bridge, which connected the east of North America to Europe via Greenland (Denk *et al.* 2011; Graham 2018; Jiang *et al.* 2019; Tiffney 1985; Wen *et al.* 2010, 2016). Zwick (2000) considered the Bering land bridge as the most probable route, whereas I instead suspect *Neoperla* spread into the Nearctic through the North Atlantic land bridge. While the Bering land bridge has acted as a route for the exchange of temperate-adapted taxa, the North Atlantic land bridge was dominated by deciduous, "Boreotropical" forests (Tiffney 1985; Wen *et al.* 2010; Xiang & Soltis 2001), which would have provided a suitable environment for the spread of *Neoperla* (Figure 4.5). In either case, no representatives are extant in the intermediate zone (i.e. northwest North America or Europe, DeWalt *et al.* 2025), suggesting a localized extinction after dispersal. This was possibly associated with decreasing temperatures, and the retreat of the boreotropical forests throughout the Miocene (Tiffney 1985; Wen *et al.* 2010, 2016; Xiang & Soltis 2001).

Both land bridges were interrupted intermittently during the Paleogene, before opening permanently in the mid-Miocene (Tiffney 1985; Wen *et al.* 2010, 2016; Xiang *et al.* 2000; Xiang & Soltis 2001). Disruption of this route, potentially due to high sea levels during the Eocene thermal maximum, may therefore have led to the initial divergence of the Nearctic species, giving rise to either *Neoperla* (*Neoperla*) or the *N. occipitalis* and *N. choctaw* species groups due to vicariance (Figure 4.5). If *Neoperla* (*Neoperla*) arose in the Nearctic, it must have spread back into the Afrotropical region and Asia, potentially via these same land bridges during the Oligocene or Miocene.

4.4.2.2. Dispersal into Africa

Most authors consider *Neoperla* a recent arrival in Africa, probably dispersing onto the continent during the Neogene alongside mammal lineages from Eurasia (Fochetti & Tierno de Figueroa 2008; Illies 1965; Sroka & Prokop 2023; Zwick 2000). Recently, Letsch *et al*. (2021) suggested that this arrival may have happened much earlier, although no specific date was suggested because no Afrotropical species were included in their analysis. Here, the origin of *Neoperla* (*Neoperla*) was dated to the early Eocene, about 50.2 Ma (55.8 – 44.7 My). The biogeographic analysis suggested that the dispersal into Africa must have occurred by this time, as it recovered a probable combined ancestral range of Congo and Upper Guinea for *Neoperla* (*Neoperla*) (Figure 4.3). However, the subgenus further diversified into at least two distinct radiations during the middle Eocene (**Clade 1**: 47.3 Ma, HPD: 52.9 – 41.9 Ma; **Clade 2:** 45.3 Ma, HPD: 50.8 – 40.1 Ma), with the Asian *Neoperla ignacsiveci* (extant range: Taiwan) placed as sister to the *spio, orthonema, dubia, arambourgana,*

sjostedti and *excisa* assemblages. While it is possible that *N. ignacsiveci* represents a migration from Africa back to Asia, it is far more likely that this ancestral range is an artifact of greater sampling from Africa, overwhelming the Holarctic signal in the analysis. In this case, the genus probably arrived in Africa later.

The endemic Afrotropical species assemblages, such as *duodeviginti, excisa* and *sjostedi*, were estimated to originate in the latest Eocene – Oligocene here. This estimate suggests that *Neoperla* dispersed into the Afrotropical region either during the Eocene, or during the Oligocene. Additionally, this division of the African taxa into two distinct radiations suggests that either multiple lineages of *Neoperla* dispersed to (perhaps different parts of) Africa simultaneously, or that multiple dispersal events occurred during the Paleogene. As discussed in Chapter 2, these two clades may represent different ancestral ranges (Asia and the Nearctic), as some African *Neoperla* seem to be more closely related to the American fauna than Asian fauna. It is therefore possible that lineages from both the Neartcic and Asia dispersed into Africa simultaneously, or separately.

As extant *Neoperla* do not occur in Europe (DeWalt *et al.* 2025; Hynes 1988; Zwick 2023; Zwick & Zwick 2023), they may have migrated directly into Africa from their ancestral range in Asia. However, as discussed above, it is also possible that ancestral *Neoperla* did occur in Europe during the Paleogene while it was warm and humid, with tropical or subtropical forests extending over much of the continent (Graham 2018; Tiffney 1985). If *Neoperla* did spread across the North Atlantic land bridge between Asia and North America, this would have brought the genus into close proximity with Africa, allowing for exchanges between all three continents simultaneously, or as a part of the same dispersal event.

Faunal exchanges of mammals and tropical plants between Africa, Europe and Asia occurred multiple times during the Paleogene (Gheerbrant & Rage 2006; Graham 2018), with four to six separate exchanges occurring from the end Cretaceous–Rupelian (Oligocene), some 33.9 Ma (Gheerbrant & Rage 2006). A Paleogene arrival of *Neoperla* into Africa would probably have coincided with one of these events. As the error margins for the origin of the Afrotropical clades of *Neoperla* cover a significant portion of the Eocene and Oligocene, their dispersal cannot be correlated to any specific dispersal phases. Nevertheless, an arrival in the late Eocene or early Oligocene, potentially aligning with the Bartonian/Priabonian (approx. 37.7 Ma) or Priabonian/Rupelian (approx. 33.9 Ma) dispersal phases (Gheerbrant & Rage 2006), is the most likely (Figure 4.5).

While there is evidence of faunal exchanges occurring prior to the Bartonian during the Early to Mid Eocene (Gheerbrant & Rage 2006; Seiffert 2012), these required crossing the Tethys Seaway, which separated the Atlantic and Indian Oceans throughout the Paleogene (Gheerbrant & Rage 2006; Palcu & Krijgsman 2023; Renner 2016; Sanders *et al.* 2004; Seiffert 2012; Tardif *et al.* 2023). Plecoptera are generally considered poor dispersers (Fochetti & Tierno de Figueroa 2008; Hynes 1976; Zwick

2000), and this large seaway would have presented a significant barrier to the spread of *Neoperla* into Africa. However, it is possible that drops in sea level, for example at the border between the Thanetian and Ypresian (approx. 56 Ma), could have exposed islands between Eurasia and Africa (Gheerbrant & Rage 2006) that acted as "stepping stones" for *Neoperla*. The genus appears to be better suited to long-distance dispersal than most Plecoptera; many species are widespread in Africa, occurring from the southern edge of the Sahara desert across the rest of the continent (Zwick & Zwick 2023). Additionally, at least one undescribed species has been recorded from Papua New Guinea (Zwick 1986, 2023), while *Neoperla burgeoni* has been recorded from Mayotte in the Comoros islands, approximately 500 km from the African mainland (Zwick & Zwick 2023). Both of these suggest trans-oceanic dispersal events (dispersal of *N. burgeoni* discussed below). Additionally, most *Neoperla* are fairly large-bodied, with a wide wingspan, which has been correlated to better dispersal ability in Plecoptera (McCulloch *et al.* 2017). A similar process of long-distance dispersal via exposed islands has also been proposed to explain disjunct distributions of closely related Asian and Madagascan or Mascarene fauna (Bradler *et al.* 2015), although most of these dispersals probably coincided with drops in sea level during the last 10 Ma (Warren *et al.* 2010), and sea levels during the Eocene were generally high (Miller *et al.* 2009; Otero 2010). A late Eocene – Oligocene arrival remains more likely because drops in sea level during the early Eocene predate the ages recovered for the radiation of *Neoperla* (*Neoperla*) (50.2 Ma, HPD: 55.8 – 44.7 Ma).

By the boundary of the Eocene and Oligocene, the Tethys seaway remained open but had begun to retreat (Bradler *et al.* 2015; Goudie 2005; Palcu & Krijgsman 2023; Tardif *et al.* 2023). This retreat led to the emergence of new land and river systems in North Africa (Goudie 2005), and the reduction of deep water barriers between the Indian and Mediterranean oceans (Palcu & Krijgsman 2023). Despite the lack of a land bridge between Africa and Eurasia, faunal exchanges, particularly of mammals, occurred throughout this period (Gheerbrant & Rage 2006; Marivaux *et al.* 2005; Sanders *et al.* 2004; Seiffert 2012; Sen 2013), possibly in two pulses at the end of the Bartonian and Priabonian eras, respectively (Gheerbrant & Rage 2006). Mechanisms behind these dispersals are poorly understood, with either sweepstake dispersal, or "island hopping" along emergent landmasses providing the likely routes (Gheerbrant & Rage 2006; Ni *et al.* 2020; Sallam *et al.* 2018; Sanders *et al.* 2004; Seiffert 2012; Sen 2013).

Two probable routes may have allowed *Neoperla* to spread into the Afrotropical region. Firstly, if *Neoperla* was present in Europe during the Oligocene, they may have dispersed into North Africa via the Iberian Peninsula (Figure 4.5). Throughout the Cenozoic, the Iberian Peninsula was tropical and covered in seasonal rainforests (Barrón *et al.* 2010; Postigo Mijarra *et al.* 2009). Extant *Neoperla* are common in tropical rainforests (Zwick & Zwick 2023), suggesting this would have provided a suitable environment for the early representatives of the genus. Many of the plant taxa present in the Oligocene fossil record of the Iberian Peninsula are today distributed either in East Asia, or the

eastern Nearctic (Postigo Mijarra *et al.* 2009), supporting the hypothesis of large, connected rainforests connecting these two disjunct regions. Additionally, from the Oligocene - Miocene, Alpine folding led to the uplift of the Atlas Mountains in North Africa, and the slow closing of the Western Tethys seaway (Dewey *et al.* 1989; Kirk-Spriggs & McGregor 2009; Schettino & Turco 2011). However, the Western Tethys seaway only closed in the Miocene, and the Mediterranean ocean remained connected to the Atlantic throughout the Oligocene (Harzhauser *et al.* 2002, 2007; Perrin & Bosellini 2012). Similarly, while the closure of this seaway allowed for many groups to disperse from Europe into North Africa, including freshwater fish (Doadrio 1994), charophytes (Soulié-Märsche *et al.* 2002), Diptera (Kirk-Spriggs & McGregor 2009), and even Plecoptera (Weiss *et al.* 2012), most of these exchanges occurred during the late Miocene, once the seaway was fully closed.

If *Neoperla* entered North Africa through this route, it probably would have occurred during the Oligocene, as increased aridity and decreasing temperatures in the Mediterranean had led to mass extinctions of Paleotropical fauna in the Iberian Peninsula by the start of the Miocene (Barrón *et al.* 2010; Postigo Mijarra *et al.* 2009). Dispersal via the Iberian Peninsula would, therefore, require crossing an oceanic seaway. Some recent reconstructions have suggested that there was at least an intermittent land bridge between the Iberian Peninsula and North Africa (Scotese *et al.* 2025), but there is little evidence of this in the biogeographic record of the Tethyan fauna (Harzhauser *et al.* 2002, 2007; Perrin & Bosellini 2012). Strong westerly currents in the Tethys during the Oligocene (von der Heydt & Dijkstra 2006) would have prevented rafting, as these currents would have carried *Neoperla* into the Atlantic ocean. Therefore, sweepstakes dispersal, possibly via "island hopping" over emergent islands in the approximately 300 km Riffian corridor (Harzhauser *et al.* 2002, 2007; Perrin & Bosellini 2012, Scotese *et al.* 2025), provides the most plausible explanation for dispersal into Africa via this route. As *N. burgeoni* has dispersed into the Comoros islands, which is almost double this distance from the African mainland, this route appears feasible.

Alternatively, *Neoperla* may have spread into Africa along the newly formed "Iranian Route" (Figure 4.5). This route formed in the Middle Eocene with the collision of the Indian plate into Asia, and allowed faunal exchanges with Africa via the Arabian plate (Abbassi *et al.* 2015; Ali & Aitchison 2008; Gheerbrant & Rage 2006). This slightly postdates the estimated times of cladogenesis in the Afrotropical *Neoperla* recovered here but falls well within the recovered error margins.

The exact timing of the Indian plate's collision with Asia remains a subject of debate, but most data point to this occurring in the middle Eocene, approximately 55-50 Ma (Jagoutz *et al.* 2015; Meng *et al.* 2023; Renner 2016). The collision led to uplift of the Tibetan plateau and large-scale environmental changes across Asia, including rainfall regimes and climate (Bondarenko & Utescher 2022, 2024; Tardif *et al.* 2023). Both Tibet and India were tropical and humid with high rainfalls (Saxena & Trivedi 2009; Su *et al.* 2020; Tardif *et al.* 2023), probably allowing *Neoperla* to inhabit

these regions, where they are still present (DeWalt *et al.* 2025). There is some evidence that the uplift of the Tibetan plateau led to the onset of the South Asian monsoon system (Beasley *et al.* 2021; Licht *et al.* 2014; Spicer *et al.* 2017; Thomson *et al.* 2021). However, this remains debated, with the monsoons potentially originating later, during the Miocene (Renner 2016; Tardif *et al.* 2023). If these monsoons had developed by the Middle Eocene, it is possible they could have aided in passively dispersing *Neoperla* from Southern Asia across the Tethys sea as aerial plankton, perhaps via the Arabian plate. Such dispersal via aerial plankton could explain the distribution of the Southern Hemisphere Antarctoperlaria (McCulloch *et al.* 2016).

If the monsoons had not yet formed, the collision of the Indian plate with Eurasia still possibly facilitated the dispersal of *Neoperla* to the Afrotropics via the Arabian plate. Prior to its collision with Asia, India was potentially much larger than today, extending an extra 2000 – 3000 km northwards (Meng *et al.* 2023). This extended region could have allowed *Neoperla* to spread southwards before crossing into the Afrotropics either via emergent volcanic islands to the south, for example from activity in the Reunion Plume (Behera & Sen 2014; Bradler *et al.* 2015), or via the Iranian route (Abbassi *et al.* 2015; Ali & Aitchison 2008; Gheerbrant & Rage 2006; Métais *et al.* 2015; Vidal *et al.* 2012). This spread would have been facilitated by large drops in sea level at the end of the Eocene-Oligocene boundary caused by the onset of the Antarctic Ice sheets (Harzhauser *et al.* 2007; Miller *et al.* 2009; Tardif *et al.* 2023), potentially allowing dispersal across the already shrinking Tethys seaway via Zagros and the Arabian plate. By this time, Anatolia and Iran had split the Tethys into two parts, the Mediterranean and Paratethys (see Fig. 1b & Fig. 10 *in* Palcu & Krijgsman 2023). It is plausible that *Neoperla* dispersed along these emergent landmasses into Africa by spreading first into Iran, then Zagros, and finally Arabia (Abbassi *et al.* 2015, 2017; Métais *et al.* 2015; Palcu & Krijgsman 2023). Fossils from both areas show evidence of faunal exchange with Southern Asia, while alluvial deposits suggest freshwater systems were present (Abbassi *et al.* 2015, 2017; Métais *et al.* 2015), potentially providing a habitat for *Neoperla.* In the Arabian plate, palaeo-ichthyofauna fossils show successive marine and freshwater environments, with freshwater fish fossils found by the early Oligocene (Otero 2010; Otero & Gayet 2001). Some of these fossils belong to Asian faunal groups (Otero 2010; Otero & Gayet 2001), suggesting that freshwater fauna were indeed crossing the Tethys along with the groups discussed above.

While I have predominantly discussed the possibility of dispersal via land bridges, ocean currents and winds, it is also possible that *Neoperla* were instead dispersed into both Africa and the Nearctic by birds. Suetsugu *et al.* (2018) demonstrated that the eggs of flightless stick insects can remain intact and even successfully hatch after being eaten and digested by birds, possibly due to the hard, impermeable chorion. This survival was correlated to the widespread distribution of one of these flightless stick insects, *Ramulus mikado* Rehn, across Japan, as birds could disperse viable eggs tens of kilometres after eating them (Suetsugu *et al.* 2023). This may have been further facilitated by

parthenogenesis, as this allows for fertile eggs to be available in the insects, which may only fertilize their eggs immediately before ovipositing (Suetsugu *et al.* 2018, 2023). *Neoperla* eggs share a hard impermeable chorion (Zwick & Zwick 2023), and females mature their eggs for a few days after mating (Hynes 1976; Zwick & Zwick 2023), suggesting that birds may be able to successfully disperse their eggs after eating gravid females. Additionally, while parthenogenesis has not yet been observed in *Neoperla*, it is known to occur in Perlidae (Harper 1973).

In freshwater organisms, endozoochory in birds occurs in Chironomids (Green & Sánchez 2006), and is at least a viable explanation for the long-distance dispersal of invasive fish over hundreds of kilometres, as they successfully hatch after long digestion times (Lovas-Kiss *et al.* 2020). The anchor cap in *Neoperla* eggs strongly adheres to substrates in fast-flowing currents (Hynes 1976), and may also allow for the eggs to become attached to the feathers or legs of wading birds (Green & Sánchez 2006; Lovas-Kiss *et al.* 2020). Several bird groups appear to have been widespread in Africa, Asia and Europe during the Paleogene - Miocene (Mayr 2005; Oliveros *et al.* 2020), including insectivorous birds like barbets (Sheldon *et al.* 2015) and waterbirds that were widely distributed around the Mediterranean (Dyke & Walker 2008; Mayr 2005). It is possible that migrations of these species contributed to the sweepstakes dispersal of *Neoperla* across the Tethys seaway.

By the end of the Cretaceous, and throughout the Paleogene, the northern reaches of Africa were tropical and wet, with large tropical rainforests covering most of West Africa and modern-day Egypt and Sudan (Jacobs 2004; Morley 2000). Several large river systems were already present, such as the Niger in the west, which may have been fed by a trans-African drainage system from Sudan and Egypt, and the Niger Delta in the east, which formed in the Benue Rift (Goudie 2005). To the south, the Palaeo-Congo River was already present, although it appears to have drained further north, in modern-day Gabon (Anka *et al.* 2010; Flügel *et al.* 2015), or to the east (Stankiewicz & de Wit 2006). By the end of the Bartonian, the retreat of the Tethys Ocean began to lead to the formation of the Nile, which started during the Eocene – Oligocene as three disconnected drainage systems (Goudie 2005). These systems would have provided an ideal environment for the newly arriving *Neoperla*, while the relatively flat landscape and low watersheds between drainage systems (Otero 2010) would have allowed them to rapidly disperse across the north of the continent.

While the hypothesis of a later dispersal of *Neoperla* during the Miocene (Figure 4.5), proposed by Illies (1965) and Zwick (2000), is not supported by the dated phylogeny recovered here, it should not be disregarded. By the end of the early Miocene, movement of the Arabian plate led to the complete closure of the Tethyan seaway and the formation of the *Gomphotherium* landbridge, which connected Africa to Eurasia via Arabia (Harzhauser *et al.* 2007; Sen 2013; Tardif *et al.* 2023). Much of this route was still humid and tropical, with heavy rainfall sustaining rainforests (Acosta *et al.* 2024; Steinthorsdottir *et al.* 2021). These conditions were ideal for range expansions in *Neoperla*.

Additionally, the South Asian Monsoon had certainly formed by the Miocene (Renner 2016; Tardif *et al.* 2023; Thomson *et al.* 2021), with winds potentially aiding dispersal into the Afrotropics. By the late Miocene the formation of the Saharo-Arabian desert caused reductions in freshwater systems along this route (Otero 2010; Otero & Gayet 2001), and probably severed this connection.

4.4.2.3. Diversification in Africa

Neoperla appears to not have immediately dispersed across the entirety of Africa after its arrival. Instead, it seems their migration into the southern reaches of the continent occurred slowly, through a complex system of changing climates and drainage systems that started in the Paleogene, and continued until the Pliocene or Pleistocene. Equatorial Africa has many species of *Neoperla*, while only seven species are known from southern Africa, all of which are widespread, with their range extending to the Sahara (Appendix 4.4, Zwick & Zwick 2023), suggesting more recent dispersals. This pattern was also recovered by my biogeographic analysis, which returned an ancestral range in a combination of the Congo, Upper Guinea and East Coast systems for all lineages.

As discussed above, it is likely that early *Neoperla* dispersed across the tropical north of the continent during the Eocene or Oligocene. However, at this point, dispersal southwards into the interior of Africa may have been prevented by a dry, "savannah-like" climate, which was experiencing increased levels of water stress (Jacobs 2004). The Eocene coincides with a depositional hiatus from the Palaeo-Congo River (Anka *et al.* 2010; Flügel *et al.* 2015), suggesting reduced flow and sediment load.

Conditions changed during the Oligocene and Miocene. The rise of the Afar Plume during the Oligocene led to regional uplift in northeast Africa, creating new river systems in the Sahara and Sudan, and altering the drainage patterns of already existing major rivers (Bussert *et al.* 2018; Flügel *et al.* 2015; Goudie 2005). In particular, sedimentation of the Congo River increased dramatically as new drainage lines into the Congo basin appeared and flow increased (Anka *et al.* 2010; Flügel *et al.* 2015; Goudie 2005). Simultaneously, the tropical rainforests begin to extend south as the African plate drifted north, although large parts of the northeast and Sahara were still humid and forested (Bussert *et al.* 2018; Goudie 2005; Jacobs 2004). These patterns intensified into the Miocene, with further uplift in the Sahara and East African Rift System (EARS) creating new river systems to the east, the reversal of drainage and flooding of the Queza-Nile in the north, and drainage captures and development of the Congo basin, leading to the large system seen today (Bakker & Mercer 1986; Bussert *et al.* 2018; Flügel *et al.* 2015; Goudie 2005; Stankiewicz & de Wit 2006). The results of my dated phylogeny and biogeographic analyses point to a period of diversification of *Neoperla* in the Congo, West Africa and East Africa during the Oligocene, extending into the Miocene (Figure 4.2 & 4.3). These geological and hydrological changes may have provided new habitat and river systems for *Neoperla* to disperse into the interior (Figure 4.6), and potentially facilitated this diversification. A

similar pattern has been observed in other Afrotropical freshwater groups such as crabs (Daniels *et al.* 2015), *Lanistes* gastropods (Mahulu *et al.* 2021), *Distichodus* fish (Arroyave *et al.* 2020), and *Synodontis* catfish (Day *et al.* 2013; Pinton *et al.* 2013).

The developing Congo basin appears to have been at the centre of this diversification because it was recovered as at least part of the ancestral range of every lineage in the biogeographic analysis (Figure 4.3). The Congo basin today is one of the largest river catchments in the world, second only to the Amazon (Flügel *et al.* 2015), and supports some of the highest biodiversity in the Afrotropical region (Cotterill 2003; Daniels *et al.* 2015; Flügel *et al.* 2015). This basin dominated the evolution of many of Africa's freshwater taxa, particularly during the Miocene, as it shaped the evolution and distribution patterns of fish (Arroyave *et al.* 2020; Goodier *et al.* 2011), gastropods (Mahulu *et al.* 2021; Schultheiß *et al.* 2014) and crabs (Daniels *et al.* 2015). Unlike many of these groups, many Afrotropical *Neoperla* species are widespread, with only a few geographically isolated clades and species (e.g. *N. tangana* and *N. massevensis* limited to the East Coast). This wide range suggests that vicariance of river basins had little effect on *Neoperla* diversification. The Congo basin may instead have facilitated connectivity between disparate river basins for *Neoperla*, as it sustained connections to the Niger, Nile, East Coast and Cueza systems until the late Miocene (Figure 4.6), when volcanic activity and rifting in the East African Rift System finally severed these connections some 12 – 3 Ma (Bauer *et al.* 2010; Flügel *et al.* 2015; Goodier *et al.* 2011; Goudie 2005; Spiegel *et al.* 2007; Stankiewicz & de Wit 2006). This rifting and separation may have led to the slightly different assortment of species found between the Upper Guinea, East Coast and Nilo-Sudan basins, although at least some species are present in all three, supporting the continued migration of species between these basins, possibly via the Congo. During the Quaternary, periods of increased humidity and rainfall, coupled with lowered evaporation rates, resulted in a "green-Sahara", with large systems of interconnected megalakes and hydrological networks (Drake *et al.* 2022; Drake & Bristow 2006). These interconnected systems may also have allowed some species to spread between these systems, and may have allowed *Neoperla* to spread south via humid pathways (Head & Gibbard 2015; Kirk-Spriggs & McGregor 2009; Trauth *et al.* 2009).

Comparatively, links to Southern Africa seem to be recent, with no evidence of *Neoperla* migrating south during the Miocene. This separation may have been caused by a combination of climatic and geological conditions. During the Paleocene, uplift along the Okavango – Kalahari – Zimbabwe axis separated river courses, and severed links between Northern Africa and the rivers of the south (Key *et al.* 2015; Moore & Larkin 2001). Amongst these, the Lower and Upper Zambezi were split into two systems, with ancient links between the upper Zambezi and the Limpopo river severed (Goudie 2005; Key *et al.* 2015; Moore *et al.* 2012; Moore & Larkin 2001). This separation persisted until the Pliocene – Pleistocene (Goudie 2005; Key *et al.* 2015; Moore *et al.* 2012; Moore & Larkin 2001). Similarly, during the Miocene most of Southern Africa was covered in savannah (Bakker & Mercer

1986; Flügel *et al.* 2015; Neumann & Bamford 2015), and an arid band (Neumann & Bamford 2015) would have prevented movement south by *Neoperla* (Figure 4.5-4.6)

This separation terminated in the Pliocene – Pleistocene, as continued volcanic activity and rifting in the EARS led to the separation of the East Coast from the Congo, creating new watersheds and forming Lake Tanganyika (Bauer *et al.* 2010; Cotterill 2003; Flügel *et al.* 2015; Goodier *et al.* 2011). Headwater erosion led to the lower and upper Zambezi reconnecting, causing heavy flow into this system, which eroded the Batoka Gorge, formed the Victoria Falls, and filled Palaeolake Makgadigadi (Cotterill 2003; Flügel *et al.* 2015; Joyce *et al.* 2005; Key *et al.* 2015; Moore *et al.* 2012). Increased sedimentation and flow along the eastern edge of the continent show increased flow in the region (Dollar 1998), while the deep erosion of the Batoka Gorge suggests a larger headwater of the Zambezi and large amounts of water flowing into the system (Key *et al.* 2015; Moore *et al.* 2012). These new river systems and geological changes have been linked to radiations in mammals and freshwater fish (Cotterill 2003; Goodier *et al.* 2011; Joyce *et al.* 2005). It is likely that these novel rivers and drainage systems, along with increased connectivity of rivers along the southern East Coast, provided a route for *Neoperla* to enter Southern Africa (Figure 4.6).

