THE PAST MEETS THE PRESENT: EXPLORING THE BIOGEOGRAPHY OF EXTANT PLECOPTERA OF SOUTH AFRICA WITH REFERENCE TO ANCIENT MIDDLE PERMIAN FOSSIL FORMS FROM THE ONDER KAROO LOCALITY NEAR SUTHERLAND.

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Abstract:

The Onder Karoo fossil locality near Sutherland, Northern Cape, recently yielded an unprecedented diversity of middle Permian insects, which were preserved in deposits of an aquatic lake margin system, in the Lowermost Abrahamskraal Formation. A large number of Plecoptera (stonefly) specimens were found, and this study represents an in-depth analysis of these species in the context of plecopteran evolution. A significant contribution to current knowledge of the Plecoptera from the middle Permian to the present has been made, utilizing a combination of fossil and phylogenetic evidence to better understand the origins, evolution, diversity and biogeography of the stoneflies. Three new plecopteran species have been described from the Onder Karoo locality, which is recognized here as the first Lagerstätte of its kind in Gondwana. Possible ecological links and relationships with the plecopterans described here have been suggested, using extant Plecoptera as analogues. An in-depth, fossil-calibrated, dated phylogeny of the Plecoptera was completed, using representatives from all three dominant lineages, the Antarctoperlaria, Systellognatha and the Euholognatha. This provides strong support for the monophyly of the Notonemouridae and the Antarctoperlaria, however the monophyly of Systellognatha and Euholognatha was not supported. The Notonemouridae were found to represent an earlier divergence than previously believed, forming a sister group to the remaining Plecoptera. Through a combination of fossil and molecular evidence, strong support was found for the current distribution of the Plecoptera worldwide being attributable to vicariance caused by the rifting of Pangea, and subsequently Gondwana and Laurasia, and long range dispersal. The focussed effort to include Southern Hemisphere stoneflies throughout this study has been a valuable step in reducing the Northern Hemisphere bias which currently dominates plecopteran research and has assisted in opening the way for future research into this important group on a global scale.

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1. Introduction

Recent excavations of the Onder Karoo locality near Sutherland, Northern Cape, South Africa have yielded an exceptional diversity of middle Permian insects. After 10 weeks of collecting, over a period of four years and less than two months of full time excavating, thousands of specimens, including over 800 insect impression fossils, have been uncovered from the locality. The exceptional concentration of the fossils, along with the high state of preservation of the specimens collected, including evidence of soft body preservation, qualify the site as the first Permian insect Lagerstätte - a taphonomic term used by palaeontologists to describe a fossil site demonstrating a high abundance and or quality of fossils preserved (e.g. Grimaldi & Engel 2005: 42) - of its kind in Gondwana. These fossils are the first middle Permian insects to be found in South Africa and they will be valuable tools in reconstructing the palaeoenvironment of the site, as well as contributing greatly to our understanding of insect evolution in South Africa and Gondwana.

The fossiliferous deposits at the Onder Karoo locality lie close to the Ecca-Beaufort Group boundary of the Karoo Supergroup, having been deposited close to the shoreline of the Karoo Sea during the Guadalupian Period, which lasted from 272.95 – 251.1 Ma (Cohen et al. 2013). The Karoo Supergroup was deposited in a retro-arc foreland basin from the Carboniferous to the Middle-Jurassic periods (Johnson 1991, Catuneanu et al. 1998, Welman et al. 2001, Rubidge et al. 2012). This retro-arc foreland basin was formed during the subduction of the Palaeo-Pacific Plate beneath the Falklands Plate, which caused crustal shortening and thickening within the Cape Fold Belt (CBF) (Catuneanu et al. 1998). The remains of this basin and others of similar age and ontogeny are today preserved in southern Africa (Karoo Basin), South America (Parana Basin), Antarctica (Beacon Basin) and Australia (Bowen Basin), having subsequently become fragmented as a result of the break-up of Gondwana (Catuneanu et al. 1998).

During the Permian, the Karoo Basin was home to a large inland continental waterbody, the Karoo Sea, fed by substantial rivers arising in the Cape Fold Mountains to the south, mountain ranges to the south-west in what is today South America and the Cargonian Highlands to the north (Rubidge 2005, Jirah & Rubidge 2014). In the southern reaches of the Karoo basin, where the Onder Karoo locality is located, these rivers would regularly burst their banks, carrying sand and mud onto the floodplains, and repeatedly burying plant and animal remains (Rubidge 2005, Jirah & Rubidge 2014). This has resulted in a high abundance of fossils from this time, and the region is particularly renowned for its wealth of fossil tetrapods (Rubidge 2005, Day & Rubidge 2014). The deposits at the Onder Karoo locality were most likely laid down in a lake on a broad prograding delta plain traversed by distributaries, close to the shoreline of the Karoo Sea.

Among the insect impression fossils found at the Onder Karoo locality were a large number of nymphs, many of which appear to be members, or at least stem-group ancestors, of a small order of insects, the Plecoptera (stoneflies). The stonefly fossils, their significance at the Onder Karoo locality and their phylogeny will be discussed in more detail throughout this dissertation.

1.1. Plecoptera

1.1.1. What are the Plecoptera?

Plecoptera (stoneflies) are a small order of ancient insects, which originated in the late Carboniferous during the Pennsylvanian Epoch (Béthoux et al. 2011). Apart from the palaeopterous Odonata and Ephemeroptera, stoneflies are some of the earliest winged insects, representing a basal branch of the Polyneoptera (Wipfler et al. 2019). This small group of insects is hemimetabolous, expressing an egg, nymph (occasionally also referred to as a naiad or larva) and adult (also referred to as an Imago) life stage (Grimaldi & Engel 2005). Throughout their lifecycles stoneflies can be identified from a combination of morphological traits, including three-segmented tarsi with paired claws, two pairs of membranous wings which are folded at rest (for which the Plecoptera are named, *plecos* = folded, *ptera* = wings) and two, long cerci (Grimaldi & Engel 2005, Fochetti & de Figueroa 2008, Thorp & Rogers 2015, DeWalt & Ower 2019).

During their immature nymphal stages Plecoptera are aquatic, generally living in rivers and streams of varying sizes, although in some rare cases they are found in lakes (Robinson et al. 1992, Fochetti & de Figueroa 2008). While most species are often characterized as cold water specialists (Hynes 1976, Brittain 1990, Fochetti & de Figueroa 2008, DeWalt & Ower 2019), some are able to tolerate a variety of environmental conditions, including large thermal ranges and variable stream permanence (Fochetti & de Figueroa 2008, Thorp & Rogers 2015, DeWalt & Ower 2019). This has resulted in a high diversity of Plecoptera recorded from tropical and warm-water rivers and it has been noted that, as more species are being discovered and described from warmer, tropical areas, they may prove to be more abundant and diverse in these areas compared to the colder, temperate areas where they are better known (Fochetti & de Figueroa 2008). The nymphal stages typically have generalist mouthparts, allowing them to feed on a variety of food sources (Hynes 1976). Most commonly, Plecoptera feed on detritus such as decaying wood and leaves, but others have specialized as scrapers, feeding on encrusting algae, and as carnivores feeding on aquatic invertebrates (Hynes 1976, Brittain 1990, Miyasaka & Genkai-Kato 2009). In some cases the nymphs are known to display ontogenetic diet shifts, especially in carnivorous species, switching to omnivory in winter despite a high availability of prey (Miyasaka & Genkai-Kato 2009). Generally, plecopteran nymphs mature slowly into adults, and the majority of species worldwide are uni-voltine (Brittain 1990), often relying on the sudden influx of resources associated with autumnal leaf fall for the nymphs to undergo rapid growth and development (Stewart & Stark 2002). A small number of species are semi-voltine (Brittain 1990), taking more than one year to develop and as long as four years in the case of *Pteronarcys californica* Newport 1848 (DeWalt & Stewart 1995), although this is relatively rare and is seen predominantly in spring-fed habitats and areas of extreme cold conditions (Thorp & Rogers 2015). There is only one species recorded as being multi-voltine, *Nemurella pictetii* Klapálek 1900, which is found in the Palearctic region, and has a remarkably flexible life cycle, varying from semi-voltine to multiple generations in a year as a result of intra-specific competition (Lieske & Zwick 2008). However, egg and nymph diapause in all Plecoptera is not uncommon, and they have been recorded as moulting up to thirty three times before maturing into the adult stage (Fochetti & de Figueroa 2008). Similarly, asynchronous diapause has been recorded in several stonefly species, with egg hatching occurring after a period ranging from three months to four years (Sandberg & Stewart 2004). This is especially common in areas which are seasonally dry or suffer from periodic droughts, and in winter-emerging species which undergo diapause during the hottest and driest parts of the year (Coleman & Hynes 1970, Snellen & Stewart 1979, Stewart & Sandberg 2004).

As adults, all plecopterans are terrestrial, with a few exceptions, such as the flightless adults of *Capnia lacustra* McLellan 1967 (Gripopterygidae), which remain underwater at a depth of 60-80 m throughout their lives (Caires et al. 2016). The adult stage in stoneflies is short-lived, usually only lasting a few days to weeks, during which many show complex mating behaviours, such as intersexual vibrational communication via an abdominal drumming apparatus; an apomorphy of the Arctoperlaria (Stewart 1994, Stewart & Sandberg 2006, Fochetti & de Figueroa 2008, Beutel et al. 2013). During this stage, approximately half of the plecopteran species feed on algae, lichen, rotting wood and detritus in order to support egg production (de Figueroa & Sánchez-Ortega 1999, Smith & Collier 2000). Mature females produce 25-3000 eggs, which they usually lay on the surface of flowing water in clutches, or occasionally individually (Fochetti & de Figueroa 2008). While some adults stoneflies do not feed at all, a large proportion of these are able to drink water (Hynes 1976). Some species have formed relationships with Hemiptera, as they are able to consume the honeydew produced by these species (Hynes 1976).

1.1.2. Significance of stoneflies

Throughout their life stages, Plecoptera fulfil a number of important ecological roles within river ecosystems and the surrounding areas. As discussed above, most stonefly nymphs can fill a diversity of generalist and specialist niches within aquatic stream environments (Hynes

1976). This activity of plecopteran nymphs in a stream assists in making way for other species which benefit from these activities. For example, the feeding of shredder species (the nymphs of a majority of stoneflies) increases the amount of fine particulate organic matter (FPOM) in river systems, on which many invertebrate and vertebrate species feed (Vannote et al. 1980). Such feeding behaviour is relatively rare, and was likely equally important among ancient, extinct species of Plecoptera which could fulfil similar roles in river systems to their extant descendants (Sinitshenkova 1997). Additionally, Plecoptera are an important source of food for insectivorous animals, such as fish and amphibians (Stewart & Stark 2002). As adults, Plecoptera provide a valuable source of protein to terrestrial predators throughout the year, as several families emerge during winter in some regions (DeWalt & Ower 2019).

Plecoptera are sensitive to environmental conditions, with many requiring specific habitats in order to survive (Hynes 1976, Robinson et al. 1992, Fochetti & de Figueroa 2008). The order is susceptible to small changes in water quality, and often disappears from freshwater systems in response to subtle changes in nutrient levels, pollution, habitat loss or changes in thermal regimes (Cuffney et al. 1984, Zwick 1992, Lenat 1993, Stewart & Stark 2002, DeWalt et al. 2005). All of these factors have led to recent large scale declines in abundance and diversity within Plecoptera populations, and even widespread species level extinction, because of anthropogenically-mediated climate change and habitat destruction (Zwick 1992, DeWalt et al. 2005, Bojková et al. 2012).

While these specific habitat requirements have resulted in a loss of plecopteran diversity globally, the sensitivity of this group makes them useful as indicators to assess the quality of water systems and environments. It is for this reason that the Plecoptera form a vital part of many biomonitoring methods, such as the South African Scoring System (SASS) (Dickens & Graham 2002) and the Ephemeroptera, Plecoptera and Trichoptera Index (EPT) (Lenat 1993). Additionally, these requirements make them an ideal order for consideration when reconstructing the taphonomy and palaeoenvironment of ancient habitats, such as the one preserved at the Onder Karoo locality. Insects, such as Plecoptera, are useful as tools in palaeontology as they have remained relatively unchanged since their emergence in the Palaeozoic Era (Labandeira & Sepkoski 1993), with common trends appearing between insect families and orders (Azar et al. 2003). As insects tend to be preserved along with plants, they have become more and more important for palaeoenvironmental reconstructions and attempts to understand the interactions between different species within these environments (Elias 1987, Wilson 1988).

1.1.3. Global Diversity and Distribution

Plecoptera are a small, ancient group within the class Insecta, accounting for less than 4000 described species globally (Fochetti & de Figueroa 2008, DeWalt et al. 2019, DeWalt & Ower 2019). Recent estimates vary between 3497 (Fochetti & de Figueroa 2008) and 3718 species (DeWalt & Ower 2019). This disparity in diversity estimates could be explained, in part, by the huge upsurge in taxonomic studies, with 435 new stonefly species recently described (between 2008 and 2019), in particular from China and the rest of Asia (DeWalt & Ower 2019). Recent work has also increased understanding of synonyms within Plecoptera, helping to alleviate some of the difficulties in distinguishing subtle species differences (DeWalt & Ower 2019). Although 260 of the known species are described from fossils, there has been considerable confusion regarding the placement of these taxa due to a number of confounding factors such as missing features (especially when preserved as rock impressions), poor preservation and alternate classification systems used within palaeoentomology (DeWalt & Ower 2019).

Despite this low number of species, stoneflies have a wide distribution and are found on every continent, with the exception of Antarctica (Fochetti & de Figueroa 2008). While there has been some evidence of fossilized plecopteran remains from Antarctica (Carpenter 1969), this record was inconclusive, as it was based on fragmentary specimens with no accompanying photographs or descriptions. However, given the close similarities between ecosystems across Gondwana, it is likely that the continent supported widespread populations of ancient Plecoptera, and may have served as a dispersal route from the southern regions of Gondwana to the north (McCulloch et al. 2016). Stoneflies are divided between two suborders, the Arctoperlaria and Antarctoperlaria (Zwick 2000) which, between them, contain 16 extant families and over 286 genera (Fochetti & de Figueroa 2008). Over a quarter of those species (1120 species) are in the largest family, the Perlidae of the Arctoperlaria (DeWalt & Ower 2019).

The two suborders of the Plecoptera are differentiated by a number of morphological characters, the strongest being the presence of a small drumming apparatus found within the Arctoperlaria, but not within the Antarctoperlaria (Stewart 1994, Zwick 2000). These small drumming organs are used for complex mate signalling, through drumming, tremulation, rubbing or a combination of the three behaviours (Zwick 2000, Stewart & Sandberg 2006). Comparatively, the Antarctoperlaria have no particularly striking external characters, and the monophyly of the group largely relies on small, difficult to identify features, such as a sternal depressor muscle of the fore trochanter and the presence of floriform chloride cells (Berthélemy 1968, Beer-Stiller & Zwick 1995, Zwick 2000). Based on the few putative apomorphies within the Antarctoperlaria, some authors have suggested that this is a polyphyletic group representing independent lineages, a theory that has been supported by some molecular evidence (Terry & Whiting 2005). However, the monophyly of both suborders has been consistently shown in recent molecular analyses of the Plecoptera (McCulloch et al. 2016, Ding et al. 2019), which supported the morphological phylogeny suggested by Zwick (2000). The two suborders also have different distributions. Antarctoperlaria, which consists of four families, is found exclusively in the Southern Hemisphere, while the Arctoperlaria is distributed predominately in the Northern Hemisphere (Zwick 2000, Fochetti & de Figueroa 2008, DeWalt & Ower 2019). However, the exception to this rule is the continent of Africa, where Antarctoperlaria are unknown, and only two families of the otherwise Northern Hemisphere-constrained Arctoperlaria occur, *viz*. Notonemouridae and Perlidae.

The African continent has an extremely low diversity, with less than 100 species recorded across the Afrotropical region, including Madagascar (95 species, Fochetti & de Figueroa

2008; 80 species, DeWalt & Ower 2019). This number is much lower than in any other region of the world where this order of insects is found. This low diversity is most likely caused by the warm temperatures found across the continent, although it may also be due to a lack of research and lack of taxonomic certainty regarding the actual numbers of species recorded across Africa. This is especially true within the Perlidae, where estimates range from one to over fifty species (Zwick 1998). There is considerable evidence that the most common African perlid species, *Neoperla spio* Newman 1839, is in fact a species complex, although it is still unclear exactly how many species are present (Picker 1980). This estimate is likely to increase in the near future, with the work of Zwick (unpublished) suggesting that at least twenty new species of the family Perlidae will be described from the region (DeWalt & Ower 2019). A recent phylogenetic study of South African stoneflies by Stevens et al. (2018) has also clearly suggested a high rate of endemism within South African stoneflies, which are biogeographically isolated within restricted watercourses across the predominantly dry habitats typical of the country. However, even with these recent increases in described species from Africa it is clear the continent has a much lower diversity than anywhere else in the world, with the next least specious region (Australasia) recording over three times the number of species (307 species) described to date (DeWalt & Ower 2019).

In addition to this trend of low diversity, the lack of Antarctoperlaria in Africa is noteworthy, although the suborder is similarly absent from India (Zwick 2000, Resh & Cardé 2003, Fochetti & de Figueroa 2008, Ramani et al. 2019). There are a number of hypotheses as to what may have caused this unexpected distribution, including the Antarctoperlaria having at some point become extinct in Africa and India (Briden et al. 1974, Thenius 1975, Campbell 1981) or the group having spread via long range dispersal after the split of Gondwana, without access to Africa and India (Zwick 2000). However, it is widely accepted that the order would likely have been present across Gondwana before it split (Illies 1965, Zwick 1990, 2000, Fochetti & de Figueroa 2008), and the disappearance of the Antarctoperlaria is likely due to extinction caused by changing environmental conditions and increasing temperatures (Banarescu 1990, Zwick 2000, 2009, Resh & Cardé 2003).

The current distribution of Notonemouridae and Perlidae is most likely explained by dispersal from the Northern Hemisphere into the Southern Hemisphere via Gondwana, and later via Africa (Cui et al. 2019, Ding et al. 2019), although it has been proposed that the hemisphere-specific distribution of the groups could have been caused by mass extinctions followed by more recent diversification events (Zwick 2000). The latter seems unlikely however, as there is evidence of stem groups of the modern, extant families from as early as 145 Ma at the Daohugou locality, from Inner Mongolia, China, in the Middle Jurassic (Cui et al. 2019).

The Notonemouridae, which are an exclusively Southern Hemisphere family of the Arctoperlaria, provide some evidence that these invasions would have happened before the breakup of Gondwana, or at least before Madagascar split from Africa. The family is found in several areas, including the Afrotropical, Australasian and Neotropical regions, suggesting that diversification may have occurred before the split of Gondwana (Sinitshenkova 1997, Fochetti & de Figueroa 2008), especially as recent phylogenetic studies of the Southern Hemisphere Plecoptera showed that the Notonemouridae are indeed a monophyletic group (McCulloch et al. 2016, Ding et al. 2019). As the Notonemouridae are present in Madagascar, whereas the Perlidae are not, it is likely the group had diversified and spread before the split between Africa and Madagascar occurred (Zwick 2000).

While there is strong evidence that the Notonemouridae are monophyletic and have dispersed over the Southern Hemisphere from a single dispersal event, some work has suggested that this took place during the Cretaceous, some 71 mya and was due to long range dispersal and not Gondwanan vicariance (McCulloch et al. 2016, Ding et al. 2019). Considering Plecoptera are notoriously poor dispersers in both their nymphal and adult stages, being inefficient flyers and swimmers, this hypothesis seems unlikely (Brundin 1967, Zwick 2000, Fochetti & de Figueroa 2008). Other authors have suggested that these findings were due to an overreliance on relatively recent fossil calibration points used for the Notonemouridae within these studies (Cui et al. 2019). Additionally, there is strong evidence that the two suborders diverged approximately 180 mya (Ding et al. 2019), which is supported by the fossil record (Cui et al. 2019). This event would then coincide with the splitting of the supercontinent Pangea during the Jurassic (McLoughlin 2001), suggesting that the unusual distribution of the Plecoptera seen today is a result of vicariance due to the rifting of Pangea (Croizat et al. 1974, Rosen 1978, Zwick 2000, Ding et al. 2019).

1.1.4. Phylogeny

The Plecoptera represent a basal branch of the Polyneoptera, one of the major lineages of winged insects, which consists of 10 orders and approximately 40 000 described species (Wipfler et al. 2019). Unfortunately, while the majority of the class Insecta is relatively well understood, the Polyneoptera remain problematic (Misof et al. 2014). The phylogeny of the group was highly controversial (Whitfield & Kjer 2008), even before the advent of molecular systematics (Kristensen 1975, 1981). While there is strong molecular evidence to support the monophyly of the Polyneoptera (Misof et al. 2014), the understanding of relationships within the group are less clear, with almost every published hypothesis disagreeing with all previous works (Wipfler et al. 2019). The most recent study combined the results of all these previous works with a large, in depth phylogenetic analysis and evaluation of morphological characters, and in the process has challenged a number of long held theories regarding this group and the relationships within (Wipfler et al. 2019). For many years the Plecoptera have been theorised to represent the earliest, most basal branch of the Polyneoptera, representing an intermediate form between the Palaeoptera (Ephemeroptera and Odonata) and all other extant winged insects (Hennig 1981, Kipling 1995, Thomas et al. 2000, Zwick 2009). This theory was largely based on the fact that the earliest winged insects, namely Ephemeroptera, Odonata and Plecoptera, all have aquatic nymphal stages, suggesting that flight may have evolved within an aquatic environment, possibly as a modification to gills in order to enable surface skimming (Kukalová-Peck 1978, 1983, Kipling 1995, Thomas et al. 2000). However, surface skimming in Ephemeroptera only occurs within Ephemeroidea (Palingeniidae) and is not present in basal extant Ephemeroptera (Siphluriscidae, Baetidae). Hence, surface skimming has to be regarded as a derived trait within Ephemeroptera (pers. comm. A. Staniczek, Department of Entomology, Stuttgart State Museum of Natural History). Additionally, Pritchard et al. (1993), Misof *et* al. (2014) and Wipfler *et* al. (2019)

suggested that this is not the case, and that the Plecoptera in fact represent a later branch of the Polyneoptera, with the Dermaptera and Zoraptera instead representing an older branch within the group. This, combined with the fact that many of the stem group representatives of the Plecoptera, Ephemeroptera and perhaps the Odonata had terrestrial immature stages (Grimaldi & Engel 2005), renders any hypothesis which relies on a shared aquatic stage obsolete, and strongly suggests that surface skimming evolving from gills cannot explain the origin of flight (Benner et al. 2013).

While their position within the Polyneoptera is hotly contested, the phylogeny of the Plecoptera themselves has also been unclear. In the past, phylogenetic analyses within the order Plecoptera were rare, with many studies having simply used the stoneflies as an outgroup or considering only a few species, especially among the Antarctoperlaria (Ding et al. 2019). However, the recent reviews of plecopteran phylogeny have allowed for a clearer image of the relationships within the order to emerge (McCulloch et al. 2016, Stevens et al. 2018, Ding et al. 2019). These studies all suggested that currently accepted phylogenies within the Plecoptera are well supported, with strong evidence for the monophyly of both suborders, the Antarctoperlaria and Arctoperlaria (McCulloch et al. 2016, Ding et al. 2019), and the monophyly of many of the extant families (McCulloch et al. 2016, Ding et al. 2019). Perhaps most interestingly, several recent studies have supported the monophyly of the Notonemouridae (McCulloch et al. 2016, Ding et al. 2019), which has often been considered a polyphyletic family consisting of several, independent lineages which dispersed into the Southern Hemisphere from the Northern Hemisphere (Zwick 1973, 1981, 2000).

1.2. Fossil Record of the Plecoptera

1.2.1. Gondwana and the rest of the world

The Plecoptera, as an ancient insect order, have a rich fossil history with stem species described from as early as the Pennsylvanian Epoch (300 Ma, late Carboniferous) (Béthoux et al. 2011). To date, 294 pre-Holocene fossil species have been described from across the world, divided between 23 families, 14 of which are now extinct (Table 1) (DeWalt et al. 2019). Unfortunately, the exact taxonomic placement of many of these fossils is uncertain, as many of the families are described from only a few species (Table 1), often with unclear or missing morphological characters (DeWalt & Ower 2019).

Following their origins during the Carboniferous period, the Plecoptera underwent a period of diversification as they radiated and spread across the world during the Permian. Although they are poorly represented in the Southern Hemisphere from this period, a large number of species are known from the Permian, with the majority of them described from the upper Permian (Sharov 1962, Sinitshenkova 1987, 2004, 2018, Sinitshenkova & Aristov 2010, Aristov et al. 2013). These species have been recorded from a variety of genera and from localities worldwide, namely Russia (Sharov 1962, Sinitshenkova 1987, 2004, 2018, Sinitshenkova & Aristov 2010, Aristov et al. 2013), Mongolia (Sinitshenkova 1992), Kazakhstan (Sinitshenkova 1987), Uruguay (Pinto et al. 2000), South Africa (Riek 1973, 1976a, 1976b, 1976c) and Argentina (Pinto et al. 2000). The diversification of the group continued throughout the Triassic Period, and through to the Middle Jurassic, where representatives of more modern, extant groups began to appear in the fossil record (Mitchell 2013). As discussed above, extant stoneflies fall within two suborders, the Arctoperlaria and Antarctoperlaria, and previous estimates, predominately based on records obtained from Baltic amber deposits, indicate that the modern stonefly orders diverged approximately 121 mya (McCulloch et al. 2016). However, the recently described *Paranotonemoura fidelis* Sinitshenkova 1987 (Daohugou locality, Inner Mongolia, China), a new stem relative of the modern Notonemouridae and a member of the Arctoperlaria, suggests that the suborders were well differentiated by at least the Middle Jurassic (Cui et al. 2019)

In total, only sixteen fossil species of Gondwanan origin have been described to date (Table 2), half of which have been found in South Africa (Table 3). These specimens are from the late Permian and Late Triassic, with a single species from the Early Cretaceous (Table 2). Five families, the Eustheniidae (1 species), Euxenoperlidae (13 species), Palaeonemouridae (1 species), Perlapsocidae (1 species) and Platyperlidae (1 species) (Table 2 & 3) are described from Gondwanan land masses. As with much of the plecopteran fossil record, the majority of these species are described from isolated wings, with only two species described from nymphs, *Platyperla marquati* (only nymph) and *Eodinotoperla duncanae* (nymph and adult). The Euxenoperlidae, an exclusively Gondwanan family (DeWalt et al. 2019), is the most species-rich family, with a total of thirteen species found from the Permian of South Africa, and the Triassic of Australia and Argentina. Despite the large gaps in time between the occurrences of many of these species, they share some interesting similarities. Apart from the pronounced morphological similarities which contributed to their classification, Lara et al. (2012) noted that the three species described from the Triassic Potrerillos Formation in Argentina (Pinto & Purper 1978, Martins-Neto et al. 2003) were largely preserved in a fluvial delta plain setting. This is very similar to the Permian delta plains along the margins of the Karoo Sea in South Africa, in which many of the South African Plecoptera were preserved, including the three new species described within this dissertation (Chapter 3).

Table 1: Plecopteran families and number of species per family, described from the pre-Holocene fossil record (older than 117 ka). Extinct families marked (†). (Counts obtained from De Walt et al. 2019)

Family	**Number of pre-Holocene species**
Golouidae †	1
Perlapsocidae †	1
Perlopseidae †	3
Incertae Sedis †	6
Suborder Antarctoperlaria	
Eustheniidae	5
Euxenoperlidae †	9
Gripopterygidae	3
Siberioperlidae †	18
Suborder Arctoperlaria	
Petroperlidae †	2
Baleyopterygidae †	16
Capniidae	2
Leuctridae	10
Mesoleuctridae †	11
Nemouridae	5
Palaeonemouridae †	50
Perlariopseidae †	54
Pronemouridae †	8
Taeniopterygidae	13
Chloroperlidae	2
Palaeoperlidae †	17
Perlidae	17
Perlodidae	5
Platyperidae †	8
Tshekardoperlidae †	9
Incertae Sedis †	21
Total	296

Table 2: Fossil plecopteran species described from Gondwanan land masses, excluding South Africa.

Genus	Species	Citation	Country	Type Stratum	Age	Specimen Type	Notes
				Eustheniidae			
Antitaxineura	*anomala*	Tillyard 1935	Australia	?	?	Wing	Originally described as Odonata, however based on a fragmentary specimen. Closer affinities with Plecoptera (Riek 1956, Rozefelds 1985)
				Euxenoperlidae			
Gondwanoperlidium	*argentinarum*	Pinto & Purper 1978	Argentina	Potrerillos Formation	Upper Middle Triassic-lower Upper Triassic	Wing	
Gondwanoperlidium	*mendozensis*	Pinto & Purper 1978	Argentina	Potrerillos Formation	Upper Middle Triassic-lower Upper Triassic	Wing	
Argentinoperlidium	*rogersi*	Martins-Neto & Gallego 2003	Argentina	Potrerillos Formation	Upper Middle Triassic-lower Upper Triassic	Wing	
Euxenoperla	*Triassicum*	Riek 1973	Australia	Ipswitch Series	Upper Triassic	Wing	Originally described *Stenoperlidium triassicum* Riek 1956
Eodinotoperla	*duncanae*	Jell & Duncan 1986	Australia	Koonwarra Fossil Bed	Lower Cretaceous	Wing and nymph	
				Perlapsocidae			
Perlapsocus	*formosoi*	Pinto *et al.* 2000	Uruguay	Melo Formation	Permian	Wing	
				Platyperlidae			
Platyperla	*marquati*	Gallego *et al*. 2011	Argentina	Potrerillos Formation	Lower Upper Triassic	Nymph	

1.2.2. Overview of Plecoptera fossils of South Africa

South Africa has a relatively low diversity of fossilized Plecoptera and their stem relatives on record, with only 16 species described to date, ranging from the upper Permian (Riek 1976a) to the Upper Triassic (Riek 1956, 1976c). Arguably, the number of true Plecoptera is in fact fewer, as seven of these species are described as Paraplecoptera (Sharov 1962, 1991) or Protoperlaria (Tillyard 1926) (Table 3). Both of these orders have since been found to likely be paraphyletic, and for many years acted as a “wastebasket group” for many nymphs from the Permian with uncertain placement (Hennig 1981). More recently, both of these groups have been included among, and become synonymous with, the Grylloblattida (Rasnitsyn & Quicke 2002). While the placement of the Protoperlaria and Paraplecoptera is still uncertain, it is unlikely that these groups do in fact represent stem Plecoptera, as there are several marked differences in the wing and body structure between the Grylloblattida and the Plecoptera. While six of the South African species were described entirely from isolated wings, a single nymph, *Thaumatophora pronotalis*, has been described as paraplecopteran (*Incertae sedis*) (Riek 1976a). It was noted that this species could also be considered plecopteran, however, this seems unlikely as a number of its features, such as the presence of prothoracic lobes, are not seen in any of the Plecoptera, and are instead usually associated with the Grylloblattida, as was the case with the other Paraplecoptera species (Riek 1976a). This is discussed in more detail in Chapter 3 (*Origin and Evolution of the Plecoptera*).