Finally, the presence of *N. burgeoni* on Mayotte, in the Comoros Islands (Zwick & Zwick 2023), can only be the result of long-distance dispersal across the Mozambique channel. The Comoros archipelago formed during the Miocene due to volcanic activity and rifting (Michon 2016; Rougeau *et al.* 2025), and has not been connected to the mainland via terrestrial land bridge since (Ali & Blair Hedges 2023, Masters *et al*. 2022, Stankiewics *et al*. 2006). *Neoperla burgeoni* must, therefore, have dispersed into the Comoros islands through sweepstakes dispersal or transport by birds. This could have occurred through rafting, but this is unlikely, as the current through the Mozambique Channel has been strongly orientated towards Southern Africa throughout the Miocene (Ali & Hedges 2023; Masters *et al.* 2021; Stankiewicz *et al.* 2006), although anticlockwise eddies in the north of the channel (Backeberg & Reason 2010) may have carried vegetation rafts to Mayotte. Alternatively, *N. burgeoni* may have been carried into the Comoros islands by tropical storms or a tornado. Tornadoes have been recorded carrying small objects >300km (Stankiewicz *et al.* 2006), and would probably be able to carry a small stonefly as aerial plankton. Alternatively, as discussed above, birds may have carried *N. burgeoni* across the Mozambique channel through endozoochory, or via eggs attached to the feathers of wading birds. Alternatively, *Neoperla burgeoni* could have spread throughout the Comoros islands by island hopping along the archipelago. At present, the species is only known from Mayotte, the easternmost island, but it is possible that the species occurred on previous, now submerged islands within the archipelago (Craig 2003; Heads 2005b).

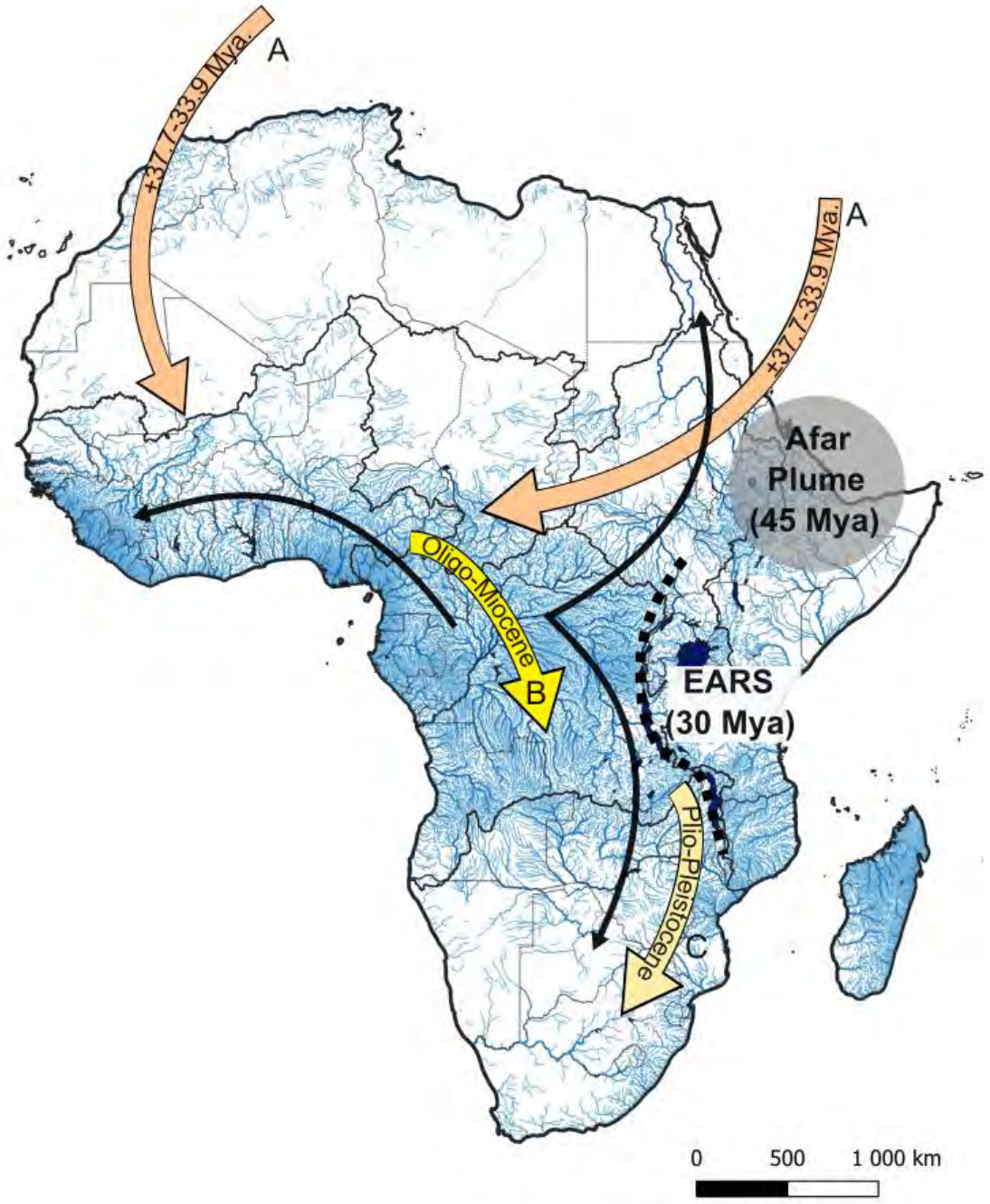

Figure 4.6: A: Map of Africa, showing a possible dispersal route of *Neoperla* into Africa based on the estimated time of cladogenesis and biogeographical analysis. **A:** *Neoperla* disperses into the Afrotropics during the late Eocene – early Oligocene, potentially via the Iranian route or the Iberian Peninsula. Uplift in the Afar Plume, and seismic activity along the East African Rift Valley (EARS: Dotted line) during the late Oligocene – Miocene leads to the formation of the Congo basin, and **B:** the dispersal of *Neoperla* into the interior. Southern Africa remains isolated after uplift along the Okavango-Kalahari-Zimbabwe axis separated river courses, until further activity of the EARS led to the recombination of the Zambezi river, allowing **C:** *Neoperla* to disperse into this region during the Pliocene and Pleistocene. River and hydrological basin data were obtained from HydroSHEDS (http://www.hydrosheds.org; Lehner & Grill 2013). Continent and country borders were obtained from Natural Earth (https://www.naturalearthdata.com/).

5. Chapter 5: The South African *Neoperla*, and a key to the mature nymphs

5.1. Introduction

While Plecoptera as an order is widespread and occurs in a range of ecological niches worldwide, most species for which such data are available are specialized and highly sensitive to environmental conditions (Brittain 1990; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008; Hynes 1976; Stewart 2009). They are often the first insects to be lost from rivers after even minor changes to water chemistry, usually caused by shifts in temperature regimes, flow rates (e.g., due to damming or decreased rainfall), nutrient availability (e.g., eutrophication), and pollution (DeWalt & Ower 2019; Hynes 1976; Lenat 1993). This sensitivity makes Plecopterans valuable as biological indicators of water quality, and they form an important component of biomonitoring methods, such as the South African Scoring System (SASS) (Dickens & Graham 2002), and the Ephemeroptera, Plecoptera and Trichoptera (EPT) index (Lenat 1993). In South Africa, Notonemouridae and Perlidae are two of the most sensitive families in the SASS v5 river assessment method, weighted 14 and 12 (on a scale of 1: highly tolerant – 15: highly sensitive), respectively (Dickens & Graham 2002).

Unfortunately, this sensitivity places many Plecoptera at risk of population decline and extinction due to anthropogenic impacts, and many species globally have shown significant range reductions and high death rates (DeWalt *et al.* 2005; DeWalt & Ower 2019; Dijkstra *et al.* 2014; Macadam *et al.* 2022; Tierno De Figueroa *et al.* 2010). For example, in Illinois, United States of America, 15 out of 77 stonefly species have been declared regionally extinct since the 1950s, with another six not recorded since 1960 (DeWalt *et al.* 2005). Two of these species were regional endemics and are now extinct (DeWalt *et al.* 2005). Pressures on stonefly populations are expected to rise with global climate change and further anthropogenic activities that affect freshwater systems (DeWalt & Ower 2019; Tierno De Figueroa *et al.* 2010), and freshwater systems as a whole continue to be increasingly disturbed (Day & Davies 2023; Dijkstra *et al.* 2014; Dudgeon *et al.* 2006; Reid *et al.* 2019; Strayer & Dudgeon 2010).

Ecological data, such as the distribution, population density, habitat preferences, and physiological limits of a species are important for predicting how changing conditions will affect species (Dallas 2016) and for the effective conservation and management of species and habitats (IUCN 2024; Macadam *et al.* 2022; Melville *et al.* 2021; Ripple *et al.* 2017; Rowntree *et al.* 2000). Similarly, these data greatly improve the accuracy of biomonitoring methods as increased knowledge of a species' ecological traits allows for greater nuance, interpretation and understanding of its significance as an indicator of environmental, ecological or biodiversity changes (Dolédec *et al.* 2000; McGeoch 1998; Menezes *et al.* 2010). Unfortunately, information on the physiological requirements, biology, and

distribution of Plecoptera is generally lacking in South Africa, even amongst the relatively well-studied Notonemouridae (Picker & Stevens 1997, 1999; Stevens *et al.* 2018; Stevens & Picker 1995, 1999).

Distribution data and emergence times are available for all 31 of the Notonemourid species (Picker & Stevens 1999; Stevens & Picker 1995, 1999, 2003), and five species were described with some behavioural and ecological notes (Balinsky 1956, 1967). Additionally, the thermal tolerance of *Aphanicerca capensis sensu lato* Tillyard was investigated (Dallas 2016; Ketley 2009), and studies examined the distribution of some Notonemourid species along river continua (Bellingan 2010; Harrison 1958; Harrison & Barnard 1972; de Moor & Bellingan 2010). However, gaps remain for many species, as new collection records have not been published since the turn of the century, or earlier, for most species (e.g., *Aphanicerca uncinata* Barnard has not been collected since its original description in 1934)(Stevens *et al.* 2018; Stevens & Picker 2003). Many nymphs remain undescribed, and their habitat preferences and ecology are mostly unknown (Picker & Stevens 1997; Stevens & Picker 2003). Additionally, Notonemourids are usually collected from clear mountain streams in forested areas (Balinsky 1956, 1967; Harrison 1958; Harrison & Barnard 1972; McLellan 1991), but recent collections have indicated that they can occur in a much wider range of habitats, as nymphs of *Afronemoura amatolae* Balinsky and *Aphanicercella cassida* Barnard were both collected from small seeps and runs (Balinsky 1956, pers. obs.), and some specimens of *Aphanicercella sp.* were collected from the lowland river sections of the Sabie River in Kruger National Park (pers. obs., LIM catalogue, Albany Museum, Makhanda, South Africa).

Compared to Notonemouridae, ecological data for the African *Neoperla* species are extremely sparse. This bias is not due to a lack of interest; the emergence periods of adults (Tjønneland 1961), their distribution (Ogbogu 2006), their ecological requirements and habitats (Arimoro *et al.* 2011; Edia *et al.* 2016; Hynes 1976; Sivec *et al.* 1988), and their responses to changes in water chemistry (Arimoro *et al.* 2011; Edia *et al.* 2016; Hynes & Williams 1962; Ito & Ugbomeh 2017) have all been examined. Many of these studies provided fascinating results and generally supported the conclusion that African *Neoperla* are sensitive to environmental changes, with increased electrical conductivity, increased temperatures, and, by extension, decreasing oxygen content, all leading to declines in their abundance (Edia *et al.* 2016; Ito & Ugbomeh 2017). While *Neoperla* generally occur in forested, stony-bottomed rivers (Chutter 1968; Dallas 2007; Hynes 1953), some specimens were found in the margins of lakes in Central Africa (Hynes 1976). However, these studies were completed while all African *Neoperla* were treated as *Neoperla spio sensu lato*, resulting in the genus being treated as a widespread and abundant generalist species in Africa. This means it is currently unclear what of this ecological data applies to each of the 93 now recognized species (83 from Zwick & Zwick, 2023; 10 more from Chapter 2). Original descriptions made before Hynes (1952) synonymized the species did not include ecological notes, and generally had only very imprecise locality information [see for example

(Barnard 1934; Enderlein 1909; Klapálek 1909, 1911, 1923; Navás 1919)]. Nevertheless, evidence from museum collections suggests that many *Neoperla* species in Africa are showing the same declines seen in other Plecoptera. Zwick & Zwick (2023) noted that "early collections were made prior to serious environmental impact, e.g., deforestation, pollution, and the systematic application of insecticides to streams in the combat against Simuliidae, the vectors of River Blindness. A few species known from the old collections are rare in recent samples or are missing completely" (Zwick & Zwick 2023, 7–8).

With these gaps in mind, an increased understanding of the distribution, habitat, physiological and ecological requirements of the species in this genus is urgently needed, both to guide conservation and red-listing efforts, and to reassess their value as biological indicators in freshwater systems. This will require large-scale assessments across South Africa, which is beyond the scope of this study. Nevertheless, in this chapter, I aim to facilitate this initiative with a baseline summary of the sparse information that is available for each of the South African species that can be elaborated through future collections and research.

Unfortunately, the collection of further data is difficult, as only adults can be identified to species level reliably using morphological characters (Zwick & Zwick 2023). Adult *Neoperla* are nocturnal and are usually caught using light traps and passive traps, meaning that collections are dominated by nymphs captured by less labour-intensive methods. For example, of the 276 sampling events from South Africa vouchered in the national collection of aquatic insects at the Albany Museum, Makhanda, South Africa, only 21 included adults. Species identifications can be confirmed using the COX1 barcode region (Zwick & Zwick 2023), but this is often expensive in large-scale studies (Reguero *et al.* 2014; Stein *et al.* 2014). Additionally, *Neoperla* species are regularly sympatric (Picker 1980; Zwick 1976; Zwick & Zwick 2023, *e.g. Neoperla panafricana* Zwick & Zwick & *Neoperla sjostedti* Klapálek both occur in Mbotyi Forest, Eastern Cape and *Neoperla transvaalensis* Enderlein and *Neoperla burgeoni* Navás coexist in the Mzimkhulu River in KwaZulu-Natal), so many specimens from a single locality may need to be sequenced to identify them accurately.

Metabarcoding of bulk samples has become increasingly popular, and offers a cost-effective approach to using the COX1 barcoding region to identify species diversity (Meyer & Paulay 2005; Pereira-da-Conceicoa *et al.* 2021; Van Der Loos & Nijland 2021). This method has many advantages, as it can differentiate cryptic species, and captures species that may be missed due to standard collecting methods (e.g., species that are only present as eggs or small nymphs during a season) (Pereira-da-Conceicoa *et al.* 2021; Van Der Loos & Nijland 2021). Additionally, these barcodes allow for the distribution and effect of environmental stressors to be identified in species that are not yet described, as data can be reanalyzed as reference libraries and species are described (Pereira-da-Conceicoa *et al.* 2021). However, these methods can still be biased by difficulties in identifying the "barcoding gap"

(Meyer & Paulay 2005) and variation in PCR methods (Van Der Loos & Nijland 2021). Additionally, voucher material is generally destroyed, or only distinct morphospecies kept (Pereira-da-Conceicoa *et al.* 2021; Van Der Loos & Nijland 2021), which prevents verification of these findings. Therefore, the development of these sampling methods should also be accompanied by an improvement of taxonomic resolution and DNA reference libraries for the targeted groups (Pereira-da-Conceicoa *et al.* 2021; Van Der Loos & Nijland 2021).

To facilitate future research, a key to the more commonly collected nymphs of the South African species was constructed. The key to adults constructed by Zwick & Zwick (2023), and amended in Chapter 2, remains the best resource for identifying adult African *Neoperla*.

5.2. Methods

5.2.1. Distribution Records

All published distribution records of *Neoperla* species in South Africa were obtained from their original descriptions (Barnard 1934; Enderlein 1909; Klapálek 1909, 1911; Zwick & Zwick 2023), and the recent revision of Afrotropical *Neoperla* (Zwick & Zwick 2023). Additionally, all South African *Neoperla* from the Albany Museum, Makhanda, South Africa (276 sampling events, predominantly nymphs), and the Department of Zoology and Entomology, Rhodes University, Makhanda, South Africa (7 adults) were reviewed. Adults were identified using the key by Zwick & Zwick (2023). Nymphs were not included unless their identification was verified using COX1 barcodes (Chapter 2). These records were supplemented with adults and nymphs collected during this study (refer to Chapter 2 for collection methods and localities).

These records were used to construct distribution maps in QGIS V3.34.12 (QGIS.org 2025), overlayed with the rivers of South Africa dataset obtained from HydroRIVERS (Lehner & Grill 2013) and HydroBASINS (Lehner & Döll 2004). As the range of these species may have changed over time (see above), records were separated into historical categories (>50 years old, 26-50 years old, 16-25 years old, 6-15 years old, 0-5 years old). Older records are considered less precise and the continued presence of the species in these regions should be verified by new collections. These distribution maps are incomplete because only 100 South African records of *Neoperla* can currently be identified to species level (Appendix 5.1). Therefore, these maps provide only an indication of distribution for each species, rather than a definitive summary of presence across the country.

5.2.2. Ecological Notes

Emergence dates for adults were estimated using the collection dates from each of the records. For all newly collected material, physical conditions and water chemistry measurements were taken on site using a Hanna Instruments HI-98130 pH meter. These measurements included temperature, electrical conductivity (EC), pH, and total dissolved solids (TDS). At localities where nymphs were collected, the habitat surrounding the river, the biotope(s) specimens were collected in (following Dickens & Graham (2002)), and river type (following the descriptions of Rowntree *et al.* (2000)) were recorded. Biotope(s) included stones in current (SIC), marginal vegetation in current (MVIC) and submerged macrophytes (Sub. Macro). Biomes surrounding the collection localities were inferred from the South African National Biodiversity Institute (SANBI) National Vegetation Map (SANBI 2012) using QGIS. In cases where specimens were found on the edge of multiple biomes (e.g., Mbotyi Forest, in the Indian Ocean Coastal Belt), each biome was included.

5.3. Results

Neoperla burgeoni Navás, 1926

Unique Collecting Events: 25

Taxonomic Notes: *Neoperla burgeoni* is morphologically heterogeneous, and molecular analyses separate the species into several lineages that may represent a species complex (Zwick & Zwick 2023; Chapter 2). Zwick & Zwick (2023) consider it likely that *N. burgeoni* is a species complex. However, the South African specimens all clustered into a single lineage with specimens from Central Africa (Zwick & Zwick 2023; Chapter 2).

Distribution: Widespread in Africa, with records in 20 countries (Figure 5.1) including South Africa, Namibia and Zimbabwe (Zwick & Zwick 2023).

Neoperla burgeoni is relatively commonly collected in South Africa and is particularly well represented in samples collected from the Kruger National Park, Limpopo and Mpumalanga provinces. Its South African distribution is apparently disjunct, with most records from the Eastern Escarpment and eastern Lowveld regions, and a single specimen from the northwest of the Northern Cape, near the Namibian border (Appendix 5.1; Figure 5.1). It is currently unclear if *N. burgeoni* occurs between these regions. A possible specimen has been identified from the Orange River in Upington based on egg morphology (Department of Zoology and Entomology, Rhodes University: M. Villet, Northern Cape, Upington, 28.27S, 21.15E, March 2002: *Neoperla ?burgeoni*, Navas, 1926: Det: A. Kirkaldy), but the spermathecal stalk was damaged during dissection, and the identification could not be confirmed.

Notes on Records: The presence of the species along the Eastern Escarpment and Lowveld has been verified by relatively recent records collected over the last 25 years (Appendix 5.1). Comparatively, *N. burgeoni* was last collected in the Northern Cape 45 years ago, in 1980 (Zwick & Zwick 2023).

Ecological Notes: *Neoperla burgeoni* is found in a wide range of environments, with specimens collected in all South African biomes except the Succulent Karoo (Table 5.1). The species is most commonly collected from Savannah landscapes. Adults were sampled from light traps in a riverine forest alongside a rocky bed foothill section of the Blyde River (surrounding biome: Savannah), while nymphs were also collected from rocky bed rivers in the Drakensberg grasslands (Figure 5.2). However, it seems *N. burgeoni* can occur in a range of river types, as specimens have also been collected from large, lowland sections of the Orange, Umkomaas, Sabie and Luvhuvu Rivers (Zwick & Zwick 2023, Appendix 5.1). All of the nymphs were collected from fast-flowing, stony-bed rivers. Nymphs were found throughout these sites, although they were most common in slightly slower currents, towards the margins of the river (Table 5.2). The water chemistry recorded for the nymphs at two sites suggests that they prefer relatively cold (approximately 18°C, recorded in autumn, March - April), clean water (Table 5.3). Because specimens have been collected from larger, slow-flowing rivers, it seems likely that they are tolerant of a wider range of water conditions.

Seasonality: Adults - present from early spring – late autumn.

Neoperla heideae Zwick & Zwick, 2023

Unique Collecting Events: 1

Taxonomic Notes: Very little ecological information is available for *Neoperla heideae*, which was only recently described (Zwick & Zwick 2023).

Distribution: *Neoperla heideae* has a disjunct distribution, with all other known records from Equatorial Africa, in Cameroon and the Democratic Republic of Congo (Figure 5.3; Zwick & Zwick 2023). A single specimen has been collected from the Waterberg district (Zwick & Zwick 2023).

Notes on Records: The specimen record from Limpopo is fairly recent (2012; Zwick & Zwick 2023), suggesting the species still occurs in this region.

Ecological Notes: *N. heideae* was collected in the Savannah biome (Table 5.1), but satellite imagery of the locality (Google Maps: https://www.google.com/maps, Accessed 28 May 2025) suggests the site is in a densely wooded valley, with steep hills flanking the river. Nymphs have not been collected.

Seasonality: In Central Africa, the species emerges throughout the year (Zwick & Zwick 2023). Its emergence period in South Africa is uncertain.

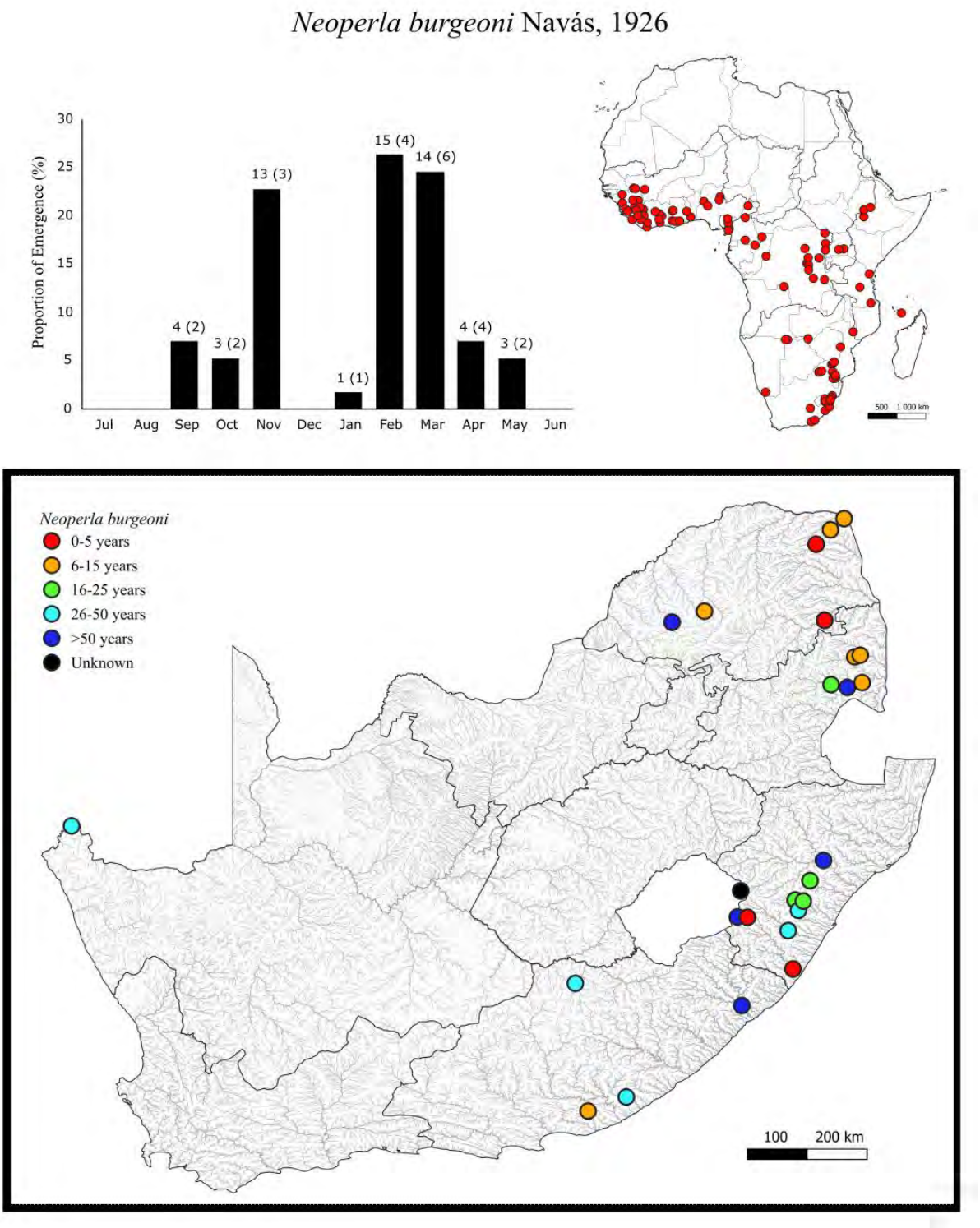

Figure 5.1: Distribution map and emergence times of *N. burgeoni* in South Africa. **Graph:** Proportion (%) of total emergence recorded in each month. Each bar is labelled with the number of specimens collected in that month, followed by the number of collecting events in brackets. **Map of Africa:** Distribution of *N. burgeoni* in Africa. Each red dot represents a collecting event. **Map of South Africa:** Distribution in South Africa. Each record is colour-coded according to the age of the sample.

Figure 5.2: Collection localities of *N. burgeoni*. **A:** Blyde River, near Blyde River Cabins, (24.29S, 30.85E); stony-bottomed, in riverine forest. **B:** Mzimkhuli River, KwaZulu-Natal, 29.75S, 29.43E, stony-bottomed, in mountain grassland. **C:** Typical Drakensberg grassland surrounding Mzimkhulu River, KwaZulu-Natal.

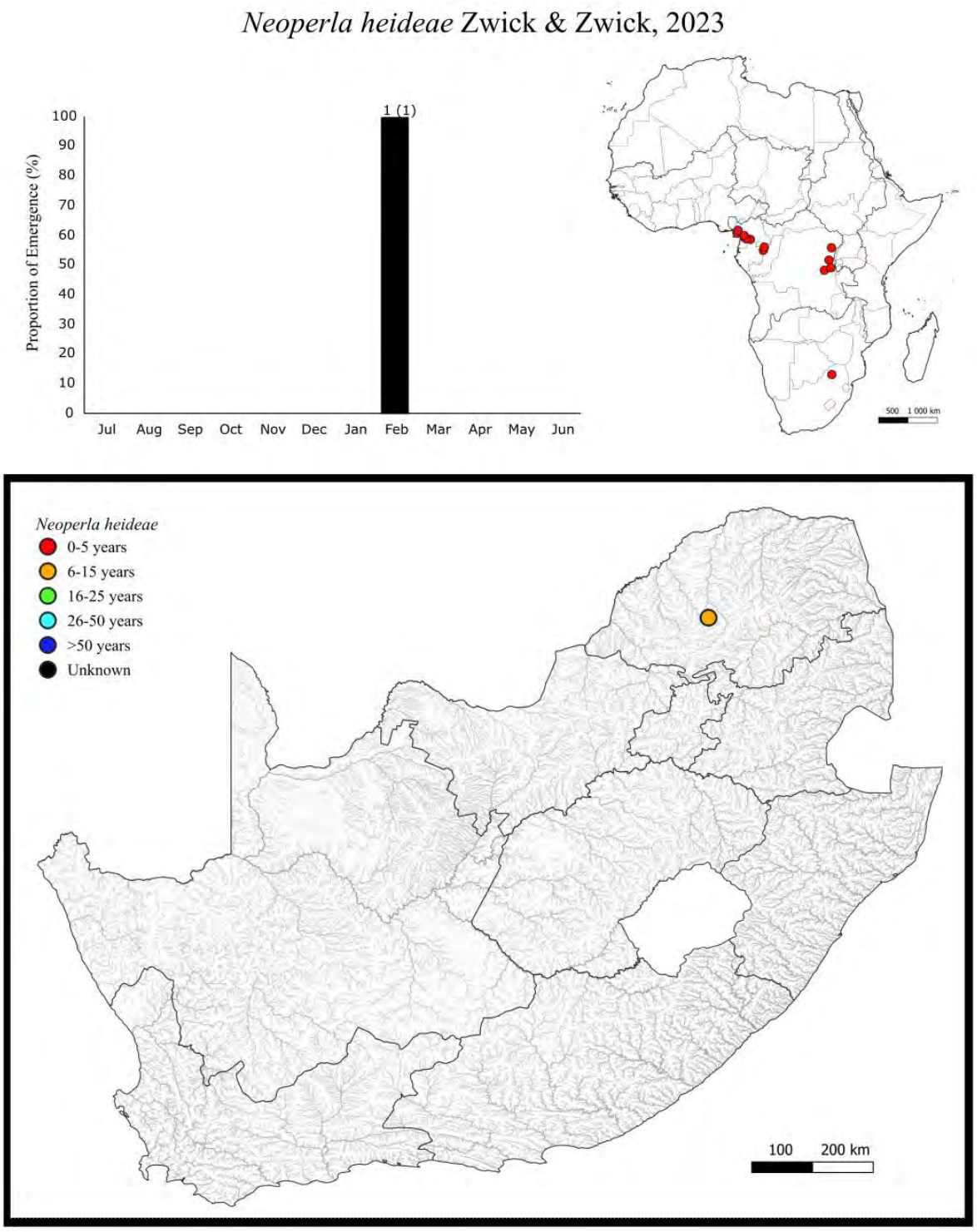

Figure 5.3: Distribution map and emergence times for *N. heideae* in South Africa. All figures follow the same format as Figure 5.1.

Neoperla leroiana Klapálek, 1911

Unique Collecting Events: 2

Taxonomic Notes: Several morphologically distinct morphs of *N. leroiana* occur in isolated regions across its range (Zwick & Zwick 2023). These morphs can be differentiated by the collar of the eggs, the nail on S8 in females, the length of the spermathecal stalk and the width of the T8 process in males (Zwick & Zwick 2023). However, monophyly of the species is strongly supported by molecular evidence (Zwick & Zwick 2023).

Distribution: *Neoperla leroiana* is widespread, occurring in 18 African countries (Figure 5.4), stretching from South Africa, to Sudan and Ethiopia in the northeast, and Senegal in the northwest (Zwick & Zwick 2023).

Neoperla leroiana is apparently rare in South Africa, with only two records from the Eastern Escarpment, near Mashishing in Mpumalanga (Zwick & Zwick 2023), and from Champagne Pools, near Giants Castle in the Drakensberg (Appendix 5.1; Figure 5.4). No recent specimens were collected from the area surrounding Mashishing, despite sampling in the region. However, *Neoperla* nymphs were common, and it is possible that this species was collected but not sequenced, preventing identification.

Notes on Records: The specimen from Champagne Pools was collected in 2024, providing a recent, confirmed record of the species in the Drakensberg. No date was recorded for the specimen from Mashishing.

Ecological Notes: In South Africa, *N. leroiana* has only been collected from mountain grasslands (Table 5.1).

Seasonality: The specimen from Champagne pools was collected in early autumn (March), however, many exuviae were found near the rivers, suggesting that most of the emergence had occurred slightly earlier, in late February (pers. comm., Dr. John Midgley, Assistant Director of Natural Science, KwaZulu-Natal Museum, Pietermaritzburg). Specimens in Zimbabwe were collected in both summer and autumn (Zwick & Zwick 2023).

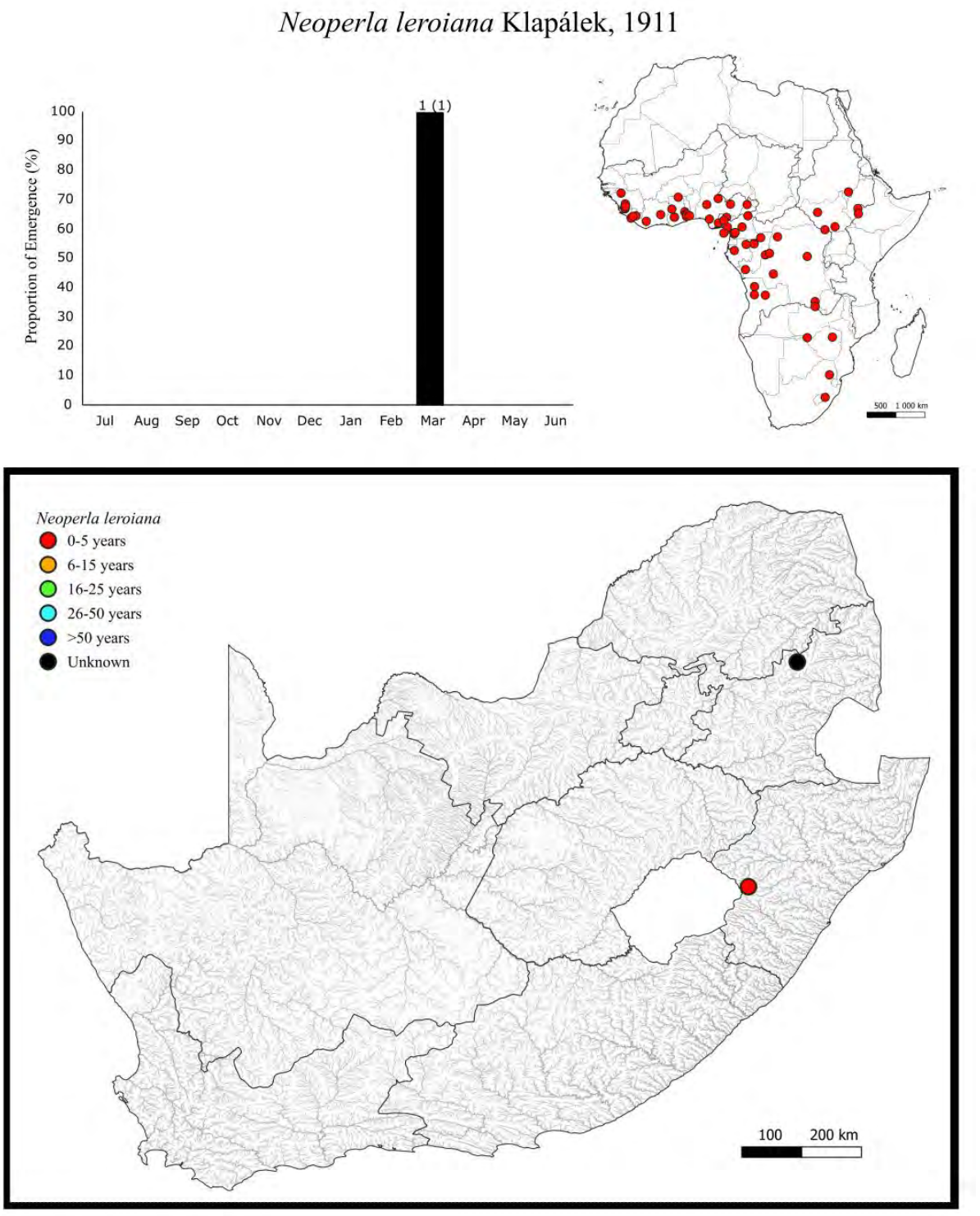

Figure 5.4: Distribution map and emergence times for *N. leroiana* in South Africa. All figures follow the same format as Figure 5.1.

Neoperla panafricana Zwick & Zwick, 2023

Unique Collecting Events: 6

Taxonomic Notes: *Neoperla panafricana* may be a species complex (Zwick & Zwick 2023). Molecular analyses using entire mitogenomes point to several distinct lineages, but specimens represented by only COX1 resolve poorly, often clustering with Northern African specimens, and even specimens of *Neoperla camerunensis* (Zwick & Zwick 2023). Morphologically, the species is homogenous across its range (Zwick & Zwick 2023). The holotype of the species was collected in South Africa (Zwick & Zwick 2023).

Distribution: *Neoperla panafricana* has a disjunct distribution, occurring in 13 countries (Figure 5.5) from sub-equatorial Africa, and parts of Northern and Western Africa (Zwick & Zwick 2023). *N. panafricana* is found from South Africa, to Guinea and Senegal in the northwest (Zwick & Zwick 2023).

Within South Africa, *N. panafricana* is predominantly distributed along the east of the country, occurring in the Lowveld, Eastern Escarpment, Wild Coast, and Waterberg mountains (Appendix 5.1, Figure 5.5) The southernmost record is from near Humansdorp on the Southern Coast, near the Tsitsikamma Forest (Zwick & Zwick 2023).

Notes on Records: Most of the records of *N. panafricana* in South Africa are relatively recent, having been collected between 1995 and 2013 (Zwick & Zwick 2023). However, the southernmost record from Humansdorp was collected in 1950, and the presence of *N. panafricana* has not been confirmed recently, despite sampling in the Eastern Cape.

Ecological Notes: *Neoperla panafricana* has been collected from five biomes in South Africa, namely Fynbos, mountain grassland, Savannah, Indian Ocean Coastal Belt and forests (Table 5.1). According to satellite imagery of the specimens with precise GPS co-ordinates (Google Maps: https://www.google.com/maps, Accessed 28 May 2025), *N. panafricana* was almost always collected from small forest patches within these broader biomes. This includes the type locality, which is Mbotyi Forest in the Wild Coast (Indian Ocean Coastal Belt).

Seasonality: In South Africa, adults have been collected from late spring – summer, with the highest emergence in late February (Zwick & Zwick, 2023; Figure 5). Adults across the rest of Southern Africa have been collected during the same period, although a few have also been found during autumn (April) and winter (July and August) in Northern Namibia and Tanzania.

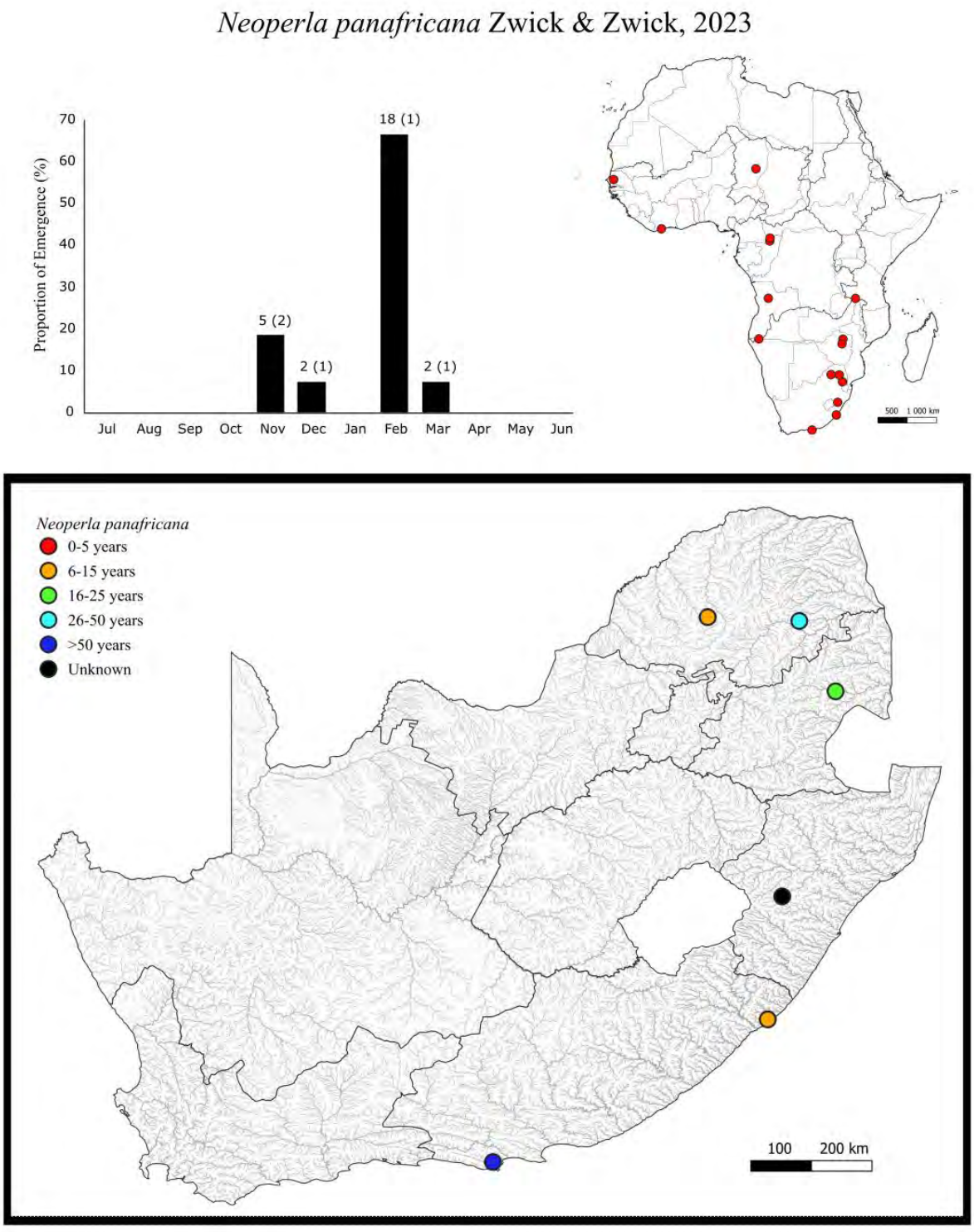

Figure 5.5: Distribution map and emergence times for *N. panafricana* in South Africa. All figures follow the same format as Figure 5.1.

Neoperla sjostedti Klapálek, 1909

Unique Collecting Events: 11

Taxonomic Notes: Zwick & Zwick (2023) suggested that *N. sjostedti* may be a species complex, and recognized two subspecies, and a third morphological variation. The elevation of all three of these to species rank was proposed in Chapter 2 based on molecular evidence. The South African representatives of *N. sjostedti* form a separate lineage that is strongly isolated from the specimens across the rest of the species' range (Zwick & Zwick 2023). Based on this, it is possible that the South African specimens are a new species, but *N. sjostedti*, as it currently stands, is monophyletic and morphologically homogeneous. The nymphs are distinctive and can be identified by the three-lobed "clover leaf" marking on the prothorax, and a banded abdomen (see Key to South African *Neoperla*: mature nymphs below).

Distribution: Compared to the other South African *Neoperla, N. sjostedti* has a relatively limited range across Africa, as it is found only in South Africa, Zimbabwe, Zambia and Kenya (Figure 5.6) in the southeast of the continent (Zwick & Zwick 2023).

Within South Africa, *N. sjostedti* occurs along the eastern and southern margins of the country, with records from the Lowveld, Eastern Escarpment, Wild Coast and Southern Coast (Appendix 5.1; Figure 5.6). It is one of only two *Neoperla* species in the Western Cape, and is the only species known around Cape Town, where 12 females were recorded from Cape Point Nature Reserve in a single collecting event (Zwick & Zwick 2023).

Two records of this species are treated as uncertain here and excluded from the distribution maps. These were recorded as having been collected at the "University of Pretoria", but the (admittedly imprecise) GPS coordinates instead point to Magoebaskloof, in Limpopo Province (Zwick & Zwick 2023). Another specimen was recorded from "Natal, … Malta Forest", but the estimated GPS coordinates placed the sample in the Free State (Zwick & Zwick 2023). Malta forest is a relatively well-known site in Limpopo province, and these locality details were instead used.

Notes on Records: The distribution of *N. sjostedti* presented here is significantly out of date. Most of the distribution records of this species are old, with eight of the eleven made in 1986 or earlier (Zwick & Zwick 2023). More recently, specimens have been collected from Mbotyi Forest in the Wild Coast (Zwick & Zwick 2023), and in the Mpumalanga Drakensberg (Appendix 5.1; Chapter 2).

Ecological Notes: *Neoperla sjostedti* has been recorded from five of South Africa's biomes (Figure 5.6). However, due to the age of these records, most are vague, with inaccurate or imprecise geoco-ordinates given, and without locality notes except for a general area. These biomes are therefore based on the dominant biomes in the region, and not necessarily accurate accounts of where this species occurs.

More recent specimens were collected from forested areas, namely Mbotyi Forest in the Wild Coast, and in forestry areas around Sabie in Mpumalanga. One of the localities where nymphs were collected, Hartebeestvlaagte, is predominantly a mountain grassland, with some dense wooded riparian vegetation along the stream (Figure 5.7). At lower elevations, the mountain is used for pine plantations.

Nymphs were collected from a small, rocky mountain stream, and a foothill river with a stony bed. Both rivers were in heavy flow after continued heavy rainfall in the area (Figure 5.7). The nymphs were present throughout both sites, and were collected from all available biotopes, although they were most commonly found clinging to stones in the fast-flowing current. *Neoperla sjostedti* was sympatric with a Notonemourid, *Aphanicercella cassida* Barnard, in both sites, although the Notonemourids were more abundant towards the sides of the river, in slower currents and marginal vegetation. The water chemistry recorded for the nymphs suggests that they prefer cold (15.9-16.6°C), very clean water, but this was based on only two samples, taken in Autumn (April). Additionally, the region received very high rainfall in the preceding weeks, which may have resulted in altered water chemistry readings.

Seasonality: Adults - present from late spring - late autumn. *Neoperla sjostedti* seems to follow the same emergence times across the rest of its distribution (Zwick & Zwick 2023). A total of 63 adults were collected in a single night from early April 1951 at Royal Natal National Park, in the Drakensberg.

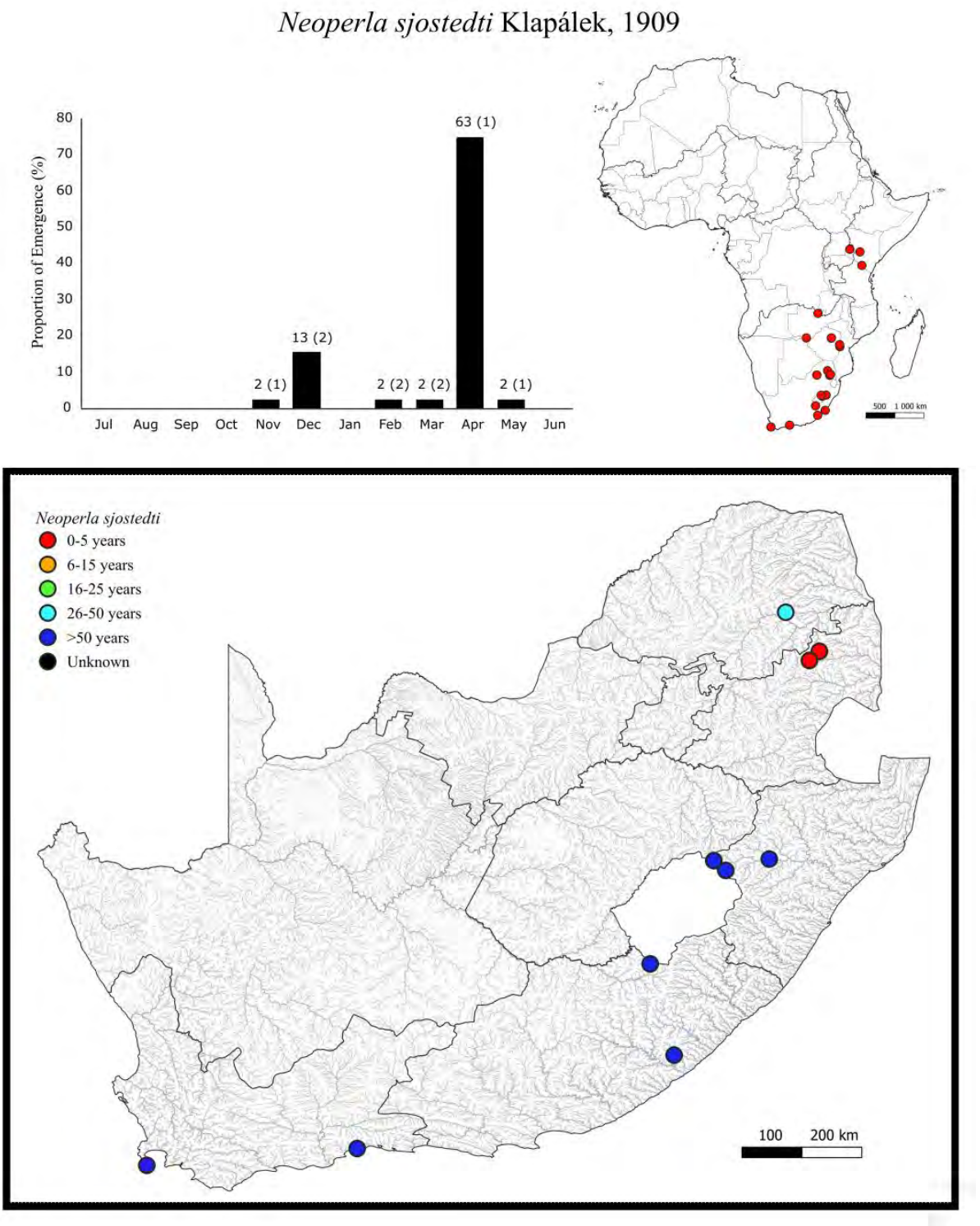

Figure 5.6: Distribution map and emergence times for *N. sjostedti* in South Africa. All figures follow the same format as Figure 5.1

Figure 5.7: Collection localities of *N. sjostedti*. **A-B:** Upper Lisbon River, Mpumalanga, 24.89S, 30.85E, in a plantation forestry area. **B:** Hartebeestvlaagte, -24.89S, 30.85E, surrounded by mountain grassland, and forestry area, stream fast, with dense riparian vegetation.

Neoperla transvaalensis Enderlein, 1909

Unique Collecting Events: 30

Taxonomic Notes: The soft endophallus and spermathecal stalk make it difficult to compare morphology across *N. transvaalensis*' range, but it is generally homogenous, although egg ornamentation varies (Zwick & Zwick 2023). Molecular evidence does not suggest any deep lineages within the species (Zwick & Zwick 2023), but the specimens from Maria Shires waterfall in Mpumalanga form a separate clade from the rest of the South African specimens (Chapter 2).

Distribution: *Neoperla transvaalensis* is widely distributed in Africa, with records from 24 countries (Figure 5.8) across the entirety of sub-Saharan Africa (Zwick & Zwick 2023). Within Southern Africa, it has been recorded from South Africa, Zimbabwe and Namibia, and is the only *Neoperla* known from Lesotho (Zwick & Zwick 2023).

In South Africa, *Neoperla transvaalensis* is widely distributed, with records from the Waterberg, Magaliesberg, Zoutpansberg, Lowveld, Eastern Escarpment, Northern Cape and the Amathole mountains (Appendix 5.1; Figure 5.8).

Notes on Records: The distribution of this species across the eastern parts of South Africa has been verified by many records taken in the last 25 years (Figure 5.8). However, records from the Western Cape, North West and Northern Cape provinces are all fairly old (> 25 years), and the continued presence of the species in these areas needs to be verified.

Ecological Notes: *Neoperla transvaalensis* has been collected from five of South Africa's biomes (Figure 5.8). Although the species apparently occurs in a range of environments, it has been collected most often in mountainous regions (Enderlein 1909; Zwick & Zwick 2023), and is common in grasslands (Table 5.1). Newly collected samples were found in mountain grasslands or forested patches (Figure 5.9). Despite being the most collected species in South Africa, it is rare among samples taken from large lowland rivers, and no specimens were collected from the Kruger National Park. This may point to a preferred distribution in mountainous regions. However, specimens of *N. transvaalensis* have been recorded from the Umkomaas and Orange Rivers, so it is unclear if this is a genuine distribution pattern or a sampling artifact.

Nymphs were collected from seven rivers, and were generally clinging to stones in the fast-flowing currents of rocky-bottomed foothill rivers (Figure 5.9; Table 5.2). They were rare in slower, marginal stretches of the river. Some specimens were collected from a small mountain stream near Karkloof (KwaZulu-Natal), clinging to rocks in slow-flowing currents. The water chemistry readings [taken in autumn (April) and spring (November)] suggest that nymphs prefer cool (16.2-20.2°C), clean water (Table 5.3). *Neoperla transvaalensis* was also collected from sites where water chemistry was good, but there were clear anthropogenic impacts, including cattle farming and forestry (Figure 5.9).

Seasonality: Adults- present from late spring - late autumn.

Neoperla ?angolana Zwick & Zwick, 2023

Unique Collecting Events: 3

Taxonomic notes: Only females of *Neoperla ?angolana* have been collected. However, there is some doubt about their assignment: the spermathecal stalk is straight and finger-like, without any internal scales (Zwick & Zwick 2023) but differs from that of *N. angolana* as the distal end is tightly coiled, forming approximately a quarter of a loop. The eggs are similar to *N. angolana*, but the costae are more strongly raised and resemble the eggs of *N. burgeoni*.

I consider it likely that this is a novel species, rather than a range expansion for the otherwise extremely rare and localised *N. angolana.* However, at present, there is insufficient evidence to separate the species. It is therefore putatively assigned to *N. angolana,* but this identification cannot be confirmed until a male of the species is collected. Molecular evidence cannot be used to confirm the identification, as *N. angolana* has not yet been sequenced (Zwick & Zwick 2023).

Distribution: A new record for South Africa, four specimens were collected from the Vaal River, in the area around Warrenton in the Northern Cape (Figure 5.10). Outside of South Africa, *N. angolana* is known only from its type locality in Angola (Zwick & Zwick 2023).