The first clear examples of Plecoptera from South Africa were recorded from the upper Permian, with the only other records from the Upper Triassic (Table 3). These Plecoptera are represented by eight species, all of which have been described from isolated wings, and have been identified as belonging to two families, the Palaeonemouridae and the Euxenoperlidae. The Euxenoperlidae were originally identified as Gripopterygidae (Tillyard 1935, Riek 1973, 1976b, 1976c), an extant family of the Plecoptera, with representatives from the upper Permian, Normandien Formation (Riek 1973, 1976b), and the Upper Triassic, Molteno Formation (Riek 1976c) (Table 3). This assignment to an extant taxon has not been satisfactorily explained to date, and indeed seems unlikely as it would have required the

Gripopterygidae to have remained unchanged since the Permian, and they have not been identified anywhere else from this period. Based on this reasoning, along with a review of the known characters of the family, a new family was erected and these seven species, along with five species from South America and Australia, were moved to the Euxenoperlidae (Sinitshenkova 1987). A single Palaeonemouridae species has been described, also from the Permian (Van Dijk & Geertsema 2004), which appears to match global trends more closely, as these have been identified from several parts of Europe from this time (Aristov et al. 2013). This is discussed in more detail in Chapter 3 (*Plecoptera Fossils of South Africa*).

Table 3: Plecopteran species described from the fossil record of South Africa

Genus	Species	Reference	Type Stratum	Age	Specimen Type	Updates	Reference
			Protoperlaria/Paraplecoptera				
			Liomopteridae				
Liomopterina	*clara*	Riek 1973	Middle Beaufort Group	Upper Permian	Wings	Description Updated	Riek 1976 b
Liomoptoides	*similis*	Riek 1973	Middle Beaufort Group	Upper Permian	Wings		
Mioloptera	*stuckenbergi*	Riek 1973	Middle Beaufort Group	Upper Permian	Wings	Description Updated	Riek 1976 b
Mioloptina	*tenuipennis*	Riek 1976 b	Middle Beaufort Group	Upper Permian	Wings		
Mioloptoides	*andrei*	Riek 1976 b	Middle Beaufort Group	Upper Permian	Wings		
Neoliomopterum	*picturatum*	Riek 1976 b	Middle Beaufort Group	Upper Permian	Wings		
			Incertae Sedis				
Thaumatophora	*pronotalis*	Riek 1976 a	Middle Ecca Series	Lower Permian	Nymph		
			Plecoptera				
			Euxenoperlidae (Updated from Gripopterygidae- Sinitshenkova 1987)				
Euxenoperla	*simplex*	Riek 1973	Middle Beaufort Group	Upper Permian	Wings		
Euxenoperla	*similis*	Riek 1973	Middle Beaufort Group	Upper Permian	Wings	More Specimens Added	Riek 1976 b
Euxenoperla	*clara*	Riek 1976 c	Molteno Formation	Upper Triassic	Wings	More Specimens Added, Range extended to Permian	Riek 1976 b
Euxenoperla	*triassicum*	Riek 1956	Upper Ipswich Series	Upper Triassic	Wings	Updated to current genus, Previously Stenoperlidium	Riek 1976 b
Euxenoperla	*spp.*	Riek 1976 b	Middle Beaufort Group	Upper Permian	Wings		
Euxenoperla	*oliveri*	Riek 1976 b	Middle Beaufort Group	Upper Permian	Wings		
Euxenoperlella	*jacquesi*	Riek 1976 b	Middle Beaufort Group	Upper Permian	Wings		
			Palaeonemouridae				
Afroperla	*Permiana*	Van Gijk & Geertseema 2004	Middle Beaufort Group	Upper Permian	Wings		

1.3. Aims

- Describe and identify the three new plecopteran species found at the Onder Karoo locality, near Sutherland, Northern Cape, South Africa (Chapter 3)
- Discuss possible relationships and links between the plecopteran species and the biotic and abiotic factors at the Onder Karoo locality. This in turn will contribute to the ongoing research into the ecology of the locality being conducted by the PEATs research team, Albany Museum, Makhanda, South Africa (Chapter 4)
- Identify the phylogenetic relationships and lineages within the Plecoptera, including species from all three major lineages, the Antarctoperlaria, Systellognatha and Euholognatha (Chapter 5)
- Use a combination of the phylogenetic relationship and fossil record of the Plecoptera to comment on and review the biogeography of both extant and fossil plecopterans (Chapter 6)

2. Methods

2.1. Fossil Taxonomy and Biostratigraphy

2.1.1. Site Details

The fossiliferous deposits at the Onder Karoo locality are currently mapped as lying within the uppermost Waterford Formation of the Ecca Group (de V. Wickens & Cole 2017), but probably fall within the lowermost Abrahamskraal Formation of the Beaufort Group, Karoo Supergroup, having been deposited close to the shoreline of the Karoo Sea (pers. comm. B.S. Rubidge, ESI, University of the Witwatersrand; M.O. Day, Natural History Museum, London). It is likely that the fossiliferous deposits of the Onder Karoo locality were deposited during the Wordian Age within the middle Permian. (Fig. 1b). All of the fossil specimens investigated in this study were collected from a single locality, a roadcutting/small roadbuilding quarry on the Onder Karoo road, which is approximately 25km southwest of Sutherland, a small town in the Northern Cape of South Africa (Fig. 1a).

The fossiliferous Onder Karoo deposits are fine-grained, finely laminated olive-grey mudrocks, suggestive of a calm lacustrine setting. Given the regional geological context, this was probably a delta-plain lake close to the Karoo Sea margin, fed by small distributaries. There are a number of small channels of slightly coarser-grained siltstone deposits within the fossiliferous mudrock sequence (pers. comm. R. Prevec, Earth Sciences Department, Albany Museum), which suggest that there were gentle distributaries feeding the lake. This input of flowing water would have introduced fine clastics, oxygen and nutrients into the system, creating a diversity of aquatic niches within the lake, and could also have introduced the remains of insects living in faster flowing water upstream.

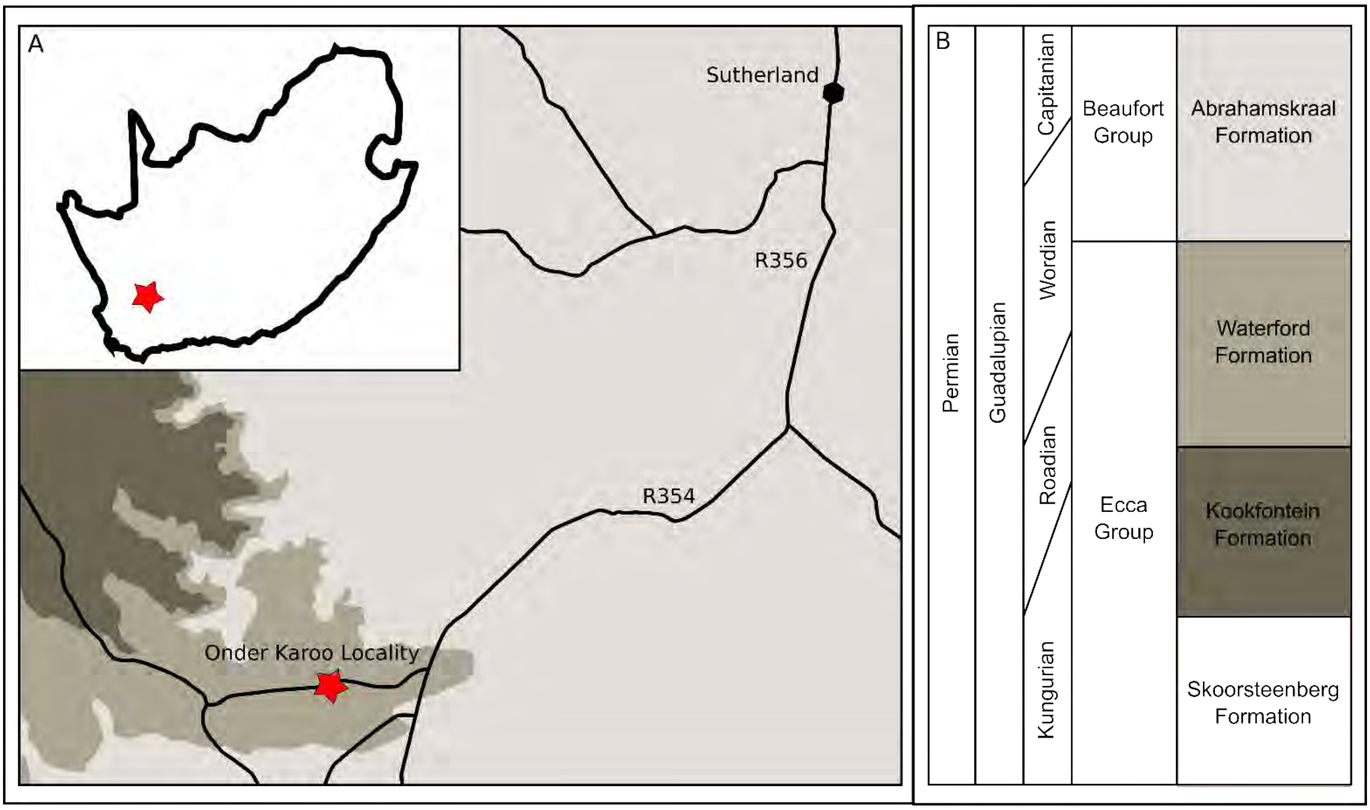

Figure 1: A: Location of the Onder Karoo Fossil Locality, near Sutherland in the Northern Cape, South Africa. B: Geological Ages of the deposits surrounding the Onder Karoo locality. Based on geological map in de V. Wickens & Cole (2017).

As the Onder Karoo locality represents a lake margin deposit, the specimens found at this locality are mainly autochthonous, or parautochthonous, with the majority of specimens probably sourced via infall from overhanging branches, windblown/drifted material settling from the water surface, or material introduced by small distributaries feeding the lake.

Multiple ash beds exposed along the Ouberg Pass, to the north of the Onder Karoo locality, within the lowermost Abrahamskraal Formation (Lanci et al. 2013), reflect a landscape regularly inundated with large ashfalls from distant volcanic eruptions. It is likely that these ashfalls had a significant effect on the terrestrial and aquatic ecosystems of the region, an avenue of investigation being pursued by the larger research team. The unusual taphonomic conditions at the site have resulted in a large number and high density of plants and insects being preserved, in exceptional detail, and this site is considered to be the first Permian Lagerstätte (sensu Seilacher 1970) from South Africa and the first insect-rich Lagerstätte from the Permian of Gondwana.

2.1.2. Site Dating

The Onder Karoo and the Ouberg Pass localities are considered to be approximately coeval, and lie close to the Ecca-Beaufort Group contact (pers. comm. B.S. Rubidge; R. Prevec). The deposits are probably within the *Eodicynodon* Assemblage Zone, or lowermost *Tapinocaninus* Assemblage Zone of the middle Permian (Rubidge 2005). Similarly, Ouberg Pass ash dates point to an age in the region of 268 Ma, which corresponds closely to the findings of Rubidge (2005), and places the localities within the Wordian Age. Recent excavations at the Onder Karoo locality have exposed what may be a reworked ash bed, and this is currently being processed for radiometric dating (Prevec et al. In preparation).

2.1.3. Collection and Curation

Since its initial discovery in 2009 (by R. Prevec) multiple collecting visits have been made at the Onder Karoo locality, with more planned in the forthcoming years. Over 800 insect fossils have been collected to date, along with a variety of other fossils including *Glossopteris* leaves and fructifications, bryophytes, sphenophytes and fish or amphibian coprolites. All of these specimens have been catalogued and are housed at the Albany Museum in Makhanda (formerly Grahamstown), South Africa. Collections were made under a South African Heritage Resource Agency (SAHRA) permit issued to Dr. Rosemary Prevec, Head of the Earth Sciences Department, Albany Museum (Permit I.D.: 2234). For all of these collections, standard equipment was used to extract the fossils from the outcrop as carefully as possible and the extracted slabs and loose scree were carefully examined for fossils, using a jeweller’s lens. Any fossil-bearing rocks were removed and labelled with location, date and collector information, before being carefully wrapped in newspaper and transported back to the Albany Museum. Once back in the museum, the specimens were labelled and placed into the Onder Karoo fossil collection, and the holotypes housed at the Albany Natural Sciences Museum (Codon: AMGS), Makhanda, South Africa.

Of the 800 specimens collected so far, 15 have been identified as nymphs of a stem group lineage of stoneflies, six of which have counterparts. These specimens represent three morphospecies, which are described in detail within this dissertation (Chapter 3: Taxonomy of the Onder Karoo Locality Plecoptera). The majority of the specimens were preserved individually, however in some cases several nymphs were preserved in small clusters on a single slab. Some of these clusters include a variety of different morphospecies and different life stages.

2.1.4. Taxonomy

Once collected, all of the plecopteran specimens were photographed under a strong, low angled, unilateral, polarized light source. Specimens were each photographed multiple times using a Zeiss discovery V8 incident light microscope, with a Nikon D90 SLR camera. For these photographs, each specimen was illuminated from the top left, and was photographed longitudinally and laterally. Additional photographs were taken at different light angles if any features of the specimen were obscured in the photograph. Each specimen was also photographed under 80% ethanol to reveal additional details, as the ethanol increased the contrast. However, use of ethanol was kept to a minimum to avoid damaging the specimens.

Standard descriptions of the morphology of the stonefly specimens were then completed, including detailed measurements and illustrations where required. The line drawings were completed in Inkscape (Version 0.92.3.0) (Harrington 2005), and measurements were made from photographs using the program ImageJ (Version 1.52) (Rueden et al. 2017). The descriptions were completed by drawing comparisons from both extant and extinct stoneflies from South African, Gondwanan and global faunas, including a variety of different nymph instars, in order to give as accurate a description and taxonomic evaluation as possible (Figs. 2 and 3). These taxonomic identifications were completed with the assistance of Dr. Arnold Staniczek (Department of Entomology, State Museum of Natural History, Stuttgart, Germany) and Dr. André Nel (Department of Systematics and Evolution, Muséum National d'Histoire Naturelle, Paris, France).

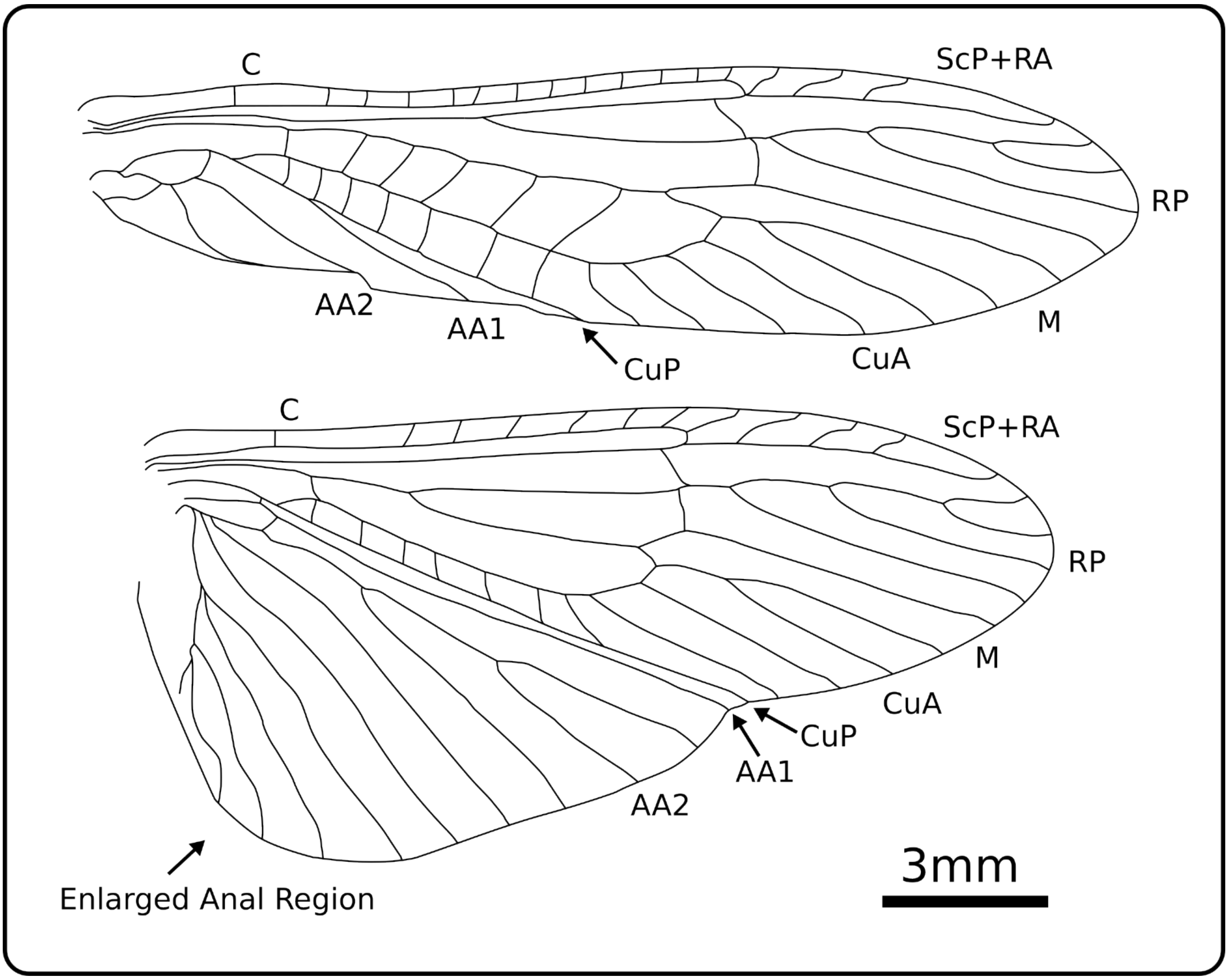

Figure 2: Generalized plecopteran wing showing the enlarged anal region and major longitudinal wing veins.C: Costal; ScP: posterior subcostal; RA: anterior radius; RP: posterior radius; M: media; CuA: anterior cubitus; CuP: posterior cubitus; AA: anterior analis. Drawing modified from Béthoux (2005)

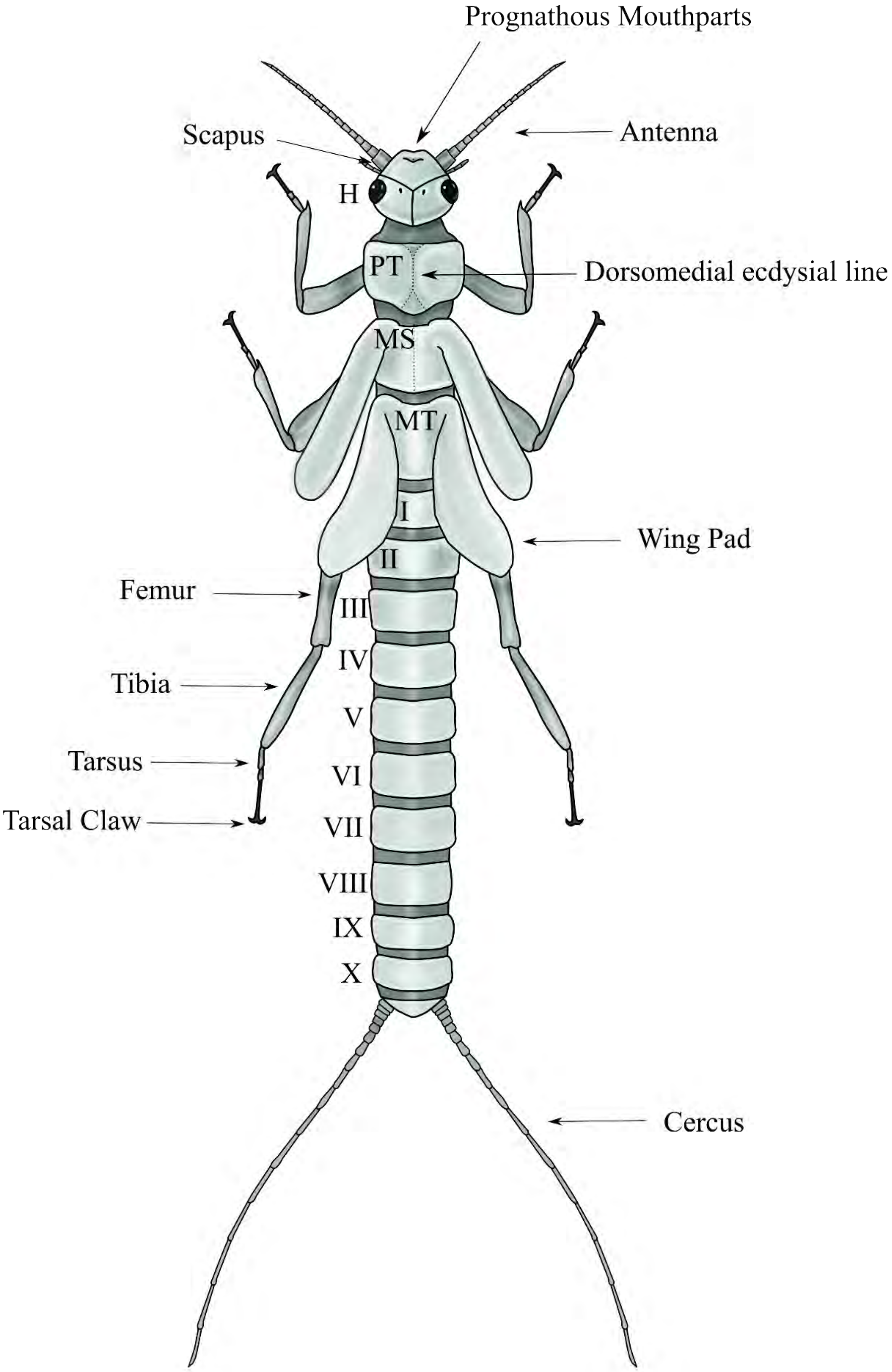

Figure 3: Generalized plecopteran nymph reconstruction, showing the long cerci and antennae, three-segmented tarsi and body shape characteristic of plecopteran nymphs. Reconstruction based on a combination of all three morphospecies described in Chapter 3. I-X = Abdominal segments 1-10, MT= metathorax, MS= mesothorax, PT= prothorax, H= head. Figure by R. Prevec.

2.2. Plecopteran Phylogeny

2.2.1. Collection of specimens of extant species

Samples for DNA analysis were taken from multiple recently collected specimens of Plecoptera in the Freshwater Invertebrate collections at the Albany Museum, with the aim of developing a more detailed phylogeny of South African stoneflies. These specimens were selected from a variety of collection sites across South Africa in order to ensure a diverse range of species and habitats were included, although in-depth comparisons between populations at the different locations within South Africa are beyond the scope of this project.

Sampled stoneflies originated from the Western Cape, the Karoo lowlands of the Northern Cape and from the Kruger National Park, which lies within both the Limpopo and Mpumalanga provinces (Table 4). In addition, several specimens were included from Angola, providing valuable insight into the relationships of the Plecoptera over a larger area in the Afrotropical region and including populations of stoneflies that have been excluded from previous research on plecopteran phylogeny (see McCulloch et al. 2016). Two species of West Palearctic Plecoptera were selected for inclusion in the phylogenetic analysis of the South African stoneflies, viz. *Isoperla grammatica* and *Protonemura intricata*. These stoneflies were selected as they belong to the Arctoperlaria, the same suborder as the Afrotropical stoneflies, and allow for comparisons between the African species and the rest of the Northern Hemisphere-constrained group.

The current study utilized a combination of the new South African sequences, as well as available GenBank data (McCulloch et al. 2016, Morinière et al. 2017, Stevens et al. 2018), to create a more complete and robust phylogeny of South African and Southern Hemisphere stoneflies. Details on all sequences used and which tests they were included in can be found in Appendix 2.

Table 4: Details of specimens used for DNA barcoding

Name	Catalogue Number	River	Province	Country
Neoperla sp.	CAW840H	Cubango River	Huambo	Angola
Neoperla sp.	CAW844	Cubango River	Huambo	Angola
Aphanicercella sp.	HMJ253Q	Seven Weeks Poort	Western Cape	South Africa
Aphanicerca	HMJ277H	Secret Falls	Western Cape	South Africa
Neoperla sp.	ISF137	Boesmans River	Kwa-Zulu Natal	South Africa
Neoperla sp.	ISF137	Boesmans River	Kwa-Zulu Natal	South Africa
Aphanicerca sp.	ISF29D	Jondershoek River	Western Cape	South Africa
Aphanicerca sp.	ISF48d	Silwer River	Western Cape	South Africa
Aphanicerca sp.	ISF49F	Millwood Creek	Western Cape	South Africa
Neoperla sp.	LIM673Q	Sabie River	Mpumalanga	South Africa
Neoperla sp.	LIM705	Sabie River	Mpumalanga	South Africa
Neoperla sp.	LIM778	Luvhuvu River	Limpopo	South Africa
Neoperla sp.	LIM803	Luvhuvu River	Limpopo	South Africa
Neoperla sp.	LIM860S	Crocodile River	Mpumalanga	South Africa
Isoperla sp.	N/A	Ach River	Bavaria	Germany
Protonemura intricata	N/A	Ach River	Bavaria	Germany
Protonemura intricata	N/A	Ach River	Bavaria	Germany
Isoperla sp.	N/A	Ach River	Bavaria	Germany
Isoperla grammatica	N/A	Ach River	Bavaria	Germany
Isoperla grammatica	N/A	Ach River	Baveria	Germany
Protonemura sp.	N/A	Ach River	Baveria	Germany

2.2.2. Identification

All of the specimens located in the Department of Freshwater Invertebrates collection at the Albany Museum were identified to genus based on morphological features using the identification key in Stevens & Picker (2009). Once identified, representatives of the different morphospecies from as many different locations as possible were selected for DNA barcoding.

2.2.3. DNA Extraction, Amplification and Sequencing

DNA was extracted from whole plecopteran nymphs using the standard spin column protocol from a DNeasy Blood and Tissue isolation kit (QIAGEN, South Africa). The tissues were dissolved, leaving intact nymphal exoskeletons which will make good voucher specimens. A 644-bp section of the COI gene was amplified using the LCO1490 (50-GGTCAACAATCATAAAGATATTGG) and HCO2198 (50-TAAACTTCGGGTGACCAAAAAATCA) primers (Folmer et al. 1994). Similarly, a 322 BP section of the H3 gene was amplified using the H3AF (50-ATGGCTCGTACCAAGCAGACVGC) and H3AR (50-TATCCTTRGGCATRATRGTGAC) primers (Colgan et al. 1998). The PCRs (25 µl) contained KAPA Taq ReadyMix (12,5 µl), the two relevant primers (3 µl of each primer), molecular grade water (3.5 µl) and the extracted DNA (3 µl). The PCR was completed under the following cycle parameters: Initial Denaturation: 95˚C for two minutes; 35 cycles of: denaturing at 95˚C for one minute, primer annealing (45˚C for COI and 50˚C for H3) for one minute, elongation at 72˚C for one minute and 30 seconds; followed by a final extension at 72˚C for seven minutes. The PCR products were confirmed by a 1% agarose gel electrophoresis using 1µl of tracking dye and stained with ethidium bromide, then visualized on a UV trans-illuminator. Successful PCR products were sent for sequencing to Macrogen (Netherlands, Europe).

Table 5: Results of jModelTest2 and the parameters which were used for Mr Bayes

	COI	H3
Number of sites	633	353
Concatenated		
AIC Model	TIM2+I+G	TIM1+I+G
p-inv	0.424	0.6
Gamma Shape	0.248	0.808
H3 Only		
AIC Model	N/A	TIM1+I+G
p-inv	N/A	0.551
Gamma Shape	N/A	0.758
COI Only		
AIC Model	GTR+I+G	N/A
p-inv	0.45	N/A
Gamma Shape	0.427	N/A

2.2.4. Data Characteristics

Newly obtained Cytochrome Oxidase Subunit 1 (COI) sequences lacked any ambiguous sites or stop codons, which suggests a true mitochondrial origin (Zhang & Hewitt 1996). Unfortunately, all new Histone 3 (H3) sequences were found to be unusable, due to the primers being contaminated by DNA belonging to *Zapada columbiana* (98% similarity) (Zhang et al. 2000), a plecopteran species from North America which was not included in this study. These primers appear to have been contaminated during production, as no *Z. columbiana* specimens were present in the laboratory where the extractions took place. The edited COI sequences (South African material and GenBank extracted sequences) were 604 BP long, and included sequences from 206 specimens (with an addition five for the outgroup), while the edited H3 sequences were 301 BP long and included sequences from 59 specimens (With an additional three for the outgroup). Once concatenated, the sequences were 905 base pairs long and included sequences from 65 specimens (with an additional 3 for the outgroup), as the majority of the species with only a single gene were excluded. Sequence statistics and nucleotide substitution models are included below.

2.2.5. Sequence Analysis

Basic Local Alignment Search Tool (BLAST)(Altschul et al. 1990) analysis was performed on all of the new sequences to see if the sequences were close to any other taxa on GenBank and to check for contamination. All new sequences obtained from the Department of Freshwater Invertebrates collection, at the Albany Museum have been submitted to GenBank (BOLD accession numbers: PLCM001-20 to PLCM017-20, GenBank accession numbers to follow). The sequences were aligned using the Multiple Alignment using Fast Fourier Transform (MAFFT), version seven online portal (Katoh et al. 2019). These alignments were then viewed and double checked by eye in BioEdit, version 7.2.5 (Hall 1999), after which the sequences were trimmed to an equal length. DAMBE, version 7 (Xia 2018) was used to test for any codon specific variation within the sequences. Models of sequence evolution were selected using jModelTest 2 (Guindon & Gascuel 2003, Darriba et al. 2012)(Table 5). Both of these tests were completed using the CIPRES science gateway platform (Miller et al. 2010).

Phylogenetic relationships for all of the stonefly specimens were first estimated using a Maximum Likelihood test of phylogeny, conducted in GARLI version 2.0.1 (Zwickl 2006) with 1000 bootstrap replicates. The bootstrap replications were then combined into a single tree using Consense (Felsenstein 1989), before being visualized in FigTree v1.4.2 (Rambaut 2006). Bayesian Interference estimates of phylogeny were then completed in MrBayes (Version 3,12) (Huelsenbeck & Ronquist 2001)☐ using the parameters identified by jModelTest (Table 5), and with the first 1250 trees discarded as "burn-in" (Defined here as the process of discarding the first 1250 replicates in a Bayesian analysis, in order to increase the accuracy of the results). The MrBayes tests of phylogeny were completed twice, once with BEAGLE enabled, however there was no variation in the phylogenies obtained. All of these tests were completed using the CIPRES science gateway platform (Miller et al. 2010). Three mayfly specimens, viz *Paraleptophlebia stricta,* were selected as an outgroup for all of these analyses in order to assist in rooting the tree.