Notes on Records: This species has only been collected three times, from June 1977 – April 1981. The current presence of *N.* ?*angolana* in Warrenton still needs to be verified.

Ecological Notes: *Neoperla ?angolana* has only been collected from the Savannah biome (Table 5.1)

Seasonality: Adults were collected throughout the year, with specimens from summer, autumn and winter (Figure 5.10; Appendix 5.1)

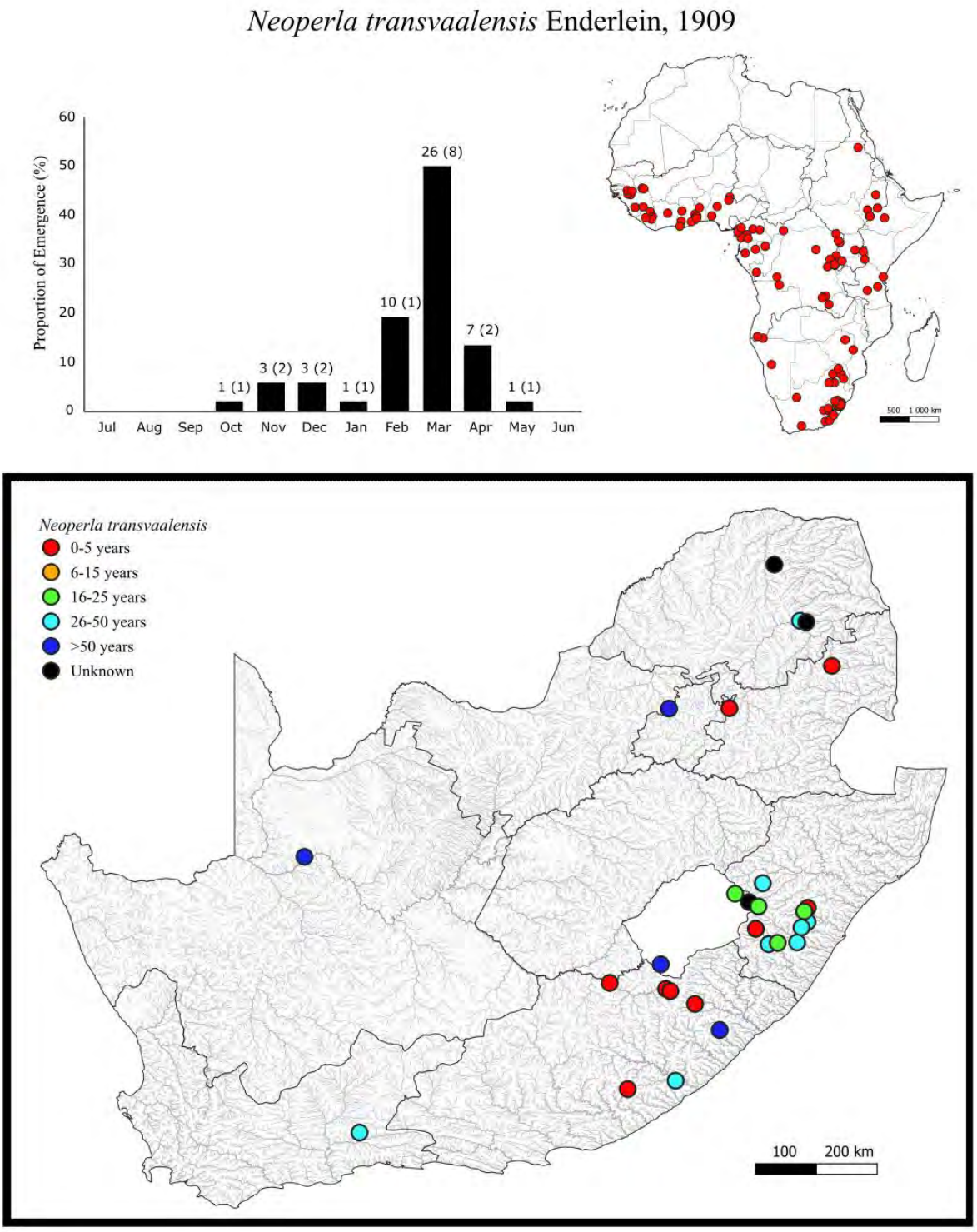

Figure 5.8: Distribution map and emergence times for *N. transvaalensis* in South Africa. All figures follow the same format as Figure 5.1.

Figure 5.9: Collection localities of *N. transvaalensis*. **A:** Bell River, southern Drakensberg, 30.84S, 27.81E, stony bottomed, surrounded by mountain grassland. **B:** Kraairiver, 30.74S, 26.78E, deep large sections sandy, *N. transvaalensis* collected amongst stones. **C:** Nculwane River, KwaZulu-Natal, 29.37S, 30.37E, forestry area. **D:** Gxucu River, Amathole Mountains, 32.66S, 27.11E, surrounded by farmland. **E:** Maria Shires Waterfall, Mpumalanga, 24.99S, 30.81E, forestry area.

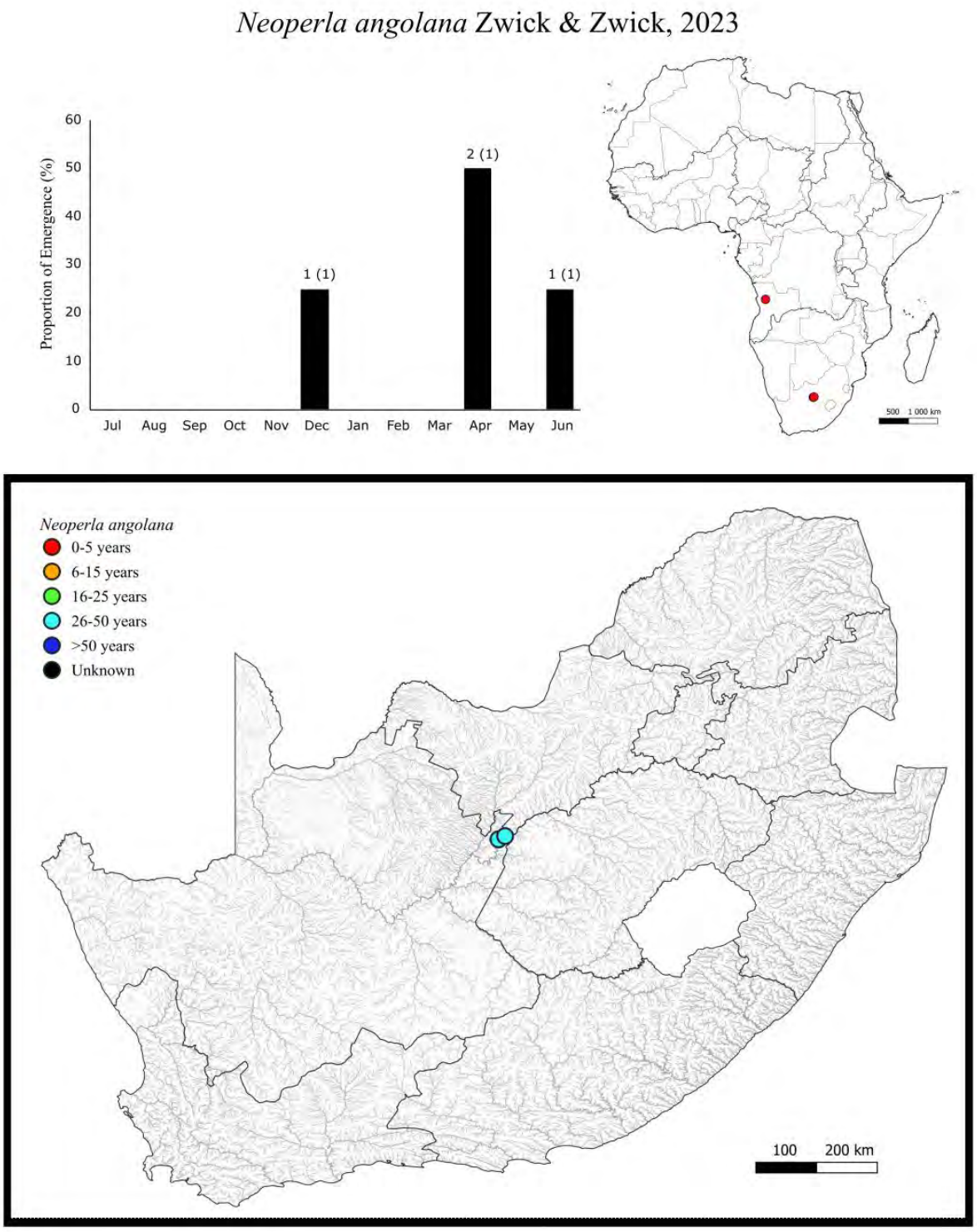

Figure 5.10: Distribution map and emergence times for *N.* ?*angolana* in South Africa. All figures follow the same format as Figure 5.1.

Neoperla sp. Afr_E

Unique Collecting Events: 1

Taxonomic Notes: The morphology of this species is discussed, and the nymph described, in Chapter 2. At present, it is not a valid species.

Distribution: This putative species is known from Makhanda, Eastern Cape (Figure 5.11).

Notes on Records: Despite recent sampling in the region, no further *Neoperla* have been collected.

Seasonality: Unknown.

Ecological Notes: This specimen was collected from a diverse region, where four biomes (Savannah, Albany Thicket, Afromontane Forest and Fynbos) meet. No site description was included with the specimen. The nearby Bloukrans River is heavily polluted below the town (Gininda 2016; Mangadze *et al.* 2019) but has seven headwater streams feeding it, which may still harbour this species.

Neoperla sp. Afr_G

Unique Collecting Events: 1

Taxonomic Notes: The morphology of this species is discussed, and the nymph described, in Chapter 2. At present, it is not a valid species.

This species is collected from the area around Mbotyi forest, where *N. panafricana* and *N. sjostedti* have both been collected. It is possible that this is the nymph of *N. panafricana*, but the COX1 sequencing of this specimen failed, preventing identification using molecular methods.

Distribution: This species is known from Hofani River, near Mbotyi Forest in the Wild Coast of the Eastern Cape.

Notes on Records: No collection date was recorded for this specimen.

Seasonality: Unknown.

Ecological Notes: The area this specimen was collected in falls broadly into the Indian Ocean Coastal Belt biome, which covers the Eastern Coast of South Africa. However, the collection notes specified that the species was collected from a dense forest patch, with wooded vegetation alongside both sides of the small stream. No biotope was recorded.

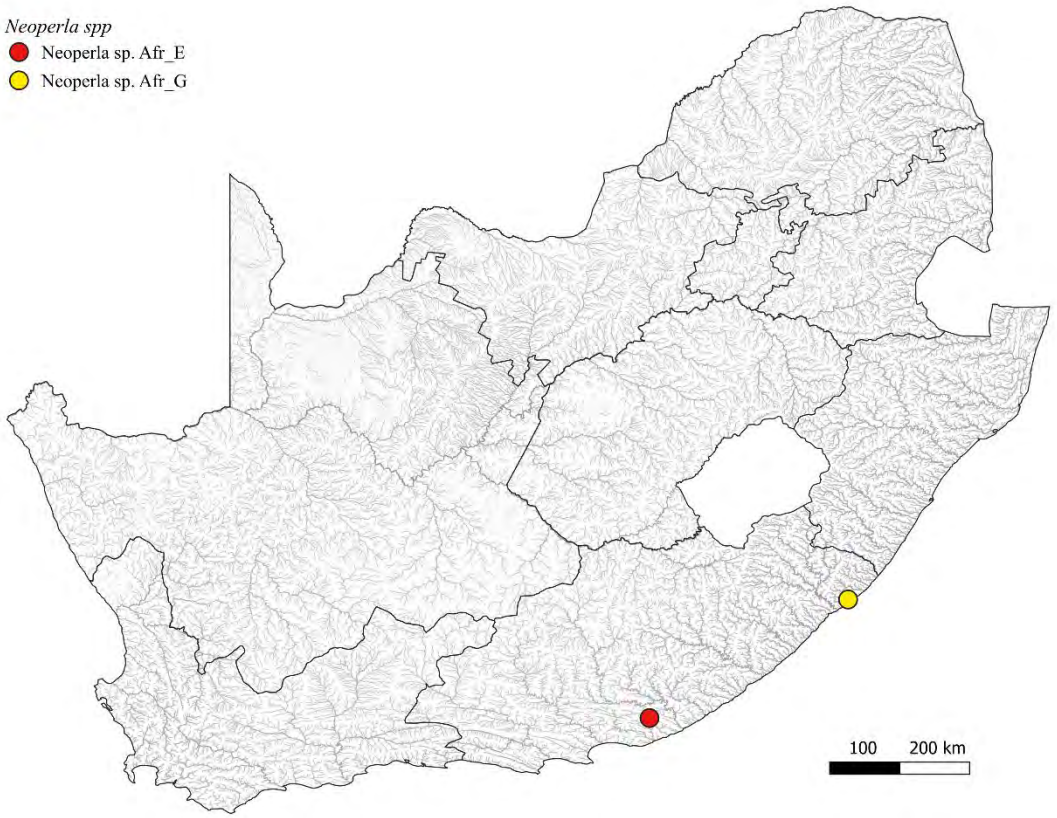

Figure 5.11: Distribution map of *Neoperla* sp. Afr_E and *Neoperla* sp. Afr_G in South Africa.

Table 5.1: Number of sampling events for each *Neoperla* species in South African Biomes.

	Desert	S. Karoo	N. Karoo	Fynbos	A. Thicket	Grassland	Savannah	IOC Belt	Forest
N. burgeoni	1		1	1	1	6	15	1	3
N. heideae							1		
N. leroiana						2			
N. panafricana				1		1	2	1	2
N. sjostedti				2		6	2	1	2
N. transvaalensis			1		2	19	9		2
N. ?angolana							3		
N. sp. Afr_E				1	1		1		1
N. sp. Afr_G								1	1

Table 5.2: Number of sampling events for each *Neoperla* species in river biotopes, following Dickens & Graham (2002).

	Riffles	SIC, fast	SIC, slow	MVIC, fast	MVIC, slow	Sub. Macro
Neoperla burgeoni	1	2	1		1	
Neoperla heideae			Unknown			
Neoperla leroiana			Unknown			
Neoperla panafricana			Unknown			
Neoperla sjostedti	1	2	1	1	1	1
Neoperla transvaalensis		6	3		1	
Neoperla ?angolana			Unknown			
Neoperla sp. Afr_E			Unknown			
Neoperla sp. Afr_G			Unknown			

Table 5.3: Water chemistry readings taken at each site where *Neoperla* specimens were collected.

	Temperature (°C)	pH	EC (µs/cm)	TDS (ppm)
Neoperla burgeoni	18.4 - 18.9 (n = 2)	7.5 (n = 1)	36 - 57 (n = 2)	18 (n = 1)
Neoperla heideae		Unknown		
Neoperla leroiana		Unknown		
Neoperla panafricana		Unknown		
Neoperla sjostedti	15.8 - 16.6 (n = 2)	6.2 - 7.5 (n = 2)	6.6 - 7.1 (n = 2)	4 - 5 (n = 2)
Neoperla transvaalensis	16.2 - 20.2 (n = 7)	6.5 - 8.2 (n = 2)	36 - 229 (n = 7)	18 - 151ppm
Neoperla ?angolana		Unknown		
Neoperla sp. Afr_E		Unknown		
Neoperla sp. Afr_G	17.1 (n = 1)	7.27 (n=1)	157.7 (n=1)	111

5.3.1. Key to South African Neoperla: Mature nymphs

The following key is designed for the identification of mature *Neoperla* nymphs in South Africa. However, identifications based on this key should be utilized carefully, as it relies on habitus characters that can be expressed to a variable level in all species (pers. obs.). Additionally, colouration patterns appear only in mature nymphs, and can change significantly in the young stages (Hynes 1953; pers. comm. Peter Zwick). This key should therefore be used only for specimens > 1 cm in length, excluding cerci and antennae, as colouration patterns are expressed by this stage (Hynes 1953).

The full range of variation within each species is not yet fully categorized. While this key allows for the identification of common species and colouration patterns, determination of specimens with

unusual or strongly differing colouration patterns should, whenever possible, be confirmed using the COX1 barcoding gene, as all of the South African species are well differentiated by this gene.

Finally, identifications based on this key should not be used outside South Africa. The vast majority of African *Neoperla* are not known as nymphs (87/93 species), and it is possible that several species share the same colouration patterns, particularly within species groups.

Neoperla heideae, N. leroiana, N. panafricana & N. ?angolana unknown.

1. Pro-, meso- and metathorax dark, with prominent, clearly delineated pale markings. Markings on meso- and metathorax either band-shaped, or forming distinct patches on either side of pale median ecdysial line. Head with dark brown markings in region between occipital ridge and eyes, posterior markings clearly separated from dark frons by a pale region, which is usually transverse or semi-circular (Figure 5.12, A-B, D) …….. **2**

1'. Prothorax brown, occasionally pale medially, but never with strongly delineated markings. Meso- and metathorax with pale markings, usually indistinct. Head either without dark markings between occipital ridge and eyes, or if present, with pale "W" marking on frons (Figure 5.12, C, E) ……. **4**

2. Prothorax with pale, clover-shaped mark. Band-like marking on meso- and meta-thorax thick, covering almost entire tergite. Abdomen distinctly banded along posterior margin of each segment. Brown marks on head fill the region between occipital ridge and ecdysial line medially, fading away approximately halfway between ocelli and eyes; ocelli covered with a triangular brown marking (Figure 5.12, D) ...…...…...……. ***Neoperla sjostedti***

2'. Markings on prothorax not clover-shaped. Markings on meso- and metathorax vary, not covering entire tergite. Abdomen either not distinctly banded, or with dark bands distally, not along the posterior margin. Ocelli with only small brown marking (Figure 5.12, A-B) …… **3**

3. Body dark, chocolate brown. Pale band on the head either transverse, or forming a semi-circle, the edges of which usually reach the margin of head in front of eyes ……. ***Neoperla transvaalensis***

3'. Body patterns as with *N. transvaalensis*, but pale, light amber brown …… ***Neoperla* sp. Afr_E**

4. Body amber. Head without markings between occipital ridge and eyes, or with indistinct, slight darkening. Strong, transverse yellow band separates occipital ridge and brown frons. Sometimes with small dark patch over ocelli. Markings on pro-, meso- and metathorax indistinct, without clear margins (Figure 5.12, C) ……. ***Neoperla burgeoni***

4'. Entire head brown, except for small pale region in front of ocelli, frons with pale "W" marking (Figure 5.12, E) …….. ***Neoperla* sp. Afr_G**

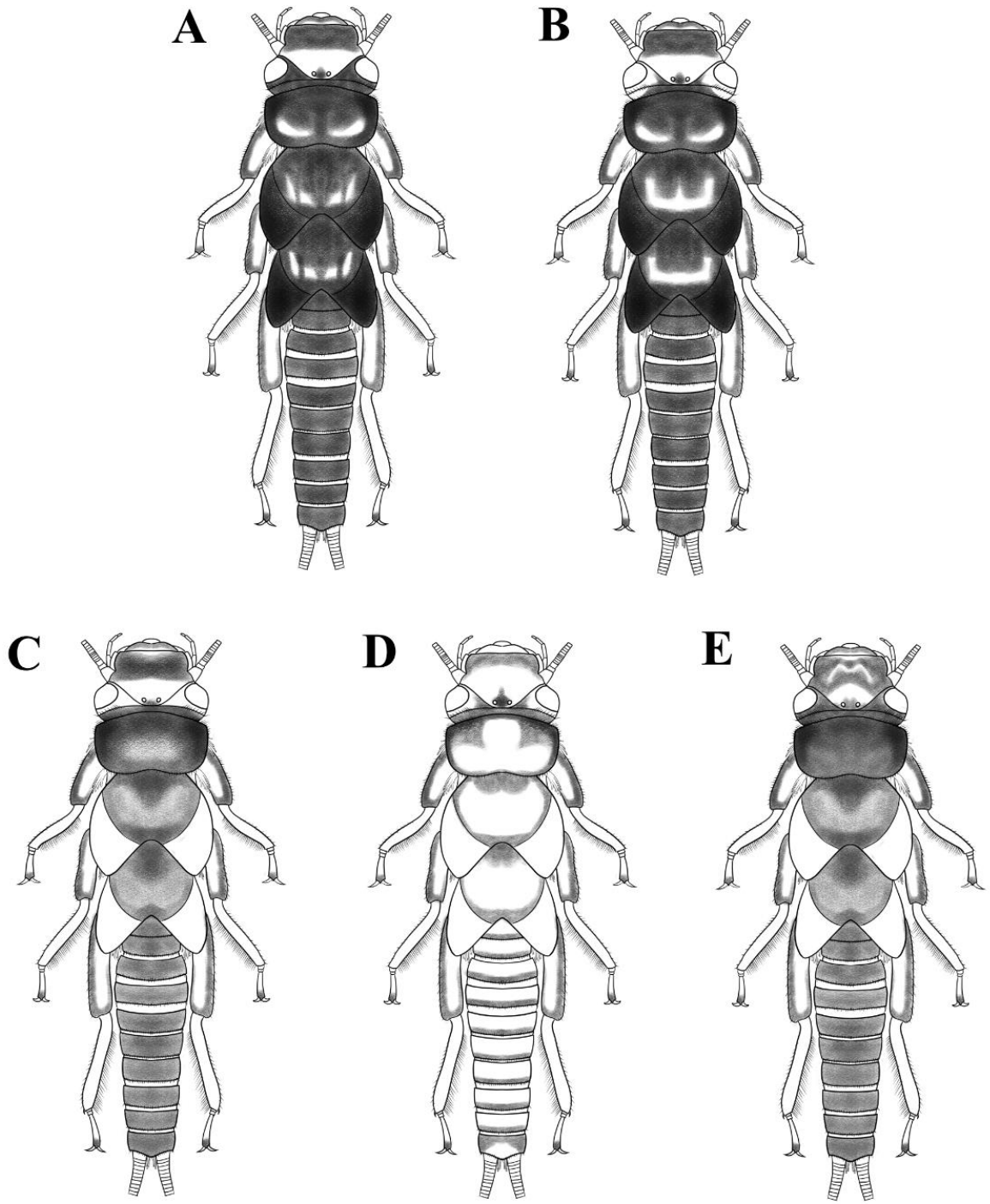

Figure 5.12: Mature nymphs. A-B:, *N. transvaalensis*, **C:** *N. burgeoni*, **D:** *N. sjostedti*, **E:** *Neoperla* sp. Afr_G.

5.4. Discussion

5.4.1. Distribution and ecology of Neoperla in South Africa

Neoperla is widespread across South Africa, with records from every province except the Free State (Appendix 5.1). Neoperlids are apparently most abundant in the summer rainfall regions of South Africa (Picker 1980; Stevens & Picker 2003), with the highest diversity recorded from the stony mountain rivers of the Lowveld and Mpumalanga Drakensberg (Zwick & Zwick 2023), and the Eastern Escarpment, particularly in the Drakensberg. As *Neoperla* do occur in high-altitude regions with cold winters, their absence from the Free State is probably due to undersampling. While there are multiple records from the Amathole and Outeniqua mountains supporting their presence along the Southern Coast (Appendix 5.1), they appear rare in these regions, which have been extensively sampled for Notonemouridae (Bellingan 2010; de Moor & Bellingan 2010; Picker & Stevens 1997, 1999; Stevens *et al.* 2018; Stevens & Picker 1995, 1999).

The Drakensberg mountain range is approximately 400 km long, covering an area of about 40 000 km^2 in the Great Escarpment on the east of South Africa (Hamer & Martens 1998). Most of the mountains are covered by temperate grassland, and small, naturally fragmented forest patches (Hamer & Martens 1998; Hamer & Slotow 2009; Scholtz & Twidwell 2022; Taylor *et al.* in press). Grasslands are arguably the most imperilled and least protected terrestrial biome worldwide (Bengtsson *et al.* 2019; Scholtz & Twidwell 2022; Taylor *et al.* in press). Only seven large, continuous grasslands remain worldwide (Scholtz & Twidwell 2022), including the Drakensberg, but these biomes are important due to their high biodiversity, endemism, and provision of ecosystem services to humans, such as water provisioning, supporting high crop yields and cultural services (Bengtsson *et al.* 2019). The Drakensberg is no different, and it harbours some of the highest biodiversity and endemism in South Africa (Armstrong & Brand 2012; Brendonck *et al.* 2000; Hamer & Martens 1998; Hamer & Slotow 2009; Matthews *et al.* 1993; Taylor *et al.* in press). In 2000, large sections of the mountain range were named a UNESCO world heritage site, due to both its cultural and biological significance (UNESCO 2000). While the highest Plecoptera diversity in South Africa occurs in the Western Cape (Stevens *et al.* 2018), the Drakensberg is a close second, as three genera of Notonemouridae, including the Drakensberg endemic *Balinskycercella* (Balinsky 1956, 1967; Stevens *et al.* 2018; Stevens & Picker 1995), and most South African *Neoperla* species occur in this region. The true diversity of this mountain range may be even higher, as large sections are inaccessible (Armstrong & Brand 2012), and many stoneflies are highly localized endemics (Stevens *et al.* 2018).

Within the Drakensberg, increases in temperature and unpredictable rainfall have already been recorded, and these trends are expected to continue, with the region around Underberg likely to see the greatest changes (Department of Environmental Affairs 2018; Jewitt *et al.* 2023). These changes

are expected to lead to range restrictions of cold-adapted species that are forced to retreat to higher elevations, leading to fragmented and isolated distributions (Jewitt *et al.* 2023; Taylor *et al.* in press). Predicted changes in rainfall due to climate change are unclear (Nel 2009; Takong & Abiodun 2023), but declines in annual rainfall and an increase in extreme events, causing flooding or droughts, are anticipated (Takong & Abiodun 2023). These will potentially lead to changes in the diversity of aquatic insects, with Ephemeroptera, Plecoptera and Trichoptera particularly negatively affected by both flooding events (Gholizadeh 2021; Greenwood & Booker 2015) and drought (Calapez *et al.* 2014; Tierno De Figueroa *et al.* 2010). Therefore, these changes may lead to range restrictions, or reductions in *Neoperla* diversity throughout the Drakensberg. However, the mid-altitude Drakensberg grasslands, where they were most commonly collected, are expected to be only marginally affected by these climatic changes (Jewitt *et al.* 2023). Comparatively, the cold-adapted Notonemouridae are likely to be strongly affected by these changes.

Only a few samples have been collected from the interior and the northwest of South Africa (Appendix 5.1), suggesting *Neoperla* are uncommon in these regions. These areas are dominated by the desert and Karoo biomes, which are hot and arid (de Coning & Poolman 2011; NCCIS 2025; Rutherford *et al.* 2006; SANBI 2012), providing poor conditions for most Plecoptera (DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008; Hynes 1976). However, the low diversity may also result from poor sampling effort, particularly in the Orange and Vaal Rivers, where *N. burgeoni*, *N. transvaalensis* and a possibly novel endemic, *N.* ?*angolana* have been collected. No recent collections have been made from this region (Appendix 5.1), and the current extent and range of *Neoperla* species are unknown over the interior. It is likely that *Neoperla* diversity in this region has already decreased, as large sections of the land have become moderately to severely degraded (Meadows & Hoffman 2003), and temperatures have increased considerably (0.025 - 0.039 °C / year, MacKellar *et al.* 2014) over the last 50 years. While the thermal tolerances of the individual species of South African *Neoperla* are unknown, the genus seems to prefer cool waters and has not been recorded in rivers above 25°C (Edia *et al.* 2016; Hynes 1976). My collections support this, as *Neoperla spp.* were often absent from warm rivers, even if specimens were collected from cooler rivers nearby (pers. obs.). The Northern Cape is predicted to become much drier and hotter (Coetzee *et al.* 2009; Department of Environmental Affairs 2018). This will probably result in significant losses of biodiversity in the area (Coetzee *et al.* 2009), and it is possible that these changes will cause significant range changes for the *Neoperla* species occurring here.

Although preliminary, the ecological notes presented here suggest that the biology of *Neoperla* varies between species. *Neoperla transvaalensis* and *N. burgeoni* seem to be widespread and occur in a range of habitats, including small streams, stony foothill rivers, and large, meandering lowland rivers. Comparatively, *N. leroiana* and *N. heideae* are more restricted and have been collected only from mountain forests and grasslands, respectively. However, in all cases, *Neoperla* species were generally

collected from cold or cool, fast-flowing sections of rivers with stoney bottoms. The genus does also occur in marginal stretches, amongst vegetation, gravel or sandy patches (Dallas 2007), but to a lesser extent (Dallas 2007, pers. obs.).

The electrical conductivity, pH, and total dissolved solids at collection sites were summarized when possible (Table 5.3). Electrical conductivity (< 250 µS/m) and TDS (< 151 ppm) were low for all species, which is usually correlated with clean water (Bispo *et al.* 2006; Kazanci *et al.* 2017; Olson & Hawkins 2017; Rychla *et al.* 2011). Sites with high TDS and electrical conductivity (500 – 1200 µS/m) were sampled, but no *Neoperla* were found in these rivers (pers. obs.). pH showed a fairly large range in the environments of multiple species (Table 5.1), suggesting it is unlikely to have a significant effect on *Neoperla* species except in extreme cases. However, very few samples were available, and in some cases, unusually heavy rainfall had occurred in the area, possibly compromising these results. These results are generally in line with observations for Plecoptera worldwide (Bispo *et al.* 2006; Edia *et al.* 2016; Kazanci *et al.* 2017; Olson & Hawkins 2017), and validate the use of *Neoperla* as a sensitive biological indicator in South Africa (Dickens & Graham 2002). *Neoperla burgeoni* and *N. transvaalensis* were found in areas impacted by agriculture and forestry, suggesting that these two species are more tolerant than currently assumed. Environmental parameters such as flow rates, substrate, shade, and neighbouring vegetation can all strongly influence Plecoptera abundance and diversity overseas (Edia *et al.* 2016; Kazanci *et al.* 2017; Macadam *et al.* 2022). This information is scarce for all species in South Africa, and will be vital for redlisting, conservation and management strategies (IUCN 2024; Macadam *et al.* 2022).

Adults from all species were recorded as emerging between early spring – late autumn, with peak emergence occurring in summer, particularly around February - April (Appendix 5.1; Figures 5.1 - 5.10, pers. obs.). No adults have been collected during winter in South Africa, although emergence seems to be throughout the year for the same species in the tropics (Zwick 1976; Zwick & Zwick 2023). These samples are biased by collecting effort, but there are currently 32 observations of adult *Neoperla* spp. from South Africa on the citizen science platform iNaturalist.com (iNaturalist 2025; searched using Species: "*Neoperla*" and Location: "South Africa"), none of which were photographed during winter. This differs from the emergence times of Notonemouridae, which usually occur from autumn to winter (e.g., *A. cassida*) or spring – summer (e.g., *Desmonemoura pulchella* Tillyard). This prolonged emergence period is common in tropical species (Hynes 1976; Zwick 1976), and has been observed for *Neoperla* in Africa, even when species were considered separately (Tjønneland 1961; Zwick 1976; Zwick & Zwick 2023). Peak emergence during summer may allow avoidance of high water temperatures (Dallas 2016), which has been suggested for two morphotypes in the *A. capensis* species complex (Dallas 2016; Ketley 2009). Water temperature is a significant driver of emergence in Plecoptera overseas (Brittain 1990; Hynes 1976; McCulloch & Waters 2018).

5.4.2. Using the Key to Mature Nymphs, and identification of Neoperla in South Africa

As mentioned above, 100 records and 260 individual specimens in total of *Neoperla* are identified to species level in South Africa. Only 20 are nymphs. However, this is a fraction of the records across the country, with 276 locality records in the Albany Museum, 24 new locality records in this study, and more than 5000 specimens from just these two collections. Even in this larger, unidentified collection, regions such as the Northern Cape and interior are poorly sampled (pers. obs.). It is therefore clear that our current inability to identify nymphs is a significant hindrance to the study of *Neoperla* in South Africa. It is also highly likely that additional species remain undiscovered, as even this review has identified three putatively novel species. While molecular methods are becoming increasingly accessible and allow for the identification of species, even in bulk samples (Antil *et al.* 2023; Pereira-da-Conceicoa *et al.* 2021; Stein *et al.* 2014; Van Der Loos & Nijland 2021), morphological identification guides remain a useful tool. A key to allow for the identification of nymphs was therefore urgently required to facilitate future research on the genus.

As discussed in Chapter 2, colouration patterns provide the only characters that can be used to differentiate *Neoperla* species as nymphs. However, these habitus characters can vary between conspecific specimens, and change during development (pers. comm. P. Zwick, pers. obs., see *N. transvaalensis* above). Despite these character limitations, the preliminary investigation of nymphs presented here suggests they are sufficiently consistent to allow identification of at least the common taxa. *Neoperla panafricana, N. leroiana, N. heideae* and *N. ?angolana* are not known as nymphs. Whenever possible, identifications should be confirmed using the COX1 barcoding gene region, focusing on new or unusual morphotypes. This will allow for the variation within each species to be categorized, and for additional species to be added.

6. Chapter 6: General Discussion and Conclusions

6.1. Biogeographical history of Plecoptera

Despite attention over the last few decades (Ding *et al.* 2019; García-Girón *et al.* 2024; Hynes 1988; Illies 1965; Letsch *et al.* 2021; McCulloch *et al.* 2016; Zwick 2000), the biogeographical history of Plecoptera is still debated. Primarily, the origin of Antarctoperlaria and Arctoperlaria, and the mechanism behind their antitropical distributions, have been attributed to both vicariance caused by the breakup of Pangaea (Ding *et al.* 2019; García-Girón *et al.* 2024; McCulloch *et al.* 2016; Zwick 2000), and dispersal after evolving in a combined range of either the Southern (Illies 1965) or Northern Hemisphere (Letsch *et al.* 2021). Similarly, the distribution of Notonemouridae across the Southern Hemisphere has been attributed to either vicariance (Letsch *et al.* 2021) or trans-oceanic dispersals after the breakup of Gondwana (García-Girón *et al.* 2024; McCulloch *et al.* 2016). The presence of Acroneuriinae and *Neoperla* in the Southern Hemisphere is consistently explained by dispersal during the Paleocene or Miocene (Fochetti & Tierno de Figueroa 2008; Illies 1965; Letsch *et al.* 2021; Zwick 2000).

This uncertainty is partially due to inconsistent taxonomic and phylogenetic systems used for the description of fossil species (Cui *et al.* 2015; Jouault *et al.* 2021; Sinitshenkova 1987, 2002; Zwick 2000). These inconsistencies confound interpretations of the fossil record, as they yield different dating estimates for cladogenesis (Crisp *et al.* 2011; Eichert *et al.* in press; Heads 2005a; Letsch *et al.* 2021; McCulloch *et al.* 2016), and contradictory patterns in the fossil record, such as the apparent presence of Antarctoperlaria and Arctoperlaria in the Northern and Southern Hemispheres, respectively (Aristov *et al.* 2013; Cui *et al.* 2015; Gallego *et al.* 2011; Sinitshenkova 1987, 2018). In Chapter 3, I reviewed the taxonomy of 107 fossil species from the Southern Hemisphere and Systellognatha from an explicitly cladistic foundation. I found that 62 of these species (58%) had insufficient evidence to support their current placement and should be reclassified (Tables 3.1-3.3). This review provided new insights into the biogeographical history of Plecoptera as a whole.

The first representatives of Systellognatha (Brauer *et al.* 1889; Sinitshenkova 1985) and Euholognatha (Cui *et al.* 2019; Sroka & Prokop 2023) were found from the Jurassic, 145 – 183 Ma (Table 3.3). There is no evidence of Antarctoperlaria in the Northern Hemisphere or Arctoperlaria in the Southern Hemisphere prior to these discoveries. Vicariance, therefore, provides the most parsimonious explanation for the formation of the suborders, rather than dispersal and reciprocal mass extinction from a shared ancestral range (Figure 3.6). However, while this vicariance is usually attributed to the breakup of Pangaea (Ding *et al.* 2019; García-Girón *et al.* 2024; McCulloch *et al.* 2016; Zwick 2000), the presence of extant groups so soon after the separation of the continent points to an earlier origin of the suborders. I proposed a novel hypothesis that Antarctoperlaria and Arctoperlaria may have

originated due to climatic vicariance on Pangaea (Chapter 3; Figure 3.6), after stem-Plecoptera became isolated in the Southern and Northern hemispheres by arid bands across the equator during the Permian and Triassic (Chaboureau *et al.* 2014; López-Gómez *et al.* 2021; Péron *et al.* 2005; Roscher & Schneider 2006).

Gripopterygidae, an extant family in Antarctoperlaria, first appears in the fossil record during the Early Cretaceous, 112.6 – 122.46 Ma (Jell 2004; Jell & Duncan 1986). This record suggests that Antarctoperlaria were present across Gondwana, and probably spread across Australia, New Zealand, and South America via land bridges over Antarctica (Letsch *et al.* 2021; Zwick 2000). It is unclear whether Antarctoperlaria ever occurred in Africa. However, as the continent remained connected to South America until the end of the Early Cretaceous (119 – 105 Ma), it is certainly possible (Figure 3.6). The apparent absence of Antarctoperlaria in Africa is therefore probably due to a mass extinction within the continent, possibly associated with hot and dry periods during the Late Cretaceous (Jacobs 2004; Myers *et al.* 2011; Rees *et al.* 2004; Sellwood & Valdes 2008). Most Antarctoperlaria are cold-adapted and prefer temperate environments (Zwick 2000), with the highest diversity occurring at lower latitudes than the southernmost point in Africa (DeWalt *et al.* 2025). However, many Gripopterygidae and Stenoperlinae (Eustheniidae) species are tropical (DeWalt *et al.* 2025; Zwick 2000). I consider it likely that at least Gripopterygidae occur in the Afrotropical region but have not yet been recorded. Sampling across most of Africa is sparse (Zwick & Zwick 2023), and if Antarctoperlarian species are present, they may be limited to cold, high-altitude mountain peaks. These are often inaccessible or difficult to sample (Armstrong & Brand 2012). The nymphs of many families look similar and may be misidentified. Additionally, most species of Plecoptera, including Antarctoperlaria, are not attracted to light traps (Zwick 2000). Therefore, even species that co-occur with *Neoperla* may have gone unnoticed, despite sampling of the genus across the continent over the last century (Hynes 1952; Picker 1980; Tjønneland 1961; Zwick & Zwick 2023).

The earliest Notonemouridae fossil is from Australia during the mid-Jurassic (Table 3.1; Figure 3.6; Sroka & Prokop 2023). This fossil provides strong evidence that the family arrived in the Southern Hemisphere during the Jurassic, and its distribution across the Southern Hemisphere is due to vicariance associated with the breakup of Gondwana (Illies 1965; Letsch *et al.* 2021; Sroka & Prokop 2023; Zwick 2000).

Unfortunately, no fossil species were found that could be assigned to Neoperlini or *Neoperla*. Therefore, the biogeographical history of *Neoperla* in the Afrotropical region was examined using a time-calibrated molecular analysis (see below).

6.2. Calibration points for phylogenetic and biogeographical analyses of Plecoptera

The uncertain taxonomy of the Plecopteran fossil record has led to inconsistent selection of calibration points for time-calibrated phylogenies (Cui *et al.* 2019; Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016). Based on the results of the literature review of 107 fossil species, I recommend eleven fossil species that can be used to calibrate future analyses of the order (Figure 3.4). These calibration points consist of a combination of stem-Plecoptera (e.g., †*G. carpenteri* and †*G. oudardi*; Carboniferous: 314.6 - 306.95 Ma), infraorders such as Systellognatha (e.g., †*P. platypoda* and †*P. conferta;* Jurassic: 164.7 – 183.0 Ma), extant families such as Gripopterygidae (e.g., †*E. duncanae*; Cretaceous: 122.46 – 112.6 Ma) and Perlidae (e,g., †*L. acus*; Cretaceous: 99.7 – 94.3 Ma), and one extant genus, *Isoperla* (e.g., *I. †baltica*; Paleogene: 37.2 – 33.9 Ma). The relationships and affinities of all eleven of these fossilized species are supported by multiple autapomorphies and will allow for increased consistency in biogeographical analyses of Plecoptera.

6.3. Taxonomy and phylogenetic placement of *Neoperla*

The taxonomy of *Neoperla* and its phylogenetic relationships within Perlidae have been reviewed (Sivec *et al.* 1988; Zwick 2023, 2024). *Neoperla* falls in Neoperlini, a tribe in Perlinae that also includes *Chinoperla*, *Phanoperla*, and *Furcaperla* (Sivec *et al.* 1988; Zwick 2023, 2024). Perlinae, Neoperlini and all four of these genera are supported by morphological autapomorphies (Sivec *et al.* 1988; Zwick 2023, 2024), and Perlinae has been monophyletic in all recent molecular analyses (Chen *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021). However, the monophyly of Neoperlini and affinities of *Neoperla* have differed in these analyses, with Neoperlini and Perlini regularly recovered as paraphyletic (García-Girón *et al.* 2024; Letsch *et al.* 2021; South *et al.* 2021; Xiang *et al.* 2021; Zwick & Zwick 2023).

Molecular analyses using mitochondrial genomes have recovered *Neoperlops* as sister to *Neoperla*, suggesting a possible close relationship with Neoperlini (Chapter 4; Figure 4.2; Zwick 2023; Zwick & Zwick 2023). While *Neoperlops* lacks autapomorphies of Neoperlini, there are definite morphological similarities to the Neoperlini genera. Namely, *Neoperlops* is biocellate (shared with *Neoperla*, *Chinoperla*, and *Phanoperla*), habitus of adults and nymphs are similar (Chen 2020b; Mo *et al.* 2020; Stark & Sivec 2008) and the base of the aedeagus is slightly sclerotized, although it does not share the autapomorphic constricted basal opening (Sivec *et al.* 1988; Zwick 2023). In fact, *Neoperlops* was at one time synonymized with *Neoperla* (Mo *et al.* 2020), and one species was recently misidentified as belonging to *Phanoperla* due to these similarities (Chen 2020b; Mo *et al.* 2020). Comparatively, its assignment to Perlini is strongly supported by autapomorphic setal patterns and sensilla basiconica on

the hemitergite apex (Sivec *et al.* 1988). The hemitergites of *Neoperla* similarly have sensilla basiconica and setae on the mediobasal callus, but not on the anterior spike-like process (Zwick 2023; Zwick & Zwick 2023).

Unfortunately, these relationships remain uncertain, as molecular analyses have had a limited sample size, with relatively few sequences or species available from across the diverse Perlinae. *Chinoperla*, *Phanoperla* and *Furcaperla* have not been included in any of these analyses, and COX1 data are available only for *Phanoperla* (Wang *et al.* 2024). While these COX1 data are from the standard barcoding region (Wang *et al.* 2024), they align poorly with *Neoperla*, introducing large indels and gaps that make molecular analyses of the two genera inconsistent (pers. obs.). Until these relationships can be investigated with greater molecular coverage, the morphological phylogeny proposed by Sivec *et al.* (1988) remains the best explanation of relationships within Perlinae.

Neoperla has been monophyletic in all recent phylogenetic analyses (Chapter 2; Chapter 4; Chen *et al.* 2019; Letsch *et al.* 2021; Xiang *et al.* 2021; Zwick & Zwick 2023). The taxonomy of the genus was reviewed recently (Zwick 2023, 2024), and four subgenera were formally recognized. These subgenera are genetically (Chapter 4; Zwick & Zwick 2023) and morphologically distinct (Zwick 2023, 2024), with autapomorphies that allow for their identification. As such, an argument could be made that these subgenera should be elevated to genus rank. In Africa and North America, this change would have no effect: all the Afrotropical and Nearctic species belong to *Neoperla* (*Neoperla*). However, relationships between many of the Indo-Malayan species are poorly understood, and the autapomorphies of each subgenus can be variable (Zwick & Zwick 2023). Molecular coverage of the Indo-Malayan species is poor, and many species are not yet assigned to a species group or subgenus (Zwick 2023). Therefore, I suggest these subgenera are maintained, at least until relationships and autapomorphies are better understood throughout the genus.

In the Afrotropical region, Zwick (2023) erected four informal species groups based on autapomorphic characters. These species groups are not recognized by the ICZN and can be altered as needed. These species groups have been recovered as paraphyletic or polyphyletic in all molecular analyses (Chapter 2; Figure 2.7; Chapter 4; Figure 4.2; Zwick & Zwick 2023). At least some of the autapomorphies suggested by Zwick to support these species groups (e.g., *N. sjostedti* species group: Female S8 with a median sclerotization or hard nail, and sulci with one regular row of microscopic punctures on either side) are either inconsistent or also occur in other species groups (e.g., see discussion on the *N. duodeviginti* species group in Chapter 2). However, Zwick & Zwick (2023) proposed several additional species assemblages in their review of the Afrotropical taxa. As these species assemblages are generally monophyletic and supported by clear morphological differences, I propose that these are used to summarize the diversity and biogeography of *Neoperla* in Africa, rather than the broader species groups.

At a species level, I agree with previous authors that habitus characters are not sufficient to readily identify and differentiate adult *Neoperla* in Africa (Hynes 1952; Picker 1980; Zwick 1973, 1976, 2023; Zwick & Zwick 2023). In males, spine patterns on the endophallus and aedeagus, coupled with the shape of the processes on T7-H10, provide the only reliable characters for species determination (Chapter 2). In females, the shape and sclerotization of the subgenital plate, the length, shape and spine patterns of the spermathecal stalk, and striation patterns of the eggs can be used to differentiate species (Chapter 2). However, I disagree with Zwick & Zwick (2023) that nymphs cannot be identified morphologically, at least to the assemblage level. While characters such as setal pattern, mouthpart shape and tooth number, gill position and structure, body size, and spine pattern on the proventriculus are similar between species (Barnard 1934; Hynes 1953; Ogbogu 2006; Zwick & Zwick 2023), colouration patterns of the ten species examined here are significantly different and consistent enough to allow for determination (Chapter 2; Chapter 5, *Key to South African Neoperla: Mature Nymphs*). Colouration patterns similarly seem to allow the Asian fauna to be differentiated (Cruz *et al.* 2018; Pelingen & Freitag 2020). However, as most African species are not yet known as nymphs, these characters should provide only auxiliary characters for species diagnoses. Nevertheless, I have provided a key to enable the identification of *Neoperla* nymphs in South Africa, where the diversity of the genus is relatively low (Chapter 5).

As the different life stages and sexes of *Neoperla* cannot be associated using morphological features and it is common to collect multiple species from the same locality (Zwick 2023), molecular methods are the most reliable way to associate different life stages (Zwick 2023; Zwick & Zwick 2023). Additionally, as very few nymphs are known or described, molecular methods remain the only way to identify the nymphs of most *Neoperla* species. For this reason, three genes (COX1, H3, and 28S) were extracted from all species described in Chapter 2, providing a molecular library of Afrotropical species. Sequence data of the COX1 barcoding region are now available for 68 of the 93 species of *Neoperla* occurring in Africa (60 from Zwick & Zwick, 2023, eight from Chapter 2). A further 12 species have either partial mitochondrial genomes or H3 and 28S genes available. DNA, and in particular barcodes, can be used to diagnose species, and upcoming versions of the ICZN will probably expand on the guidelines of how these should be applied (Rheindt *et al.* 2023). As COX1 data exist for most of the African species, novel species, including those represented by nymphs alone, can be described and named based on genetic evidence. While this practice does run the risk of introducing synonyms of species that have not been sequenced, the same is true of describing adults, as the males or females are occasionally not described for these species.

6.4. Diversity of *Neoperla* in Africa

Until the cryptic diversity of *Neoperla* was recognized, stonefly diversity in Africa was thought to be low, with only 40 species in two families recognized from sub-Saharan Africa (DeWalt *et al.* 2025; DeWalt & Ower 2019; Fochetti & Tierno de Figueroa 2008). This was a significant underestimate, and Zwick & Zwick (2023) recognized 82 novel species of *Neoperla*. I have augmented the species list, and in Chapter 2 I described a further ten species, predominantly from Central Africa. In total, 132 stonefly species comprised of Notonemouridae (39 spp) and Perlidae (93 spp) are now recognized from the Afrotropical region, more than tripling the known species. Even this new total is probably an underestimate. Six more putative species are currently unnamed, but have been identified and informally designated (Chapter 2; Zwick & Zwick 2023). Additionally, molecular evidence suggests some of the most widespread species, such as *N. burgeoni* and *N. amoena*, may be species complexes (Zwick & Zwick 2023). Therefore, undescribed species of *Neoperla* probably remain across the continent.

Beyond *Neoperla*, I suspect that the diversity of Plecoptera in general has been underestimated in Africa. I have already discussed the possibility that at least the Antarctoperlarian family Gripopterygidae may occur on the continent. Additionally, Notonemouridae may still be found outside South Africa, possibly limited to high-altitude mountain streams and rivers (Stevens & Picker 2003). As most Notonemouridae are highly localized endemics (Stevens *et al.* 2018), these would potentially be new species. Sampling and taxonomic expertise across much of Tropical Africa are scarce (Slade & Ong 2023), and in many cases suitable identification guides do not exist (Ochieng *et al.* 2019), so researchers rely on identification guides from Southern Africa, or even other continents, which lead to acute misidentifications (Ochieng *et al.* 2019).

Some evidence of this hidden family diversity in Tropical Africa is present on the citizen science platform iNaturalist (Daniels *et al.* 2022; iNaturalist 2025; Mesaglio & Callaghan 2021; White *et al.* 2023), as a likely Nemouroidea has been recorded from West Africa (https://www.inaturalist.org/observations/219204283, Accessed 19 June 2025).

6.5. Biogeography of *Neoperla*

The biogeographical history of *Neoperla* has not been investigated in detail before, and explanations for its disjunct distribution in the Indo-Malayan, Nearctic and Afrotropical regions are usually cursory (Hynes 1988; Illies 1965; Letsch *et al.* 2021; Zwick 2000). The genus probably originated in Asia, and later dispersed into the Nearctic and Afrotropical regions (Chapter 4; Zwick 2000, 2023; Zwick & Zwick 2023). The presence of *Neoperla* in the Nearctic is usually attributed to vicariance, either due to the opening of the Atlantic (Hynes 1988), or the Bering land bridge (Zwick 2000). Its presence in

Africa is attributed to dispersal from Asia during the Miocene or Pleistocene (Fochetti & Tierno de Figueroa 2008; Illies 1965; Zwick 2000). However, none of these hypotheses have been tested by time-calibrated phylogenies (Ding *et al.* 2019; García-Girón *et al.* 2024; Letsch *et al.* 2021; McCulloch *et al.* 2016).

In Chapter 4, I investigated these hypotheses using a time-calibrated phylogeny of Perlidae, focusing on the Afrotropical *Neoperla* species. However, my results did not support these explanations, and I presented several novel hypotheses for the unusual, disjunct distribution of *Neoperla*. The origin of *Neoperla* was estimated from the border of the Cretaceous and the Paleogene, approximately 66.2 Ma (Figure 4.2, 72.9 – 59.3 Ma). This post-dates the breakup of Eurasia and formation of the Atlantic Ocean by over 40 million years (Scotese *et al.* 2025), suggesting the genus could not have been widespread on Eurasia during the Cretaceous as proposed by Hynes (1988). Zwick (2000) suggested that *Neoperla* spread into America later via the Bering land bridge. While the Bering land bridge has played a role in the dispersal of temperate-adapted fauna and flora into North America, I consider it far more likely that *Neoperla* instead dispersed via the boreotropical forests of the North Atlantic land bridge (Figure 4.5; Tiffney 1985; Wen *et al.* 2010, 2016; Xiang & Soltis 2001). If *Neoperla* did spread into America via Europe, it probably went extinct in the region during the Miocene, as decreasing temperatures led to the disappearance of these boreotropical forests (Tiffney 1985; Wen *et al.* 2010; Xiang & Soltis 2001).

Most authors consider *Neoperla* a recent arrival in Africa, possibly arriving in the Miocene or Pliocene via a land bridge over the Arabian plate (Fochetti & Tierno de Figueroa 2008; Illies 1965; Zwick 2000). However, the purely Afrotropical clades in my phylogeny were dated earlier, with the earliest cladogenesis of the species groups estimated at 42.9 - 35.8 Ma (Figure 4.2). This timeline points to a dispersal of the genus in either the late Eocene, or during the Oligocene. I proposed two possible routes that *Neoperla* could have used to cross the retreating Tethys seaway, either arriving via the Iberian Peninsula or “Iranian route” (Figure 4.5 - 4.6). These dispersals could have occurred during the Bartonian/Priabonian (37.7 Ma) or Priabonian/Rupelian (33.9) dispersal phases (Gheerbrant & Rage 2006). The results of time-calibrated phylogenies can be controversial and can vary even when the same molecular datasets and calibration points are used (Guindon 2020; Heads 2005a; Hipsley & Müller 2014; Klopfstein 2021). Considering this uncertainty, a Miocene dispersal via the *Gomphotherium* landbridge (Harzhauser *et al.* 2007; Sen 2013; Tardif *et al.* 2023) cannot be disregarded, although I found no evidence to support this possibility.