2.2.6. Molecular Clock Analysis

The molecular clock approach is a widely used tool to estimate lineage divergence times. While a molecular phylogeny based on a number of different genes gives a useful reflection of relationships between taxa, the results should be treated with some caution, as the dates produced by molecular clocks still show wide error margins. Pulquerio & Nichols (2007) found that different molecular clock methods can give dates that differ up to 20-fold. However, the relaxed clock method introduced by Drummond & Rambaut (2003, 2007) and Drummond et al. (2006) provides more accurate estimates than some of the earlier methods. The relaxed clock method does not rely only on assumptions of nucleotide substitution rate, which differs between organisms, but allows specific dating of nodes. In this case, using the age of the fossil stoneflies found in this study gives a good calibration for dating.

A final estimate of phylogenetic relationships within the Plecoptera was obtained through Bayesian analyses using the program BEAST 1.4.8 (Bouckaert et al. 2014). The sequences were partitioned by gene, and by codon position (although codon position 1 and 2 were combined in H3 to allow for a low number of active sites), with a separate GTR + I + γ model of sequence evolution applied to each partition and a speciation: Yule Process tree prior. The analysis was run 10 times, each one requiring 50 million generations per run, before the separate runs were combined in LogCombiner (Bouckaert et al. 2014). Tracer 1.4 (Bouckaert et al. 2014) was used to monitor convergences within the phylogenetic model to ensure that all sample sizes were over 200, and to select an appropriate number of burn-in cycles. Finally, a maximum clade credibility tree was generated in TreeAnnotator (Bouckaert et al. 2014) and visualized in FigTree v1.4.2 (Rambaut 2006).

2.2.7. Molecular Clock Calibration

The molecular clock calibration dates were based on a number of insect fossils (Table 6), which unfortunately introduced potential uncertainties, such as inaccurate dating and equivocal identification of the fossils. For this reason, soft upper bounds were used, which allow for the possibility of much older ages, but are also associated with drastically reduced probabilities (see Sanders & Lee 2007, McCulloch et al. 2016). Featured among the calibrations is the oldest definitive evidence of a stem-relative Plecopteran, which is from the late Carboniferous, approximately 320 Ma (Béthoux et al. 2011)□. This takes the potential time frame much further back than previous molecular clocks of the stonefly phylogeny, which dated the oldest stonefly fossil to 250-300 Ma (Sinitshenkova 1997, Zwick 2000). For this reason, the maximum age constraint of the Plecoptera was recalculated, so that 95% of the prior probability density is bounded by the new constraints.

Table 6: Fossil calibration points used to constrain the molecular clock. Oldest fossil upper limit constrained by Béthoux et al., lower limit set according to fossil specimens identified in Chapter 3.

Calibration Point	Age (Ma)	Taxa	Reference
Oldest Fossil	320-268	*Gulou carpenteri*	Béthoux et al. 2011
Impression Fossil	165	Notonemouridae	Cui et al. 2019
Amber Fossil	16	*Aucklandobious gresseti*	Hoernle et al. 2006
Amber Fossil	38	Perlidae	Hagen 1863
Amber Fossil	16	*Zelandobius wardi*	McLellan 1993
Amber Fossil	7.5	*Rungaperla longicauda*	Hoernle et al. 2006

3. Taxonomy of Fossil Plecoptera

3.1. Aims:

The aims for this chapter are to:

- Describe and identify the three new plecopteran species found at the Onder Karoo locality, near Sutherland, Northern Cape, South Africa.
- Compare and discuss their significance in the context of the plecopteran fossil record.

3.2. Plecoptera within insect evolution

The Plecoptera represent a fascinating step in insect evolution, as they are a basal form of the Polyneoptera, an early, species-rich branch of the Neoptera (new winged insects). The Neoptera, which include all modern winged insect orders except for the Odonata and the Ephemeroptera, originated in the late Carboniferous (during the early Bashkirian Age, approximately 323.2 to 315.2 Ma) before undergoing intensive radiation and spreading into a large diversity of terrestrial and freshwater niches (Grimaldi & Engel 2005). Neoptera are characterized by a number of features, the most significant of which includes the presence of a wing flexion, which allows the insects to fold their wings horizontally over the abdomen when at rest and the formation of a median plate in the wing base (Grimaldi & Engel 2005, Beutel et al. 2013). Additionally, female Neoptera have paired so-called third valvulae, or gonopacs, in their ground plan, which serve as a sheath for the paired first and second valvulae. Together they form a characteristic ovipositor, which is secondarily lost in the Polyneoptera (Grimaldi & Engel 2005, Beutel et al. 2013).

Amongst the earliest representatives of the Neoptera in the fossil record are specimens classified in an extinct order, the Protoptera (Sharov 1966), which occurred from the mid- to late Carboniferous (Grimaldi & Engel 2005). This order consists solely of the ancient, basal family Paoliidae, which represents a stem group of the Neoptera (Grimaldi & Engel 2005, Rasnitsyn & Aristov 2016). These large insects are scarce in the fossil record, having been described from only a few, rarely found and isolated species across the world (Rasnitsyn 2002a, Rasnitsyn & Aristov 2016). They are characterized only by plesiomorphies for the Polyneoptera, and have been described solely on their wing venation, with little knowledge of their ontogeny (Rasnitsyn 2002a, Rasnitsyn & Aristov 2016). They were, however, able to fold their wings horizontally over the body, as in all Polyneoptera (Rasnitsyn 2002a). Recently, an alternative diagnosis for the Paoliidae has suggested that they may not represent the most basal Pterygota, instead forming a sister group to the Dictyoptera, an extant group which includes the Blattodea (cockroaches and termites) and Mantodea (praying mantids) (Prokop et al. 2014). This was suggested based on similarities in the wing venation between the groups, most notably in the presence of anterior concave branches of CuA (Prokop et al. 2014). Along with the Paoliidae, a number of stem Polyneoptera, the oldest of the modern Neoptera, are preserved from the late Carboniferous, including the earliest known plecopteran, along with a number of Palaeodictyoptera (Béthoux et al. 2011).

3.2.1. What defines Polyneoptera?

The Polyneoptera are a large, species-rich group, which includes approximately 40 000 described species within ten insect orders (Wipfler et al. 2019). These orders include a variety of diverse, well known groups such as the Orthoptera (grasshoppers and crickets), Plecoptera (stoneflies), Dermaptera (earwigs), Blattodea (termites and cockroaches), Mantodea (mantids) and Phasmatodea (stick insects); along with rare, species-poor groups such as the Grylloblattodea (ice-crawlers), Embioptera (web-spinners), Mantophasmatodea (heel-walkers) and Zoraptera (ground lice) (Beutel et al. 2013, Wipfler et al. 2019). The monophyly of the group has been contested for some time, but has been supported by recent morphological and molecular studies (Ishiwata et al. 2011, Yoshizawa 2011, Beutel et al. 2013, Misof et al. 2014). Several morphological apomorphies of the group support a monophyletic origin: an enlarged anal field in the hind wings (secondarily missing in

Zoraptera, Embioptera, and Isoptera, while the wings are absent in Grylloblattodea and Mantophasmatodea), the presence of euplantulae on the tarsi and a heart with excurrent ostia (Beutel et al. 2013). Additionally, Yoshizawa (2011) noted four modifications to the wing base, which could be considered autapomorphies. However, three of these traits show considerable variation or homoplasy within the group, with only a single clear apomorphy being identified, specifically elongation of the first antenodal cross vein, which is articulated with the notum (Yoshizawa 2011).

The Polyneoptera are diverse in appearance, diet, habitat, life history and social behaviours, and the evolution of this diversity within the Polyneoptera is still poorly understood. As such, the evolution of the group is considered to be one of the major unsolved issues in studies of insect evolution (Whitfield & Kjer 2008). This uncertainty of the evolutionary relationships within the Polyneoptera dates back to well before the advent of molecular systematics (Kristensen 1975, 1981). More recent molecular analyses were unable to reach consensus on the ordinal relationships of this group, as each study seems to contradict previous work (Whitfield & Kjer 2008 for an in depth discussion). However, recent work by Wipfler *et al*. (2019) has initiated the process of combining these phylogenies to create a more complete and reliable phylogeny of the Polyneoptera, focussing on the evolution of characters throughout the group, an element which has been largely neglected.

A number of long held beliefs exist regarding the phylogeny of the Polyneoptera, including the idea that Plecoptera represented the sister group to all insects with a wing flexion (Zwick 2000, 2009, Beutel & Gorb 2006). Wichard (1997) argued that aquatic nymphal stages in most primitive pterygote orders, along with the presence of homologous chloride cells in Ephemeroptera and Plecoptera, point to an aquatic lifestyle in the ground plan of Neoptera. Zwick (2009) consequently argued that accordingly the absence of such unique pluricellular chloride cells in all groups of Neoptera except for Plecoptera, would instead suggest the Plecoptera form the sister group to all other Neopterans. Similarly, an aquatic nymphal stage, as seen in the mayflies and dragonflies, was assumed in the ground plan of the Polyneoptera (Kukalová-Peck 1978, 1983, Thomas et al. 2000). However, recent studies suggest that this is not the case, and instead the Zoraptera and Dermaptera are thought to represent the earliest split of the Polyneoptera, followed by the Plecoptera (Wipfler et al. 2019). As neither Zoraptera or Dermaptera have aquatic nymphal stages, it appears that this feature was instead evolved independently within the stoneflies, and is an apomorphy of the Plecoptera (Wipfler et al. 2019), which contradicts the assumption of an aquatic nymph in the ground plan of all Pterygota as argued by Kukalová-Peck (1978, 1983) and Wichard (1997).

This finding poses the question as to whether or not ancient Plecoptera were likely to possess the filamentous, abdominal gills typical of the extant nymphs of the group (Štys & Soldán 1980, Zwick 1980, Shepard & Stewart 1983). If an aquatic nymph stage was included in the groundplan of the Plecoptera it would be highly likely that these gills would have been present, and that they would be analogous to the gills of the Ephemeroptera and Odonata. However, this appears to not be the case, as none of the early plecopteran fossils from the Permian are preserved with any evidence of gills, even though there is strong evidence these species were already aquatic (Sinitshenkova 1987, 2003, Gallego et al. 2011). Additionally, there are marked differences in the structure and functions of the gills of Plecoptera, Ephemeroptera and Odonata, once again suggesting independent evolution of these structures (Pritchard et al. 1993, Grimaldi & Engel 2005). Considering these factors, it seems likely that gills within the Plecoptera developed later, most likely as an adaptation for ion uptake and as an osmoregulation device (Shepard & Stewart 1983, Sroka & Staniczek 2020).

Finally, the Grylloblattida were thought by some to represent the stem ancestors of the Perlidea, a paraphyletic group that includes the Grylloblattodea, Plecoptera, Dermaptera and Embioptera (Rasnitsyn 2002b). However, there is little support for this idea and recent phylogenetic analyses support the division of the Pterygota into the Palaeoptera and Neoptera (Haas & Kukalová-Peck 2001, Hovmöller et al. 2002, Kjer et al. 2006, Misof et al. 2014). Additionally, the analysis of Wipfler *et al*. 2019, who suggested that Grylloblattodea branched off much later as the sister group of the Embioptera, did not support this relationship. Nevertheless, among the earliest winged insects and as the only Polyneoptera with an aquatic stage, Plecoptera represent a fascinating step in the evolution of insects.

3.2.2. Origin and evolution of the Plecoptera

Fossil plecopteran nymphs are difficult to identify with confidence, as most previous diagnoses have been based on the isolated wings of adults, with little information on the nymphal stages (Sinitshenkova 1987). A major contributor to this difficulty is the lack of any strong, easily identified autapomorphies, especially of plecopteran nymphs (Beutel et al. 2013). Many of the most reliable autapomorphies of the Plecoptera are internal, or limited to adults, such as anteriorly paired internal genital organs, which form a closed loop, or the presence of two pairs of loop-shaped seminal vesicles in males (Beutel et al. 2013). These features are rarely preserved in fossil specimens. The few apomorphies present in nymphs, such as the presence of specialized intersegmental muscles in the thorax and abdomen, are also generally not visible in fossilized specimens (Grimaldi & Engel 2005, Beutel et al. 2013). Interestingly, one of the most significant apomorphies for identifying plecopteran nymphs may be their habitat, as the stoneflies have an aquatic nymphal stage (Zwick 2000, 2009, Beutel & Gorb 2006). Although this feature is found among other insect orders, it has been independently derived each time (Wipfler et al. 2019), and is unique to the Plecoptera among the Polyneoptera (Zwick 2000, 2009, Grimaldi & Engel 2005, Beutel & Gorb 2006). The remaining orders with an aquatic nymphal stage, Odonata, Ephemeroptera, Trichoptera, and Megaloptera, fall within other broad divisions of the Class Insecta (Odonata and Ephemeroptera are Palaeoptera and Hemimetabola, while Trichoptera and Megaloptera are Holometabola; Grimaldi & Engel 2005, Beutel et al. 2013). The placement of the Palaeodictyopterida is more uncertain, and it has been suggested that they are likely the sister group of the Palaeoptera or of the Polyneoptera, as they lack a number of characters associated with the Polyneoptera (Prokop et al. 2019). Comparatively, Sroka et al. (2015) found that the Palaeodictyopterida was more likely to represent a sister group to the Neoptera using an extensive cladistic analysis. This analysis included stem group representatives from a number of palaeopterous insects, and strongly suggested that the Neopterygota needs to be redefined to include the monophyletic groupings of

Palaeodictyopterida + Neoptera, which in turn would invalidate the Palaeoptera as a paraphyletic grouping (Sroka et al. 2015). An aquatic lifestyle, however, is again difficult to identify in fossil plecopteran nymphs, as many families are lacking, or possess greatly reduced, gills and instead respire through the cuticle (Nagell & Larshammar 1981).

This lack of strong apomorphies has been limiting to the understanding of stem Plecoptera, especially in fossil remains from the late Carboniferous and through much of the Permian. As with many of the Polyneoptera, stem representatives of the Plecoptera date back to the late Carboniferous, with the earliest known species, *Gulou carpenteri* Béthoux et al. (2011), collected from the Tupo Formation (Pennsylvanian, China), and estimated to be approximately 320 million years old (Béthoux et al. 2011). This identification was made largely based on wing venation (distally branched CuA, presence of a hind wing arculus, and a ScP which reaches RA [Refer to materials and methods for definitions, page 23]), amongst others), which had a mixture of plesiomorphies and synapomorphies of the Plecoptera (Béthoux et al. 2011). However, the authors noted that a major complication in the ordinal assignment of *G. carpenteri* was uncertainty regarding the relationships of the Plecoptera and their stem relatives with a number of stem groups preserved throughout the Carboniferous and the Permian, viz. the Protoperlaria (Tillyard 1926), Paraplecoptera (Sharov 1962, 1991) and stem Grylloblattida (Storozhenko 1997, 2002). The Protoplecoptera and the Protoperlaria have both been theorized to represent the stem ancestors of the Plecoptera. However, both have since been shown to be paraphyletic, and have since been incorporated into the Grylloblattida with few revisions (Rasnitsyn 2002b).

Seven Paraplecoptera have been described from South Africa (Riek 1973, 1976a, 1976b), however, none of these species are likely to be plecopteran and instead share a number of features with the Grylloblattida. With the exception of a single species described from a nymph, *Thaumatophora pronatalis* (Riek 1976a), the remaining six South African species are all described from isolated wings (Riek 1973, 1976a, 1976b). While there are some features present in these wings that are found in both orders, such as the presence of an “arculus”, or strong cross vein (Rasnitsyn & Quicke 2002), the wings differ considerably in a number of other features. All of the wings have the distinctive character of a basally branched CuA, where CuA2 is simple, a common trait of the Grylloblattida (Rasnitsyn & Quicke 2002), but that is absent in the Plecoptera, which have a distally forked CuA. In addition, plecopteran wings are often defined by a broad region between the veins M and CuA, which is devoid of cross veins, whereas all eight South African species have a large number of well-developed cross veins. Several other features one would expect to see in the Plecoptera, such as ScP reaching RA, Ma simple, and CuA with few distal branches, were not present in any of the six species. While it is possible that these wings could represent basal, ancestral forms of the Plecoptera, this seems unlikely as many of the earliest stem Plecoptera, such as *Gulou carpenteri* Béthoux et al. 2011, *Palaeoperla exacta* Sharov 1962 and *Perlopsis filicornis* Martynov 1940 include the majority of these traits, and are considerably older than the South African fossils.

The single paraplecopteran species described from a nymph in South Africa, *Thaumatophora pronotalis* (Riek 1976a) is an oddity, as it shares many features with the Plecoptera, including several phenetic characters, and most importantly a tarsus with three tarsomeres, an apomorphy seen in the Plecoptera and modern Dermaptera (Grimaldi & Engel 2005, Beutel et al. 2013). Interestingly, this is not a feature seen in the Grylloblattida, which have the more ancestral five-segmented tarsi (Rasnitsyn & Quicke 2002). *Thaumatophora pronotalis* has abdominal gills present on abdominal segments I-VI, a feature that is only observed in a few families of the Plecoptera, such as the Eustheniidae and Pteronarcyidae (Riek 1976a). However, while these features suggest that the species could be plecopteran, *T. pronotalis* has large, prominent, prothoracic lobes preserved, a feature associated with the nymphs of a number of groups, including the Palaeodictyoptera and the Grylloblattida (Grimaldi & Engel 2005, Beutel et al. 2013), but not seen in Plecoptera. In addition, a darkened protrusion is observed on abdominal segment X of *T. pronatalis*, emerging between the two cerci that was interpreted as “an extrusion from the anus” (Riek 1976a) but which may also have been the remnants of a terminal filament, not present in the stoneflies. Taken together, these features make it unlikely that *T. pronotalis* is a stem-group plecopteran, as there is no evidence of any of these features being preserved within the stoneflies. If it were a stonefly, it would represent the earliest plecopteran from

South Africa, as it was found in the lower to middle Permian, Ecca Group deposits of the northern Karoo Basin (Belica et al. 2017).

3.2.3. Plecoptera fossils of South Africa

Within South Africa, eight species of Plecoptera from the Lopingian (upper Permian) and Upper Triassic have been identified (Tillyard 1935, Riek 1973, 1976b, 1976c, Van Dijk & Geertsema 2004). All of these have been described from isolated wings, based on key characters expressed in their wing venation, such as the fore wing with two rows of cross veins aligned to the CuA, and an often branching CuA, all of which are commonly seen in Plecoptera across the world (Sinitshenkova 1987).

Of these eight species, seven were originally described as Gripopterygidae (Tillyard 1935, Riek 1973, 1976b, 1976c), a modern extant family of Plecoptera found across the Southern Hemisphere, although not currently present in South Africa. However, while these species are likely to be Plecoptera, this familial assignment is more questionable. Fossil representatives of the Gripopterygidae are rare, with only these species and two from Argentina described worldwide (Sinitshenkova 1987). The Argentinian specimens were assigned to the Gripopterygidae entirely on the basis of their similarity to Riek's specimens (Pinto & Purper 1978). Additionally, the Gripopterygidae are an extant family, and it seems unlikely that the modern plecopteran orders have remained unchanged since the Permian. Further evidence for this is found in the current best estimate of divergence between the two plecopteran suborders, Antarctoperlaria and Arctoperlaria, within the early Mesozoic (Cui et al. 2019). Riek (1973), when assigning these species, did so with some trepidation, noticing considerable differences between the wings of the fossilized insects and those of the extant Gripopterygidae, such as a wide apical cell formed by the upturning of R and the presence of cross veins within the distal half of the wing. Indeed, these features were compared to those within the family Perlopsidae (Martynov 1940). This is a lower Permian family found within the Ural Mountains, but which was ignored based on a basally branched M, similar to the Gripopterygidae. However, once more species and representatives were found and these differences became clearer, they were reassigned to a new family, the Euxenoperlidae within the infraorder Gripopterygomorpha (Sinitshenkova 1987). Another South African species, identified from the uppermost Permian, is *Afroperla permiana* Van Dijk & Geertsema 2004. The wing is notably different from those described by Riek (1973, 1976b,c), lacking the wide apical cell between R and Rs and with a four branched CuA (Van Dijk & Geertsema 2004). However, there are distinct similarities in the wings to those of the *Palaeonemoura*, and based on these features the genus was placed within the Palaeonemouridae (Van Dijk & Geertsema 2004).

Aside from the representatives of the two families mentioned above, three Gondwanan species have been described, *Antitaxineura anomala* (Tillyard 1935)*, Perlapsocus formosoi* (Pinto et al. 2000)*,* and *Platyperla marquati* (Gallego et al. 2011). All three of these species have been assigned to different families, all of which are represented by only a single specimen. While both *A. anomala* and *P. formosoi* were described from wing remains, *P. marquati* was identified from nymphal remains. It is currently the only Gondwanan stonefly described exclusively from nymphal remains, which makes it particularly interesting for comparisons with the species described here.

The ongoing study of an insect Lagerstätte near Sutherland, South Africa has revealed an unprecedented diversity and abundance of middle Permian stonefly nymphs. Over 150 nymphal and wing remains were examined to date. Unfortunately, all of the potentially plecopteran wings discovered so far lack clear characters for reliable classification, and because of this no wings were considered in this study. The present study provides a strong contrast to previous work, as the new species described in Chapter 3 were all described from nymphal remains. Despite their lack of strong comparative features with the currently described South African species, they are still important to the understanding of the diversity and distribution of the Plecoptera. What follows is a taxonomic description of fourteen specimens that have been identified as three new species of basal Plecoptera.

3.3. Systematic Palaeontology

Superclass: **Hexapoda** Latreille, 1825

Class: **Insecta** Linneaus, 1758

Order: **Plecoptera** Latreille, 1810

Incertae sedis

Morphospecies 1. *sp. nov*

Diagnosis: Long and narrow body shape, approximately 10 times as long as wide. Distinct prothorax, with shield like appearance due to the presence of highly convex posterior and lateral margins. Abdomen 2.2 times as long as thorax. Cerci half the length of the body (5.1 mm; 1:2 ratio with body length) when complete. Antennae incomplete, but longer than 1 mm, at least 1:10 ratio with body, but likely much longer.

***Holotype*:** AM 12952 (Fig. 4, Fig.5)

Additional Material: AM 11268a & AM 11268b

Type Locality: Onder Karoo Locality. South-western Karoo Basin, Northern Cape, South Africa.

Type Stratum: Close to the contact between the Waterford Formation of the Ecca Group and the overlying Abrahaamskraal Formation of the Beaufort Group, Karoo Supergroup.

Age: Middle Permian (Wordian Age, approximately 268 Ma)

***Material**:* AM 12952. Incomplete, well preserved impression fossil of a nymph

Description:

Species with an elongated, slender body shape. 9.8 to 11.2 mm long, approximately 10 times as long as wide. Oval head 0.6 mm long and 1.03 mm wide, 1.5 times wider than long.

Mouth parts and labrum not visible, mouthpart orientation unclear. However, head held parallel to the body axis, which suggests prognathous mouthparts. Coronal suture present and well developed, with the frons and vertex clearly differentiated. Complex eyes and ocelli are not preserved in the examined specimens. Incomplete antennae long (1.29 mm, partial length due to incomplete preservation) suggesting long filiform antennae when complete. Large scapus (0.15 mm long), three times wider (0.21 mm) than the remainder of the filiform antenna. Scapus protruding anteriorly from the head capsule at a 45˚ angle to the body axis, remainder of the antenna extending perpendicular to the body axis.

Pro-, meso- and metathorax are long and slender. The prothorax has a highly convex posterior margin that extends into the lateral margins, which are also rounded. The anterior margin, however, is straight, giving the prothorax a shield-like appearance. The mesothorax is square in shape and is approximately as long (0.88 mm) as it is wide (0.86 mm). The metathorax is wider (0.93 mm) than it is long (0.64 mm), but maintains the slender shape of the mesothorax. Dorsomedial longitudinal structure is visible on all three thoracic segments. This may be the remains of a medial cuticular crest or a dorsomedial ecdysial line. Both right wing pads well preserved, but without any clear venation, while only partial left hind wing pad present, additionally without clear venation. The wing pads are held posteriolaterally to the body axis. The fore wing pad is long (2,4 mm), but slender (0,33 mm); the hind wing bud is much shorter (1,95 mm), but considerably wider (0,53 mm). This is further accentuated by a large bulge on the basal posterior margin of the wing, which suggests the presence of a large anal field, an important diagnostic feature of the Plecoptera. Legs not completely preserved, with only small regions of three legs visible (fore- and midleg on the left, midleg on the right). Preservation of the legs limited to femora, with no remnants of tibia or tarsi in any specimens. Represents thin, long walking legs, with no visible modifications towards specialized life histories.

The ten-segmented abdomen is long (6.3-7.5 mm), more than double the length of the thorax (2.8-3.7 mm). Each abdominal segment is twice as wide as long. Abdominal segment X with distinct and well preserved paraprocts. Medial, elongated carbonized structure throughout the abdomen and extending into the thorax, may be the remains of gut contents. No evidence of abdominal gills preserved. Left cercus incomplete, with only a small section poorly preserved. Right cercus long, measuring almost as long as the abdomen and almost double the length of the thorax (5.1 mm); consists of twenty cercomeres, which are short basally, before rapidly becoming elongated at cercomere five. No evidence of a terminal filament.

Measurements: **Body Length (excl. cerci and antennae):** 10.33 mm; **Head length:** 0.66 mm; **Thorax Length:** 2.82 mm; **Abdomen Length:** 6.31 mm

Full measurements, and measurements of other material available in Appendix 1.

Remarks: Morphospecies 1 is similar to the species of the genus *Platyperla*. It resembles *Platyperla platypoda* in its elongated body shape, along with the proportions of its thorax and abdomen, but has considerably narrower fore wing pads. Also has a distinctly shield-like prothorax, which is not present in the members of the *Platyperla*. Similar appearance to *Palaeonemoura* which has a rounded, shield like prothorax, along with long antennae (1:3 antenna to body ratio). Abdomen to thorax ratio longer in Morphospecies 1. Likely stem Plecoptera, as supported by large anal region on hind wing, and a combination of phenetic characters, such as two long, multi-segmented cerci, wing pad shape and orientation. Not placed within the two modern suborders, as present before their hypothesised divergence (McCulloch et al. 2016, Cui et al. 2019, Ding et al. 2019) and without any apomorphies of the suborders preserved. Large number of representatives appear to be exuviae.

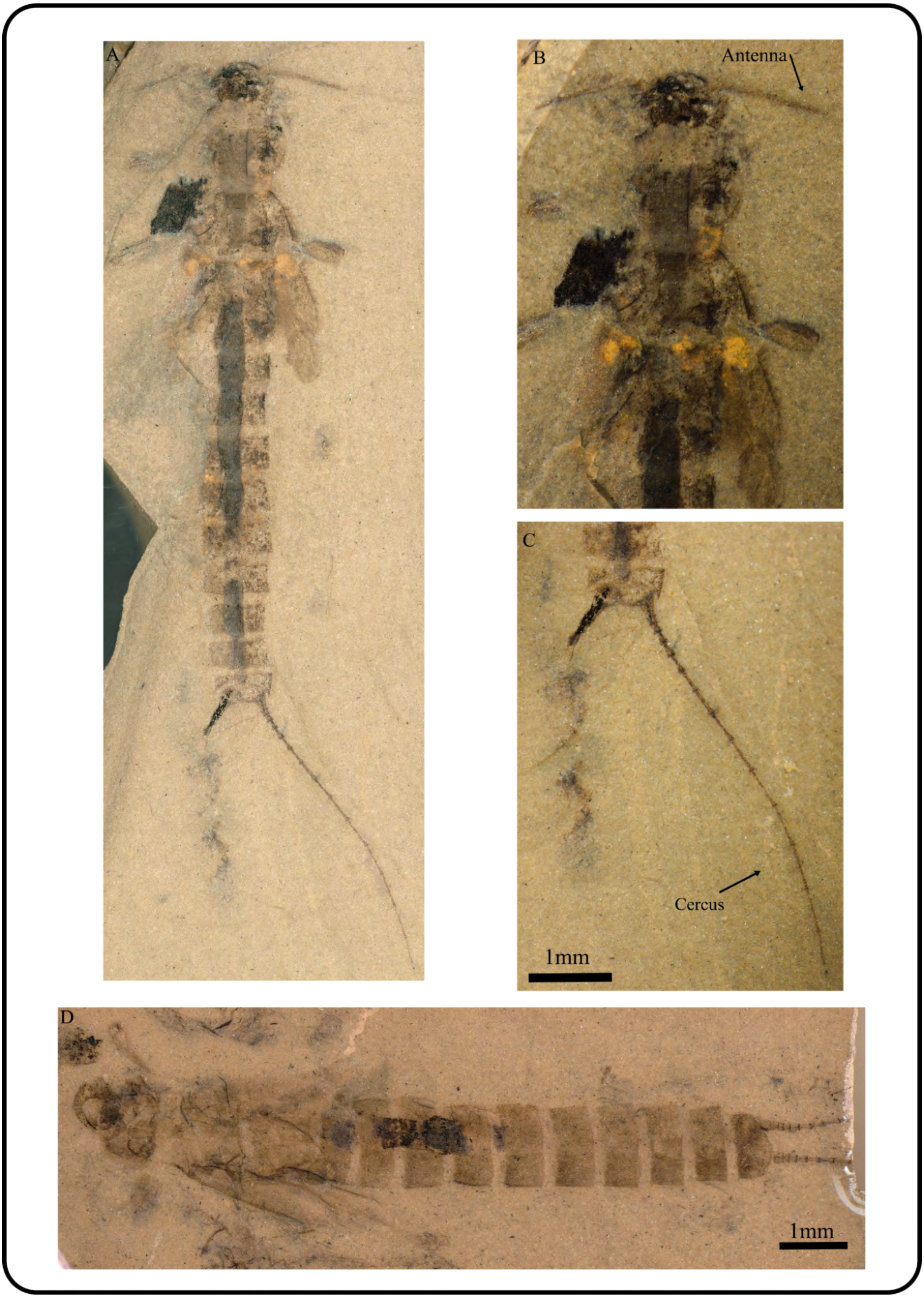

Figure 4: Morphospecies 1 sp. nov; A: AM 12952, holotype. Photograph of the specimen taken under alcohol. B: AM 12952, showing head and thorax, photograph taken under alcohol. C: AM 12952, Long right cercus. D: AM 12109b, abdomen and thorax of exuviae.

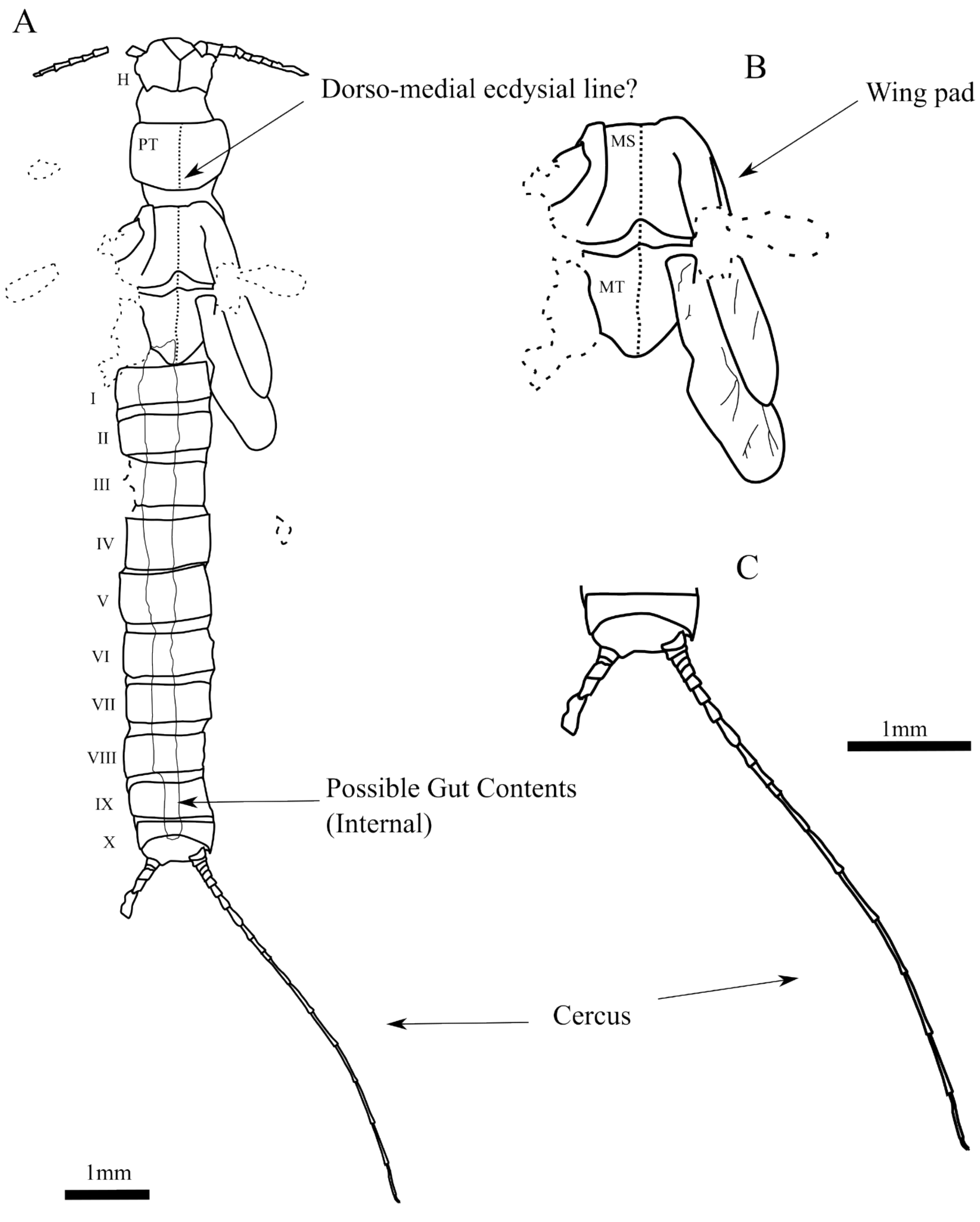

Figure 5: Morphospecies 1 sp. nov; A: AM 12952, holotype. B: AM 12952, showing wing pads with preserved venation. C: AM 12952, Long right cercus, 5.1 mm long. I-X = Abdominal segments 1-10, MT= Metathorax, MS= Mesothorax, PT= prothorax, H= head. Dotted lines represent incomplete or unclear regions.

Morphospecies 2 *sp. nov.*

Diagnosis: Elongated, slender nymph. Large scapus, more than double the length of the pedicellus. Prothorax rectangular, with small medial bulge on anterior margin. Meso- and metathorax short and square.

Holotype: AM11311. (Fig. 6, Fig. 7)

Paratype: AM13109a Specimen 1 (Fig. 6, Fig. 7)

Additional Material*:* AM 13214, AM 13109a, AM 13265a, AM 13265b

Type Locality: Onder Karoo Locality. South-western Karoo Basin, Northern Cape, South Africa.

Type Stratum: Close to the contact between the Waterford Formation of the Ecca Group and the overlying Abrahaamskraal Formation of the Beaufort Group, Karoo Supergroup.

Age: Middle Permian (Wordian Age, approximately 268 Ma)

Material*:* AM 11311. Incomplete, well preserved impression of a nymph

Description:

Nymph with an elongate body shape; 8.6-10.24 mm long, body 9 times as long as wide. Head rounded, slightly wider (1.1 mm) than long (0.9 mm). Mouth parts not visible, except for poorly preserved labrum. Head flat, in combination with posture of head and orientation of labrum suggest a prognatheous orientation. Coronal suture present and well developed. Vertex and frons clearly visible. Complex eyes and ocelli not discernible. Antennae incomplete, right antenna (0.69 mm, partial measurement as antenna not fully preserved) with 5 antennomeres and left antenna longer (1.47 mm, partial measurement as antenna not fully preserved) with unclear number of antennomeres. Scapus large, more than double the width of the pedicellus and the antennal flagellum. Although incomplete, antennae likely long.

Thorax short, ranging from 2.06 to 3.38 mm long. Pro-, meso- and metathorax distinctive and clearly separated. Rectangular prothorax; 1.8 times as wide (1.64 mm) as long (0.87 mm). The posterior margin of the prothorax convex, with a short, rounded protrusion medially. Anterior and lateral margins slightly convex, creating a shield-like appearance. Meso- and metathorax shorter and thicker than in Morphospecies 1, with a squatter body shape. Mesothorax short (0.85 mm long) and rectangular (1.38 mm wide), wider than long, while metathorax is square, as long as wide (1.03 mm long, 1.08 mm wide). Dorsomedial longitudinal structure is visible on the mesothorax and on the metathorax. May be the remains of a medial cuticular crest or a dorsomedial ecdysial line. In some specimens evidence of the exoskeleton splitting along this region is preserved (Fig. 7.b), on the basis of which this structure is interpreted as a dorsomedial ecdysial line, although not well developed or clearly defined. All four wing pads are visible and stained, darkened and with evidence of organic residue. May indicate thicker, more coriaceous wing pads compared to Morphospecies 1, although also present in Morphospecies 3. They are held posteriolaterally at a 45˚ angle from the body, rather than parallel to the body axis. The fore wing pad is long and thin (2.52 mm long and 0.45 mm wide), while the hind wing pad is shorter (2.26 mm long), but is approximately double the width of the fore wing (0.84 mm), mainly due to posterior expansion of the basal half. This suggests an enlarged hind wing in the adults, possibly due to an enlarged anal field. Legs are not preserved in holotype, but visible in paratype. Left and right hind legs visible, but incomplete. Left leg with femur, tibia and incomplete tarsus, while right leg with only femur. Legs long and slender. Left tarsus incomplete, with two long, thin tarsomeres, although unclear how many in complete tarsus. There is no evidence of thoracic gills.

The elongated abdomen (3.94-6.52 mm long) consists of 10 segments and is approximately double the length of the thorax. Each abdominal segment is twice as wide as long, and two paraprocts are visible on the tenth abdominal segment. All with fringe of setae on posterior margin. All abdominal segments rectangular, abdominal segment 10 with an enlarged posterior, medial bulge. This may be due to the presence of an epiproct, however this is not clearly preserved. Cerci not completely preserved in holotype, but incomplete cerci visible in paratype (Left: 1.26 mm, Right: 0.7-1.67 mm; partial length as cerci not preserved).

However, there is no evidence of a terminal filament and abdomen without lateral expansions, or evidence of abdominal gills.

Measurements: **Body Length (excl. cerci and antennae):** 10.24 mm; **Head length:** 0.97 mm; **Thorax Length:** 2.76 mm; **Abdomen Length:** 6.51 mm

Full measurements, and measurements of other material available in Appendix 1.

Remarks: Morphospecies 2 distinct from Morphospecies 1, as it lacks the strongly rounded posterior margin of the prothorax, but still with a medial bulge in most of the specimens. However, similarly reminiscent of both the *Platyperla* and the *Palaeonemoura*. Likely stem Plecoptera, and not placed within the two modern suborders, as predates their hypothesised divergence (McCulloch et al. 2016, Cui et al. 2019, Ding et al. 2019). Assigned to the Plecoptera based on a combination of features. Wing pads held posteriolaterally to the body, hind wing pads much wider than the forewing, suggesting an enlarged anal region commonly seen within the Plecoptera. Evidence of two long, multi-segmented cerci is preserved, with no terminal filament or abdominal gills. Large number of representatives appear to be exuviae.

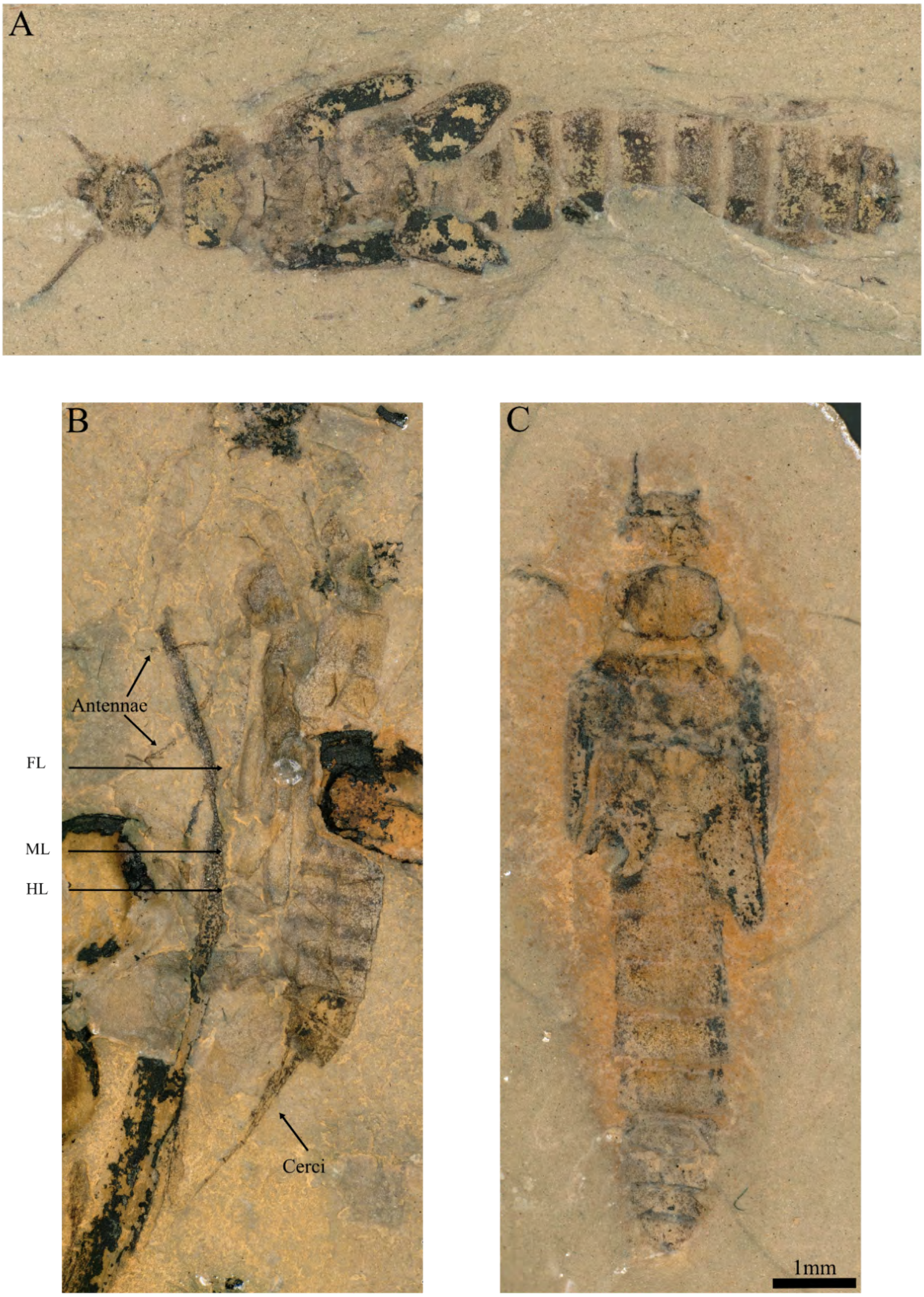

Figure 6: Morphospecies 2 sp. nov.; A: AM 11311, holotype. Photograph of the specimen taken under alcohol. B: AM 13109b Specimen 2. Lateral preservation of Morphospecies 2 exuvae. Thorax split through dorsomedial ecdysial line. C:AM 13265b. Photograph taken under alcohol. MT= Metathorax, MS= Mesothorax, PT= prothorax.

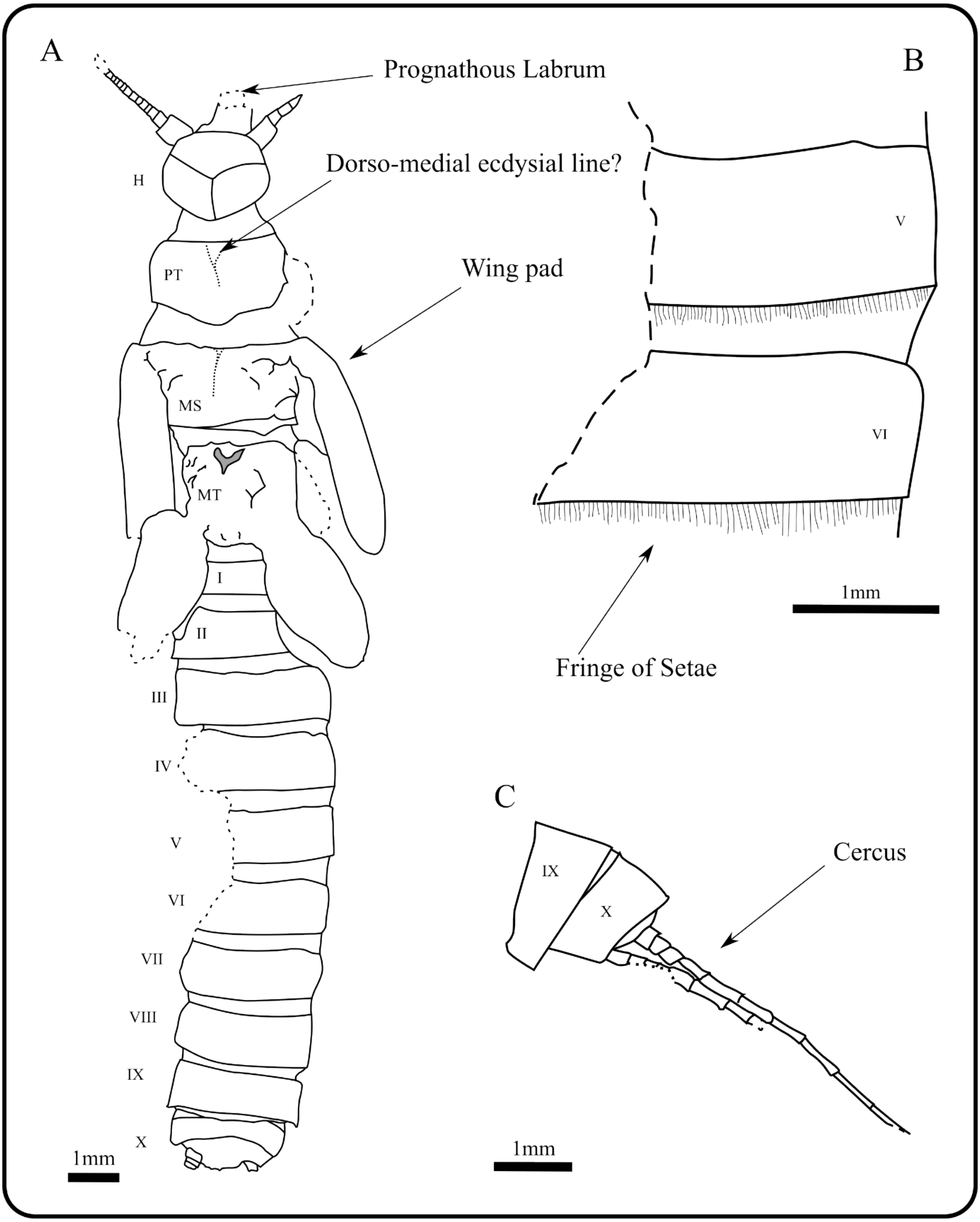

Figure 7: Morphospecies 2 sp. nov., A: AM 11311, holotype. B: AM 11311, Abdominal segments 5-6. Shows long fringe of cerci, observed on all ten abdominal segments. C: AM 13109b. Abdominal segments IX-X, with two incomplete filamentous cerci. I-X = Abdominal segments 1-10, MT= Metathorax, MS= Mesothorax, PT= Prothorax, H= head. Dotted lines represent incomplete or unclear regions.

Morphospecies 3 ***sp. nov.***

Diagnosis: Small size for Plecoptera, 8.71 mm long. Similar characters to Morphospecies 2, but with enlarged abdominal segments 7, 8 and 9, which are 3 times as wide as long, creating a distinctive bulge.

Holotype**:** Holotype AM 12497 (Fig. 8, Fig. 9, Fig. 10)

Type Locality: Onder Karoo Locality. South-western Karoo Basin, Northern Cape, South Africa.

Type Stratum: Close to the contact between the Waterford Formation of the Ecca Group and the overlying Abrahaamskraal Formation of the Beaufort Group, Karoo Supergroup.

Age: Middle Permian (Wordian Age, approximately 268 Ma)

Material*:* AM 12497. Incomplete, well preserved impression of a nymph

Description:

Nymph. Long, slender body shape, dorsoventrally compressed. 8.73 mm long, and 10 times as long as wide. Partial head capsule visible (0.42 mm long), poorly preserved, including left vertex and gena, with no additional features. Coronal suture not preserved, and no evidence of complex eyes or ocelli preserved. Antennae and mouthparts are not preserved, however, the orientation of the flat head suggests they are prognathous.

Elongated thorax well preserved, with distinct pro-, meso- and metathorax. Prothorax, with rounded, convex margins; lacking medial posterior bulge which is present in Morphospecies 2. Approximately as long (0,79 mm) as it is wide (0.77 mm), forming a square, slightly rounded shape. Meso- (1.01 mm long and 0.83 mm wide) and metathorax (0.95 mm long and 0.87 mm wide) with an oval shape; in both segments slightly longer than wide, but with rounded margins. Thicker and squatter than in Morphospecies 1, but more slender than those present in Morphospecies 2. Darkened wing pads are preserved, with left wing pads and the right hind wing pad complete. Right forewing is only partially preserved, predominately hidden within the rock matrix. Wing venation is not preserved in any of the wing pads. Wing pads are held posteriolaterally, at a 45˚ angle from the body and not parallel to the body axis. Fore wing is long (2.37 mm) and slender (0.42 mm), whilst the hind wing is shorter (1.92 – 1.99 mm), but considerably broader (0.62 – 0.76 mm). The hind wing is further basally enlarged, with a large bulge present posteriorly. This likely indicates an enlarged anal field. All three legs on the left of the specimen are present, however all of these legs are incomplete. Foreleg with coxa and femur preserved, midleg with tarsomeres preserved and hind leg unclear, but likely to be partial femur and tibia. Legs are long and slender, with an elongated femur that is less than double the width of the tibia in the hind leg. It is difficult to interpret the length of the femur as none of the legs are complete, but it would appear the preserved femora are of a similar length to the single preserved tibia (Femur: 0.97 – 1.11 mm, Tibia: 1.33 mm). The coxa of the foreleg is visible, and small. It is approximately the same length as the trochanter, and is slightly narrower than the femur. Pretarsus is simple and two-clawed. Tarsomeres are poorly preserved and borders between individual tarsomeres are not clearly visible. However, considering the length of the tarsus it is likely to have more than three tarsomeres. No euplantulae preserved on tarsomeres.

Long, ten-segmented abdomen, 1.7 times as long (0.56 mm) as thorax (3.18 mm). Abdomen simple, with no evidence of abdominal gills or leglets preserved. First 6 abdominal segments appear slightly dorsoventrally flattened, and are slightly damaged. Abdominal segments VII-IX distinctively enlarged and thickened, with segment 8 the largest, creating a prominent bulge near the posterior of the abdomen. This is found in a large number of specimens and appears to be a distinctive feature of Morphospecies 3, suggesting it is not simply a preservational artefact. Tergites and sternites sclerotized, but no evidence of sclerotization of pleurites in any of the abdominal segments. There is no evidence of lateral expansions or abdominal gills. The bases of two incomplete cerci are present (Left: 1 cercomere, Right: 3 cercomeres), and there is no evidence of a terminal filament. Long, incomplete cerci present in paratype, suggesting long cerci when complete (left: 1.84 mm, right: 3.67 mm; almost as long as abdomen). No epiproct or paraprocts visible.

In some cases multiple specimens found in small clusters, associated with *Glossopteris* seeds. Largest with 18 nymphs of Morphospecies 3 (Fig. 9). Likely to be stem Plecoptera, and not placed within the two modern suborders, as specimens predate the hypothesised divergence of these groups (McCulloch et al. 2016, Cui et al. 2019, Ding et al. 2019) and no apomorphies are apparent.

Measurements: **Body Length (excl. cerci and antennae):** 8.73 mm; **Head length:** 0.42 mm; **Thorax Length:** 3.19 mm; **Abdomen Length:** 5.56 mm

Full measurements, and measurements of other material available in Appendix 1.

Remarks: Morphospecies 3 with longer thorax (Thorax to Abdomen ratio 1:1.7) relative to Morphospecies 1 (1:2.03) and Morphospecies 2 (1:2), and slightly smaller than other stonefly species (1-2 mm shorter). However, with few complete specimens, most commonly without thorax and head perhaps due to moulting, may represent an earlier ontogenic stage. Similar to Dermaptera in presence of large bulge in posterior abdominal segments, but lacking any other apomorphies of the Dermaptera. Long cerci and antennae relative to body size present in some specimens, almost as long as abdomen. Relationship with *Glossopteris* seeds likely to be taphonomic. Although the association is common at the Onder Karoo locality, many nymphs are preserved isolated, or in clusters without seeds present. Large number of representatives appear to be exuviae.

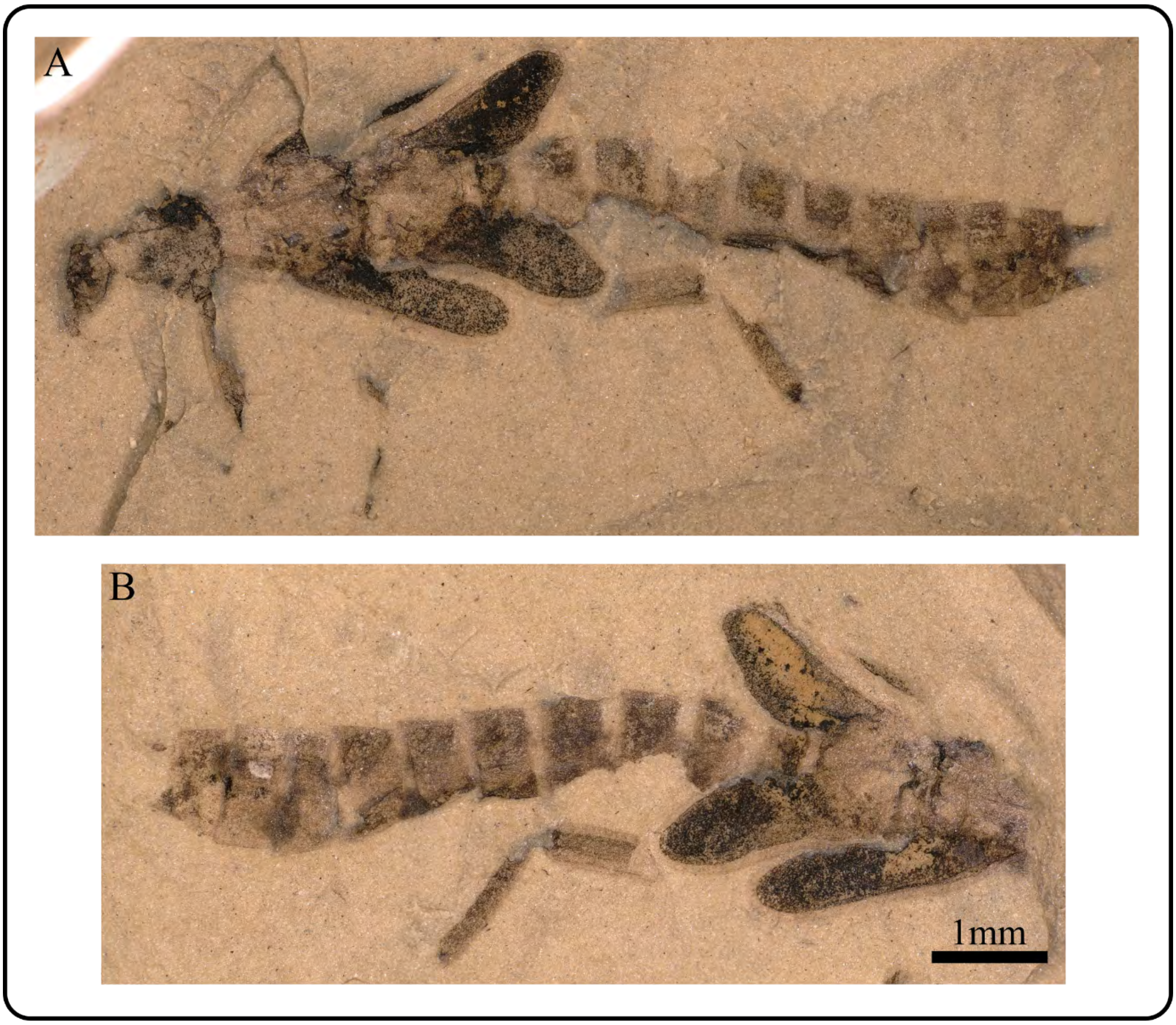

Figure 8: Morphospecies 3 sp. nov. A: AM 12497, holotype. Photograph of the specimen taken under alcohol. B: AM 12498. Counterpart of AM 12497. Photograph of the specimen taken under alcohol

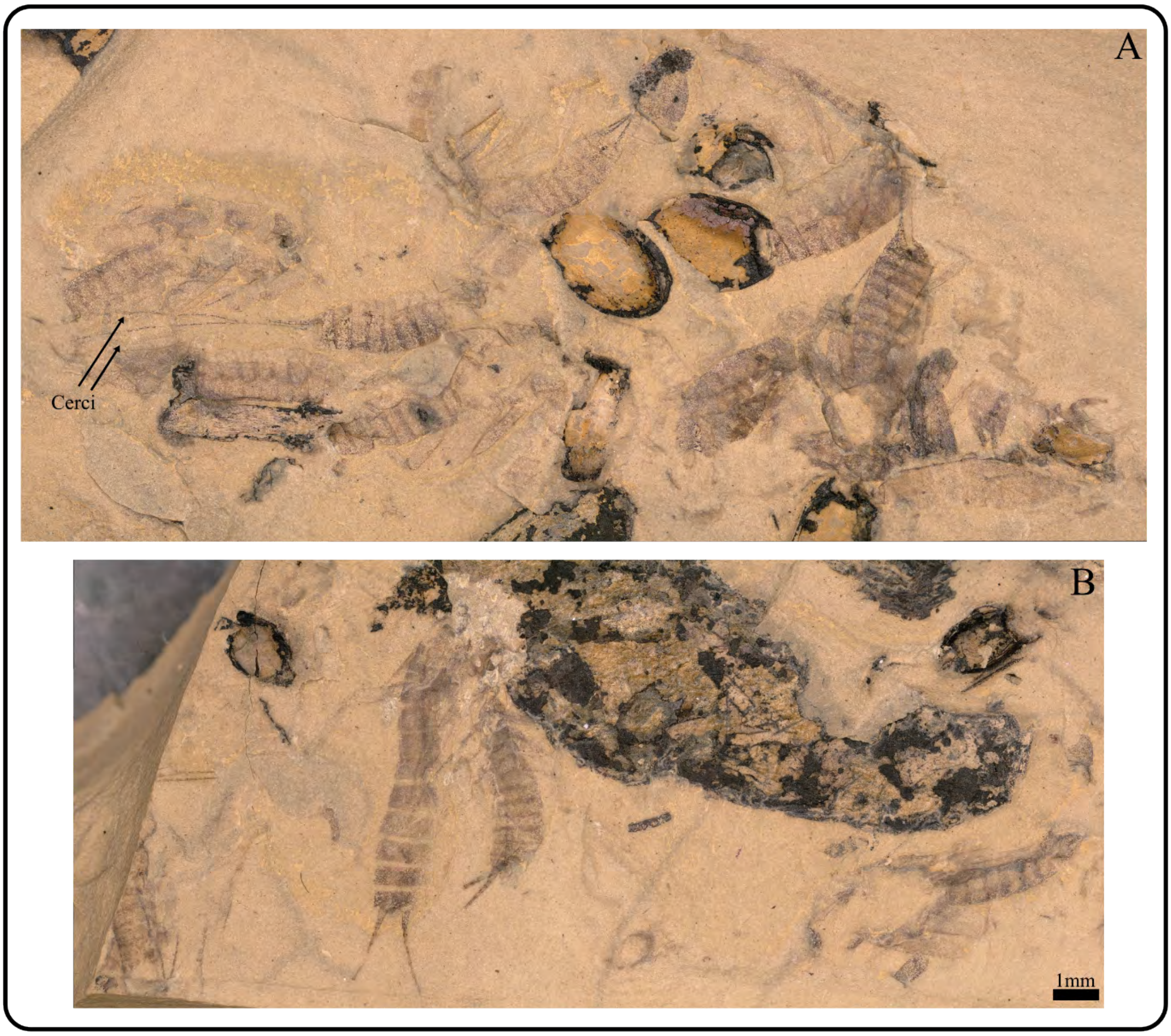

Figure 9: Morphospecies 3 sp. nov.; A: AM 11348a. Cluster of 18 nymphs scattered over 5 slabs, including specimens with legs, cerci and antennae. Photograph of the specimen taken under alcohol. B: AM 11348b. Counterpart of AM 11348a. Photograph of the specimen taken under alcohol

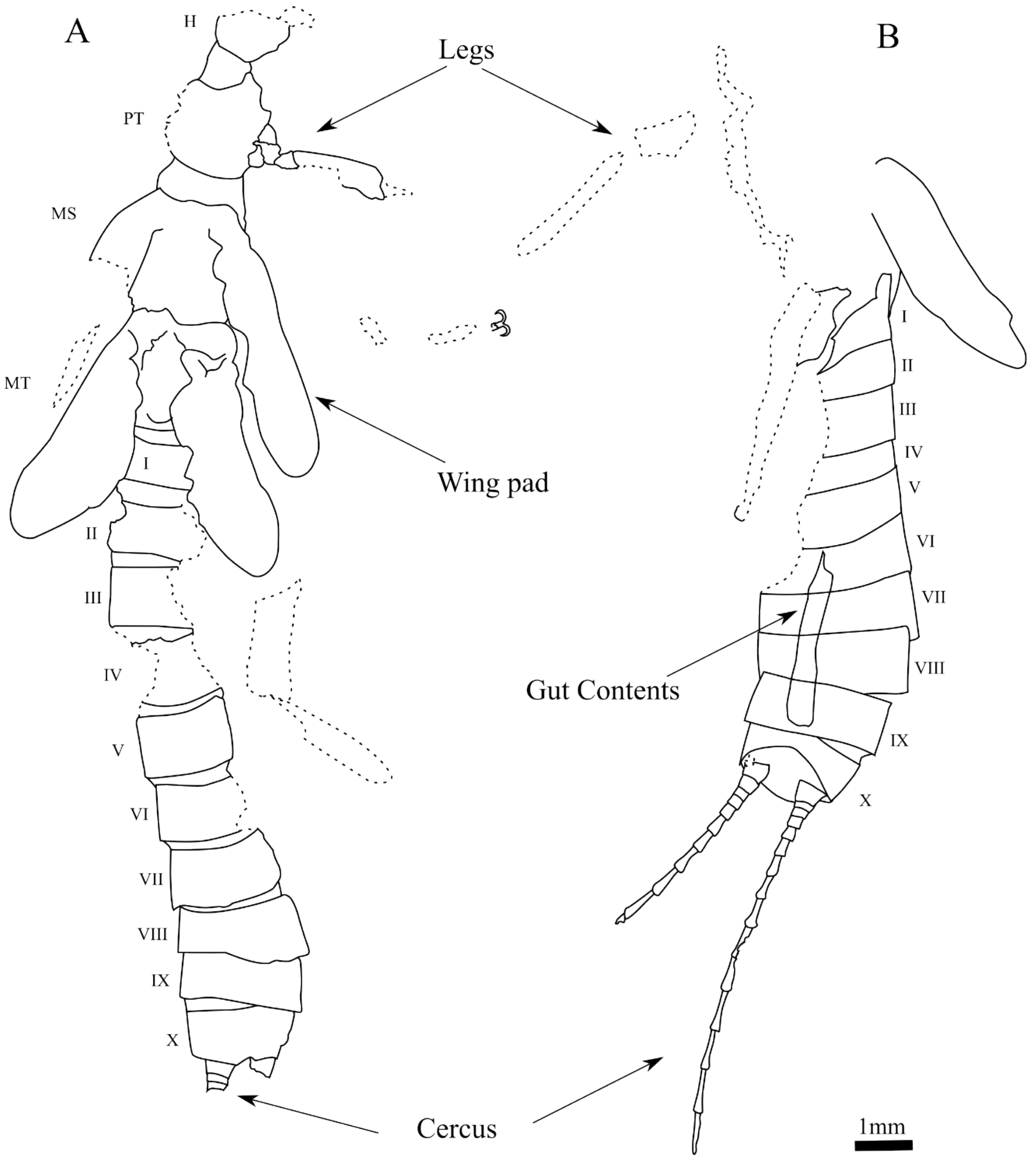

Figure 10: Morphospecies 3 sp. nov. A: AM 12497, holotype**.** B: AM 11458. With long cerci and possible gut contents preserved. I-X = Abdominal segments 1-10, MT= Metathorax, MS= Mesothorax, PT= Prothorax, H= head. Dotted lines represent in

3.4. Discussion

Morphospecies 1 *sp. nov***.***,* Morphospecies 2 *sp. nov***.** and Morphospecies 3 *sp. nov.* are here assigned to the stem group of the Plecoptera, making them the oldest stoneflies in South Africa. Previously, plecopteran fossils have been found exclusively in the upper Permian and Triassic deposits of South Africa (Tillyard 1935, Riek 1973, 1976b, 1976c).