The processes that led to the diversification and distribution patterns of *Neoperla* across the African continent have not been examined previously. I argue that *Neoperla* probably did not spread across the continent immediately, and instead spread slowly southwards as shifting climates and drainage patterns created suitable environments for the genus. Most of these changes were coincident with

seismic activity of the East African Rift (EARS), starting with the rise of the Afar plume in northeast Africa during the Oligocene (Bussert *et al.* 2018; Flügel *et al.* 2015; Goudie 2005). Early diversification of the genus may have occurred in North Africa, in a humid, tropical rainforest that covered northeast Africa and large swathes of the Sahara (Figure 4.5 - 4.6; Bussert *et al.* 2018; Goudie 2005; Jacobs 2004). Comparatively, the southern interior was largely dry and arid (Jacobs 2004). During the Miocene, the Congo basin formed due to activity in the EARS (Bakker & Mercer 1986; Bussert *et al.* 2018; Flügel *et al.* 2015; Goudie 2005; Stankiewicz & de Wit 2006), and *Neoperla* probably spread into this system. The Congo basin appears to be the center of *Neoperla* diversity in Africa, and may have facilitated connections and dispersal of the genus between river catchments in Central, West and East Africa, explaining the lack of any geographically limited species assemblages or groups in the Afrotropics (Figure 4.6). The diversity of *Neoperla* in Southern Africa is low (Chapter 5; Zwick & Zwick 2023), with only a few widespread species found south of the Zambezi River. Dispersal of *Neoperla* into this region was probably prevented by an arid band (Neumann & Bamford 2015) and the separation of the Zambezi River, which limited dispersal from rivers in Central Africa to the south until the Pleistocene or Pliocene (Key *et al.* 2015; Moore & Larkin 2001).

6.6. Ecology of South African *Neoperla*

The ecology and distribution of *Neoperla* across the Afrotropical region is poorly understood, as they have been treated as a single, widespread and generalist species for the last 70 years (Arimoro *et al.* 2011; Chutter 1968; Edia *et al.* 2016; Hynes 1952, 1976; Hynes & Williams 1962; Tjønneland 1961), which precluded nuanced study. Because more than one species as we now understand them can occur at a locality, distribution, emergence times, ecological requirements and habitat preferences are ambiguous for most species. These gaps limit conservation and biological monitoring efforts, for which species-specific ecology data are required (Dallas 2016; IUCN 2024; Macadam *et al.* 2022; Ripple *et al.* 2017; Rowntree *et al.* 2000).

In Chapter 5, I provided a preliminary review of the ecological information available for nine *Neoperla* species occurring in South Africa (seven species, two informally designated). Most species were sensitive to pollution, and all species were most commonly found in cool mountain rivers, usually amongst stones in the faster-flowing current (Dallas 2007, pers. obs.). This information validates their use as biological indicators in South Africa, as *Neoperla* is considered one of the more sensitive taxa in the SASS v5 scoring system (Dickens & Graham 2002). However, the ecology of species appeared to vary, with *N. burgeoni* and *N. transvaalensis* found in a greater range of environments, including meandering lowland rivers, and areas that have been impacted by agriculture (Chapter 5). All species were most commonly collected in Afromontane forests, mountain grasslands

and savannah biomes, which receive high rainfall throughout the year (Rutherford *et al.* 2006), although some species occur in a range of habitats. For example, *N. burgeoni* was found in the dry and hot Nama Karoo and Desert biomes, usually in large, lowland sections of the Orange River (Chapter 5, Figure 5.1). Species could be plurivoltine or univoltine but mature in cohorts throughout the year, as nymphs of multiple size classes were syntopic (pers. obs.). The developmental times of *Neoperla* have been investigated in only a single North American species, *N. clymene*, which is univoltine (Vaught & Stewart 1974). I suspect the African species are similar, as emergence and mating throughout most of the year (excluding winter in South Africa) would explain the cohorts of different-sized nymphs.

The highest diversity of *Neoperla* in South Africa occurs in the mountain grasslands of the Drakensberg, a UNESCO World Heritage site that harbours some of South Africa's highest biodiversity and endemism (Armstrong & Brand 2012; Brendonck *et al.* 2000; Hamer & Martens 1998; Hamer & Slotow 2009; Matthews *et al.* 1993; UNESCO 2000). However, the genus is found across the country, with some species also occurring in the Mediterranean climates of the Western Cape, and in the hot and dry Northern Cape (Chapter 5; Zwick & Zwick 2023). It is possible that changes in temperature regimes and rainfall associated with climate change, particularly in the Northern Cape and Drakensberg (Department of Environmental Affairs 2018; Jewitt *et al.* 2023; MacKellar *et al.* 2014) may affect these species. The ecology of the Notonemouridae is similarly poorly known. As most Notonemouridae are highly-localized, cold-water endemics, occasionally known only from a single river or stream (Stevens *et al.* 2018; Stevens & Picker 2003), they are probably highly sensitive to climate change. The same may be true of the Afrotropical *Neoperla*, as 33% are currently only known from a single locality (Chapter 2; Chapter 5; Zwick & Zwick 2023), but this is possibly due to undersampling.

6.7. Final Thoughts

Our knowledge of the Afrotropical Plecoptera has fascinating implications for the biogeographic history of Plecoptera, and the diversity of the order in the tropics, where stoneflies are poorly studied. Despite recent taxonomic reviews (Picker & Stevens 1997, 1999; Stevens *et al.* 2018; Stevens & Picker 1995, 1999; Zwick 2015; Zwick & Zwick 2023), we know remarkably little about the true diversity of the order in Africa, or the ecology of individual species. In this thesis, I aimed to address some of these gaps by investigating the biogeographical history, taxonomy and ecology of members of the most widespread African genus, *Neoperla*. I presented novel and improved hypotheses for the biogeographical history of Plecoptera and *Neoperla* worldwide, described ten new species of *Neoperla* from Africa, provided the first morphological identification resources for *Neoperla* nymphs in South Africa, and summarized the known ecology of nine South African species. Despite this

progress, I have created more questions that call for investigation. I am confident that species, and perhaps even families, remain undiscovered across the continent. The ecology of most species is poorly known, and the likely effects of climate change and anthropogenic impacts on the order are speculative. Clearly, Plecoptera are in need of further study across the Afrotropical region. It is my hope that the body of work presented here, combined with recent work (Picker & Stevens 1997, 1999; Stevens *et al.* 2018; Stevens & Picker 1995, 1999; Zwick 2015; Zwick & Zwick 2023), will provide a strong base to allow for future research on the order, both in South Africa and the wider Afrotropical region.

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8. Appendices

Appendix 1.1: Ethical Approval

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18 February 2025

PROF Martin Villet

Email: M.Villet@ru.ac.za

Review Reference: 2025-5420-9401

Dear PROF Martin Villet

Re: Animal ethics application: Plecoptera (stoneflies) of

southern AfricaJan Principal Investigator:

Collaborators: ,

This letter confirms that the above Annual Report has been reviewed and **APPROVED** by the Rhodes University Animal Research Ethics Committee (RU-AREC). Your Approval number is: 2025-5420-9401**.**

Approval has been granted for one year from the date of this letter. **An annual progress report** will be required in order to renew approval for an additional period of one year. Ethics approval for a project can be renewed twice for a project (i.e. the maximum number of years that a project can receive ethics approval for is 3 years). Thereafter, the project will need to undergo a full application should you require ethics clearance for longer than 3 years.

Sincerely

Dr. Roman Tandlich

Chair: Rhodes University Animal Research Ethics Committee, RU-AREC

cc: Ethics Coordinator

Appendix 2.1: GenBank and BOLD accession numbers for all COX1 sequences used to identify novel OTUs. Newly sequenced material is referred to by voucher material codes, and will be updated to GenBank accession numbers upon submission for publication.

Species	Source	COX1
Neoperla catharae	BOLD	GLMCR35020
Neoperla catharae	BOLD	INHSP08009
Neoperla clymene	BOLD	GLMCR34120
Neoperla clymene	BOLD	MDA09108
Neoperla clymene	BOLD	MDA79309
Neoperla coosa	BOLD	INHSP28609
Neoperla coosa	BOLD	INHSP29309
Neoperla dao	BOLD	VNEIN19423
Neoperla dao	BOLD	VNEIN19523
Neoperla formosita ussurica	BOLD	INHSP28709
Neoperla formosita ussurica	BOLD	INHSP29409
Neoperla gaufini	BOLD	INHSP07409
Neoperla harpi	BOLD	GLMCR32820
Neoperla idella	BOLD	VNEIN19623
Neoperla monacha	BOLD	VNEIN19723
Neoperla occipitalis	BOLD	MDA79409
Neoperla occipitalis	BOLD	PKSTO09108
Neoperla occipitalis	BOLD	PKSTO09208
Neoperla spinaloba	BOLD	VNEIN19823
Neoperla spinaloba	BOLD	VNEIN19923
Neoperla stewarti	BOLD	PKSTO01308
Neoperla stewarti	BOLD	PKSTO09908
Neoperla stewarti	BOLD	PKSTO16208
Neoperla yentu	BOLD	VNEIN20123
Neoperla zonata	BOLD	VNEIN20223
Neoperla annulatispina	Genbank	MZ172768
Neoperla annulatispina	Genbank	MZ172770
Neoperla annulatispina	Genbank	MZ172771
Neoperla bimaculata	Genbank	OL693682
Neoperla catharae	Genbank	HQ568848
Neoperla clymene	Genbank	JN200654
Neoperla clymene	Genbank	JN200656
Neoperla dao	Genbank	OR753679
Neoperla idella	Genbank	OR753656
Neoperla ignacsiveci	Genbank	KX091858
Neoperla mindoroensis	Genbank	MT547994
Neoperla mindoroensis	Genbank	MT547995
Neoperla mindoroensis	Genbank	MT547996
Neoperla monacha	Genbank	OR753655
Neoperla nigromarginata	Genbank	MZ172756
Neoperla nigromarginata	Genbank	MZ172763
Neoperla nigromarginata	Genbank	MZ172764

Neoperla niponensis	Genbank	LC815971
Neoperla recta	Genbank	MZ224446
Neoperla sabang	Genbank	MZ224447
Neoperla salakot	Genbank	MZ329648
Neoperla sp.	Genbank	KX091859
Neoperla spinaloba	Genbank	OR753675
Neoperla stewarti	Genbank	JN200659
Neoperla tuberculata	Genbank	OQ791986
Neoperla yentu	Genbank	OP160252
Neoperla zonata	Genbank	OP160248
Neoperlops gressitti	Genbank	MN400756
Neoperlops huanghuye	Genbank	CZT009
Neoperlops vietnamellus	Genbank	OR753625
Neoperla burgeoni	Newly Collected	CAW 966D
Neoperla burgeoni	Newly Collected	LIM 209B
Neoperla burgeoni	Newly Collected	LIM 322F
Neoperla burgeoni	Newly Collected	LIM 323W
Neoperla burgeoni	Newly Collected	LIM 774R
Neoperla burgeoni	Newly Collected	LIM 796M
Neoperla burgeoni	Newly Collected	PLC 15A
Neoperla burgeoni	Newly Collected	PLC 20A1
Neoperla burgeoni	Newly Collected	PLC 25A1
Neoperla burgeoni	Newly Collected	PLC 27A1
Neoperla burgeoni	Newly Collected	PLC 30A1
Neoperla burgeoni	Newly Collected	PLC 30A2
Neoperla burgeoni	Newly Collected	PLC 31A1
Neoperla burgeoni	Newly Collected	PLC 35A
Neoperla burgeoni	Newly Collected	PLEC 017D
Neoperla burgeoni	Newly Collected	PLEC 36H
Neoperla crenulata	Newly Collected	PLEC 36B
Neoperla crenulata	Newly Collected	PLEC 47AM
Neoperla crenulata	Newly Collected	PLEC 47AN
Neoperla dianae	Newly Collected	PLEC 34D
Neoperla dundoana	Newly Collected	CAW 779A
Neoperla excisa	Newly Collected	PLEC 35L
Neoperla excisa	Newly Collected	PLEC 35M
Neoperla excisa	Newly Collected	PLEC 37K
Neoperla larvata	Newly Collected	CAW 912B
Neoperla leroiana	Newly Collected	PLEC 59A
Neoperla sjostedji sjostedji	Newly Collected	PLEC 056A
Neoperla sjostedji sjostedji	Newly Collected	PLEC 54B
Neoperla sjostedti sjostedti	Newly Collected	PLEC 54A
Neoperla sp. Afr_C	Newly Collected	PLEC 67A
Neoperla sp. Afr_C	Newly Collected	PLEC 67C
Neoperla sp. Afr_D	Newly Collected	PLEC 68A
Neoperla sp. Afr_D	Newly Collected	PLEC 68C
Neoperla sp. nov . 2	Newly Collected	PLEC 32S
Neoperla sp. nov . 2	Newly Collected	PLEC 35K

Neoperla sp. nov . 2	Newly Collected	PLEC 35N
Neoperla sp. nov . 3	Newly Collected	PLEC 37L
Neoperla sp. nov . 5	Newly Collected	PLEC 005A
Neoperla sp. nov . 5	Newly Collected	PLEC 005B
Neoperla sp. nov . 5	Newly Collected	PLEC 005C
Neoperla sp. nov . 5	Newly Collected	PLEC 005D
Neoperla sp. nov . 5	Newly Collected	PLEC 29A
Neoperla sp. nov . 5	Newly Collected	RWA 37A
Neoperla sp. nov . 5	Newly Collected	RWA14A P22
Neoperla sp. nov . 8	Newly Collected	PLEC 69A
Neoperla sp. nov . 8	Newly Collected	PLEC 70A
Neoperla sp. nov . 8	Newly Collected	PLEC 72A
Neoperla sp. nov . 8	Newly Collected	PLEC 74A
Neoperla sp. nov. 1	Newly Collected	PLEC 69B
Neoperla sp. nov. 1	Newly Collected	PLEC 69C
Neoperla sp. nov. 1	Newly Collected	PLEC 69C
Neoperla sp. nov. 1	Newly Collected	PLEC 76A
Neoperla sp. nov. 4	Newly Collected	PLEC 005F
Neoperla sp. nov. 4	Newly Collected	PLEC 67B
Neoperla sp. nov. 4	Newly Collected	PLEC 68B
Neoperla sp. nov. 4	Newly Collected	PLEC 68D
Neoperla sp. nov. 4	Newly Collected	PLEC 77A
Neoperla sp. nov. 4	Newly Collected	RWA 34A
Neoperla sp. nov. 6	Newly Collected	PLEC 29B
Neoperla sp. nov. 7	Newly Collected	PLEC 005E
Neoperla transvaalensis	Newly Collected	CAW 775F
Neoperla transvaalensis	Newly Collected	CAW 918AK
Neoperla transvaalensis	Newly Collected	CAW 937G
Neoperla transvaalensis	Newly Collected	PLC 4A
Neoperla transvaalensis	Newly Collected	PLEC 012B
Neoperla transvaalensis	Newly Collected	PLEC 014C
Neoperla transvaalensis	Newly Collected	PLEC 015A
Neoperla transvaalensis	Newly Collected	PLEC 015C
Neoperla transvaalensis	Newly Collected	PLEC 015E
Neoperla transvaalensis	Newly Collected	PLEC 016A
Neoperla transvaalensis	Newly Collected	PLEC 016C
Neoperla transvaalensis	Newly Collected	PLEC 017B
Neoperla transvaalensis	Newly Collected	PLEC 018B
Neoperla transvaalensis	Newly Collected	PLEC 019A
Neoperla transvaalensis	Newly Collected	PLEC 019B
Neoperla transvaalensis	Newly Collected	PLEC 019C
Neoperla transvaalensis	Newly Collected	PLEC 53A
Neoperla transvaalensis	Newly Collected	PLEC 53B
Neoperla transvaalensis	Newly Collected	PLEC018A
Neoperla aethiopica	Zwick & Zwick 2023	NEOP074
Neoperla africana	Zwick & Zwick 2023	NEOP101
Neoperla africana	Zwick & Zwick 2023	NEOP103
Neoperla africana	Zwick & Zwick 2023	NEOP108

Neoperla africana	Zwick & Zwick 2023	NEOP112
Neoperla amoena	Zwick & Zwick 2023	NEOP070
Neoperla amoena	Zwick & Zwick 2023	NEOP071
Neoperla amoena	Zwick & Zwick 2023	NEOP072
Neoperla arambourgana	Zwick & Zwick 2023	NEOP269
Neoperla arambourgana	Zwick & Zwick 2023	NEOP271
Neoperla arambourgana	Zwick & Zwick 2023	NEOP272
Neoperla arambourgana	Zwick & Zwick 2023	NEOP273
Neoperla arambourgana	Zwick & Zwick 2023	NEOP274
Neoperla bareensis	Zwick & Zwick 2023	NEOP227
Neoperla bella	Zwick & Zwick 2023	NEOP172
Neoperla bella	Zwick & Zwick 2023	NEOP173
Neoperla bella	Zwick & Zwick 2023	NEOP174
Neoperla benti	Zwick & Zwick 2023	NEOP159
Neoperla beta	Zwick & Zwick 2023	NEOP198
Neoperla beta	Zwick & Zwick 2023	NEOP200
Neoperla beta	Zwick & Zwick 2023	NEOP201
Neoperla bipolaris	Zwick & Zwick 2023	NEOP175
Neoperla bipolaris	Zwick & Zwick 2023	NEOP176
Neoperla burgeoni	Zwick & Zwick 2023	NEOP203
Neoperla burgeoni	Zwick & Zwick 2023	NEOP208
Neoperla burgeoni	Zwick & Zwick 2023	NEOP210
Neoperla burgeoni	Zwick & Zwick 2023	NEOP211
Neoperla burgeoni	Zwick & Zwick 2023	NEOP212
Neoperla burgeoni	Zwick & Zwick 2023	NEOP217
Neoperla burgeoni	Zwick & Zwick 2023	NEOP222
Neoperla burgeoni	Zwick & Zwick 2023	NEOP224
Neoperla burgeoni	Zwick & Zwick 2023	NEOP344
Neoperla caeleps	Zwick & Zwick 2023	NEOP067
Neoperla caeleps	Zwick & Zwick 2023	NEOP068
Neoperla camerunensis	Zwick & Zwick 2023	NEOP125
Neoperla camerunensis	Zwick & Zwick 2023	NEOP126
Neoperla cataractae	Zwick & Zwick 2023	NEOP056
Neoperla claviger	Zwick & Zwick 2023	NEOP292
Neoperla coffea	Zwick & Zwick 2023	NEOP046
Neoperla conradti	Zwick & Zwick 2023	NEOP229
Neoperla conradti	Zwick & Zwick 2023	NEOP230
Neoperla costata	Zwick & Zwick 2023	NEOP006
Neoperla crenulata	Zwick & Zwick 2023	NEOP304
Neoperla crustata	Zwick & Zwick 2023	NEOP073
Neoperla decorata	Zwick & Zwick 2023	NEOP053
Neoperla dianae	Zwick & Zwick 2023	NEOP293
Neoperla dianae	Zwick & Zwick 2023	NEOP295
Neoperla dolium	Zwick & Zwick 2023	NEOP296
Neoperla dubia	Zwick & Zwick 2023	NEOP252
Neoperla dubia	Zwick & Zwick 2023	NEOP253
Neoperla dundoana	Zwick & Zwick 2023	NEOP267
Neoperla dundoana	Zwick & Zwick 2023	NEOP268

Neoperla duodeviginti	Zwick & Zwick 2023	NEOP049
Neoperla duodeviginti	Zwick & Zwick 2023	NEOP050
Neoperla erinaceus	Zwick & Zwick 2023	NEOP120
Neoperla erinaceus	Zwick & Zwick 2023	NEOP121
Neoperla erinaceus	Zwick & Zwick 2023	NEOP122
Neoperla excavata	Zwick & Zwick 2023	NEOP299
Neoperla excisa	Zwick & Zwick 2023	NEOP234
Neoperla excisa	Zwick & Zwick 2023	NEOP235
Neoperla excisa	Zwick & Zwick 2023	NEOP236
Neoperla gibbosa	Zwick & Zwick 2023	NEOP297
Neoperla gibbosa	Zwick & Zwick 2023	NEOP298
Neoperla gordius	Zwick & Zwick 2023	NEOP248
Neoperla heideae	Zwick & Zwick 2023	NEOP242
Neoperla heideae	Zwick & Zwick 2023	NEOP243
Neoperla heideae	Zwick & Zwick 2023	NEOP246
Neoperla juxtadidita	Zwick & Zwick 2023	NEOP042
Neoperla kalengonis	Zwick & Zwick 2023	NEOP012
Neoperla kalengonis	Zwick & Zwick 2023	NEOP013
Neoperla larvata	Zwick & Zwick 2023	NEOP239
Neoperla larvata	Zwick & Zwick 2023	NEOP240
Neoperla larvata	Zwick & Zwick 2023	NEOP241
Neoperla leroiana	Zwick & Zwick 2023	NEOP276
Neoperla leroiana	Zwick & Zwick 2023	NEOP277
Neoperla leroiana	Zwick & Zwick 2023	NEOP278
Neoperla leroiana	Zwick & Zwick 2023	NEOP279
Neoperla lineata	Zwick & Zwick 2023	NEOP228
Neoperla lujana	Zwick & Zwick 2023	NEOP014
Neoperla lujana	Zwick & Zwick 2023	NEOP015
Neoperla massevensis	Zwick & Zwick 2023	NEOP334
Neoperla multiserrata	Zwick & Zwick 2023	NEOP231
Neoperla nichollsi	Zwick & Zwick 2023	NEOP057
Neoperla nigricauda	Zwick & Zwick 2023	NEOP180
Neoperla nigricauda	Zwick & Zwick 2023	NEOP181
Neoperla nigricauda	Zwick & Zwick 2023	NEOP182
Neoperla nigricauda	Zwick & Zwick 2023	NEOP186
Neoperla nigricauda	Zwick & Zwick 2023	NEOP187
Neoperla nigricauda	Zwick & Zwick 2023	NEOP189
Neoperla occulta	Zwick & Zwick 2023	NEOP061
Neoperla occulta	Zwick & Zwick 2023	NEOP062
Neoperla occulta	Zwick & Zwick 2023	NEOP063
Neoperla orthonema	Zwick & Zwick 2023	NEOP161
Neoperla orthonema	Zwick & Zwick 2023	NEOP164
Neoperla orthonema	Zwick & Zwick 2023	NEOP165
Neoperla orthonema	Zwick & Zwick 2023	NEOP166
Neoperla pallidogigas	Zwick & Zwick 2023	NEOP008
Neoperla panafricana	Zwick & Zwick 2023	NEOP135
Neoperla panafricana	Zwick & Zwick 2023	NEOP137
Neoperla pickeri	Zwick & Zwick 2023	NEOP043

Neoperla pilulifera	Zwick & Zwick 2023	NEOP076
Neoperla pilulifera	Zwick & Zwick 2023	NEOP088
Neoperla pilulifera	Zwick & Zwick 2023	NEOP093
Neoperla pirus	Zwick & Zwick 2023	NEOP064
Neoperla planidorsum	Zwick & Zwick 2023	NEOP054
Neoperla planidorsum	Zwick & Zwick 2023	NEOP055
Neoperla plicata	Zwick & Zwick 2023	NEOP065
Neoperla plicata	Zwick & Zwick 2023	NEOP066
Neoperla proxima	Zwick & Zwick 2023	NEOP258
Neoperla proxima	Zwick & Zwick 2023	NEOP259
Neoperla proxima	Zwick & Zwick 2023	NEOP261
Neoperla pusilla	Zwick & Zwick 2023	NEOP247
Neoperla quadrata	Zwick & Zwick 2023	NEOP002
Neoperla quadrata	Zwick & Zwick 2023	NEOP003
Neoperla rostrata	Zwick & Zwick 2023	NEOP005
Neoperla schuelei	Zwick & Zwick 2023	NEOP177
Neoperla schuelei	Zwick & Zwick 2023	NEOP178
Neoperla schuelei	Zwick & Zwick 2023	NEOP179
Neoperla serrula	Zwick & Zwick 2023	NEOP009
Neoperla serrula	Zwick & Zwick 2023	NEOP010
Neoperla sjostedti	Zwick & Zwick 2023	NEOP308
Neoperla sjostedti cf needhami	Zwick & Zwick 2023	NEOP326
Neoperla sjostedti cf needhami	Zwick & Zwick 2023	NEOP327
Neoperla sjostedti needhami	Zwick & Zwick 2023	NEOP312
Neoperla sjostedti needhami	Zwick & Zwick 2023	NEOP314
Neoperla sjostedti needhami	Zwick & Zwick 2023	NEOP323
Neoperla sjostedti needhami	Zwick & Zwick 2023	NEOP324
Neoperla sjostedti sjostedti	Zwick & Zwick 2023	NEOP309
Neoperla socia	Zwick & Zwick 2023	NEOP305
Neoperla socia	Zwick & Zwick 2023	NEOP306
Neoperla socia	Zwick & Zwick 2023	NEOP307
Neoperla socia	Zwick & Zwick 2023	NEOP338
Neoperla spectabilis	Zwick & Zwick 2023	NEOP007
Neoperla spio	Zwick & Zwick 2023	NEOP192
Neoperla spio	Zwick & Zwick 2023	NEOP193
Neoperla spio	Zwick & Zwick 2023	NEOP194
Neoperla tangana	Zwick & Zwick 2023	NEOP300
Neoperla tangana	Zwick & Zwick 2023	NEOP301
Neoperla transvaalensis	Zwick & Zwick 2023	NEOP023
Neoperla transvaalensis	Zwick & Zwick 2023	NEOP025
Neoperla transvaalensis	Zwick & Zwick 2023	NEOP028
Neoperla transvaalensis	Zwick & Zwick 2023	NEOP031
Neoperla transvaalensis	Zwick & Zwick 2023	NEOP032
Neoperla transvaalensis	Zwick & Zwick 2023	NEOP035
Neoperla ussurica	Zwick & Zwick 2023	NEOP004
Neoperla vicina	Zwick & Zwick 2023	NEOP249
Neoperla vicina	Zwick & Zwick 2023	NEOP250
Neoperla vicina	Zwick & Zwick 2023	NEOP251

Appendix 2.2: Models selected by ModelFinder for all phylogenetic analyses in Chapter 2.