Despite the large abundance of relatively well-preserved specimens the assignment of these species was a difficult task. The placement of early insects from the late Carboniferous and the Permian is notoriously difficult. The earliest winged insects, including the Paoliida, and the remaining Pterygota, underwent a period of rapid diversification throughout the late Carboniferous and early Permian, with many of the ancient insect groups appearing over a short interval (Gorochov 2001). This rapid early evolution of the Polyneoptera has made the interpretation of relationships between these groups difficult, and only recently has the phylogeny of these insects become clearer (Wipfler et al. 2019). In addition, many of the early Polyneoptera are similar in appearance, a problem heightened by the fact that they have few synapomorphies, many of which are hard to identify and are often not preserved.

All Polyneoptera have unspecialized biting mouthparts, a state which was present in the ground plan of the group and has not been secondarily modified since (Wipfler et al. 2019). However, the ancestral orientation of the mouthparts is orthognathous (mouthparts held beneath head capsule), and prognathy (mouthparts held anteriorly) has evolved independently at least four times within the group (Wipfler et al. 2019). Additionally the Polyneoptera are defined by relatively few apomorphies (wings with enlarged anal field, presence of euplantulae, small pads on the tarsi, heart with excurrent ostia, and modifications of the wing joint, discussed above), but can show considerable variation in these features. The anal field has been secondarily lost in five of the ten orders, as it was reduced in Zoraptera, Embioptera and Isoptera (termites, today also classified as superfamily Termitoidae within Blattodea), while the Grylloblattodea and Mantophasmatodea have secondarily lost their wings altogether (Grimaldi & Engel 2005, Beutel et al. 2013). Additionally, the anal field is heavily modified in the Plecoptera, which do not have the “fan-like” folding pattern associated with this region and that is observed among the remaining Polyneoptera (Grimaldi & Engel 2005, Beutel et al. 2013). The remaining apomorphies of the group are often not visible in fossil species, either as they are internal (excurrent ostia) or because they are associated with small, fragile sections of the body which are often poorly preserved, such as the tarsi (euplantulae). This is particularly unfortunate, as in several cases modifications of these characters can be important diagnostically, such as the secondary loss of euplantulae in the Plecoptera or the modification of the euplantulae and first tarsomere into silk glands observed in the Embioptera (Beutel et al. 2013).

The nymphs have been placed within the Polyneoptera on the basis of a number of features, including the presence of the external wing pads, held posteriolaterally to the body, that were visible in all of the specimens. These wing buds clearly show that the species had a hemimetabolous life history, which is characteristic for the Polyneoptera (Beutel et al. 2013).

In addition, it seems unlikely that these species represent Odonata (dragonflies) or Ephemeroptera (mayflies). The wing pads all have a posterior bulge on the hind wing which suggests an enlarged anal region (Fig. 2, Chapter 2, pg 24), a feature that is consistently absent in the Ephemeroptera, Odonata and Palaeodictyoptera, and which has been secondarily lost amongst a number of the Polyneoptera (Hennig 1969, Kukalová-Peck 1991, Blanke et al. 2012, 2012, Beutel et al. 2013). Morphospecies 1, 2 and 3 all have long, filamentous antennae, features that are reduced in both mayflies and dragonflies (Beutel et al. 2013). Comparatively, the Palaeodictyoptera have commonly been found to have long, filamentous cerci and antennae similar to the Plecoptera and the species described here (Carpenter 1992, Grimaldi & Engel 2005). However, there are some notable differences between Morphospecies 1, 2 and 3 and the Palaeodictyoptera. Notably, none of the specimens provided any evidence of a terminal filament (interpreted as an ovipositor by Prokop et al. 2019) on the tenth abdominal segment, which is commonly visible in the

Palaeodictyoptera (Carpenter 1992, Grimaldi et al. 2005, Prokop et al. 2019). Additionally, there was no evidence of prothoracic lobes, or “winglets”, which are small membranous structures emerging from the prothorax that are superficially similar to wings. These are generally present in both the adult and nymphal forms of the Palaeodictyoptera (Carpenter 1992, Grimaldi & Engel 2005). Finally, the nymphs lack the abdominal gills, lateral expansions of the first seven abdominal segments and terminal filament commonly found amongst the Permian mayflies and some Palaeodicytoptera (Carpenter 1992, Kluge & Sinitshenkova 2002, Grimaldi & Engel 2005, Sinitshenkova et al. 2015), suggesting that it is highly unlikely these species represent any of the palaeopteran groups.

While the species described here can be placed amongst the early Polyneoptera, based on the features discussed above, the ordinal assignment of Morphospecies 1, Morphospecies 2 and Morphospecies 3 proved quite difficult. The final ordinal assignment relies heavily on phenetic characters, as many of the reliable synapomorphies of the Plecoptera are not visible in nymphal stages. Alternative affiliations of the nymphs with the stem Grylloblattida, or the Dermaptera have been considered, although these options are less likely, as discussed below in more detail.

The first major stumbling block for the placement of these species was the possibility of their affiliation with the Grylloblattida Walker, 1914 (also referred to as Protoperlaria and Paraplecoptera). Modern, extant Grylloblattodea are a rare group of wingless, terrestrial insects, which are adapted to survival in severely cold environments (Beutel et al. 2013). However, many species have been described from the Permian based on wing venation, amongst several other traits, as it was theorised that the stem representatives of the group were still winged, only secondarily losing this feature at a later date (Storozhenko 2002). While extant Grylloblattodea are a monophyletic group (Beutel et al. 2013), it has been suggested that they may be members of the extinct order Gryoblattida (Storozhenko 2002). However, the order Grylloblattida is difficult to describe as it lacks any strong synapomorphies, especially in the nymphal stages, and is theorized to represent a basal, stem group which eventually gave rise to all Plecoptera, Dermaptera, Embioptera and

Grylloblattodea (Storozhenko 1997, 2002). This assumption has also led to the inclusion of two paraphyletic groups, Sharov’s Paraplecoptera (Sharov 1962, 1991), and Tillyard’s Protoperlaria (Tillyard 1926). However, the link between the Plecoptera and the Grylloblattida is tenuous at best, as there are no synapomorphies amongst the nymphs, or indeed in the adult wing venation to strongly suggest the inclusion of stem Plecoptera amongst the Grylloblattida (see Béthoux et al. 2011 for a full discussion of this topic). Additionally, recent re-examinations of the phylogeny of the Polyneoptera strongly suggest that Grylloblattida is a polyphyletic group, as all of the orders theorized to be included within the group are from different lineages within Polyneoptera (Misof et al. 2014, Wipfler et al. 2019).

None of Morphospecies 1, 2 and 3 should be assigned to the Grylloblattida, as they are lacking prothoracic lobes, or “wing flaps” which have been described in the majority of Grylloblattida, among both adults and nymphs, and on the basis of which Tillyard originally described the Protoperlaria (Tillyard 1926, Carpenter 1935, 1992). However, as with many of these plesiomorphies, their absence alone is not enough to unequivocally state that these species are not Grylloblattida, instead providing two options. If the possession of these prothoracic lobes is a plesiomorphy common to a number of the stem lineages of the Polyneoptera, including the Grylloblattida and the Palaeodictyoptera (which have been found at the Onder Karoo Locality with prothoracic lobes present; Prevec et al. in preparation), the absence of these features in the newly described nymphs reduces the likelihood of affiliation with the Grylloblattida. If, however, the presence of these lobes is a synapomorphy of the Grylloblattida it is possible that the species described here simply represent a stem, basal form of the Grylloblattida. However, there are no other features preserved which suggests this is the case, and indeed the balance of features supports an affiliation with the Plecoptera.

Plecoptera are an order with many plesiomorphic characters retained, and relatively few changes to their body plan since they first evolved (Zhiltzova 2010). Comparisons have been made here with a number of Permian Plecoptera, all of which have been identified based on their shared derived characters to modern, extant species, and including specimens from South Africa and the rest of the world. Amongst the South African fossil Plecoptera there are few nymphs, with all but one having been described from isolated wings, or adults with wings (Tillyard 1935, Riek 1956, 1976a, 1976b, Van Dijk & Geertsema 2004). This made comparisons with South African species difficult as these descriptions largely relied on wing venation, with few comments on characters which would be present on nymphs. A single species, *Thaumatophora pronotalis* was described from the lower Permian as a paraplecopteran (Riek 1976a). The species is markedly different from the Onder Karroo nymphs, as it has gills present on the first six abdominal segments, as well as prothoracic lobes. Once again this suggests that the lack of these prothoracic lobes precludes placement of Morphospecies 1, 2 and 3 within the Grylloblattida, as the prothoracic lobes are already present in older, more basal forms.

There are marked similarities between the new nymphs and a number of Plecoptera species described from the Permian. These are largely phenetic characters, but the long, multi-attenulated antennae, long cerci with multiple, small cercomeres, the presence of paraprocts, and a distinct, well developed, shield like prothorax, prognathous mouthpart orientation and a well-developed meso- and metathorax, with smaller, narrow wing pads are strongly reminiscent of modern Plecoptera. These features are also reminiscent of those in a number of other nymphal forms of Plecoptera from the Permian and younger deposits, such as *Platyperla marquati* (Gallego et al. 2011), *Ecdyoperla fairlightensis* (Sinitshenkova 1998), *Issadoperla permiana* (Aristov et al. 2013)*,* amongst many others (Sinitshenkova 1987, 1990, 1992, Nam 2017), all of which were described as Plecoptera based on one or all of these characteristics.

Another common apomorphy which is used in the identification of stoneflies is the presence of a three-segmented tarsus (Grimaldi & Engel 2005, Zwick 2009). Unfortunately none of the new specimens has well preserved tarsi, making it unclear whether or not this apomorphy is present, although in those where partial tarsi are preserved it seems unlikely that they are long enough when complete to be five-segmented, which is the plesiomorphic character state commonly found in the Grylloblattida and other Polyneoptera (Storozhenko 2002).

This reliance on phenetic characters however, did present a serious alternative for the placement of Morphospecies 1, 2 and 3. Many of these features are similar to those found in the Dermaptera, and phenetically these species appear superficially similar to a number of basal representatives of this group recently uncovered from mid-Cretaceous amber deposits in Myanmar (Ren et al. 2018). A number of these earwigs had many of the features seen in the Onder Karoo nymphs, including long antennae, and in some cases within the dermapteran families Diplatyidae and Karschiellidae, elongated and multi-segmented cerci, which is rare among the Dermaptera (Ren et al. 2018). In addition, Morphospecies 3 has a distinctive bulge on abdominal segments VII, VIII and IX, a feature commonly found in Dermaptera, especially amongst the Forficulina, although this feature may be due to a dilation of the tenth abdominal segment (Haas 1995). However, upon closer inspection it remains more likely that Morphospecies 1, 2 and 3 are Plecoptera and not stem Dermaptera, as none of the autopomorphies of the Dermaptera are preserved. There is no evidence of the forewings being modified as sclerotized tegmina as, while many of the specimens were preserved with staining and carbonised residue on the wing pads which may suggest a sclerotized structure, these stains were found on both the fore- and hind wings. Additionally, the hindwing pads do not have the characteristic fan shape, with large fold lines expected of the Dermaptera, and all of the cerci are long and multisegmented with a large number of cercomeres, rather than modified into pincer like, single segmented cerci (Beutel et al. 2013). Although the new species could represent a highly basal form of Dermaptera, there is little evidence to suggest that this is in fact the case. While the Diplatyidae and Karschiellidae have longer, multi segmented cerci, they are robust and thickened consisting of few cercomeres, generally only one or two (Ren et al. 2018). This is not the case in the Onder Karoo specimens, which tend to have long cerci with upwards of 20 cercomeres preserved, many of which are shortened. There is also no evidence of the wing modifications typical of the Dermaptera. For this reason, it seems more likely that Morphospecies 1, 2 and 3 are instead Plecoptera.

A final apomorphy of the stoneflies is the presence of an aquatic nymphal stage (Grimaldi & Engel 2005). Unfortunately, despite the large number of Plecoptera identified from the Permian, many do not demonstrate any obvious visible modifications directly attributable to an aquatic life style, such as gills, and their aquatic nature is largely inferred through comparisons with the extant stoneflies (Sinitshenkova 1987, 2003). This is similarly true of Morphospecies 1, 2 and 3*,* all of which lack any preserved gills. However, all three share features with *Barathronympha victima* (Sinitshenkova 1987), which demonstrates features typical of organisms adapted to fast running, rhithral streams. This includes a slender, streamlined body, a flattened and elongated femora and widely spread cerci, all features which are present in the three new species described here. For this reason, it seems likely that all three of these species were in fact aquatic, living within the waters of the Onder Karoo locality delta floodplain, which would also go some distance to explaining the abnormally large proportion of nymphs preserved at the Onder Karoo locality in subaqueous deposits. Taphonomically, the probability of such large numbers of nymphs being preserved in close association as an allochthonous contribution to the fine grained deposit (indicative of calm water environments) is very slim, and the nymphs were most likely living in the same setting in which they are so abundantly preserved. However, the calm, lake setting of the Onder Karoo locality does not initially appear to match the hypothesised adaptations to fast flowing water observed in Morphospecies 1, 2 and 3. All of the species are represented by a high number of exuviae. This suggests the organisms may have lived in the lake distributaries, and their exuviae were washed into the calm lake setting where they were buried. Further evidence is provided by their close association with seed clusters, which indicate some kind of winnowing effect and concentration of the remains by gentle water currents within the lake (see Gedney & Lick 1972, Bennett 1974, Witten & Thomas 1976, Simons & Schertzer 1987 for similar examples). These currents could have been caused by the emptying of the distributaries into the lake, or by wind.

4. Inferences regarding the ecology of the Plecoptera at the Onder Karoo locality

4.1. Aims

The aims for this chapter are to:

- Infer possible relationships between the Plecoptera and other organisms found at the Onder Karoo locality, using knowledge of modern stoneflies and freshwater ecosystems as an analogue.

4.2. Inferred ecological interactions at the Onder Karoo locality

4.2.1. Autumnal Infall

Feeding behaviour in plecopteran nymphs ranges from specialist carnivores, scrapers and shredders, to detritivory, with the majority of species feeding on detritus such as decaying wood and leaves (Hynes 1976, Miyasaka & Genkai-Kato 2009). Interestingly, the life cycles of Plecoptera appear to be closely tied to their diets, as most species are uni-voltine and rely on the sudden influx of nutrients associated with autumnal leaf-fall for the nymphs to undergo rapid growth and development into adults (Stewart & Stark 2002).

This is particularly fascinating in the context of the Onder Karoo locality, as the vegetation that contributed the plant fossil component of the assemblage was dominated by the deciduous *Glossopteris* tree (Prevec et al. in preparation). Within the fossiliferous horizon, dense mats of *Glossopteris* leaves have been found, which were likely deposited as a result of seasonal leaf-fall. These autumnal leaf deposits would have provided an abundant food source for invertebrates, and it is likely that the plecopteran nymphs would have utilized this resource, perhaps undergoing periods of rapid growth and moulting in response. This may explain, at least in part, the dense concentrations of plecopteran exuviae that have been found at the Onder Karoo locality, frequently in association with *Glossopteris* leaf mats.

4.2.2. Aquatic lifestyle

While all three of the plecopteran species described here lacked any obvious modifications to an aquatic life style, such as gills, they likely had an aquatic nymphal stage, based on their similarities with the Russian stonefly fossils *Barathronympha victima* (Sinitshenkova 1987), hypothesised to have been adapted to fast flowing rithral zones of rivers (based on the streamlined body, widely spread cerci and flattened/widened femora). Extant Plecoptera generally prefer fast running, well oxygenated rivers and streams (Hynes 1976, Robinson et al. 1992, Fochetti & de Figueroa 2008), and although there are some lacustrine species, these are rare (Hynes 1976, Robinson et al. 1992, Fochetti & de Figueroa 2008).

However, the fine grained lithologies and sedimentology at the Onder Karoo locality, that has yielded such a staggering diversity and abundance of fossil plecopteran nymphs, are indicative of a calm, lacustrine environment in a delta plain setting (Prevec et al. in preparation). One possible explanation for this is that the input of fresh oxygenated water from small distributaries flowing into the lake (as evident through several fine siltstone channels within the fossiliferous horizon) could have introduced oxygen and nutrients into the system, creating a diversity of aquatic niches near the lake margin (Wagener et al. 1998). Another possibility is that the plecopteran fossils are parautochthonous. Considering that the majority of plecopteran fossils found are exuviae, it is possible that the stoneflies lived and reproduced further upstream, in the distributaries of the Onder Karoo lake environment, and that their exuviae were washed into the calm lake setting where they were buried and preserved (pers. comm. R. Prevec; H. Barber-James, Department of Freshwater Invertebrates, Albany Museum).

Whether these stoneflies inhabited the distal parts of the distributaries or the margins of the lake itself, the abundance of plecopteran fossils at the Onder Karoo locality does suggest an aquatic lifestyle among these middle Permian representatives. The question as to whether the aquatic life style of stoneflies is an apomorphy of the group or present in the ground plan of all Polyneoptera and Plecoptera is a hotly debated topic (Grimaldi & Engel 2005, Zwick 2009, Wipfler et al. 2019). In either case, the fossils described here, along with several other Permian Plecoptera (Sinitshenkova 1987, 2003) suggest that this trait was evolved and present in this group by at least the Wordian Age.

4.2.3. Mite Fossils

The Plecoptera, along with many aquatic insects, have been identified as important hosts for water mites (Smith & Oliver 1986, Yasick et al. 2003). Although these parasites most commonly targeted Diptera larvae, there are strong associations between water mites and stoneflies (Mullen & Vercammen-Grandjean 1980, Yasick et al. 2003). Indeed, the mite superfamily Stygothrombioidea has been recorded as exclusively parasitizing the stoneflies (Mullen & Vercammen-Grandjean 1980). While there is no direct evidence of mite attachment to the plecopteran nymphs in the fossils found, the Onder Karoo locality has revealed the earliest water mite records in the world, in a high abundance (Prevec et al. in preparation). It is entirely possible that the larvae of the water mites may have parasitized Plecoptera found at the site, or some of the other aquatic insects that have been identified, such as the Palaeodictyopterida. Generally, water mites seek out the larval stages of the Plecoptera, and attach to the nymphs, as well as the adults as they emerge (Mullen & Vercammen-Grandjean 1980), although these mites appear to have a minimal effect on Plecoptera survival or fitness (Yasick et al. 2003). As the majority of plecopterans identified at the Onder Karoo locality are exuviae, the absence of attached water mites is perhaps unsurprising. It is likely that the mites would have transferred to the next instar during moulting. Considering this, it is still possible that evidence of water mite parasitism may be found on plecopterans identified from the locality in the future.

4.2.4. Hemiptera fossils

As the Onder Karoo locality is still relatively new and actively being researched and assessed, accurate and detailed entomofauna compositions do not yet exist. However, preliminary investigations have suggested that amongst the insects fossils preserved at the Onder Karoo locality were a high number of Hemiptera, including Cicadamorphs. Comparatively, there have been no Blattodea found to date, which are often more dominant in Permian sites, and have even been used in Permian biostratigraphy (Schneider & Werneburg 2006).

While about half of the known extant species of Plecoptera do not feed as adults, many of them are able to drink water (Hynes 1976). In some cases, this drinking behaviour has developed into a relationship with Hemiptera, as some species are able to consume the honeydew produced by the feeding of Hemipterans (Hynes 1976). It is possible that the adults of the species described here may have shown similar behaviour during the Permian. Additionally, some evidence of feeding scars from piercing and sucking mouthparts have been found on leaves at the Onder Karoo locality.

4.2.5. Service Providers

A large proportion of extant Plecoptera nymphs are shredders, and the feeding of these species has been shown to increase the amount of fine particulate matter (FPOM) in river systems (Vannote et al. 1980). In turn, this allows for more invertebrate and vertebrate species to survive in the river systems, as many rely on the FPOM to feed (Vannote et al. 1980). While none of the species described here were preserved with well-developed and clear mouthparts, it is likely that the ancient stoneflies fulfilled a similar role in the Onder Karoo locality lake and distributaries, as it has been noted that many ancient Plecopterans fed in similar ways to their extant ancestors (Sinitshenkova 1997).

5. Phylogeny of the Plecoptera

5.1. Aims:

The aims for this chapter are to:

- Identify common patterns and divergences between the suborders and families of the Order Plecoptera using a phylogeny including mitochondrial and nuclear genes from three Plecopteran lineages.
- Investigate the biogeography and evolutionary processes of the Order Plecoptera using a combination of molecular and fossil evidence.

5.2. Introduction

5.2.1. Relationships within the Class Insecta

Since the Plecoptera were first described in 1592, they have proven an enigmatic group, as it was difficult to identify what distinguished the group from other insects, and where in the evolution of insects the group fell (Zwick 2000, 2009). As discussed in Chapter 3, the biggest hurdle was identifying morphological characters which could be used to define the group, as the order has largely been described from a combination of primitive characters (Boudreaux 1979, Hennig 1981, Zwick 2000). While the monophyly of the group has been supported by several morphological apomorphies, such as the presence of three tarsal segments and the presence of a closed, anterior seminal vesical loop (Zwick 2000, Grimaldi & Engel 2005, Beutel et al. 2013), their relationships with other insects has remained much more problematic (Zwick 2000, 2009).

Several theories regarding the higher placement of the stoneflies have been suggested, and the confusion regarding the relationships of the Plecoptera has been further heightened by the repeated failure to identify a sister group for them, a problem which has remained prevalent to this day (Zwick 2009, Ding et al. 2019). Hennig (1969, 1981) suggested that the Plecoptera most likely represent a sister group to all other Neoptera, including the Polyneoptera and the Holometabola. Zwick (1973, 2000, 2009) agreed with this assessment, citing a number of features including the lack of many synapomorpies of the Neoptera, which would have to all have been secondarily lost in the stoneflies, and the aquatic lifestyle of the nymphs (and morphological modifications for this), a feature which is shared by the Ephemeroptera and Odonata (Zwick 2009) (refer to Chapter 3 for more in depth discussion of this). However, several other authors have argued that the Plecoptera are more likely to represent a sister group of the Polyneoptera (Kristensen 1991, Grimaldi & Engel 2005). Grimaldi *et al*. (2005) argue this on the basis of several apomorphies of the Polyneoptera, such as an enlarged anal region on the wings along with sperm and ovarian ultrastructure, which are also present within the Plecoptera.

While morphological studies of the group have struggled to find consensus on the relationships of the Plecoptera, an increase in molecular studies have started to resolve some of the issues. The majority of these studies have supported the Plecoptera as a sister group to the Dermaptera within the monophyletic Polyneoptera (Misof et al. 2007, Ma et al. 2014, Song et al. 2016). Unfortunately, many of these have relied on few representatives of the Plecoptera, especially of the Antarctoperlaria (Ding et al. 2019). However, more recent molecular phylogenies, starting with a comprehensive transcriptomics analysis of Insecta as a whole, support an alternative phylogeny (Misof et al. 2014). These analyses have suggested that the Plecoptera form an early divergence within the Polyneoptera, as an intermediate group between the earlier branch of Dermaptera and Zoraptera, and the later divergence of the remaining Polyneoptera (Misof et al. 2014, Wipfler et al. 2019). This interpretation of the Plecoptera within the Polyneoptera is already well supported by a combination of molecular (using proteins and nucleotides) and morphological methods (Misof et al. 2014, Wipfler et al. 2019).

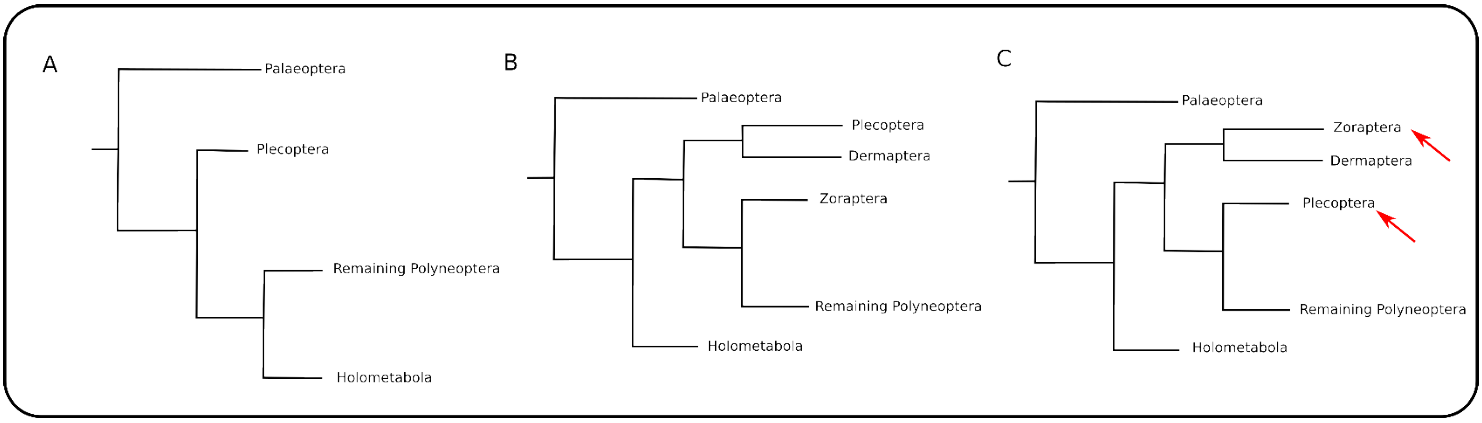

Figure 11: A: Interpretation of the Plecoptera as intermediate group between Palaeoptera and the remaining insects, suggested by Hennig (1969, 1981) and Zwick (1973, 2000, 2003), based on morphological evidence; B: Interpretation of the Plecoptera as sister group to the Dermaptera, suggested by Misof et al. (2007) Ma et al. (2014) and Song et al. (2016), based on genetic evidence. C: Interpretation of Plecoptera as an intermediate group between Dermaptera + Zoraptera and the remaining Polyneoptera, suggested by Misof et al. (2014) and Wipfler et al. (2019), based on a combination of genetic and morphological evidence.

5.2.2. Phylogenetic Relationships within Plecoptera

In addition to the difficulties in identifying ordinal relationships of the Plecoptera, the evolutionary patterns within the order have proven to be equally controversial. Stoneflies are divided into two geographically distinct suborders, the Antarctoperlaria which is distributed across the Southern Hemisphere (excluding Africa and Antarctica) and the Arctoperlaria, which is mostly distributed across the Northern Hemisphere, but is also found in Africa. These two groups have an unusual anti-tropical distribution, with the majority of the almost 4000 species (Fochetti & de Figueroa 2008, DeWalt & Ower 2019) found at higher latitudes (Zwick 2000). A similar distribution has been seen in the sparse fossil record of the group (Illies 1965, Sinitshenkova 1987), however this pattern may be due to the incompleteness of the fossil record (Zwick 2000). Based on this, the two suborders are generally theorized to have formed during the end Triassic as a result of vicariance caused by the breakup of the super continent Pangaea (Illies 1965, Banarescu 1990, Zwick 2000).

The Antarctoperlaria is distributed across the Southern Hemisphere, and all four of the extant families are found throughout Australia, New Zealand, South America and several subantarctic islands (Illies 1965, Zwick 2000, Fochetti & de Figueroa 2008, DeWalt & Ower

2019). This distribution has generally been attributed to vicariance caused by the breakup of Gondwana (Illies 1965, Zwick 2000, Fochetti & de Figueroa 2008), however some recent work has suggested that this divergence occurred at least 20 million years later (McCulloch et al. 2016). This suggests that stoneflies have undergone several dispersal events between continents and islands across the Southern Hemisphere, most likely via strong winds surrounding the continent of Antarctica (McCulloch et al. 2016). As Plecoptera are notoriously poor dispersers in both their nymphal and adult stages, being inefficient flyers and swimmers, this hypothesis is questionable (Brundin 1967, Zwick 2000, Fochetti & de Figueroa 2008). Thus, a high degree of endemism is seen within Plecoptera, with only one arctoperlarian genus present on more than a single continent (*Nemoura*) (Fochetti & de Figueroa 2008), and a high degree of spatial variation present even in individual species (Stevens et al. 2018). Regardless of the cause of their distribution patterns, the Antarctoperlaria are absent from the Afrotropical region (Fochetti & de Figueroa 2008, DeWalt & Ower 2019). This is generally ascribed to periods of climate change causing large scale extinctions, which in turn opened the way for dispersal from the Northern Hemisphere of the Arctoperlaria (Banarescu 1990, Resh & Cardé 2003).