Gene	Model
Identify OTUs	
COX1	
1st Codon Position	TIMe+I+G4
2nd Codon Position	TN+F+I+G4
3rd Codon Position	GTR+F+ASC+G4
Three Gene Analysis	
28S	TIM2+F+I+G4
COX1	
1st Codon Position	TNe+G4
2nd Codon Position	F81+F+I
3rd Codon Position	TIM3+F+G4
H3	
1st Codon Position	F81+F+I
2nd Codon Position	K2P+I
3rd Codon Position	GTR+F+G4

Appendix 2.3: c

Species	Source	COX1	H3	28S
Acroneuria lycorias	Genbank	EF622961	EF622649	HQ568944*
Agnetina capitata	Genbank	EF622950	EF622640	GU712520*
Eccoptura xanthenes	Genbank	EF622962	EF622650	HQ568852*
Neoperla clymene	Genbank	-	EF622652	JN200654*
Paragnetina media	Genbank	EF622963	EF622651	GU713228*
Paragnetina tinctipennis	Genbank	-	MK774425	MK774337
Paragnetina tinctipennis	Genbank	-	MK774451	MK774332
Neoperla arambourgana	Newly Collected	-	-	PLEC 35G
Neoperla arambourgana	Newly Collected	-	CAW 934D	-
Neoperla burgeoni	Newly Collected	CAW 966D	CAW 966D	-
Neoperla burgeoni	Newly Collected	LIM 209B	LIM 209B	LIM 209B
Neoperla burgeoni	Newly Collected	LIM 322F	LIM 322F	-
Neoperla burgeoni	Newly Collected	LIM 323W	LIM 323W	LIM 323W
Neoperla burgeoni	Newly Collected	LIM 774R	LIM 774R	LIM 774R
Neoperla burgeoni	Newly Collected	LIM 796M	LIM 796M	-
Neoperla burgeoni	Newly Collected	PLC 15A	PLC 15A	PLC 15A
Neoperla burgeoni	Newly Collected	PLC 20A1	PLC 20A1	PLC 20A1
Neoperla burgeoni	Newly Collected	PLC 25A1	PLC 25A1	PLC 25A1
Neoperla burgeoni	Newly Collected	PLC 27A1	PLC 27A1	PLC 27A1
Neoperla burgeoni	Newly Collected	PLC 30A1	PLC 30A1	PLC 30A1
Neoperla burgeoni	Newly Collected	PLC 30A2	PLC 30A2	PLC 30A2
Neoperla burgeoni	Newly Collected	PLC 31A1	PLC 31A1	PLC 31A1
Neoperla burgeoni	Newly Collected	PLC 35A	PLC 35A	PLC 35A
Neoperla burgeoni	Newly Collected	PLEC 017D	-	PLEC 017D
Neoperla burgeoni	Newly Collected	PLEC 36H	PLEC 36H	PLEC 36H
Neoperla crenulata	Newly Collected	PLEC 36B	PLEC 36B	-
Neoperla crenulata	Newly Collected	PLEC 47AM	-	-
Neoperla crenulata	Newly Collected	PLEC 47AN	PLEC 47AN	PLEC 47AN
Neoperla dianae	Newly Collected	PLEC 34D	PLEC 34D	PLEC 34D
Neoperla dundoana	Newly Collected	-	CAW 918AK_P12	-
Neoperla dundoana	Newly Collected	CAW 779A	CAW 779A	-
Neoperla excisa	Newly Collected	PLEC 35L	-	PLEC 35L
Neoperla excisa	Newly Collected	PLEC 35M	PLEC 35M	PLEC 35M
Neoperla excisa	Newly Collected	PLEC 37K	-	PLEC 37K
Neoperla larvata	Newly Collected	CAW 912B	CAW 912B	-
Neoperla leroiana	Newly Collected	-	PLEC 35G	PLEC 35G
Neoperla leroiana	Newly Collected	PLEC 59A	PLEC 59A	-
Neoperla sjostedji sjostedji	Newly Collected	PLEC 056A	PLEC 056A	PLEC 056A
Neoperla sjostedji sjostedji	Newly Collected	PLEC 54B	PLEC 54B	PLEC 54B
Neoperla sjostedti sjostedti	Newly Collected	PLEC 54A	PLEC 54A	PLEC 54A
Neoperla sp. Afr_C	Newly Collected	PLEC 67A	PLEC 67A	PLEC 67A
Neoperla sp. Afr_C	Newly Collected	PLEC 67C	PLEC 67C	-
Neoperla sp. Afr_D	Newly Collected	PLEC 68A	PLEC 68A	PLEC 68A
Neoperla sp. Afr_D	Newly Collected	PLEC 68C	PLEC 68C	-
Neoperla sp. Afr_E	Newly Collected	-	-	PLEC 021A

Neoperla sp. Afr_F	Newly Collected	-	PLEC 31C	-
Neoperla sp. Afr_F	Newly Collected	-	PLEC 31C	PLEC 31C
Neoperla sp. Afr_G	Newly Collected	-	-	ECR859D
Neoperla sp. nov . 2	Newly Collected	PLEC 32S	-	PLEC 32S
Neoperla sp. nov . 2	Newly Collected	PLEC 35K	PLEC 35K	PLEC 35K
Neoperla sp. nov . 2	Newly Collected	PLEC 35N	-	PLEC 35N
Neoperla sp. nov . 3	Newly Collected	PLEC 37L	PLEC 37L	PLEC 37L
Neoperla sp. nov . 5	Newly Collected	PLEC 005A	-	PLEC 005A
Neoperla sp. nov . 5	Newly Collected	PLEC 005B	-	PLEC 005B
Neoperla sp. nov . 5	Newly Collected	PLEC 005C	-	-
Neoperla sp. nov . 5	Newly Collected	PLEC 005D	-	PLEC 005D
Neoperla sp. nov . 5	Newly Collected	PLEC 29A	-	PLEC 29A
Neoperla sp. nov . 5	Newly Collected	RWA 14A	RWA 14A	RWA 14A
Neoperla sp. nov . 5	Newly Collected	RWA 37A	RWA 37A	-
Neoperla sp. nov . 8	Newly Collected	PLEC 69A	PLEC 69A	-
Neoperla sp. nov . 8	Newly Collected	PLEC 70A	PLEC 70A	-
Neoperla sp. nov . 8	Newly Collected	PLEC 72A	PLEC 72A	PLEC 72A
Neoperla sp. nov . 8	Newly Collected	PLEC 74A	PLEC 74A	-
Neoperla sp. nov. 1	Newly Collected	-	PLEC 42F	PLEC 42F
Neoperla sp. nov. 1	Newly Collected	-	PLEC 42F	PLEC 42F
Neoperla sp. nov. 1	Newly Collected	PLEC 69B	PLEC 69B	PLEC 69B
Neoperla sp. nov. 1	Newly Collected	PLEC 69C	PLEC 69C	-
Neoperla sp. nov. 1	Newly Collected	PLEC 69C	PLEC 69C	-
Neoperla sp. nov. 1	Newly Collected	PLEC 76A	PLEC 76A	PLEC 76A
Neoperla sp. nov. 10	Newly Collected	-	PLEC 32R	PLEC 32R
Neoperla sp. nov. 4	Newly Collected	PLEC 005F	PLEC 005F	PLEC 005F
Neoperla sp. nov. 4	Newly Collected	PLEC 67B	PLEC 67B	-
Neoperla sp. nov. 4	Newly Collected	PLEC 68B	PLEC 68B	PLEC 68B
Neoperla sp. nov. 4	Newly Collected	PLEC 68D	PLEC 68D	-
Neoperla sp. nov. 4	Newly Collected	PLEC 77A	PLEC 77A	PLEC 77A
Neoperla sp. nov. 4	Newly Collected	RWA 34A	RWA 34A	-
Neoperla sp. nov. 6	Newly Collected	PLEC 29B	PLEC 29B	PLEC 29B
Neoperla sp. nov. 7	Newly Collected	PLEC 005E	-	PLEC 005E
Neoperla sp. nov. 8	Newly Collected	-	PLEC 31J	-
Neoperla sp. nov. 9	Newly Collected	-	PLEC44A	-
Neoperla sp. nov. 9	Newly Collected	-	PLEC 31H	-
Neoperla transvaalensis	Newly Collected	-	CAW 937E	CAW 937E
Neoperla transvaalensis	Newly Collected	-	PLEC 0124	PLEC 0124
Neoperla transvaalensis	Newly Collected	-	PLEC 012A	PLEC 012A
Neoperla transvaalensis	Newly Collected	-	PLEC 16D	PLEC 16D
Neoperla transvaalensis	Newly Collected	-	PLEC 32M	PLEC 32M
Neoperla transvaalensis	Newly Collected	-	TUG 164A1	TUG 164A1
Neoperla transvaalensis	Newly Collected	-	TUG 164A2	TUG 164A2
Neoperla transvaalensis	Newly Collected	-	TUG 164A3	TUG 164A3
Neoperla transvaalensis	Newly Collected	CAW 775F	-	-
Neoperla transvaalensis	Newly Collected	CAW 918AK_P13	CAW 918AK_P13	-
Neoperla transvaalensis	Newly Collected	CAW 937G	CAW 937G	-
Neoperla transvaalensis	Newly Collected	PLC 4A	PLC 4A	-

Neoperla transvaalensis	Newly Collected	PLEC 012B	PLEC 012B	PLEC 012B
Neoperla transvaalensis	Newly Collected	PLEC 014C	PLEC 014C	PLEC 014C
Neoperla transvaalensis	Newly Collected	PLEC 015A	PLEC 015A	PLEC 015A
Neoperla transvaalensis	Newly Collected	PLEC 015C	PLEC 015C	PLEC 015C
Neoperla transvaalensis	Newly Collected	PLEC 015E	PLEC 015E	PLEC 015E
Neoperla transvaalensis	Newly Collected	PLEC 016A	-	PLEC 016A
Neoperla transvaalensis	Newly Collected	PLEC 016C	PLEC 016C	PLEC 016C
Neoperla transvaalensis	Newly Collected	PLEC 017B	PLEC 017B	PLEC 017B
Neoperla transvaalensis	Newly Collected	PLEC 018B	-	PLEC 018B
Neoperla transvaalensis	Newly Collected	PLEC 019A	PLEC 019A	-
Neoperla transvaalensis	Newly Collected	PLEC 019B	PLEC 019B	PLEC 019B
Neoperla transvaalensis	Newly Collected	PLEC 019C	PLEC 019C	PLEC 019C
Neoperla transvaalensis	Newly Collected	PLEC 53A	-	-
Neoperla transvaalensis	Newly Collected	PLEC 53B	-	-
Neoperla transvaalensis	Newly Collected	PLEC018A	PLEC018A	PLEC018A
Neoperla transvaalensis	Newly Collected	-	PLEC 020A	-
Neoperla amoena			PLEC 32N	PLEC 32N

Appendix 3.1: Character present in the fossilized species of Plecoptera supporting their assignments, as discussed in Chapter 3.

	Arcto.		Systellognatha						Pteronarcyidae				Peltoperlid.		Perloidea			Perlodidae		Perlidae		Acroneur.		Perl.
	Adult		All			Adult			All	Adult			N.	A.	All	Nymph		Adult		All	N.	All	N.	A.
	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii	xiii	xiv	xv	xvi	xvii	xviii	xix	xx	xxi	xxii	xxiii	xxiv
Plecoptera Burmeister, 1839																								
Systellognatha Enderlein, 1909																								
incertae sedis																								
†Perlisca Sinitshenkova 1985																								
†Perlisca aufuga			✓																					
†Chloroperloides Sinitshenkova 1985																								
†Chloroperloides fusiformis			✓																					
†Bestioperlisca Sinitshenkova 1990																								
†Bestioperlisca inulta			✓													?								
†Dipsoperla Sinitshenkova 1987																								
†Dipsoperla kunikanensis			✓																					
†Dipsoperla serpentis			✓																					
†Isoperlodes Sinitshenkova 1992																								
†Isoperlodes perstrictus			✓																					
†Perlitodes Sinitshenkova 1987																								
†Perlitodes aenigmaticus			✓																					
†Perlitodes borealis			✓																					
†Savina Sinitshenkova 1987																								
†Savina laeta			✓																					
†Sinosharaperla Liu et al. 2007																								
†Sinosharaperla zhaoi	✓					✓	✓																	
†Platyperlidae Sinitshenkova 1982 (part)																								

†Platyperla Brauer et al. 1889																					
†Platyperla platypoda		✓												?							
†Platyperla conferta		✓												?	✓						
†Petroperlidae Sroka et al. 2018																					
†Branchioperla Sroka & Staniczek 2019																					
†Branchioperla ianstewarti	✓	✓	✓	✓	✓	✓	✓														
†Lapisperla Sroka et al. 2018																					
†Lapisperla keithrichardsi	✓	✓	✓	✓	✓	✓	✓														
†Petroperla Sroka et al. 2018																					
†Petroperla michjaggeri	✓	✓	✓	✓		✓	✓														
Pteronarcyoidea Newman, 1853																					
†Cavoperlidae Chen 2023																					
†Cavoperla Chen 2023																					
†Cavoperla excavata	X		X		✓	✓		✓													
Pteronarcyidae Newman 1853 (part)																					
†Pteroliriope Cui et al. 2016																					
†Pteroliriope sinitshenkovae	X				✓	✓			✓	✓	✓										
Peltoperlidae Claassen 1931 (Part)																					
†Ecdyoperla Sinitshenkova 1998																					
†Ecdyoperla fairlightensis		✓									✓										
†Borisoperla Chen & Xu 2020																					
†Borisoperla kondratieffi	✓	✓	X	✓	✓	✓	✓					✓									
†Dewaltoperla Chen 2023																					
†Dewaltoperla edwardi	✓	✓	X	✓	✓	✓	✓					✓									
†Zwickoperla Chen & Wang 2020																					
†Zwickoperla brevicauda	✓	✓	X	✓	✓	✓	✓					✓									

Perloidea Latreille, 1802																						
incertae sedis																						
†Trianguliperla Sinitshenkova 1985 (part)																						
†Trianguliperla quassa			✓								✓	✓										
†Derancheperla Sinitshenkova 1990																						
†Derancheperla collaris			✓									✓	✓									
†Burmacroneuria Chen 2019																						
†Burmacroneuria projecta	✓	✓	✓		✓	✓	✓	✓			✓											
†Pinguisoperla Chen 2018																						
†Pinguisoperla yangzhouensis	✓		✓		✓	✓	✓	✓			✓											
†Starkoperla Chen & Wang 2020																						
†Starkoperla longusocollum	✓	✓	✓	X	✓	✓	✓	✓			✓											
†Kachinoperlidae Chen 2022																						
†Kachinoperla Chen 2022																						
†Kachinoperla zwicki			✓								✓	✓	✓									
Perlodidae Klapálek, 1909 (Part)																						
Isoperla Banks 1906																						
Isoperla †baltica			✓								✓	✓	✓	✓	✓							
Perlidae Latreille 1802 (Part)																						
†Euperlida Cifuentes-Ruiz 2007																						
†Euperlida parvicercifera												✓	✓			✓	✓					
Acroneuriinae Klapálek 1914																						
†Burmaperla Jouault & Nel 2021																						
†Burmaperla pouilloni	✓	✓	✓	✓	✓	✓	✓	✓			✓							✓				
†Burmesoperla Chen 2019																						
†Burmesoperla expansa	✓	✓	✓		✓	✓	✓	✓			✓							✓*				
†Cretacroneuria Chen 2020																						

†Cretacroneuria angularis	✓	✓	✓		✓	✓	✓	✓					✓						✓			
†Electroneuria Sroka et al. 2018																						
†Electroneuria ronwoodi			✓										✓	✓	✓		✓	✓		✓		
†Largusoperla Chen 2018																						
†Largusoperla acus	✓	✓	✓		✓	✓	✓	✓					✓						✓			
†Largusoperla arcus	✓	✓	✓		✓	✓	✓	✓					✓						✓			
†Largusoperla billwymani	✓	✓	✓	✓	✓	✓	✓	✓					✓				✓		✓			
†Largusoperla borisi	✓	✓	✓		✓	✓	✓	✓					✓						✓*			
†Largusoperla brianjonesi	✓		✓	✓	✓	✓	✓	✓					✓				✓					
†Largusoperla charliewattsi	✓	✓	✓	✓	✓	✓	✓	✓					✓				✓		✓			
†Largusoperla crassus	✓	✓	✓		✓	✓	✓	✓					✓						✓			
†Largusoperla dewalti	✓	✓	✓		✓	✓	✓	✓					✓						✓*			
†Largusoperla difformitatem	✓	✓			✓	✓	✓	✓					✓						✓*			
†Largusoperla flata	✓	✓	✓		✓	✓	✓	✓					✓						✓			
†Largusoperla micktaylori	✓		✓	✓	✓	✓	✓	✓					✓				✓					
†Largusoperla reni	✓	✓	✓		✓	✓	✓	✓					✓						✓			
†Dominoperla Stark & Lentz 1992																						
†Dominiperla antigua	✓					✓	✓															
Perlinae Latreille, 1802																						
Perla Geoffroy 1792 (part)																						
"Perla" †prisca	✓		✓			✓	✓													✓		

Key

✓ = Present

X = Plesiomorphic form retained
? = Present, but differs from apomorphic state

i = Absence of crossveins in distal half of (ScP+) RA-RP.

ii = Drumming Hammer

iii = Short basal tarsomere

iv = Setose Arolium

v = Euplantulae present

vi = Basal crossvein between ScP and anterior margin of wing short, opposed obliquely, stronger than remaining crossveins
vii = Area between ScP and anterior margin
narrow
viii = Numerous crossveins in basal region of the costal
field

ix = Laterally expanded Arolium

x = M with more than two branches

xi = Numerous crossveins between M and CuA

xii = Crossveins between branches of AA2

xiii = Cockroach like body
xiv = Shortened head, inserted into an angular
prothorax

xv = Long, whip like palps
xvi = Evidence of carnivory, slender mandibles without
mola

xvii = Frontoclypeal suture absent
xviii = Paraprocts modified into simple upcurved
hooks

xix = *Isoperla* like wing venation

xx = Tufted thoracic and/or anal gills

xxi = Occipital row of short spinules
xxii = Paraprocts modified into anteriorly upcurved hooks. *Not upcurved, but enlarged and otherwise
modified.
xxiii = Occipital row of spinules incomplete, or
interrupted
xxiv = Hemitergites (H10) modified: Deeply divided or
cleft

Appendix 4.1: Models selected by ModelFinder for all phylogenetic analyses in Chapter 4.

Gene	Model
Mitogenome Approach	
ATP6	
1st Codon Position	TIM2e+I+G4
2nd Codon Position	TVM+F+I+G4
3rd Codon Position	TPM2+F+I+G4
ATP8	
1st Codon Position	TIM2e+I+G4
2nd Codon Position	TPM3+F+G4
3rd Codon Position	TIM2+F+ASC+G4
COX1	
1st Codon Position	SYM+I+G4
2nd Codon Position	TPM3u+F+I+G4
3rd Codon Position	TPM2+F+G4
COX2	
1st Codon Position	TIM2e+I+G4
2nd Codon Position	TPM3u+F+I+G4
3rd Codon Position	TPM2u+F+I+G4
COX3	
1st Codon Position	SYM+I+G4
2nd Codon Position	K3Pu+F+I+G4
3rd Codon Position	TPM2u+F+I+G4
CYTB	
1st Codon Position	TIM2e+I+G4
2nd Codon Position	TPM3u+F+I+G4
3rd Codon Position	GTR+F+I+G4
ND1	
1st Codon Position	TN+F+I+G4
2nd Codon Position	GTR+F+I+G4
3rd Codon Position	TN+F+I+G4
ND2	
1st Codon Position	GTR+F+I+G4
2nd Codon Position	GTR+F+I+G4
3rd Codon Position	TPM2u+F+I+G4
ND3	
1st Codon Position	TIM2+F+I+G4
2nd Codon Position	GTR+F+G4
3rd Codon Position	TPM2u+F+ASC+G4
ND4	
1st Codon Position	TN+F+I+G4
2nd Codon Position	GTR+F+I+G4
3rd Codon Position	TIM3+F+G4
ND4L	
1st Codon Position	HKY+F+G4

2nd Codon Position	TVM+F+I+G4
3rd Codon Position	TN+F+G4
ND5	
1st Codon Position	TIM3+F+I+G4
2nd Codon Position	GTR+F+I+G4
3rd Codon Position	TIM3+F+I+G4
ND6	
1st Codon Position	GTR+F+I+G4
2nd Codon Position	GTR+F+I+G4
3rd Codon Position	TPM2u+F+G4

Three Gene Approach

COX1	
1st Codon Position	TNe+G4
2nd Codon Position	F81+F+I
3rd Codon Position	TPM3+F+G
H3	
1st Codon Position	F81+F+I
2nd Codon Position	K2P+I
3rd Codon Position	TVM+F+G4
28S	
Whole gene	TIM2+F+G4

Appendix 4.2: Genbank Accession numbers for all species used in the Mitochondrial Approach analysis in Chapter 4.

Species	Source	ATP6	ATP8	COX1	COX2	COX3	CYTb	NAD1	NAD2	NAD3	NAD4	NAD4L	NAD5	NAD6
Perlodes microcephalus	Genbank	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621	MT483621
Isoperla eximia	Genbank	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457	MG910457
Suwallia teleckojensis	Genbank	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754	NC037754
Sweltsa sp.	Genbank	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266	OL351266
Flavoperla hatakeyamae	Genbank	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010	MN821010
Niponiella limbatella	Genbank	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067	MK686067
Acroneuria hainana	Genbank	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685	KM199685
Sinacroneuria dabieshana	Genbank	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253	MK492253
Acroneuria carolinensis	Genbank	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989	MN969989
Claassenia magna	Genbank	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914	MN419914
Claassenia sp.	Genbank	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652	OK021652
Oyamia seminigra	Genbank	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578	OL505578
Oyamia nigribasis	Genbank	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290	MN548290
Dinocras cephalotes	Genbank	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757	KF484757
Kamimuria klapaleki	Genbank	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755	MN400755
Kamimuria chungnanshana	Genbank	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102	KT186102
Kamimuria wangi	Genbank	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944	KC894944
Togoperla limbata	Genbank	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990	MN969990
Paragnetina indentata	Genbank	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431	MN627431
Togoperla sp.	Genbank	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708	KM409708
Ectocorema hochii	Genbank	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888	MK905888
Neoperlops gressitti	Genbank	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756	MN400756
Neoperla sp.	Genbank	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859	KX091859
Neoperla bimaculata	Genbank	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682	OL693682
Neoperla ignacsiveci	Genbank	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858	KX091858
Neoperla costata	NEOP 006	OR034303	OR034368	OR031161	OR034431	OR035034	OR034499	OR034567	OR034635	OR034703	OR034770	OR034837	OR034905	OR034969

Neoperla spectabilis	NEOP 007	OR034346	-	OR031201	-	OR035074	-	-	-	OR034743	OR034813	OR034878	OR034946	-
Neoperla kalengonis	NEOP 013	OR034344	OR034407	OR031199	OR034471	OR035072	OR034539	OR034607	OR034675	OR034741	OR034811	OR034876	OR034944	OR035006
Neoperla transvaalensis	NEOP 024	OR034284	OR034349	OR031142	OR034412	OR035015	OR034480	OR034548	OR034616	OR034684	OR034751	OR034818	OR034886	OR034950
Neoperla nichollsi	NEOP 057	OR034293	OR034358	OR031151	OR034421	OR035024	OR034489	OR034557	OR034625	OR034693	OR034760	OR034827	OR034895	OR034959
Neoperla plicata	NEOP 065	-	-	OR031135	OR034409	-	OR034475	OR034543	OR034611	OR034679	OR034746	OR034816	OR034881	OR034949
Neoperla caeleps	NEOP 067	OR034342	OR034405	OR031134	OR034469	OR035070	OR034537	OR034605	OR034673	OR034739	OR034809	OR034874	OR034942	OR035004
Neoperla amoena	NEOP 071	OR034319	OR034383	OR031176	OR034447	OR035050	OR034515	OR034583	OR034651	OR034719	OR034786	OR034853	OR034921	OR034985
Neoperla crustata	NEOP 073	OR034348	-	OR031203	OR034474	OR035076	OR034542	OR034610	OR034678	OR034745	OR034815	OR034880	OR034948	OR035008
Neoperla aethiopica	NEOP 074	OR034320	OR034384	OR031177	OR034448	OR035051	OR034516	OR034584	OR034652	OR034720	OR034787	OR034854	OR034922	OR034986
Neoperla pilulifera	NEOP 093	OR034340	OR034403	OR031197	OR034467	OR035068	OR034535	OR034603	OR034671	OR034738	OR034807	OR034873	OR034940	OR035003
Neoperla panafricana	NEOP 130	OR034291	OR034356	OR031149	OR034419	OR035022	OR034487	OR034555	OR034623	OR034691	OR034758	OR034825	OR034893	OR034957
Neoperla camerunensis	NEOP 125	OR034305	OR034370	OR031163	OR034433	OR035036	OR034501	OR034569	OR034637	OR034705	OR034772	OR034839	OR034907	OR034971
Neoperla coffea	NEOP 046	OR034343	OR034406	OR031198	OR034470	OR035071	OR034538	OR034606	OR034674	OR034740	OR034810	OR034875	OR034943	OR035005
Neoperla juxtadidita	NEOP 042	OR034295	OR034360	OR031153	OR034423	OR035026	OR034491	OR034559	OR034627	OR034695	OR034762	OR034829	OR034897	OR034961
Neoperla beta	NEOP 198	OR034317	OR034381	OR031174	OR034445	OR035048	OR034513	OR034581	OR034649	OR034717	OR034784	OR03485	OR034919	OR034983
Neoperla burgeoni	NEOP 217	OR034307	OR034372	OR031165	OR034435	OR035038	OR034503	OR034571	OR034639	OR034707	OR034774	OR034841	OR034909	OR034973
Neoperla schuelei	NEOP 178	OR034287	OR034352	OR031145	OR034415	OR035018	OR034483	OR034551	OR034619	OR034687	OR034754	OR034821	OR034889	OR034953
Neoperla bella	NEOP 173	OR034315	OR034379	OR031172	OR034443	OR035046	OR034511	OR034579	OR034647	OR034715	OR034782	OR034849	OR034917	OR034981
Neoperla nigricauda	NEOP 180	OR034292	OR034357	OR031150	OR034420	OR035023	OR034488	OR034556	OR034624	OR034692	OR034759	OR034826	OR034894	OR034958
Neoperla orthonema	NEOP 161	OR03432	OR034385	OR031178	-	-	OR034517	-	-	OR034721	OR034788	-	OR034923	-
Neoperla benti	NEOP 159	OR034316	OR034380	OR031173	OR034444	OR035047	OR034512	OR034580	OR034648	OR034716	OR034783	OR034850	OR034918	OR034982

Neoperla vicina	NEOP 250	OR034335	OR034398	OR031192	OR034462	OR035064	OR034530	OR034598	OR034666	OR034734	OR034802	OR034868	OR034935	OR034998
Neoperla gordius	NEOP 248	OR034298	OR034363	OR031156	OR034426	OR035029	OR034494	OR034562	OR034630	OR034698	OR034765	OR034832	OR034900	OR034964
Neoperla arambourgana	NEOP 271	OR034285	OR034350	OR031143	OR034413	OR035016	OR03448	OR034549	OR034617	OR034685	OR034752	OR034819	OR034887	OR034951
Neoperla dundoana	NEOP 267	OR034299	OR034364	OR031157	OR034427	OR035030	OR034495	OR034563	OR034631	OR034699	OR034766	OR034833	OR034901	OR034965
Neoperla sjostedti needhami	NEOP 323	OR034294	OR034359	OR031152	OR034422	OR035025	OR034490	OR034558	OR034626	OR034694	OR034761	OR034828	OR034896	OR034960
Neoperla socia	NEOP 305	OR034332	OR034395	OR031189	OR034459	OR035061	OR034527	OR034595	OR034663	OR034731	OR034799	OR034865	OR034932	OR034995
Neoperla tangana	NEOP 300	OR034313	OR034377	OR031170	OR034441	OR035044	OR034509	OR034577	OR034645	OR034713	OR034780	OR034847	OR034915	OR034979
Neoperla massevensis	NEOP 334	OR034347	-	OR031202	OR034473	OR035075	OR034541	OR034609	OR034677	OR034744	OR034814	OR034879	OR034947	-
Neoperla crenulata	NEOP 304	OR034333	OR034396	OR031190	OR034460	OR035062	OR034528	OR034596	OR034664	OR034732	OR034800	OR034866	OR034933	OR034996
Neoperla claviger	NEOP 292	OR034304	OR034369	OR031162	OR034432	OR035035	OR034500	OR034568	OR034636	OR034704	OR034771	OR034838	OR034906	OR034970
Neoperla dianae	NEOP 293	OR034300	OR034365	OR031158	OR034428	OR035031	OR034496	OR034564	OR034632	OR034700	OR034767	OR034834	OR034902	OR034966
Neoperla gibbosa	NEOP 298	OR034297	OR034362	OR031155	OR034425	OR035028	OR034493	OR034561	OR034629	OR034697	OR034764	OR034831	OR034899	OR034963
Neoperla multiserrata	NEOP 231	OR034339	OR034402	OR031196	OR034466	OR035067	OR034534	OR034602	OR034670	OR034737	OR034806	OR034872	OR034939	OR035002
Neoperla excisa	NEOP 235	OR034337	OR034400	OR031194	OR034464	OR035066	OR034532	OR034600	OR034668	OR034736	OR034804	OR034870	OR034937	OR035000
Neoperla simplex	NEOP 232	OR034283	-	-	-	OR035012	-	-	-	OR034682	-	-	-	-
Neoperla conradti	NEOP 229	OR034302	OR03436	OR031160	OR034430	OR035033	OR034498	OR034566	OR034634	OR034702	OR034769	OR034836	OR034904	OR034968
Neoperla bareensis	NEOP 227	OR034314	OR034378	OR031171	OR034442	OR035045	OR034510	OR034578	OR034646	OR034714	OR034781	OR034848	OR034916	OR034980

Appendix 4.3: Genbank accession numbers for all species used for the Three-gene phylogeny in Chapter 4. Voucher accession numbers are given for newly sequenced material, and will be replaced with GenBank accession numbers of submission for publication.