The Arctoperlaria have an interesting distribution (refer to DeWalt et al. 2019 for detailed distribution maps), as the majority of the families within the suborder are found exclusively in the Northern Hemisphere, and only two (Notonemouridae and Perlidae) are found in the Southern Hemisphere (Fochetti & de Figueroa 2008, McCulloch et al. 2016). Both of these families most likely spread into the Southern Hemisphere through recent independent invasions through Africa (Illies 1965, Stark & Gaufin 1976, Zwick 2000, Fochetti & de Figueroa 2008). The Perlidae are found in Southern Africa, Central America and across the northern hemisphere, making it the only family that are found in both hemispheres (Fochetti & de Figueroa 2008). The Notonemouridae, despite being within the Arctoperlaria, are found exclusively in the Southern Hemisphere, across all continents apart from Antarctica, and with a distribution reminiscent of the Antarctoperlaria, but including Africa and Madagascar (Fochetti & de Figueroa 2008). This “Gondwanan” distribution could be explained by vicariance, if the Notonemouridae had already spread into Gondwana before the supercontinent split (Illies 1965, Zwick 1981, 1990, Cui et al. 2019), or could be explained by dispersal via Antarctica, perhaps using the strong winds which surround the continent (Illies 1965, Zwick 1981, 1990, McCulloch et al. 2016). Additionally, the monophyly of the Notonemouridae has repeatedly been questioned, suggesting that the group may have instead been formed through multiple independent dispersal events from the north (Zwick 1981, 1990, 2000, McLellan 2000, Fochetti & de Figueroa 2008). In contrast, recent molecular studies of the group show evidence of a monophyletic origin of the group, suggesting that a single dispersal event is much more likely (McCulloch et al. 2016, Ding et al. 2019).

5.2.3. Knowledge gaps

Research on Plecoptera phylogeny has increased within the last two decades (Misof et al. 2014, McCulloch et al. 2016, Wipfler et al. 2019, Ding et al. 2019). Prior to this, inferences had to be made through studies on other related orders (Zwick 2000, 2009). Unfortunately, most of these studies continued to examine the Plecoptera in the context of other insect orders, with few considering the actual relationships within the Plecoptera (Ding et al. 2019). This means that many of these studies have shared low taxon numbers, especially among the Antarctoperlaria, and molecular phylogenetic studies on Plecoptera generally have not included all three lineages, the Systellognatha, Euholognatha and the Antarctoperlaria (Zwick 2009, Ding et al. 2019). This lack of knowledge regarding the relationships of Plecoptera as a whole is detrimental to our understanding of the evolutionary development of the group, as it is unclear whether many of the families are monophyletic, and whether their distribution is due to vicariance associated with the fragmentation of Gondwana and Pangaea, or due to long distance dispersal (Zwick 2000, McCulloch et al. 2016).

5.3. Results

Detailed below are the results of seven different tests of phylogeny, conducted using two genes, one mitochondrial and one nuclear, viz. Cytochrome Oxidase Subunit 1 (COI) and

Histone 3 (H3) respectively. For full details on the methodology used in obtaining these results please refer to Chapter 2.

5.3.1. Maximum Likelihood

Three maximum likelihood phylogenies were created here, a concatenated test which included both the COI gene and the H3 gene (Fig. 12), one with the COI gene only (Fig. 13), and finally, one with the H3 gene only (Fig. 14).

The concatenated tree (Fig. 12) revealed three dominant lineages between the suborders and the families of the Plecoptera. The earliest branch of the tree consisted of a monophyletic Notonemouridae, which formed a sister group to all remaining Plecoptera (bootstrap replicates = 284, <700). The remaining Plecoptera then split into two further clades, which broadly matched the divide between the two suborders, the Arctoperlaria (bootstrap replicates = 251, <700) and the Antarctoperlaria (bootstrap replicates = 390, <700). However, while the divides between the suborders and families of the Plecoptera were not significantly supported, strong support was found for species level relationships throughout the phylogeny. The monophyly of all families was shown, except for the Austroperlidae, which was found to appear within the Gripopterygidae. Additionally, the monophyly of all genera was supported, and all of the lineages were found to broadly match their current distributions (bootstrap replicates > 700).

The same patterns seen in the concatenated tree were also observed in the COI gene phylogeny (Fig. 13). Once again, three dominant lineages were seen, with the Notonemouridae emerging as a monophyletic sister group to the remaining Plecoptera (bootstrap replicates = 185, <700), which further split into two lineages broadly matching the Antarctoperlaria (bootstrap replicates = 46, <700) and the Arctoperlaria (bootstrap replicates = 171, <700). While the majority of the families were found to be monophyletic, the Perlidae was found to be polyphyletic, with two distinct lineages forming around the

Nemouridae. One of these lineages included only West Palaearctic representatives of the family (bootstrap replicates = 813, >700), while the other included both Afrotropical and West Palaearctic Perlidae (bootstrap replicates = 97, <700). While the divides between the different suborders and families of the group were poorly supported, strong bootstrap values were found for many of the relationships between species and genera (bootstrap replicates > 700). The monophyly of all genera were supported, and all of these lineages broadly matched their current distribution.

The H3 gene phylogeny (Fig. 14) did not show the same lineages identified in both the COI gene and concatenated phylogenies. The monophyly of the Antarctoperlaria was shown (bootstrap replicates = 292, <700), although not significantly supported. With the exception of the Austroperlidae, which appeared nestled within the Gripopterygidae, the monophyly of all families were supported (Gripopterygidae: bootstrap replicates = 301, <700, Diamphipnoidae: bootstrap replicates = 1000, >700, Eustheniidae: bootstrap replicates = 1000, >700). A monophyletic Notonemouridae was not supported, as the family showed a distinctive “step-like” pattern, consisting of several poorly resolved nodes. The monophyly of all genera were significantly supported (bootstrap replicates >700), including the monophyly of the Notonemouridae. Additionally, these lineages broadly matched the current distribution of the Plecoptera, with all species from a country forming monophyletic lineages.

All three maximum likelihood phylogenies (COI only, H3 only, COI and H3 combined) had high support and resolution for species level relationships within the Plecoptera (Figs. 12, 13 and 14). In all three trees strong support was uncovered for the species-level relationships within the Antarctoperlaria, and all of the genera were found to be monophyletic, with the lineages broadly matching their current distributions (Bootstrap replicates >70%, Fig. 12, 13 and 14). Both the COI only and the concatenated tree found the same pattern in the Notonemouridae, in which strong support was found for all Notonemouridae genera to be monophyletic, and these lineages broadly matched current global distributions (Figs. 12, 13 and 14).

Maximum likelihood analyses of the phylogeny of the Plecoptera only provided weak support for the relationships between the plecopteran species at family level. Both the COI specific and the concatenated gene revealed two dominant lineages, which did not fully correspond to the two suborders of the Plecoptera, the Antarctoperlaria and the Arctoperlaria.

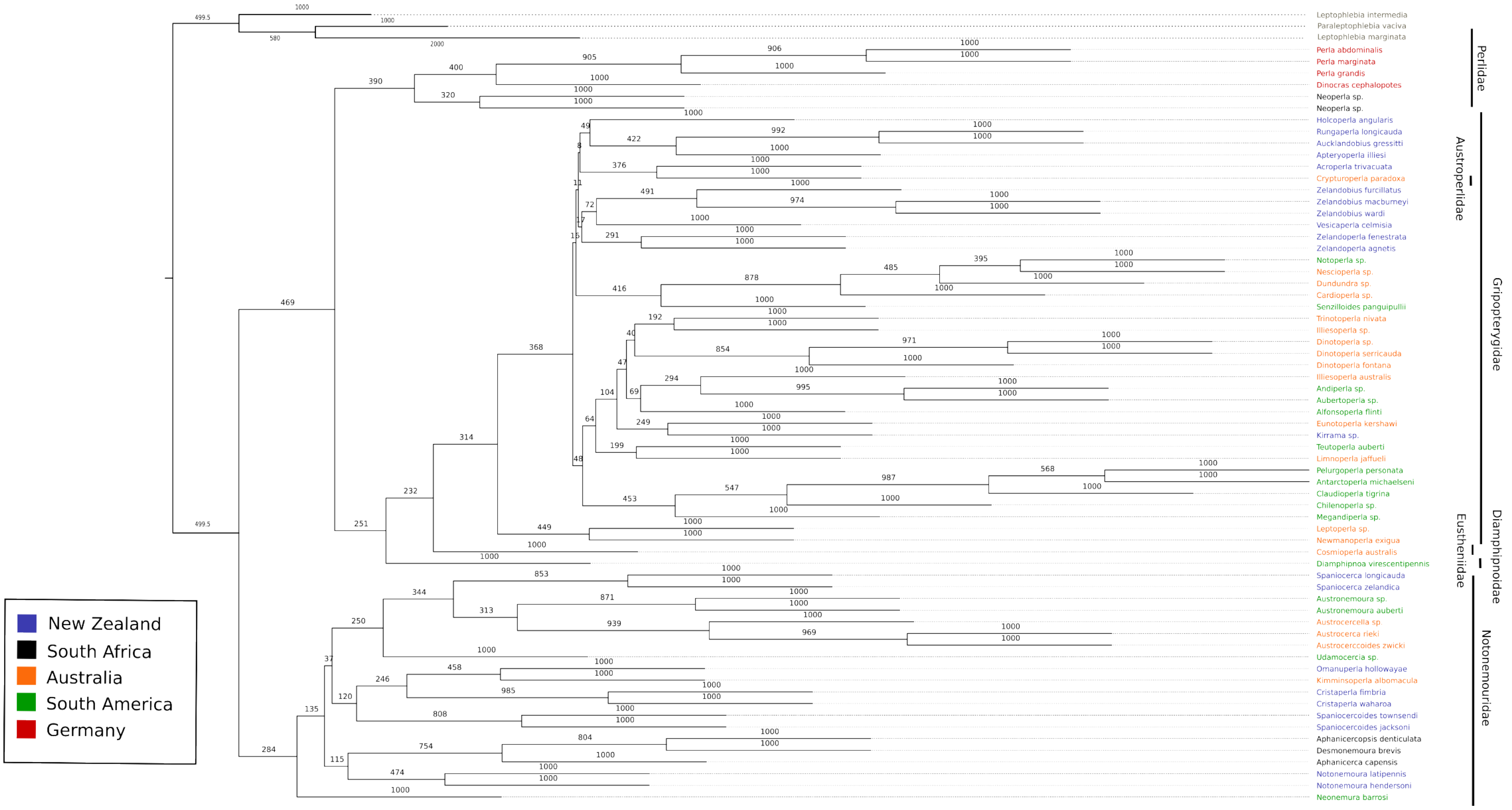

Figure 12: Concatenated Maximum likelihood phylogeny of the Plecoptera constructed using a combination of Cytochrome Oxidase Subunit I (COI) and Histone 3 (H3) genes. Numbers on each branch represent bootstrap replications, and are significant when >700. Species are coloured according to country of origin, and families are represented by black bars on the right side of the tree.

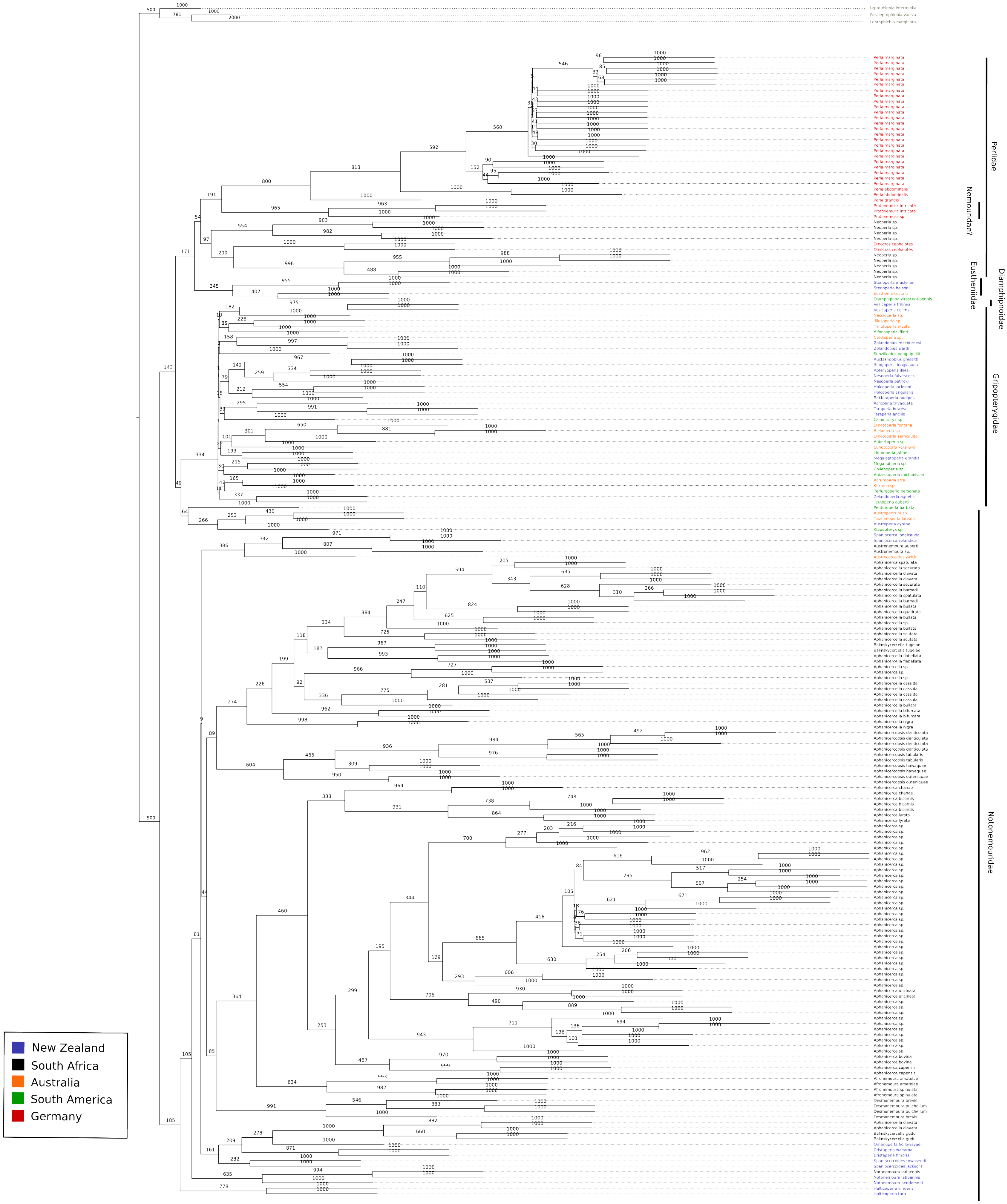

Figure 13: Maximum Likelihood phylogeny of the Plecoptera constructed using Cytochrome Oxidase Subunit 1 (COI) genes. Numbers on each branch represent bootstrap replicate values, and are significant when >700. Species are coloured according to country of origin, and families are represented by black bars on the right side of the tree.

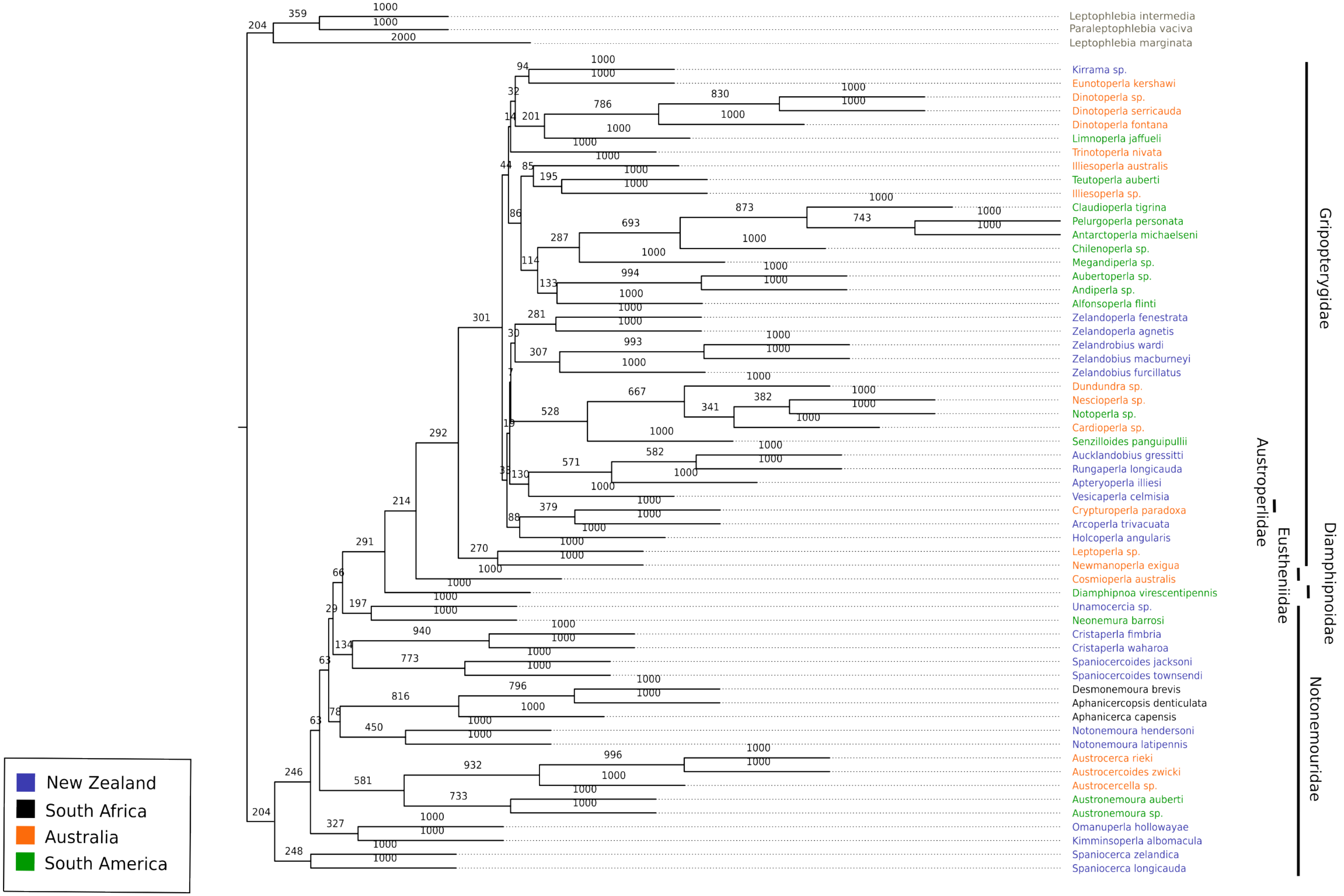

Figure 14: Maximum likelihood phylogeny of the Plecoptera constructed using Histone 3 (H3) genes. Numbers on each branch represent bootstrap replication values, and are significant when >700. Species are coloured according to country of origin, and families are represented by black bars on the right side of the tree.

5.3.2. Bayesian Interference

Three Bayesian interference phylogenies were created here, a concatenated test with both the COI gene and the H3 gene combined (Fig. 15), one with the COI gene only (Fig. 16), and one with the H3 gene only (Fig. 17).

As with the maximum likelihood concatenated tree, the concatenated Bayesian phylogeny (Fig. 15) revealed three dominant lineages between the suborders and the families of the Plecoptera. The first branch of the tree consisted of a monophyletic Notonemouridae, which formed a well-supported sister group to all of the remaining Plecoptera (posterior probability = 1, >0.95). The remaining Plecoptera then split into two further clades, which broadly matched the divides between the two suborders, the Antarctoperlaria (posterior probability = 0.73, < 0.95) and the Arctoperlaria (posterior probability = 0.66, < 0.95). With the exception of the early branch of the Notonemouridae, none of the family and sub-ordinal relationships was significantly supported in the concatenated tree. The monophyly of all families was shown, except for the Austroperlidae which was found to appear among the Gripopterygidae, similar to what was observed in the maximum likelihood analyses (Gripopterygidae: posterior probability = 0.66, < 0.95, Diamphipnoidae: posterior probability = 1, > 0.95, Eustheniidae: posterior probability = 1, > 0.95, Perlidae: posterior probability = 0.66, < 0.95, Notonemouridae: posterior probability = 1, > 0.95). Additionally, strong support was shown for the monophyly of all genera included within the phylogeny (posterior probability > 0.95). However, while relationships within genera were well supported, the relationships between genera were generally marked by low resolution and formed “comb-like” structures, where multiple clades originate from a single node with no clear evolutionary patterns shown.

For the most part, the same patterns seen in the concatenated tree were also observed in the COI gene Bayesian phylogeny (Fig. 16). Once again, the Notonemouridae emerged as a monophyletic sister group to the remaining Plecoptera (posterior probability = 1, > 0.95). However, unlike the concatenated tree and the maximum likelihood phylogeny, the remaining Plecoptera did not divide into the two suborders, with most families instead forming individual, monophyletic and well supported clades (Gripopterygidae: posterior probability = 0.83, < 0.95, Diamphipnoidae: posterior probability = 1, > 0.95, Eustheniidae: posterior probability = 0.97, > 0.95, Austroperlidae: posterior probability = 0.97, > 0.95). However, the relationships between the families had poor resolution, and formed “comb-like” structures with no clear evolutionary patterns shown. While the majority of the families were found to be monophyletic, the Perlidae was found to be polyphyletic, with two distinct lineages forming around the Nemouridae (posterior probability = 0.96, > 0.95). One of these lineages included only West Palaearctic representatives of the family (posterior probability = 1, > 0.95), while the other included both Afrotropical and West Palaearctic Perlidae (posterior probability = 0.98, > 0.95). Strong support was shown for the monophyly of all genera included within the phylogeny (posterior probability > 0.95). However, while relationships within genera were well supported, the relationships between genera in a family once again formed “comb-like” structures with low resolution.

As seen in the maximum likelihood trees, the H3 gene phylogeny (Fig. 17) did not show the same lineages identified in both the COI gene and concatenated phylogenies. The monophyly of the Antarctoperlaria was shown, although not significantly supported (posterior probability = 0.88, < 0.95). With the exception of the Austroperlidae, which appeared nestled within the Gripopterygidae, the monophyly of all families were significantly supported (Gripopterygidae: posterior probability = 0.99, > 0.95, Diamphipnoidae: posterior probability = 1, > 0.95, Eustheniidae: posterior probability = 1, > 0.95). A monophyletic Notonemouridae was not supported, as the family showed a distinctive “step-like” pattern, consisting of several poorly resolved nodes. The monophyly of all genera were significantly supported (bootstrap replicates >700), including in the Notonemouridae. Unfortunately, the relationships between genera in a family were poorly resolved, as the families generally had “comb-like” structures with no clear evolutionary patterns shown.

All three Bayesian interference phylogenies (COI only, H3 only, COI and H3 combined) had similar topologies to the same maximum likelihood trees, but had high support and resolution for family-level relationships within the Plecoptera (Figs. 15, 16 and 17). The monophyly of the Antarctoperlaria was supported by both the concatenated and H3 tree, and the monophyly of the Antarctoperlarian families was supported in all three trees. Both the COI only and the concatenated tree found the same pattern in the Notonemouridae, which formed a strongly supported monophyletic, sister group to all other Plecoptera. Additionally, strong support was found for all genera included to be monophyletic. While the monophyly of the different genera were well supported, relationships within families generally formed "comb-like" structures, with low resolution.

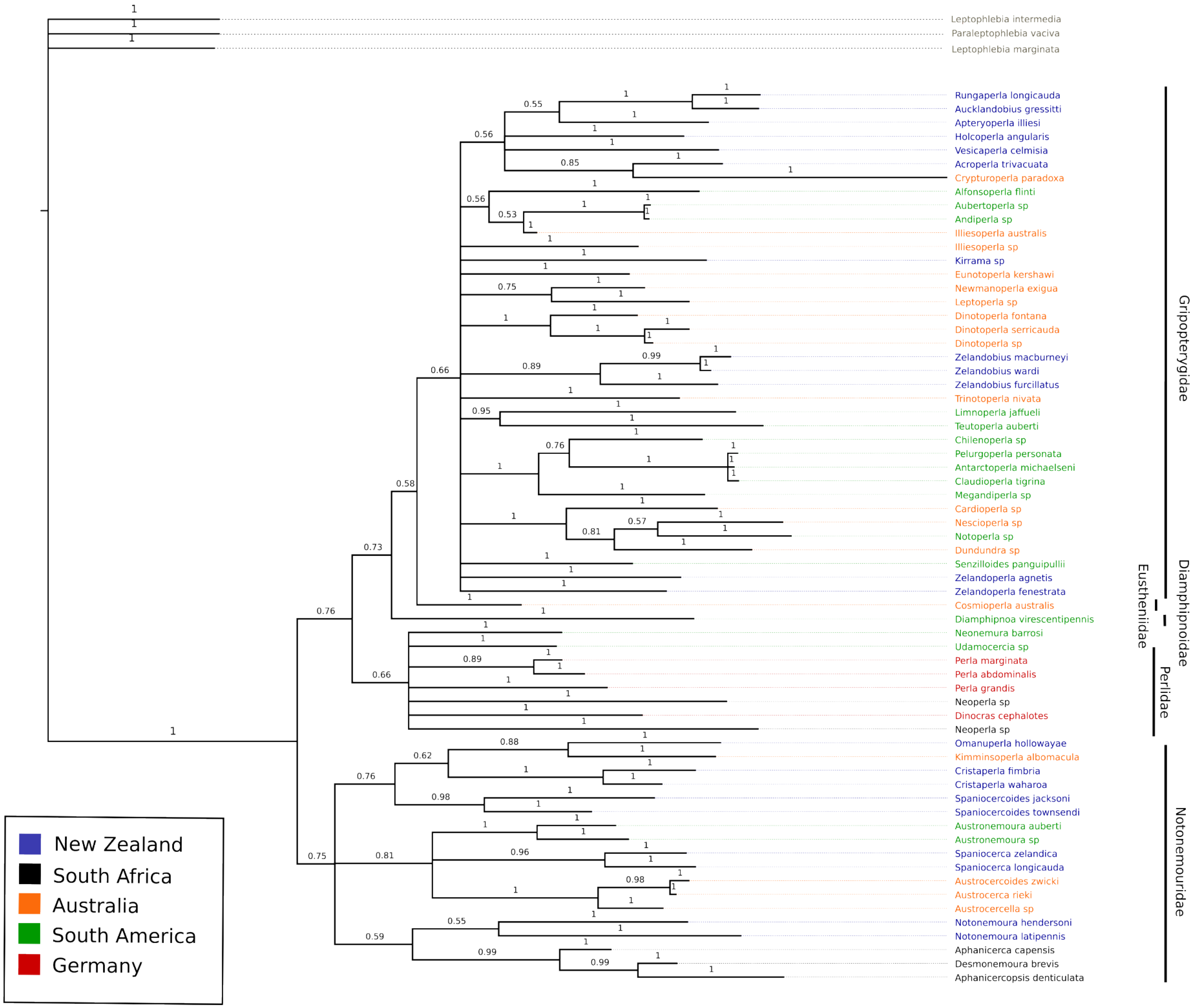

Figure 15: Concatenated Bayesian Interference phylogeny of the Plecoptera constructed using a combination of Cytochrome Oxidase Subunit I and Histone 3 genes. Numbers on each branch represent posterior probabilities, and are significant when >0.95. Species are coloured according to country of origin, and families are represented by black bars on the right side of the tree.

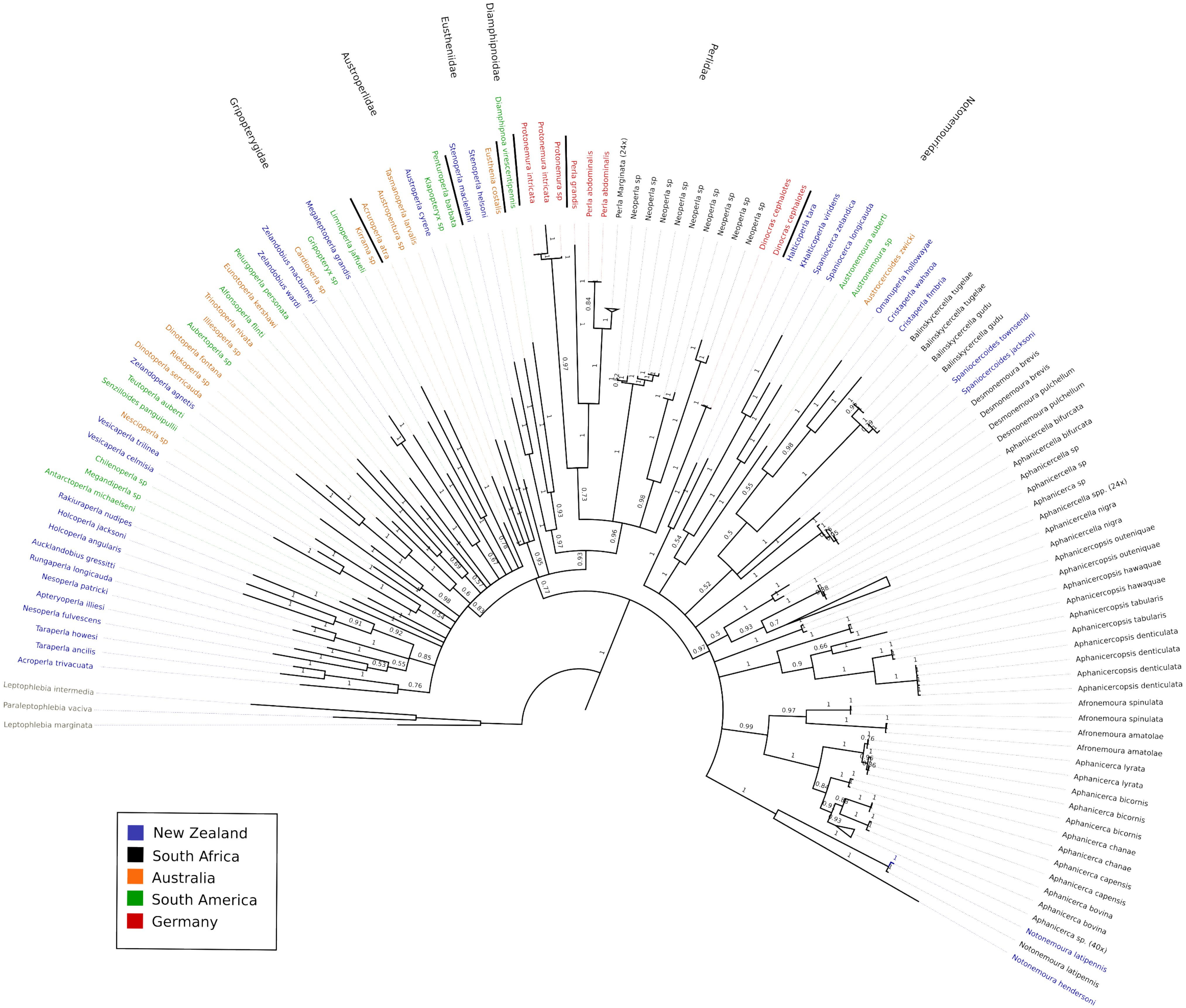

Figure 16: Bayesian Interference phylogeny of the Plecoptera constructed using Cytochrome Oxidase Subunit 1 (COI). Numbers on each branch represent posterior probabilities, and are significant when >0.95. Species are coloured according to country of origin, and families are represented by black bars interspersed throughout the tree

Figure 17: Bayesian Interference phylogeny of the Plecoptera constructed using Histone 3 (H3) genes. Numbers on each branch represent posterior probabilities, and are significant when >0.95. Species are coloured according to country of origin, and families are represented by black bars on the right side of the tree.