Species	Source	COX1	H3	28S
Acroneuria lycorias	Genbank	EF622961	EF622649	HQ568944*
Agnetina capitata	Genbank	EF622950	EF622640	GU712520*
Eccoptura xanthenes	Genbank	EF622962	EF622650	HQ568852*
Paragnetina media	Genbank	EF622963	EF622651	GU713228*
Neoperla arambourgana	Newly Collected	-	-	PLEC 35G
Neoperla burgeoni	Newly Collected	LIM 774R	LIM 774R	LIM 774R
Neoperla crenulata	Newly Collected	PLEC 47AN	PLEC 47AN	PLEC 47AN
Neoperla dianae	Newly Collected	PLEC 34D	PLEC 34D	PLEC 34D
Neoperla excisa	Newly Collected	PLEC 35M	PLEC 35M	PLEC 35M
Neoperla larvata	Newly Collected	CAW 912B	CAW 912B	-
Neoperla leroiana	Newly Collected	PLEC 59A	PLEC 59A	-
Neoperla sjostedji sjostedji	Newly Collected	PLEC 056A	PLEC 056A	PLEC 056A
Neoperla sp. Afr_C	Newly Collected	PLEC 67A	PLEC 67A	PLEC 67A
Neoperla sp. Afr_D	Newly Collected	PLEC 68A	PLEC 68A	PLEC 68A
Neoperla sp. Afr_E	Newly Collected	-	-	PLEC 021A
Neoperla sp. Afr_F	Newly Collected	-	PLEC 31C	PLEC 31C
Neoperla sp. Afr_G	Newly Collected	-	-	ECR859D
Neoperla sp. nov . 2	Newly Collected	PLEC 35K	PLEC 35K	PLEC 35K
Neoperla sp. nov . 3	Newly Collected	PLEC 37L	PLEC 37L	PLEC 37L
Neoperla sp. nov . 5	Newly Collected	RWA 14A	RWA 14A	RWA 14A
Neoperla sp. nov . 8	Newly Collected	PLEC 72A	PLEC 72A	PLEC 72A
Neoperla sp. nov . 8	Newly Collected	PLEC 74A	PLEC 74A	-
Neoperla sp. nov. 1	Newly Collected	PLEC 76A	PLEC 76A	PLEC 76A
Neoperla sp. nov. 10	Newly Collected	-	PLEC 32R	PLEC 32R
Neoperla sp. nov. 4	Newly Collected	PLEC 005F	PLEC 005F	PLEC 005F
Neoperla sp. nov. 6	Newly Collected	PLEC 29B	PLEC 29B	PLEC 29B
Neoperla sp. nov. 7	Newly Collected	PLEC 005E	-	PLEC 005E
Neoperla sp. nov. 8	Newly Collected	-	PLEC 31J	-
Neoperla transvaalensis	Newly Collected	PLEC018A	PLEC018A	PLEC018A
Neoperla amoena			PLEC 32N	PLEC 32N

Appendix 4.4: African Icthyological Provinces that each species of *Neoperla* has been recorded in.

	Nilo-Sudan	U. Guinea	L. Guinea	Congo	Quanza	East Coast	Zambezi	Southern
Neoperla transvaalensis group								
Neoperla transvaalensis	✓	✓	✓	✓	✓	✓	✓	✓
Neoperla laticollis	✓		✓					
Neoperla lujana	✓		✓	✓		✓		
Neoperla costata	✓	✓	✓			✓		
Neoperla serrula	✓	✓						
Neoperla kalengonis	✓			✓		✓		
Neoperla rostrata					✓			
Neoperla spectabilis			✓					
Neoperla luhohonis				✓				
Neoperla luluana				✓				
Neoperla ivanloebli	✓							
Neoperla pallidogigas			✓					
Neoperla duodeviginti group								
Neoperla angolana					✓			
Neoperla pickeri						✓		
Neoperla coffea			✓					
Neoperla duodeviginti	✓	✓	✓	✓				
Neoperla didita			✓					
Neoperla juxtadidita			✓					
Neoperla africana group								
Neoperla planidorsum		✓						
Neoperla decorata			✓					
Neoperla cataractae		✓						
Neoperla erinaceus			✓					
Neoperla africana	✓	✓	✓	✓	✓	✓		
Neoperla panafricana	✓	✓	✓	✓	✓	✓	✓	✓

Neoperla camerunensis	✓	✓	✓	✓	✓			
Neoperla caeleps	✓	✓	✓					
Neoperla plicata			✓	✓				
Neoperla pirus			✓					
Neoperla amoena	✓	✓		✓				
Neoperla pilulifera	✓	✓	✓	✓	✓			
Neoperla aethiopica	✓							
Neoperla crustata	✓	✓						
Neoperla occulta	✓	✓	✓	✓				
Neoperla nichollsi		✓						
Neoperla pusilla		✓						
Neoperla multiserrata		✓						
Neoperla brachyphallus		✓						
Neoperla spio complex								
Neoperla burgeoni	✓	✓	✓	✓	✓	✓	✓	✓
Neoperla beta	✓	✓	✓	✓				
Neoperla sassandrae	✓							
Neoperla benti		✓						
Neoperla bipolaris				✓	✓			
Neoperla schuelei					✓			
Neoperla nigricauda			✓			✓		
Neoperla spio	✓	✓						
Neoperla bella	✓			✓				
Neoperla funiculata				✓				
Neoperla biserrata	✓							
Neoperla transanica						✓		
Neoperla usambara						✓		
Neoperla sambarua						✓		
Neoperla orthonema complex								
Neoperla orthonema	✓	✓	✓					

Neoperla filamentosa	✓	✓	✓					
Neoperla spironema	✓	✓	✓					
Neoperla dubia complex								
Neoperla dubia	✓		✓	✓	✓			
Neoperla proxima	✓	✓	✓	✓	✓			
Neoperla vicina				✓				
Neoperla gordius				✓				
Neoperla spaghetti		✓						
Neoperla excisa complex								
Neoperla bareensis			✓					
Neoperla lineata			✓					
Neoperla conradti		✓	✓	✓				
Neoperla muyukae			✓					
Neoperla excisa		✓	✓	✓	✓			
Neoperla heideae			✓	✓				✓
Neoperla larvata	✓	✓		✓	✓			
Neoperla sorella		✓						
Neoperla simplex			✓	✓				
Neoperla sjostedti complex								
Neoperla claviger			✓					
Neoperla dianae			✓	✓				
Neoperla gibbosa				✓	✓			
Neoperla dolium			✓					
Neoperla excavata			✓					
Neoperla crenulata	✓		✓	✓				
Neoperla massevensis						✓		
Neoperla tangana				✓		✓		
Neoperla socia	✓			✓		✓		
Neoperla s. sjostedti						✓	✓	✓
Neoperla s. needhami	✓	✓	✓	✓	✓	✓		

Neoperla s. cf. needhami			✓			✓		
				Neoperla arambourgana complex				
Neoperla arambourgana				✓		✓		
Neoperla dundoana				✓	✓		✓	
Neoperla leroiana	✓	✓	✓	✓	✓		✓	✓

Appendix 5.1: All species records of *Neoperla* in South Africa.

Source	Species	Number	Sex	Province	Locality Notes	Latitude	Longitude	Notes	Date
DZE, Rhodes Uni.	*Neoperla burgeoni*	1	♀	Eastern Cape	Grahamstown	-33.3175	26.5075		7 April 2010
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♂	Eastern Cape	Port St. Johns, Pondoland	-31.38	29.33		25-31 March 1923
Albany Museum	*Neoperla burgeoni*	1	♀	Eastern Cape	Pot river, at Oakleigh	-30.97167	26.273056	ECR 131	27 March 1993
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Eastern Cape	Ravens Wood 125meters Keiskama River, nr Rt N2 bridge,	-33.06	27.21		5 March 1978
Albany Museum	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Confluence of the Umkomaas and Lufafa River	-30.00217	30.182222	UMK 115E	14 October 1996
Albany Museum	*Neoperla burgeoni*	1	♂	Kwa-Zulu Natal	Confluence of the Umkomaas and Lufafa River	-30.00217	30.182222	UMK 116E	14 October 1996
Albany Museum	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Confluence of the Umkomaas and Lufafa River	-30.00217	30.182222	UMK 71Y	5 May 1996
Albany Museum	*Neoperla burgeoni*	1	♂	Kwa-Zulu Natal	Confluence of the Umkomaas and Lufafa River	-30.00217	30.182222	UMK 71Y	5 May 1996
Zwick & Zwick, 2023	*Neoperla burgeoni*	3	♀	Kwa-Zulu Natal	Drakensberg Garden Natal	-29.75	29.25		22 March 1968
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Drakensberge, Lotheni	-29.27	29.31		No date
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Drakensberge, Lotheni	-29.27	29.31		No date
Albany Museum	*Neoperla burgeoni*	2	♂	Kwa-Zulu Natal	Karkloof River, near Albert Falls Dam	-29.44	30.31	UMG 1065B	30 March 2004
Albany Museum	*Neoperla burgeoni*	1	♂	Kwa-Zulu Natal	Karkloof River, near Albert Falls Dam	-29.44	30.31	UMG 1065B	30 March 2004
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	M’fongosi	-28.71	30.83		October 1911
Newly collected	*Neoperla burgeoni*	1	Nymph	Kwa-Zulu Natal	Near Badam Coffee shop, Trout River	-29.75678	29.434139	PLEC 17D	4 April 2024
Newly collected	*Neoperla burgeoni*	5	♂	Kwa-Zulu Natal	Oribi Gorge	-30.7065	30.270306		15-17 March 2025

Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Pietermaritzburg	-29.63	30.37		1 May 1979
DZE, Rhodes Uni.	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Twist Farm	-29.08333	30.583333		20 February 2005
DZE, Rhodes Uni.	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal	Twist Farm	-29.08333	30.583333		10 January 2005
Albany Museum	*Neoperla burgeoni*	2	♀	Kwa-Zulu Natal	Umgeni below Mpolweni, Site 12, Umgeni River	-29.46	30.46	UMG 1058C	3 February 2004
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Kwa-Zulu Natal					1872
Newly collected	*Neoperla burgeoni*	1	♀	Limpopo	Blyde River Cabins	-22.89933	30.695972	PLC 15A	20 November 2022
Newly collected	*Neoperla burgeoni*	1	♀	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 20	23 November 2022
Newly collected	*Neoperla burgeoni*	1	♀	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 30	24 November 2022
Newly collected	*Neoperla burgeoni*	1	♂	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 30	24 November 2022
Newly collected	*Neoperla burgeoni*	1	♂	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 25	24 November 2022
Newly collected	*Neoperla burgeoni*	1	Nymph	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 27	24 November 2022
Newly collected	*Neoperla burgeoni*	1	Nymph	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 31	24 November 2022
Newly collected	*Neoperla burgeoni*	1	♂	Limpopo	Blyde River Cabins	-24.29703	30.851361	PLC 35	25 November 2022
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♂	Limpopo	Dwars R. at Vaalwater, Mogol river	-24.33333	28.05		1 February 1961
Albany Museum	*Neoperla burgeoni*	1	♀	Limpopo	Luvuvhu River, Shidzivani Site	-22.63492	30.95915	LIM 275Z	19 April 2016
Albany Museum	*Neoperla burgeoni*	1	♀	Limpopo	Luvuvhu River, Shidzivani Site	-22.63492	30.95915	LIM 774R	15 September 2016

Zwick & Zwick, 2023	*Neoperla burgeoni*	2	♂	Limpopo	Mogol R. at vaalwater & Dwars R. ca 3mls from Mogol sampling point, Mogol River	-24.33333	28.05		1 February 1961
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♂	Limpopo	Mogol R. at vaalwater & Dwars R. ca 3mls from Mogol sampling point, Mogol River	-24.33333	28.05		1 February 1961
Albany Museum	*Neoperla burgeoni*	2	♀	Limpopo	Mutale Tributary, Outport site	-22.42773	31.20942	LIM 259	11 September 2015
Albany Museum	*Neoperla burgeoni*	1	♂	Limpopo	Mutale Tributary, Outport site	-22.42773	31.20942	LIM 259	11 September 2015
Zwick & Zwick, 2023	*Neoperla burgeoni*	7	♂	Limpopo	Waterberge, Mapote Farm, Kleinsterkrivier	-24.13095	28.644689	NEOP 218-226	22-24 February 2012
Zwick & Zwick, 2023	*Neoperla burgeoni*	2	♀	Limpopo	Waterberge, Mapote Farm, Kleinsterkrivier	-24.13095	28.644689	NEOP 218-226	22-24 February 2012
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Mpumalanga	5 km w.Kaapmuiden	-25.53	31.27		26 January 1974
DZE, Rhodes Uni.	*Neoperla burgeoni*	1	♀	Mpumalanga	Leopard Creek	-25.44389	31.540556		2 April 2010
Zwick & Zwick, 2023	*Neoperla burgeoni*	6	♀	Mpumalanga	Pullen Farm, near Nelspruit	-25.48	30.97		24 November 2004
Albany Museum	*Neoperla burgeoni*	1	♂	Mpumalanga	Sabie River, at Lisbon	-24.97	31.4039	LIM 322F	25 April 2016
Albany Museum	*Neoperla burgeoni*	1	Nymph	Mpumalanga	Sabie River, Tinga, Kruger National Park	-24.94067	31.50496	LIM 209B	4 September 2015
Zwick & Zwick, 2023	*Neoperla burgeoni*	1	♀	Northern Cape	Grootpens Richtersveld	-28.07825	17.022083		2 November 1980
Albany Museum	*Neoperla burgeoni*	1	Nymph					LIM 223W	
Zwick & Zwick, 2023	*Neoperla heideae*	1	♀	Limpopo	Waterberge, Mapote Farm	-24.13095	28.644689	slide SMNS.036	22-24 February 2012

Zwick & Zwick, 2023	*Neoperla leroiana*	1	♀	Mpumalanga	Transvaal Lydenburg [Mashishing]	-25.08	30.45	South African Museum, <1964 when Barnard died.	No date
Newly collected	*Neoperla leroiana*	1	♂	Kwa-Zulu Natal	Champagne Pools, Giants Castle, Drakensburg Mountains, Boesmansrivier	-29.22164	29.550611	Collected by J. Midgley	9 March 2024
Zwick & Zwick, 2023	*Neoperla panafricana*	1	♂	Eastern Cape	Mbotyi Forest, Mkozi River	-31.4465	29.7372	NEOP 158, type locality	14-15 November 2013
Zwick & Zwick, 2023	*Neoperla panafricana*	1	♀	Eastern Cape	Mbotyi Forest, Mkozi River	-31.4465	29.7372	NEOP 156, type locality	14-15 November 2013
Zwick & Zwick, 2023	*Neoperla panafricana*	2	♂	Limpopo	Legalameetse	-24.2	30.31	NEOP 154, NEOP 155	15 December 1995
Zwick & Zwick, 2023	*Neoperla panafricana*	13	♂	Limpopo	Waterberge, Mapote Farm, Kleinsterkrivier	-24.13095	28.644689	NEOP 136-153	22-24 February 2012
Zwick & Zwick, 2023	*Neoperla panafricana*	5	♀	Limpopo	Waterberge, Mapote Farm, Kleinsterkrivier	-24.13095	28.644689	NEOP 136-153	22-24 February 2012
Zwick & Zwick, 2023	*Neoperla panafricana*	3	♀	Mpumalanga	Pullen Farm, near Nelspruit	-25.48	30.97		24 November 2004
Zwick & Zwick, 2023	*Neoperla panafricana*	1	♀	Kwa-Zulu Natal	Mooi River	-29.21	30		No date
Zwick & Zwick, 2023	*Neoperla panafricana*	2	♂	Eastern Cape	8 Miles West of Humansdorp	-34.04	24.74		1 March 1950
Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♀	Eastern Cape	Butterworth	-32.32	28.18	GPS estimated, not given	3 March 1951
Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♀	Eastern Cape	Lundean's Nek [Lundin's Nek], 20 Miles NW Rhodes	-30.64	27.74	GPS estimated, not given	11 March 1951
Zwick & Zwick, 2023	*Neoperla sjostedti*	2	♂	Eastern Cape	Mbotyi Forest, Mkozi River	-31.4465	29.7372		14-15 November 2013

Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♂	Free State	Malta Forest	-24.16667	30.233333		4 December 1976
Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♂	Gauteng	University of Pretoria	-25	28	Collection locality somewhat uncertain, GPS are given as -24, 30, but stated as University of Pretoria	May 1976
Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♂	Gauteng	University of Pretoria	-25	28	Collection locality somewhat uncertain, GPS are given as -24, 30, but stated as University of Pretoria	May 1976
Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♀	Kwa-Zulu Natal	Cathedral Peak	-28.92	29.13	GPS estimated, not given	February 1954
Zwick & Zwick, 2023	*Neoperla sjostedti*	50	♀	Kwa-Zulu Natal	Royal Natal national Park, the Hostel	-28.71	29.93	GPS estimated, not given	3 April 1951
Zwick & Zwick, 2023	*Neoperla sjostedti*	13	♀	Kwa-Zulu Natal	Royal Natal national Park, Tugela Valley	-28.74	28.91	GPS estimated, not given	3 April 1951
Newly collected	*Neoperla sjostedti*	1	N.	Mpumalanga	Upper Blyde, Haartebeestvlaagte.	-25.05367	30.668694	PLEC 56A	8 April 2024
Newly collected	*Neoperla sjostedti*	1	N.	Mpumalanga	Upper Lisbon, before the falls	-24.88592	30.851667	PLEC 54A	7 April 2024
Newly collected	*Neoperla sjostedti*	1	N.	Mpumalanga	Upper Lisbon, before the falls	-24.88592	30.851667	PLEC 54B	7 April 2024
Zwick & Zwick, 2023	*Neoperla sjostedti*	12	♀	Western Cape	Cape Peninsula, Cape Point Nature reserve	-34.35	18.48	GPS estimated, not given	10 December 1950

Zwick & Zwick, 2023	*Neoperla sjostedti*	1	♀	Western Cape	Cape Province, George	-34.04	22.35	GPS estimated, not given	27 February 1951
Newly collected	*Neoperla transvaalensis*	1	N.	Eastern Cape	Keiskammahoek, River under bridge, Gxulu River	-32.66239	27.109911	PLC 4A	9 July 2022
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	Western Cape	Schoemanspoort ca. 18km N of Oudtshoorn ca. 700m, Grobbelaarsrivier	-33.45	22.25		17 March 1978
Newly collected	*Neoperla transvaalensis*	3	N.	Eastern Cape	Steepside Farm, Next to Campsite, Bell river	-30.84506	27.806444	PLEC 15A,B, E	10 November 2023
Newly collected	*Neoperla transvaalensis*	1	N.	Eastern Cape	Toll in Guestfarm, Large river, passing under 2x bridges, with heavy flow in the centre, Kraairiver	-30.737	26.784139	PLEC 14A	10 November 2023
Newly collected	*Neoperla transvaalensis*	2	♀	Eastern Cape	Tortoni Caravan Park	-31.11694	28.331667	PLEC 18A-B	15 November 2023
Zwick & Zwick, 2023	*Neoperla transvaalensis*	3	♀	Eastern Cape	Transkei Border Station, Great Kei River	-32.51	27.98		5 March 1978
Newly collected	*Neoperla transvaalensis*	1	N.	Eastern Cape	Trout filled river, next to bridge across road, Bokspruit river	-30.88486	27.884083	PLEC 12B	9 November 2023
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Eastern Cape	Umtata	-31.59	28.78		18 February-18 March 1923
Zwick & Zwick, 2023	*Neoperla transvaalensis*	2	♂		Malta Forest	-24.16667	30.233333		3 December 1976
Newly collected	*Neoperla transvaalensis*	3	N.	Gauteng	Renosterpoort Reserve, Wilge River	-25.75286	28.954889	PLEC 19A-C	10 October 2023
Albany Museum	*Neoperla transvaalensis*	1	♀	Kwa-Zulu Natal	Confluence of the Umkomaas and Lufafa River, Umkomaas River	-30.00217	30.182222	UMK 116E	14 October 1996
Albany Museum	*Neoperla transvaalensis*	6	♀	Kwa-Zulu Natal	Creighton, Heiston farm, collected from the lights at the farm house at 10pm	-30.03333	29.666667	GEN 1383H	22 April 1995

Department of Zoology and Entomology, Rhodes University	*Neoperla transvaalensis*	1	♀	Kwa-Zulu Natal	Creighton	-30.01	29.83		8 January 2007
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	Kwa-Zulu Natal	Drakensbergen, Natal	-	-		4 April 1942
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	Kwa-Zulu Natal	Lindeque spruit	-28.93	29.56		19 February 1979
Zwick & Zwick, 2023	*Neoperla transvaalensis*	3	♀	Kwa-Zulu Natal	Lindeque spruit	-28.93	29.56		19 February 1979
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Kwa-Zulu Natal	Lotheni, Drakensberg	-29.27	29.31		No date
Newly collected	*Neoperla transvaalensis*	1	N.	Kwa-Zulu Natal	Nculwane River	-29.37397	30.373278	PLEC 16A-C	5 April 2024
Newly collected	*Neoperla transvaalensis*	1	N.	Kwa-Zulu Natal	Near Badam Coffee shop, Trout River, Mzimkhulu River	-29.75678	29.434139	PLEC 17B	4 April 2024
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	Kwa-Zulu Natal	Pietermaritzburg	-29.63	30.37		13 May 1976
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	Kwa-Zulu Natal	Ukhalamba-Drakensberg NP, Giant's Castle, Tugela river Castle, River Forest Trail	-29.35	29.48		23 March 2005
Zwick & Zwick, 2023	*Neoperla transvaalensis*	15	♀	Kwa-Zulu Natal	Ukhalamba-Drakensberg NP, Giant's Castle, Tugela river Castle, River Forest Trail	-29.35	29.48		23 March 2005
Albany Museum	*Neoperla transvaalensis*	1	♀	Kwa-Zulu Natal	Karkloof River, near Albert Falls Dam	-29.44	30.31	UMG 1065B	30 March 2004
Albany Museum	*Neoperla transvaalensis*	1	♂	Kwa-Zulu Natal	Umlaas, Nr Pietermaritzburg	-29.73	30.26	REA 1269	March 1998
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Lesotho	Basutoland, Quthing	-30.4	27.71		12-13 March 1951
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Lesotho	Basutoland, Quthing	-30.4	27.71		12-13 March 1951
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Lesotho	Injasuti Drakensberg, Wazni River	-29.11837	29.057783		15 March 2002

Albany Museum	*Neoperla transvaalensis*	1	♀	Limpopo	Mukhutswi River, at Bamboo Site	-24.19244	30.347889	LIM 139A	No date
Zwick & Zwick, 2023	*Neoperla transvaalensis*	3	♂	Limpopo	Waterberge, Mapote Farm, Kleinsterkrivier	-24.13095	28.644689	NEOP 36-41	22-24 February 2012
Zwick & Zwick, 2023	*Neoperla transvaalensis*	3	♀	Limpopo	Waterberge, Mapote Farm, Kleinsterkrivier	-24.13095	28.644689	NEOP 36-41	22-24 February 2012
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Limpopo	Zoutpansberg	-23.15	29.77		No date
Newly collected	*Neoperla transvaalensis*	2	N.	Mpumalanga	Maria Shires Waterfall, rather polluted stream next to the road	-24.98519	30.811028	PLEC 53A-B	7 April 2024
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	North West	Magaliesberg, Bartlett's Stream	-25.76	27.86		26 November 1971
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♂	North West	Tonquani Gorge	-25.76	27.86		21 December 1971
Zwick & Zwick, 2023	*Neoperla transvaalensis*	1	♀	Northern Cape	Uppington, Orange River	-28.45	21.25		March 1919
Albany Museum	*Neoperla ?angolana*	1	♀	Northern Cape	Vaal River, Near Warrenton, on Witrand farm	-28.15	24.76	WR13	3 December 1980
Albany Museum	*Neoperla ?angolana*	2	♀	Northern Cape	Vaal River, Near Warrenton, on Witrand farm. Light trap at farmhouse	-28.15	24.76		April 1981
Albany Museum	*Neoperla ?angolana*	1	♀	Northern Cape	Vaal River, Near Warrenton, on Witrand farm, at Railway bridge	-28.09	24.88		9 June 1977
Newly collected	*Neoperla sp. Afr_E*	1	N.	Eastern Cape	Fairy Vale Farm, Grahamstown	-33.32272	26.590361	PLEC 021A	15 February 2015
Newly collected	*Neoperla sp. Afr_G*	1	N.	Eastern Cape	Hofani River	-31.45653	29.718472	ECR 859	No date