5.3.3. Time Calibrated Phylogeny

The final test of phylogeny used the relaxed molecular clock model in order to estimate the relationships within the stoneflies and the ages of the lineages (Fig. 18). As the relaxed molecular clock method equates older dates with exponentially lower probabilities, the dates recovered were not normally distributed around the mean; therefore the mean did not fall within the centre of the time ranges recovered. The phylogeny uncovered here showed the same topology as was uncovered by the Bayesian interference and maximum likelihood trees at both the family and species level, although with greater resolution at the species level than was seen in the Bayesian trees. Once again, the Notonemouridae were shown to represent a well-supported earlier divergence than the remaining families, forming a monophyletic sister group to all of the remaining stoneflies (posterior probability = 1, >0.95). This divergence was estimated to occur at 106.61 Ma (mean: 106.61 Ma, 95% confidence interval: 166.39 - 71.43 Ma). As with the previous trees, the second lineage, consisting of the remaining stoneflies, was split into two additional clades which broadly matched the divide between the Antarctoperlaria and Arctoperlaria, although these relationships were weakly supported (posterior probability = 0.7 and 0.3 respectively, < 0.95). This split was estimated to take place at approximately 88.53 Ma (mean: 88.55 Ma; 95% confidence interval: 126.38 - 56.81 Ma), followed by the Antarctoperlaria and Arctoperlaria further diverging at 74.84 Ma (mean: 74.84 Ma; 95% confidence interval: 104.23 - 46.58 Ma) and 45.41 Ma (mean: 45.41 Ma; 95% confidence interval: 80.36 - 35.52 Ma) respectively. The monophyly of all genera included in the study were strongly supported, although relationships between genera were generally weak and suffered from low resolution in some lineages. The majority of these genera were found to have evolved relatively recently, predominantly having emerged within the last 50 million years.

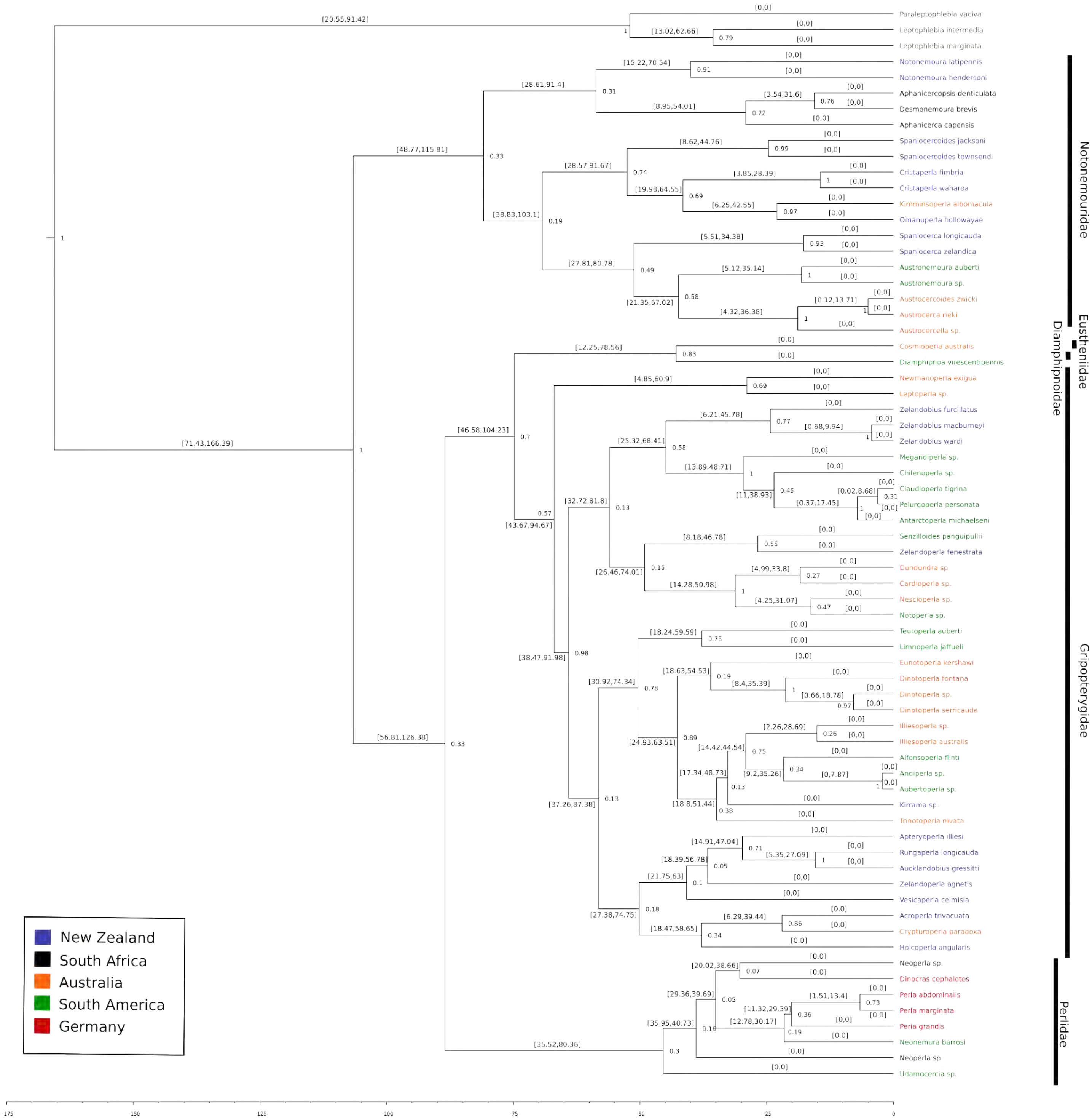

Figure 18: Concatenated Bayesian Interference phylogeny completed in BEAST of the Plecoptera constructed using a combination of Cytochrome Oxidase Subunit I (COI) and Histone 3 (H3) genes. Numbers on each node represent posterior probabilities, and are significant when >0.95. Estimated divergence time ranges are reported in each branch and are in million years. Species are coloured according to country of origin, and families represented by black bars on the right side of the tree.

5.4. Discussion

5.4.1. Major lineages within the Plecoptera

Higher level relationships among the stoneflies were intensely debated throughout the last century, with two main competing subordinal concepts (Klapálek 1905, Enderlein 1909). Both of these concepts were based on a combination of derived and primitive traits, with little consideration for the different taxonomic value of these traits (Zwick 2000). Additionally, both concepts were often treated as mutually exclusive “sides” of an argument, with Klapálek’s Subulipalpia and Filipalpia opposing Enderlein’s Systellognatha and Holognatha with little to no overlap considered (Zwick 2000). Neither of these concepts has persisted in their entirety, and it has since become widely accepted that the Plecoptera are divided into two suborders, the Antarctoperlaria and Arctoperlaria, the latter of which is further divided into the Systellognatha and Euholognatha (Zwick 1973, 1980). These suborders have a predominantly disjunct, non-tropical distribution, and the monophyly of these groups has been supported by limited morphological characters (Zwick 2000), and more recently by several phylogenetic analyses of the group (McCulloch et al. 2016, Ding et al. 2019). The phylogenetic analysis completed here (Chapter 5: Results) did not support these divisions, as the monophyly of the Arctoperlaria was unsupported. The Notonemouridae were found to diverge much earlier than the Perlidae and the Nemouridae, forming a sister group to all remaining Plecoptera. However, the remaining stoneflies were found to broadly support these divisions, as the Antarctoperlaria formed a single, monophyletic clade separate from the remaining Arctoperlaria, although this was supported overall by low posterior probabilities.

These results seemed unusual when compared to recent literature on the phylogeny of the stoneflies. Recent, taxa-rich molecular analyses of the Plecoptera have supported the monophyly of the Arctoperlaria and Antarctoperlaria (McCulloch et al. 2016, Ding et al. 2019). Additionally, support for the monophyly of both of the arctoperlarian superfamilies, the Euholognatha (Ding et al. 2019) and Systellognatha (Chen et al. 2018, Ding et al. 2019) has been found, although a consensus has not been reached on the exact relationships within the group.

The starkly different results obtained here (Chapter 5: Results) compared to recent phylogenies of the stoneflies is peculiar, especially as the majority of sequences from GenBank were obtained from a study by McCulloch et al. (2016), and all of the phylogenies constructed in this thesis used a large number of these sequences. For example, the concatenated trees were constructed using almost exclusively those sequences with only a small number of Perlidae from South Africa, Angola and Germany added. Yet the results obtained above (Chapter 5: Results) did not show the same clear divide between the suborders and the support for the monophyletic Antarctoperlaria was considerably lower than indicated by McCulloch et al. (2016). However, this discrepancy may be due to the differing number of species included in each phylogeny, and the representation of each gene. Many of the species included in McCulloch *et al*. (2016) were represented only by COI and not H3 genes or 18S, and most of these species, along with the 18S gene, were removed from my analysis, as these information gaps can reduce the reliability and resolution of the results. However, this resulted in a much lower taxon number being included in the final analysis (65 compared to 105), and considerably fewer species represented by only COI included in the final concatenated tree. Mitochondrial and COI genes appear to better identify deeper level relationships within the Plecoptera than nuclear genes (Ding et al. 2019), suggesting that the differing ratios of genes may have caused the differences in results. However, at the same time the reduced taxon numbers did cause lower posterior probabilities and resolution throughout the tree, making it unclear whether a more similar topology would have been uncovered if more representatives had been included.

5.4.2. Notonemouridae

In all of the phylogenies included here, it was found that Notonemouridae formed a sister group to all other Plecoptera and did not fall within the Arctoperlaria. This finding contradicts the accepted evidence that the Notonemouridae represent the sister group of the Nemouridae (Zwick 2009, Ding et al. 2019). However, this finding is not unprecedented, as Misof et al. (2014) identified the same deeper divergence of the Notonemouridae, before the split of the two plecopteran suborders. Additionally, recent molecular evidence has suggested that the Arctoperlaria are not monophyletic, or at least that the relationships within the superfamilies are not as simple as currently believed (Terry & Whiting 2005, Chen & Du 2017, Wang et al. 2017a, 2017b, Chen et al. 2018)

Many authors have considered Notonemouridae to represent a polyphyletic lineage, made up of separate arctoperlarian lineages, which have repeatedly and independently dispersed into the Southern Hemisphere through the African continent (Zwick 1990, 2000, McLellan 2000, Fochetti & de Figueroa 2008, Ding et al. 2019). However, the results detailed here (Chapter 5: Results), which included over 100 notonemourid species from 19 of the 25 genera, found strong support for the monophyly of the family in all phylogenies, with the exception of the H3 gene. The H3 tree lacked any clear resolution or significant support regarding the relationships of the family, and featured a “step-like” topology, which is not consistent with either multiple independent dispersal events, or with a single dispersal event. This variation in the phylogenies is likely a result of the different genes being used. While the COI regions of mitochondrial DNA is quite informative at species level, H3 genes are generally used at the supraspecific level, as they evolve more slowly (Ogden & Whiting 2003). This conservation of the H3 gene makes it more useful in ancient lineage delineation and at higher taxonomic levels (Ogden & Whiting 2003), but may explain the poor resolution of the Notonemouridae lineages encountered here (Chapter 5: Results). Therefore, it seems more likely that the Notonemouridae represent a monophyletic lineage rather than a polyphyletic group. Evidence of a monophyletic Notonemouridae was also found by McCulloch *et al*. (2016), many of whose sequences were included in the current study (Chapter 2). It is possible that this explains the similar results found here. In an effort to counter this, a large number of South African notonemourid sequences, sourced from GenBank (Stevens et al. 2018) and newly extracted (Chapter 2) were included in all of the analyses. The results matched the relationships found by McCulloch et al. (2016) previously, suggesting that using the same sequences as McCulloch et al. (2016) did not have a significant effect on this finding.

Extant Notonemouridae have a “Gondwanan” distribution, as they are found across the Southern Hemisphere, on every continent and major island except for Antarctica (Fochetti & de Figueroa 2008, DeWalt & Ower 2019). This distribution has been suggested to be the result of vicariance following the break-up of Gondwana (Illies 1965, McLellan 1993, Zwick 2000). The early divergence of the Notonemouridae uncovered here (Chapter 5: Results) was dated to approximately 106.61 Ma (166.39 - 71.43 Ma), which coincided with the rifting of Pangea in the Jurassic (McLoughlin 2001). Misof et al. (2014) identified the same deeper divergence of the Notonemouridae, although they dated it to 180 Ma. Furthermore, evidence for the early origin of the Notonemouridae is found in the fossil record, as stem representatives of the group have been recorded from as early as 145 Ma, Daohugou locality, from Inner Mongolia (China) in the Middle Jurassic (Cui et al. 2019). These early dates provide strong evidence that the Notonemouridae may have developed earlier than currently believed, and where likely well established on Gondwana by the end of the Jurassic, supporting the theory of vicariance playing an important role in the distribution of extant Notonemouridae. Additionally, the early divergence of the Notonemouridae, before the split of the other two suborders, may suggest a Gondwanan origin of the group, rather than the currently accepted theory of dispersal into the Southern Hemisphere through the African continent (Zwick 1990, 2000, McLellan 2000, Fochetti & de Figueroa 2008, Ding et al. 2019).

These results disagree with those of McCulloch et al. (2016) and Ding et al. (2016), who found the Notonemouridae to have diverged more recently, at approximately 76 Ma and 71 Ma respectively. These results suggest that the family would have spread into the Southern Hemisphere during the late Cretaceous, post-dating the breakup of Gondwana (McCulloch et al. 2016, Ding et al. 2019). If this was the case the distribution of the extant representatives of the group would be due to long distance dispersal, most likely via Antarctica in the strong winds that surround the continent (Illies 1965, Zwick 1981, 1990, McCulloch et al. 2016). However, both of these time calibrated molecular clocks have relied on conservative calibration dates from recent, amber fossils, which in some cases have resulted in disparities with the fossil record of up to 93 million years (Cui et al. 2019). These discrepancies likely contributed to the later dates found in these phylogenies, as there is clear evidence that many of the plecopteran lineages are older than suggested. For example, both studies placed the origin of the Plecoptera as a whole at 251 Ma (Sinitshenkova 1997, 2004), despite several records of fossil Plecoptera from up to 60 million years earlier (Béthoux et al. 2011, Sinitshenkova 2018).

While the Notonemouridae have been in existence for some 106 mya, Fig. 18 shows that the lineage leading to the extant species in South Africa today only arose some 54-8 mya, meaning that these are relatively young. While this age range is too broad to be truly meaningful, these dates could coincide with late Paleocene glaciation events, which were associated with large changes to sea levels and river inundation (Mercer 1983, Leckie et al. 1995, Abreu & Anderson 1998). It is possible that these glaciation events may have resulted in the isolation of the South African Notonemouridae, resulting in the relatively recent evolution and divergence of these genera.

5.4.3. Split between the Antarctoperlaria and Arctoperlaria

The monophyly of the Antarctoperlaria and Arctoperlaria has been supported by a few limited morphological characters (Zwick 2000), and more recently by several phylogenetic analyses of the group (McCulloch et al. 2016, Ding et al. 2019). However, the phylogenetic analysis completed here (Chapter 5: Results) largely did not support this division, as the Notonemouridae were found to form an earlier, deeper sister group to the remaining Plecoptera. Nevertheless, the remaining stoneflies then split into two further clades, which matched the divide between the two suborders. This divide was supported by the concatenated and COI gene trees, in all three tests of phylogeny, although it was not seen in the H3 gene. As discussed above, the only Arctoperlarian representatives included in the H3 gene trees were the Notonemouridae, which were marked by poor resolution.

It has been widely accepted that the hemisphere specific distributions of the two extant plecopteran suborders is due to vicariance caused by the breakup of Pangea, and subsequently Laurasia and Gondwana (Illies 1965, Banarescu 1990, Zwick 1990). This theory has received strong support from both fossil and molecular evidence. There is evidence that the Plecoptera were widespread by the Permian (Riek 1973, 1976b, Sinitshenkova 1987, 1997, 2004, 2018, Sinitshenkova & Aristov 2010, Aristov et al. 2013), which suggests that the hemisphere-specific suborders would have likely originated during a period of diversification following the break-up of Pangea (Illies 1965, Banarescu 1990, Zwick 1990, 2000). Additionally, McCulloch et al. (2016) and Ding et al. (2019) both used fossil-calibrated molecular clock dated phylogenies to date the split of the suborders to approximately 121 Ma and 181 Ma respectively. Misof et al. (2014) found a similar result, dating the split between the suborders to 110 Ma, but also identified the same deeper, older divergence of the Notonemouridae at 180 Ma. Comparatively, the time calibrated phylogeny completed here (Chapter 5: Time Calibrated Phylogeny) estimated that the suborders diverged more recently (at approximately 89 Ma). This significantly post-dates the breakup of Pangea and seems to contradict the theory that vicariance formed the suborders of the stoneflies. However, the later dates seen in the phylogenies are still consistent with vicariant formation of the suborders. Zwick (2000) argues that although the Plecoptera are ancient, the extant families of the order are not necessarily old themselves and may have diverged from a common stock relatively recently, after the rifting of Pangea. Further evidence of this is seen in the phylogenies of closely related species and genera worldwide, which reflect recent speciation (Illies 1966, Zwick 1973, Michaelis 1988, McLellan 1993). Finally, the monophyletic lineages of both suborders seen here (Chapter 5: Results), with the exception of the Notonemouridae, is consistent with independent, geographically isolated divergence after the split of the supercontinent.

5.4.4. Antarctoperlaria

In all of the Bayesian interference and maximum likelihood phylogenies presented here (Chapter 5: Results), the monophyly of the Antarctoperlaria, and its included families, is supported, albeit by low probabilities.

Extant Antarctoperlaria have an unusual distribution, as every family is found in all of the Southern Hemisphere regions, with the exception of the Afrotropical region (Fochetti & de Figueroa 2008, DeWalt & Ower 2019). However, while the families are wide-spread, only a few species from a single genus, *Notonemoura*, are found on more than one continent, namely Australia and New Zealand (Fochetti & de Figueroa 2008, McCulloch et al. 2016, DeWalt et al. 2019). It is commonly held that the disjunct distribution of the suborder across the Southern Hemisphere is due to vicariance caused by the fragmentation and break up of Gondwana (Illies 1965, Zwick 2000, Fochetti & de Figueroa 2008). However, a recent time-calibrated phylogeny of this group by McCulloch et al. (2016) suggests instead that the majority of cladogenesis within the group occurred after the fragmentation of Gondwana (McCulloch et al. 2016). This suggests that several post-breakup dispersal events between the continents occurred. It has been theorized that these dispersal events likely occurred via Antarctica until 41 Ma, although the continents would already be separated by ocean by this time (Scher & Martin 2006, McCulloch et al. 2016).

The results of the Bayesian phylogenies (Chapter 5: Bayesian Interference) seem to be consistent with long range dispersal, as individual, geographically isolated clades including all of the species within a family were not identified. This suggests that a number of trans-continental dispersal events occurred after the breakup of Gondwana, preventing the groups becoming geographically isolated until more recently. These relationships remain unclear due to poor resolution and support values in all three Bayesian trees (Chapter 5: Bayesian Interference). However, the fossil-calibrated molecular clock (Chapter 5: Time Calibrated Phylogeny) estimated that the divergence of the Antarctoperlaria only occurred at 75 Ma, significantly post-dating the split of Gondwana. Comparatively, the maximum likelihood phylogenies, which were distinctly more supported at the species level, showed geographical groupings within all of the families, with only a few species not falling within these clades (Figs. 12-14). This highly specific, endemic diversity seems to be indicative of vicariance, with the Antarctoperlarian families being widespread before the split of Gondwana, and undergoing a period of diversification in geographical isolation afterwards (Illies 1965, Zwick 2000, Fochetti & de Figueroa 2008), contrary to the findings of McCulloch et al. (2016). Considering all of the evidence above, it seems likely that while vicariance has probably played an important role in determining the distribution patterns of extant Plecoptera, this unusual distribution was likely caused by a combination of vicariance and long-distance dispersal.

As shown previously (Chapter 1), the stoneflies have limited swimming and flight abilities as nymphs and adults respectively, which severely limits their distribution capacity (Brundin 1967, 1972, Zwick 2000, Schultheis et al. 2002, Fochetti & de Figueroa 2008, McCulloch et al. 2009). This, in turn, has resulted in a high degree of endemism within countries (Stevens et al. 2018), and between continents (Fochetti & de Figueroa 2008, DeWalt & Ower 2019), suggesting that long range dispersal is probably rare. Considering this, it is highly unlikely that this long range dispersal has occurred as a result of active dispersal mechanisms (e.g. flying) but rather as a result of passive methods, such as wind dispersal (McCulloch et al. 2016). It has been shown that larger insects, such as Plecoptera, can be dispersed via strong winds (Bilton et al. 2001, Kelly et al. 2001). In particular, the strong westerly winds surrounding Antarctica have been identified as a possible circumpolar dispersal mechanism (Sanmartíin & Ronquist 2004, Waters 2008), and have been posited to have played an important role in the dispersal of stoneflies across the Southern Hemisphere (McCulloch et al. 2016) since the separation of Antarctica from South America and Australia (Scher & Martin 2006).

The Antarctoperlaria, or at least their stem ancestors, were likely present across Gondwana before it split throughout the Jurassic, making it unusual that they are not present in either modern-day Africa or India (Fochetti & de Figueroa 2008, DeWalt & Ower 2019). Currently, it is theorized that the disappearance of this group from these regions was brought about by mass extinctions due to changing climatic conditions (Banarescu 1990, Resh & Cardé 2003, Fochetti & de Figueroa 2008). These changing conditions are still reflected today, as both regions have large, dry areas marked by low rainfall, making it an inhospitable environment for Plecoptera reflected the low species number found in both areas (DeWalt & Ower 2019).

Their absence, however, did open the way for the dispersal of two families throughout the Afrotropical region, *viz.* Notonemouridae and Perlidae.

5.4.5. Arctoperlaria in the Southern Hemisphere

The monophyly of the Arctoperlaria was not supported in any of the phylogenies completed here (Chapter 5: Results). The early divergence of the Notonemouridae as a sister group to the remaining Plecoptera has already been discussed above. However, there was also little evidence for the monophyly of the Arctoperlarian superfamilies, Euholognatha and Systellognatha, in most of the trees created here. In the COI gene trees the Perlidae and Nemouridae clumped together, with the Nemouridae nestled within the Perlidae (Figs. 13 and 16), in contrast to the majority of molecular studies of these families (Thomas et al. 2000, Terry & Whiting 2005, Davis 2013). While this lineage did include all three of the Nemouridae species, it only included some of the Palaearctic Perlidae species, with the Afrotropical Perlidae and a single Palaearctic species, *viz. Dinocras cephalotes,* remaining separate and forming an earlier branch in the tree. This could suggest the presence of two separate lineages of Perlidae, which broadly match the divide between the Northern Hemisphere and Southern Hemisphere stoneflies. Some evidence for this could be found in the behaviour of the Afrotropical *Neoperla*. The Arctoperlaria are united by shared drumming behaviour, an audible form of communication between the adults which is generally created by rubbing, tapping or scraping the abdomen across a hard surface, such as a rock or log (Zwick 1973, 1980, Stewart & Sandberg 2006). However, this drumming has not been recorded in Afrotropical *Neoperla*, and it is unclear if they share this behaviour (pers. comm. P. Zwick, Schlitz, Germany). This variation could be explained as arising from geographic isolation, resulting in the two groups diverging independently within the different Hemispheres. Alternatively, this pattern could be explained by different lineages of the Perlidae existing in localized pockets and dispersing into other areas more recently, perhaps in response to extinction events. This would make sense in the light of the Palaearctic stoneflies splitting into two, separate clades.

5.4.6. Conclusions

Three dominant lineages between the suborders and the families of the Plecoptera were identified here. The earliest branch of the tree consisted of a monophyletic Notonemouridae, which formed a sister group to all remaining Plecoptera. The remaining Plecoptera then split into two further clades, which broadly matched the divide between the two suborders, the Arctoperlaria and the Antarctoperlaria. Based on a combination of these findings and the fossil record, it seems likely that the distribution of extant Plecoptera is due to a combination of vicariance, caused by the rifting of Pangea, and subsequently Laurasia and Gondwana. Unfortunately, most phylogenies which include the Plecoptera rely on only a few representatives, especially of the Antarctoperlaria, resulting in the phylogeny of the group and its relationships with other extant insects remains uncertain (Ding et al. 2019). More large scale, taxon-rich molecular phylogenies including species from both the Northern and Southern Hemisphere, and both mitochondrial and nuclear genes are clearly required to improve our understanding of the evolutionary pathways within this group.

6. General Discussion

The study completed here is the first of its kind in Gondwana, as it utilized both fossil evidence and detailed phylogenetic analyses to better understand the origins, evolution and modern diversity of the Plecoptera. This thesis represents a significant contribution to the current understanding of the Plecoptera from the Permian to the present. Additionally, it has opened the way for significant future applications of the work in reconstructing ancient food-webs, palaeohabitats, palaeoclimate and palaeobiogeography, in conjunction with the wealth of information emerging from the Onder Karoo fossil locality.

There is a significant Northern Hemisphere bias in knowledge regarding the biodiversity and evolution of extant and fossil Plecoptera. Of the 260 plecopteran species described from fossils prior to this study, only 16 were from Gondwanan land masses. The Southern Hemisphere-specific suborder Antarctoperlaria and the Afrotropical members of the Arctoperlaria, families Notonemouridae and Perlidae, are severely underrepresented or entirely excluded in molecular phylogenies of this important group (Ding et al. 2019). This study represents an important step towards understanding the significance of South African Plecoptera in a global context. Three new fossil plecopteran species have been described from a new middle Permian Lagerstätte, a significant contribution to the Gondwanan fossil record and the understanding of early insect evolution. A phylogeny of the Plecoptera including representatives from all three lineages of the order has been completed.

6.1. Ecology of the Plecoptera at the Onder Karoo locality

The extraordinary preservation, both in terms of the quantity and quality of fossils emerging from the site, has led our research team to class the Onder Karoo locality among the rare Lagerstätten of the world. The fine-grained, finely laminated olive-grey mudrocks, suggestive of a calm lacustrine setting, are typical features of fossiliferous insect and plant localities elsewhere in South Africa, such as those of the upper Permian in KwaZulu-Natal

(Anderson & Anderson 1984, Geertsema et al. 2002, Prevec et al. 2009). However, why the incidence of generally rare elements, such as insect remains, plant fertile structures and attached plant organs, is so high at the Onder Karoo site is a taphonomic question currently under consideration.

It can be inferred from a preliminary geological study of the site (pers. comm. M. Day) combined with the types of fossil evidence found, that the fossils are mainly autochthonous or parautochthonous (Prevec et al. in preparation). The majority of parautochthonous specimens were probably windblown, infall from overhanging branches or introduced via the small distributaries feeding the lake, where the organics and fine clastics settled not far from the lake margin. An additional factor that may have contributed to the unusually fine preservation of the material, and which may have even acted as a periodic kill-mechanism in aquatic ecosystems at the time, is ashfall from volcanic eruptions. There is evidence of repeated and regular ashfall events occurring only 30 km away from the Onder Karoo locality, at the Ouberg Pass (Lanci et al. 2013).

Volcanic ash fall in modern ecosystems has been shown to have significant effects on the aquatic insects living within them, as it alters the habitat and can be a direct cause of death in many species (Brusven & Hornig 1984, Meyerhoff 1991, Elizalde 2015). In Plecoptera, the changes to the pH of water caused by ashfall can result in changes to their behaviour, survivability and in rare cases their physiology (Brusven & Hornig 1984, Lechleitner et al. 1985). In addition to these direct effects, ash tends to seal the soils in rivers and streams, making it harder for the plecopteran nymphs to remain attached to the substrate, often resulting in behavioural and microhabitat shifts (Brusven & Hornig 1984). The introduction of ash to an aquatic system may also cause eutrophication, leading to the development of anoxic conditions in water bodies and mass death of aquatic organisms.

All of these factors have been observed in the wake of recent volcanic eruptions, such as after the eruption of Mt. St. Helens in the USA (Meyerhoff 1991, Elizalde 2015). Following this eruption, a distinct decline in stonefly populations and densities were observed, along with many other aquatic invertebrates (Meyerhoff 1991). In the years that followed however, stoneflies recovered quickly, reaching much higher population densities than were present before the eruption due to increased nutrients and minerals introduced by the ash, which increased primary production and food availability (Meyerhoff 1991). As the numbers recovered, their trophic interactions contributed towards stabilization of the environment (Meyerhoff 1991). If the Onder Karoo locality was subject to regular volcanic eruptions, it may in part explain the large abundance of Plecoptera identified from the site, as they became more and more dominant in the wake of previous eruptions, recovering faster than other aquatic insects, as was observed at Mt. St. Helens. The extent and effects of the ashfall events on the terrestrial and aquatic environments of the region is being pursued by the larger research PEATs research team.

6.2. Significance of Fossil Specimens

The three new species of Plecoptera described here represent the earliest fossil stoneflies from Gondwana. Although not the earliest stonefly fossils recorded globally, these species have been dated to approximately 268 Ma (Prevec *et* al. in preparation), making them vital to understanding the spread and diversification of the ancient stoneflies in Pangaea and Gondwana. Additionally, all three species are described from the remains of nymphs, which are severely underrepresented in the fossil record.

While three new plecopteran species have been identified from the Onder Karoo locality, it is likely that more stoneflies remain undescribed within the large collection of nymph remains. Many of these were examined in the process of describing the species presented here, but most of the specimens seem to be exuviae and in most cases lack a preserved thorax or head region. The absence of these details makes it difficult to confidently describe new species, but it is likely that careful re-examination of these slabs will reveal even more Plecoptera from the Onder Karoo site. The Albany Museum collection appears to contain a diverse basal fauna, since it includes other early insect groups such as the

Palaeodictyoptera, which have been tentatively identified from the locality and will be described in other research being conducted by the larger Albany Museum PEATs research team.

A major component of the research being conducted by the Albany Museum PEATs research team is the reconstruction of an in-depth ecological food web for the Onder Karoo collection. Several possible relationships of the Plecoptera with both biotic and abiotic factors were inferred and discussed in Chapter 4 of this dissertation. This reconstruction relies on using extant relatives of the Plecoptera as analogues for fossil species, which is based on the assumption that fossil and extant species vary and behave in similar ways (Ackermann 2003). Unfortunately, these assumptions remain intrinsic to these studies, even though there is a growing understanding that they can be misleading (Wood 1991, Collard & Wood 2000, Ackermann 2002). However, insects such as Plecoptera to a greater extent, perhaps than any other animal group, have remained relatively unchanged since their evolution in the Palaeozoic Era (Labandeira & Sepkoski 1993). It is therefore perhaps more justifiable to make inferences regarding habitat, functional morphology and ecological interactions using insects rather than other animals.

6.3. Phylogeny of the Plecoptera

The phylogenies uncovered within this dissertation repeatedly revealed several patterns. The broad sub-ordinal divisions suggested by Zwick (1973, 1980, 2000), into the Antarctoperlaria and Arctoperlaria, were supported here, with the exception of the Notonemouridae. However, the Notonemouridae were found to form a monophyletic, deeper divergence, forming a sister group to all other Plecoptera included in this study. This earlier branch of the Notonemouridae suggests a much earlier origin of the group than is currently believed and that the Notonemouridae may be an old cold-adapted gondwanan relict group, with similar distributions to the Teloganodidae (Ephemeroptera) (Pereira da Conceicoa 2016), instead of their distribution being due to the currently proposed dispersal from the Northern Hemisphere (Illies 1965, Stark & Gaufin 1976, Zwick 2000, Fochetti & de Figueroa 2008).

While the monophyly of the Antarctoperlaria and its included families were predominantly supported, along with the earlier divergence of the Notonemouridae, another pattern was identified among the super-families of the Arctoperlaria (Systellognatha and Euholognatha), the monophyly of which was not supported. Two lineages of the Perlidae were identified, one which included only West Palaearctic species and one which included a combination of Palaearctic and Afrotropical species. This suggested that two separate lineages of Perlidae may be present, which broadly match the divide between the Northern and Southern Hemispheres. Some evidence may additionally be seen in the behaviour of the Perlidae. Drumming in African Perlidae has never been recorded and it is currently assumed that it does not occur in the African species, but this has yet to be convincingly tested (pers. comm. P. Zwick).

The phylogenies included here also filled some of the gaps seen in most current phylogenies of the Plecoptera. The inclusion of representatives from all three major lineages (Antarctoperlaria, Systellognatha and Euholognatha), using a combination of nuclear and mitochondrial genes (COI and H3) and shifting the focus to predominantly Southern Hemisphere species is an important step in addressing the strong Northern Hemisphere bias which has been prevalent in Plecoptera phylogeny studies to date.

6.4. Plecopteran phylogeny in relation to the fossil record

The phylogenies completed here showed clear monophyletic clades delimiting the suborders and families of the Plecoptera, which broadly matched their current geographical ranges. This strongly suggests that vicariance played an important role in explaining the relationships and biogeography of the stoneflies. Some additional evidence was found in the time-calibrated, molecular clock phylogeny of the Plecoptera and in the fossil record of the group. The fossil species identified here, in combination with other species identified worldwide (see Chapter 1), provide evidence that the Plecoptera were widespread by the middle Permian. As the groups were widespread before the split of Pangea, and later Gondwana and Laurasia, it seems likely that vicariance would indeed have had a significant impact on the group as they became geographically isolated.

Interestingly, while the majority of the lineages uncovered here supported vicariance as the major factor influencing global distributions of extant Plecoptera, in several examples evidence of long-range dispersal was found. For example, while two separate lineages of Perlidae were identified, broadly dividing between the Northern and Southern Hemisphere, some German species were found to be more closely related to the Afrotropical species. This suggests that while an initial divide due to vicariance may have resulted in divergent evolution, some species have undergone long range dispersal since. A similar pattern was seen in several families in the Southern Hemisphere between continents, including the Notonemouridae (for example the South African *Notonemoura latipennis* emerging amongst the New Zealand Notonemouridae), again supporting the theory that long range dispersal since the split of Pangea and Gondwana has played an important role in the distribution of stoneflies.

As a part of understanding the evolution of the Plecoptera, a fossil-calibrated, dated phylogeny of the stoneflies was completed here. Fossils remain the only physical evidence of ancient life and evolution, and therefore remain vital for studies of past life. Thus, fossil evidence must take precedence over purely molecular reconstructions of the evolutionary development of insect lineages, and it is vitally important to include all forms of information available (fossil and molecular data), when considering a topic as vast and complex as evolution.

The results detailed here have shown the importance of using multiple methods to examine the origin and evolution of the Plecoptera, and other invertebrate species. While both molecular and morphological studies can provide interesting information regarding the origin and evolution of these groups, they both have some pitfalls, and it is only in combination that a holistic view can be achieved. Furthermore, the addition of more genes, or even whole genome studies using next generation sequencing may give further refinement of the molecular phylogeny.

6.5. Future Research

This study represents an important step in the study of the origin and evolution of the Plecoptera, revealing biogeographic patterns that have not been recognized previously. It is clear that a large amount of work is still needed to further elucidate the distribution patterns seen in this important order.

As more species within the Plecoptera and other insect orders are identified and described from the Onder Karoo locality, phylogeographic patterns in other taxa may help shed light on the patterns discerned so far. This site has revealed the only insects from the middle Permian in South Africa and represents an important and fascinating stage in the evolution of early insects. Three new fossil plecopteran species were described within this dissertation, barely scratching the surface of potential new information which can be gleaned from the incredible fossils found at this site. Further studies conducted at this locality will help identify and reconstruct interactions between stem Plecoptera and other species during the Permian. Additionally, a large number of plant fossil remains have been recovered from the Onder Karoo locality, particularly *Glossopteris* leaves, and it is important to understand the plant-insect interactions that occurred in these environments. All of this information will feed into a food-web reconstruction currently being tackled by the broader PEATs group at the Albany Museum.

More in-depth molecular phylogenies of the Plecoptera are desperately needed. Phylogenetic analyses within the order Plecoptera remain rare, with many studies simply using the stoneflies as an outgroup and considering only a few species, especially among the Antarctoperlaria (Ding et al. 2019). While this has improved in recent years (McCulloch et al. 2016, Ding et al. 2019), it is still uncommon to find a phylogeny which includes all three lineages of the stoneflies, and the majority still have a strong Northern Hemisphere bias. The addition of Southern Hemisphere stoneflies in this thesis has uncovered several unusual relationships within the Plecoptera, which contradict the currently accepted phylogeny of the group. For this reason, it is important that a large scale, species-rich phylogenetic analysis of the Plecoptera, including species from both hemispheres and using a combination of mitochondrial and nuclear genes, is completed. While it is important that a variety of South African species from different families are included in these phylogenies, perhaps the most significant group is the Perlidae. The phylogenies included here, along with the lack of resolution in the species complex *Neoperla spio* (Picker 1980), make it abundantly clear that the phylogeny and relationships of this family are still poorly understood. The inclusion of this group will therefore serve a dual purpose - not only will it assist in clarifying the biogeography and evolution of the Perlidae, but it will also significantly contribute towards the identification and description of new species from the Afrotropical region. Zwick (pers. comm.) has indicated that there are over 70 species of African Perlidae soon to be described.

While several molecular clock dated phylogenies have been completed on the Plecoptera (McCulloch et al. 2016, Ding et al. 2019), several of the calibration points used in these studies excluded recent discoveries of fossil taxa. Additionally, several discoveries, including the Plecoptera described here, suggest that the dates uncovered within those phylogenies significantly underestimated divergence times within the order. It is important that accurate and up-to-date phylogenies are completed, to allow for a clearer understanding of the origin, evolution and biogeography of this order.

Finally, while many of these suggestions have been made with the goal of increasing understanding of the lineages and divergences within the Plecoptera on a global scale, a recent study conducted by Stevens et al. (2019) showed that a high degree of endemism can be seen in Plecoptera even within a country. This suggests that more detailed regional studies of species are important and are likely to reveal more divergences and lineages of the stoneflies than are currently known.

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Appendix 1

	Morphospecies 1			Morphospecies 2						Morphospecies 3							
	AM 11268a	AM 11268b	AM 12952	AM 11311	AM 13214	AM 13109a	AM 13109 b	AM 13265a	AM 13265b	AM 11284a	AM 11458	AM 12465	AM 12497	AM 12498	AM 13075	AM 13085 1	AM 13085 2
Abdomen	6062 (Incomplete)	7555	6308	6517	4937	3940	3941	5064	4898	3976	4071	3650	5558	5065	4817	3149 (Incomplete)	2510 (Incomplete)
Segment Length																	
1	N/A	521	536	450	665	462	462	683	413	368	N/A	359	368	390	529	N/A	N/A
2	N/A	558	468	582	482	460	460	511	509	440	461	403	479	467	542	388	N/A
3	544	640	526	516	506	432	432	544	573	412	404	284	528	487	503	346	N/A
4	690	636	550	647	586	461	461	621	571	415	394	422	582	559	489	358	432
5	681	625	538	581	551	501	501	520	573	475	493	425	516	489	521	387	400
6	619	648	472	621	505	341	341	528	560	442	369	343	529	492	423	269	325
7	552	594	467	555	548	329	329	435	522	364	354	291	465	490	482	302	315
8	512	549	445	664	N/A	436	436	292	390	325	442	302	477	522	464	357	289
9	513	525	449	502	N/A	315	215	495	383	409	422	360	368	464	571	379	333
10	628	613	466	525	N/A	363	323	435	404	346	485	461	473	522	293	363	416
Segment Width																	
1	N/A	?	1349	606 (Obscured)	992	800	800	633 (Obscured)	548 (Obscured)	810	N/A	603 (Obscured)	540 (Obscured)	402 (Obscured)	853	N/A	N/A
2	N/A	1442	1239	976 (Obscured)	1349	869	869	1477	1233	905	655 (Incomplete)	846 (Obscured)	850	842	1023	446 (Incomplete)	N/A
3	1379	1460	926	1608	1394	848	848	1412	1344	926	589 (Incomplete)	863 (Obscured)	597 (Obscured)	574 (Obscured)	1021	607 (Incomplete)	N/A
4	1291	1318	1020	1578	1314	778	778	1383	1413	967	699 (Incomplete)	656 (Obscured)	689 (Obscured)	726 (Obscured)	1099	510 (Incomplete)	454 Iincomplete)
5	1339	1341	1079	1031 (Incomplete)	1337	872	872	1402	1453	1046	863 (Incomplete)	677 (Obscured)	809	794	1204	761 (Incomplete)	774
6	1293	1303	1099	1186 (Incomplete)	1266	1124	1124	1308	1344	1049	1246	1111	755	724	1201	676	1006
7	1345	1339	1001	1423 (Incomplete)	1362	1144	1144	1161	1164	1128	1310	1250	940	938	1238	678	1096
8	1290	1300	1003	1652	N/A	1089	1089	1114	985	1216	1268	1189	1088	1074	1292	679	1048
9	1215	1197	945	1347	N/A	902	902	1008	909	1188	1141	1097	1061	1052	1149	548	971
10	1049	1047	928	1168	N/A	589	589	622	627	966	939	944	880	849	871	295	878
Cerci																	
Left	1667 (incomplete)	1584 (incomplete)	744 (incomplete)	N/A	N/A	1257	1257 (Incomplete)	N/A	N/A	383	1838 (Incomplete)	865 (Incomplete)	467	N/A	553 (Incomplete)	N/A	1331 (Incomplete)
Right	1407 (incomplete)	1335 (incomplete)	5129	N/A	N/A	692	692	1670	N/A	1131	3672 (Incomplete)	2169 (Incomplete)	453	N/A	834 (Incomplete)	N/A	630 (Incomplete)
Thorax	N/A	3734	2815	2753	3295	2064	2382	2735	3378	1914 (Incomplete)	N/A	1194 (Incomplete)	3178	1953 (Incomplete)	1566 (Incomplete)	N/A	N/A
Segment Length																	
Metathorax	N/A	1299	638	1032	819	733	733	811	1143	166	N/A	836	947	872	533	N/A	N/A
Mesothorax	N/A	1151	884	847	883	738	738	1016	976	1149	N/A	358 (Incomplete)	1007	885	1033	N/A	N/A
Prothorax	N/A	991	819	874	876	911	911	908	922	N/A	N/A	N/A	785	N/A	N/A	N/A	N/A
Segment Width																	
Metathorax	N/A	1089	931	1084	1160	846	846	817 (Obscured)	809 (Obscured)	693	N/A	446 (Incomplete)	865	826	781	N/A	N/A
Mesothorax	N/A	735	861	1382	1246	884	884	1098	1505	872	N/A	348 (Incomplete)	827	775 (Incomplete)	1032	N/A	N/A
Prothorax	N/A	1095	1344	1636	1206	713 (Incomplete)	713 (Incomplete)	1269	1437	N/A	N/A	N/A	770	N/A	N/A	N/A	N/A
Wing Length																	
Left Hind	N/A	2199 (inc)	N/A	2343	1885	1841	1841	2191	2361	N/A	N/A	N/A	1988	1908	N/A	N/A	N/A
Right Hind	N/A	2271	1953	2262	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1923	2019	N/A	N/A	N/A
Left Fore	N/A	2986 (inc)	?	2080	2511	1770	1770	2591	2659	N/A	N/A	N/A	2373	N/A	N/A	N/A	N/A
Right Fore	N/A	3243	2404	2521	N/A	2107	2107	2393	2480	N/A	N/A	1351 (Incomplete)	2379	2393	N/A	N/A	N/A
Wing Width																	
Left Hind	N/A	424	N/A	754	641	548	548	822	732	N/A	N/A	N/A	764	801	N/A	N/A	N/A
Right Hind	N/A	326	325	835	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	615	783	N/A	N/A	N/A
Left Fore	N/A	534	?	568	509	428	428	611	513	N/A	N/A	N/A	337 (Incomplete)	N/A	N/A	N/A	N/A
Right Fore	N/A	363	527	446	N/A	357	357	406 (Incomplete)	470	N/A	N/A	569	421	487	N/A	N/A	N/A
Head																	
Length	N/A	N/A	648	972	1010	N/A	N/A	810	897	N/A	N/A	N/A	422 (Incomplete)	N/A	N/A	N/A	N/A
Width	N/A	N/A	1025	1100	619 (Incomplete)	N/A	N/A	1104	1261	N/A	N/A	N/A	466	N/A	N/A	N/A	N/A
Antennae																	
Left	N/A	N/A	1291 (Incomplete)	1467 (Incomplete)	1161 (Incomplete)	N/A	N/A	384 (Incomplete)	249 (Incomplete)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Right	N/A	N/A	1291 (Incomplete)	685 (Incomplete)	861 (Incomplete)	N/A	N/A	877 (Incomplete)	923 (Incomplete)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Total Length (Excl Antennae and Cerci:	6062	11289	9771	10242	9242	6004	6323	8609	9173	5890	4071	4844	8736	7018	6383	3149	2510

Appendix 2

Family	Genus	Species	Accession Number	Reference	Country	COI	H3	COI + H3
Austroperlidae	Austropentura	sp	KU366132.1	McCulloch et al. 2016	Australia	X		
Austroperlidae	Austroperla	cyrene	KU366131.1	McCulloch et al. 2016	New Zealand	X		
Austroperlidae	Crypturoperla	paradoxa	KU366174.1	McCulloch et al. 2016	Australia		X	X
Austroperlidae	Klapopteryx	sp	KU366134.1	McCulloch et al. 2016	Argentina	X		
Austroperlidae	Penturoperla	barbata	KU366135.1	McCulloch et al. 2016	Chile	X		
Austroperlidae	Tasmanoperla	larvalis	KU366133.1	McCulloch et al. 2016	Australia	X		
Diamphipnoidae	Diamphipnoa	virescentipennis	KU366116.1	McCulloch et al. 2016	Chile	X		X
Diamphipnoidae	Diamphipnoa	virescentipennis	KU366181.1	McCulloch et al. 2016	Chile		X	X
Eustheniidae	Cosmioperla	australis	KU366180.1	McCulloch et al. 2016	Australia		X	X
Eustheniidae	Eusthenia	costalis	KU366138.1	McCulloch et al. 2016	Australia	X		
Eustheniidae	Stenoperla	helsoni	KU366137.1	McCulloch et al. 2016	New Zealand	X		
Eustheniidae	Stenoperla	maclellani	KU366136.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Acroperla	trivacuata	KU366094.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Acroperla	trivacuata	KU366143.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Acruroperla	atra	KU366105.1	McCulloch et al. 2016	Australia	X		
Gripopterygidae	Alfonsoperla	flinti	KU366095.1	McCulloch et al. 2016	Chile	X		X
Gripopterygidae	Alfonsoperla	flinti	KU366195.1	McCulloch et al. 2016	Chile		X	X
Gripopterygidae	Andiperla	sp	KU366139.1	McCulloch et al. 2016	Argentina		X	X
Gripopterygidae	Antarctoperla	michaelseni	KU366109.1	McCulloch et al. 2016	Argentina	X		X
Gripopterygidae	Antarctoperla	michaelseni	KU366147.1	McCulloch et al. 2016	Argentina		X	X
Gripopterygidae	Apteryoperla	illiesi	KU366091.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Apteryoperla	illiesi	KU366165.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Aubertoperla	sp	KU366096.1	McCulloch et al. 2016	Argentina	X		X
Gripopterygidae	Aubertoperla	sp	KU366158.1	McCulloch et al. 2016	Argentina		X	X
Gripopterygidae	Aucklandobius	gressitti	KU366107.1	McCulloch et al. 2016	Auckaland Island	X		X
Gripopterygidae	Aucklandobius	gressitti	KU366170.1	McCulloch et al. 2016	Auckland Island		X	X
Gripopterygidae	Cardioperla	sp	KU366088.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Cardioperla	sp	KU366155.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Chilenoperla	sp	KU366104.1	McCulloch et al. 2016	Chile	X		X
Gripopterygidae	Chilenoperla	sp	KU366157.1	McCulloch et al. 2016	Chile		X	X
Gripopterygidae	Claudioperla	tigrina	KU366156.1	McCulloch et al. 2016	Peru		X	X
Gripopterygidae	Dinotoperla	fontana	KU366082.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Dinotoperla	fontana	KU366164.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Dinotoperla	serricauda	KU366081.1	McCulloch et al. 2016	Australia	X		X

Gripopterygidae	Dinotoperla	serricauda	KU366141.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Dinotoperla	sp	KU366142.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Dundundra	sp	KU366144.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Eunotoperla	kershawi	KU366083.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Eunotoperla	kershawi	KU366160.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Gripopteryx	sp	KU366103.1	McCulloch et al. 2016	Brazil	X		
Gripopterygidae	Holcoperla	angularis	KU366092.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Holcoperla	angularis	KU366161.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Holcoperla	jacksoni	KU366093.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Illiesoperla	australis	KU366140.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Illiesoperla	sp	KU366086.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Illiesoperla	sp	KU366173.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Kirrama	sp	KU366084.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Kirrama	sp	KU366162.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Leptoperla	sp	KU366153.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Limnoperla	jaffueli	KU366097.1	McCulloch et al. 2016	Argentina	X		X
Gripopterygidae	Limnoperla	jaffueli	KU366166.1	McCulloch et al. 2016	Argentina		X	X
Gripopterygidae	Megaleptoperla	grandis	KU366110.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Megandiperla	sp	KU366100.1	McCulloch et al. 2016	Chile	X		X
Gripopterygidae	Megandiperla	sp	KU366159.1	McCulloch et al. 2016	Chile		X	X
Gripopterygidae	Nescioperla	sp	KU366145.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Nescioperla	sp.	KU366079.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Nesoperla	fulvescens	KU366111.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Nesoperla	patricki	KU366112.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Newmanoperla	exigua	KU366151.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Notoperla	sp	KU366182.1	McCulloch et al. 2016	Argentina		X	X
Gripopterygidae	Pelurgoperla	personata	KU366080.1	McCulloch et al. 2016	Chile	X		X
Gripopterygidae	Pelurgoperla	personata	KU366146.1	McCulloch et al. 2016	Chile		X	X
Gripopterygidae	Rakiuraperla	nudipes	KU366108.1	McCulloch et al. 2016	New.Zealand	X		
Gripopterygidae	Riekoperla	sp	KU366113.1	McCulloch et al. 2016	Australia	X		
Gripopterygidae	Rungaperla	longicauda	KU366106.1	McCulloch et al. 2016	Campbell Island	X		X
Gripopterygidae	Rungaperla	longicauda	KU366171.1	McCulloch et al. 2016	Campbell Island		X	X
Gripopterygidae	Senzilloides	panguipullii	KU366101.1	McCulloch et al. 2016	Argentina	X		X
Gripopterygidae	Senzilloides	panguipullii	KU366175.1	McCulloch et al. 2016	Argentina		X	X
Gripopterygidae	Taraperla	ancilis	KU366114.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Taraperla	howesi	KU366115.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Teutoperla	auberti	KU366102.1	McCulloch et al. 2016	Chile	X		X
Gripopterygidae	Teutoperla	auberti	KU366167.1	McCulloch et al. 2016	Chile		X	X

Gripopterygidae	Trinotoperla	nivata	KU366085.1	McCulloch et al. 2016	Australia	X		X
Gripopterygidae	Trinotoperla	nivata	KU366154.1	McCulloch et al. 2016	Australia		X	X
Gripopterygidae	Vesicaperla	celmisia	KU366089.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Vesicaperla	celmisia	KU366172.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Vesicaperla	trilinea	KU366090.1	McCulloch et al. 2016	New Zealand	X		
Gripopterygidae	Zelandobius	furcillatus	KU366163.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Zelandobius	macburneyi	KU366099.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Zelandobius	macburneyi	KU366169.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Zelandobius	wardi	KU366098.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Zelandobius	wardi	KU366168.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Zelandoperla	agnetis	KU366087.1	McCulloch et al. 2016	New Zealand	X		X
Gripopterygidae	Zelandoperla	agnetis	KU366148.1	McCulloch et al. 2016	New Zealand		X	X
Gripopterygidae	Zelandoperla	fenestrata	KU366149.1	McCulloch et al. 2016	New Zealand		X	X
Nemouridae	Protonemura	intricata	PLCM012-20	Kirkaldy 2020	Germany	X		
Nemouridae	Protonemura	intricata	PLCM011-20	Kirkaldy 2020	Germany	X		
Nemouridae	Protonemura	sp	PLCM013-20	Kirkaldy 2020	Germany	X		
Notonemouridae	Afronemoura	amatolae	MH575281.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Afronemoura	amatolae	MH575280.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Afronemoura	spinulata	MH575283.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Afronemoura	spinulata	MH575282.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	bicornis	MH575246.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	bicornis	MH575245.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	bicornis	MH575244.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	bovina	MH575239.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	bovina	MH575238.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	capensis	MH575241.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	capensis	MH575240.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	capensis	KU366198.1	McCulloch et al. 2016	South Africa		X	X
Notonemouridae	Aphanicerca	chanae	MH575243.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	chanae	MH575242.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	lyrata	MH575248.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	lyrata	MH575247.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	sp	PLCM002-20	Kirkaldy 2020	South Africa	X		X
Notonemouridae	Aphanicerca	sp	MH575235.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	sp	MH575234.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	sp	MH575232.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	sp	MH575225.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicerca	sp	MH575224.1	Stevens et al. 2019	South Africa	X		

Notonemouridae	Aphanicerca	sp	MH575218.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575217.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575216.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575215.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575223.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575222.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575221.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575220.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575219.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575209.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575208.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575213.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575212.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575211.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575210.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575207.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575205.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575204.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575202.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575201.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575200.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575199.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575198.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575206.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575203.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575214.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	PLCM001-20	Kirkaldy 2020	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575231.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575229.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575228.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575227.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575226.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	MH575230.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	sp	PLCM007-20	Kirkaldy 2020	South Africa	X
Notonemouridae	Aphanicerca	sp	PLCM009-20	Kirkaldy 2020	South Africa	X
Notonemouridae	Aphanicerca	uncinata	MH575237.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicerca	uncinata	MH575236.1	Stevens et al. 2019	South Africa	X
Notonemouridae	Aphanicercella	barnardi	MH575252.1	Stevens et al. 2019	South Africa	X

Notonemouridae	Aphanicercella	barnardi	MH575251.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	bifurcata	MH575273.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	bifurcata	MH575272.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	bullata	MH575265.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	bullata	MH575262.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	bullata	MH575259.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	bullata	MH575255.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	cassida	MH575269.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	cassida	MH575268.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	cassida	MH575267.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	cassida	MH575266.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	clavata	MH575257.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	clavata	MH575256.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	clavata	MH575191.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	clavata	MH575190.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	flabellata	MH575264.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	flabellata	MH575263.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	nigra	MH575197.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	nigra	MH575196.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	quadrata	MH575258.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	scutata	MH575261.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	scutata	MH575260.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	securata	MH575254.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	securata	MH575250.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	sp	MH575271.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	sp	MH575270.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	sp.	PLCM004-20	Kirkaldy 2020	South Africa	X		
Notonemouridae	Aphanicercella	spatulata	MH575253.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercella	spatulata	MH575249.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	denticulata	MH575274.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	denticulata	MH575192.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	denticulata	MH575194.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	denticulata	MH575193.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	denticulata	KU366197.1	McCulloch et al. 2016	South Africa		X	X
Notonemouridae	Aphanicercopsis	hawaquae	MH575277.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	hawaquae	MH575276.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	outeniquae	MH575279.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	outeniquae	MH575278.1	Stevens et al. 2019	South Africa	X		

Notonemouridae	Aphanicercopsis	tabularis	MH575275.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Aphanicercopsis	tabularis	MH575195.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Austrocerca	rieki	KU366184.1	McCulloch et al. 2016	Australia		X	X
Notonemouridae	Austrocercella	sp	KU366183.1	McCulloch et al. 2016	Australia		X	X
Notonemouridae	Austrocercoides	zwicki	KU366117.1	McCulloch et al. 2016	Australia	X		X
Notonemouridae	Austrocercoides	zwicki	KU366185.1	McCulloch et al. 2016	Australia		X	X
Notonemouridae	Austronemoura	auberti	KU366119.1	McCulloch et al. 2016	Chile	X		X
Notonemouridae	Austronemoura	auberti	KU366179.1	McCulloch et al. 2016	Chile		X	X
Notonemouridae	Austronemoura	sp	KU366118.1	McCulloch et al. 2016	Argentina	X		X
Notonemouridae	Austronemoura	sp	KU366186.1	McCulloch et al. 2016	Argentina		X	X
Notonemouridae	Balinskycercella	gudu	MH575289.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Balinskycercella	gudu	MH575288.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Balinskycercella	tugelae	MH575291.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Balinskycercella	tugelae	MH575290.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Cristaperla	fimbria	KU366126.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Cristaperla	fimbria	KU366188.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Cristaperla	waharoa	KU366125.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Cristaperla	waharoa	KU366187.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Desmonemoura	brevis	MH575287.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Desmonemoura	brevis	MH575286.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Desmonemoura	brevis	KU366196.1	McCulloch et al. 2016	South Africa		X	X
Notonemouridae	Desmonemoura	pulchellum	MH575285.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Desmonemoura	pulchellum	MH575284.1	Stevens et al. 2019	South Africa	X		
Notonemouridae	Halticoperla	tara	KU366130.1	McCulloch et al. 2016	New Zealand	X		
Notonemouridae	Halticoperla	viridens	KU366129.1	McCulloch et al. 2016	New Zealand	X		
Notonemouridae	Kimminsoperla	albomacula	KU366152.1	McCulloch et al. 2016	Australia		X	X
Notonemouridae	Neofulla	sp	KU366191.1	McCulloch et al. 2016	New Zealand		X	
Notonemouridae	Neonemura	barrosi	KU366150.1	McCulloch et al. 2016	Chile		X	X
Notonemouridae	Neoperla	sp	PLCM006-20	Kirkaldy 2020	South Africa	X		
Notonemouridae	Notonemoura	hendersoni	KU366121.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Notonemoura	hendersoni	KU366192.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Notonemoura	latipennis	KU366120.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Notonemoura	latipennis	MH575189.1	McCulloch et al. 2016	New Zealand	X		
Notonemouridae	Notonemoura	latipennis	KU366190.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Omanuperla	hollowayae	KU366122.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Omanuperla	hollowayae	KU366193.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Spaniocerca	longicauda	KU366127.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Spaniocerca	longicauda	KU366176.1	McCulloch et al. 2016	New Zealand		X	X

Notonemouridae	Spaniocerca	zelandica	KU366128.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Spaniocerca	zelandica	KU366177.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Spaniocercoides	jacksoni	KU366124.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Spaniocercoides	jacksoni	KU366189.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Spaniocercoides	townsendi	KU366123.1	McCulloch et al. 2016	New Zealand	X		X
Notonemouridae	Spaniocercoides	townsendi	KU366178.1	McCulloch et al. 2016	New Zealand		X	X
Notonemouridae	Udamocercia	sp	KU366194.1	McCulloch et al. 2016	New Zealand		X	X
Perlidae	Dinocras	cephalotes	KY261656.1	Morinière et al. 2017	Germany	X		X
Perlidae	Dinocras	cephalotes	KY261163.1	Morinière et al. 2017	Germany	X		
Perlidae	Neoperla	sp	PLCM015-20	Kirkaldy 2020	South Africa	X		X
Perlidae	Neoperla	sp	PLCM016-20	Kirkaldy 2020	South Africa	X		
Perlidae	Neoperla	sp	PLCM017-20	Kirkaldy 2020	South Africa	X		
Perlidae	Neoperla	sp	PLCM010-20	Kirkaldy 2020	Angola	X		X
Perlidae	Neoperla	sp	PLCM008-20	Kirkaldy 2020	Angola	X		
Perlidae	Neoperla	sp	PLCM014-20	Kirkaldy 2020	South Africa	X		
Perlidae	Neoperla	sp	PLCM005-20	Kirkaldy 2020	South Africa	X		
Perlidae	Neoperla	sp.	PLCM003-20	Kirkaldy 2020	South Africa	X		
Perlidae	Perla	abdominalis	KY261924.1	Morinière et al. 2017	Germany	X		X
Perlidae	Perla	abdominalis	KY261418.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	grandis	KY262081.1	Morinière et al. 2017	Germany	X		X
Perlidae	Perla	marginata	KY262574.1	Morinière et al. 2017	Germany	X		X
Perlidae	Perla	marginata	KY262496.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261839.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262309.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262303.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262253.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262205.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262187.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262097.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261775.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261554.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261423.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261410.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261213.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261586.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261309.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY261211.1	Morinière et al. 2017	Germany	X		
Perlidae	Perla	marginata	KY262397.1	Morinière et al. 2017	Germany	X		

Perlidae	Perla	marginata	KY262372.1	Morinière et al. 2017	Germany	X
Perlidae	Perla	marginata	KY262105.1	Morinière et al. 2017	Germany	X
Perlidae	Perla	marginata	KY261198.1	Morinière et al. 2017	Germany	X
Perlidae	Perla	marginata	KY262572.1	Morinière et al. 2017	Germany	X
Perlidae	Perla	marginata	KY262069.1	Morinière et al. 2017	Germany	X
Perlidae	Perla	marginata	KY262370.1	Morinière et al. 2017	Germany	X