# A systematic revision of *Aprionyx* Barnard, an endemic South African genus of Leptophlebiidae (Ephemeroptera)

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Department of Zoology and Entomology
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South Africa

by

Ina Susan Ferreira

ORCID: https://orcid.org/0000-0002-4971-9652

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# Abstract

*Aprionyx* Barnard, 1940 is the most speciose genus of Leptophlebiidae in South Africa and endemic to the southern region. Like the other African Leptophlebiidae, *Aprionyx* has received limited research. The earliest described *Aprionyx* species were based only on adults that were then placed in *Atalophlebia* Eaton, 1881. The first descriptions of nymphs were contributed by Barnard (1932), with the smooth claws of the nymphs being the distinctive feature that allowed him to create *Aprionyx* for all of the South African species placed in *Atalophlebia* at that time.

This research aimed to improve knowledge of *Aprionyx* species by revising its molecular and morphological systematics. Molecular phylogenetic analysis of the COI, 16S and 28S genes generated a species tree illustrating the species' relationships within *Aprionyx*. Species delimitation analyses (bPTP and GMYC methods) were generally congruent with the molecular phylogenetic species composition. The phylogeny identified up to ten potentially new species, a substantial increase in the species diversity of *Aprionyx*.

Review of the descriptions of the eight named *Aprionyx* species resulted in major nomenclatural changes concerning *A. tabularis* (Eaton, 1884), *A. phoeocera* (Lestage, 1924) stat. rev. and *A. intermedius* Barnard, 1932 n. syn. With the redescriptions of all currently known species, the nymph of *A. rubicundus* Barnard, 1932 was described for the first time. An identification key to the species of *Aprionyx* was compiled from newly recognized descriptive characteristics and, for the first time, includes both imagos and nymphs.

Molecular and morphological phylogenetic analyses recovered *Aprionyx* as monophyletic, although this was poorly supported morphologically. The phylogenies were topologically congruent and identified two clear geographically separated clades, a western and eastern *Aprionyx* group. Molecularly, morphologically and geographically, these two clades were recovered as two clearly distinct groups within the genus. The analyses further identified three lineages in *Aprionyx*: *rubicundus*, *pellucidulus* and eastern *Aprionyx*.

The estimated age of *Aprionyx* indicated that initial diversification occurred during the Cretaceous (approximately 100 ma). *Aprionyx peterseni* (Lestage, 1924) was shown as the oldest of the analysed species, and the main drivers to species diversification were possibly the glacial cycles of the Quaternary period, through the mechanism of river anastomosis during

fluctuating sea levels. Further research is needed to strengthen the support for the hypothesis identified in this study, but a substantial foundation has been laid by this work for future research, conservation and environmental decision-making.

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# Declaration

The following manuscript has not been submitted to a university other than Rhodes University, Makhanda, South Africa. The work presented here is that of the author unless otherwise stated.

This work is explicitly and entirely disclaimed as a nomenclatural work for the purposes of zoological nomenclature, and is not published within the meaning of the International Code of Zoological Nomenclature, in line with Article 8 ("What constitutes published work") of that Code (ICZN 2000):

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# Chapter 1: Introduction and background to Afrotropical Leptophlebiidae

## 1.1 General introduction

Mayflies (Ephemeroptera) have been documented since the renowned *Systema Naturae* of Linnaeus, at which time all mayflies were classified under Ephemera: Neuroptera (Linnaeus 1758). In the Afrotropical region, the first mayfly was described in 1839 (Burmeister 1839) – not considering *Prosopistoma variegatum* Latreille, 1833, which was described as a Madagascan crustacean at the time (Latreille 1833).

Globally, Leptophlebiidae is one of the most speciose Ephemeropteran families, demonstrated by its diverse morphology, especially of the nymphal stages. In the Afrotropical region, however, the more than 50 discovered species are only a fraction of at least 420 members, yet it is the most diverse Afrotropical mayfly family after Baetidae (Barber-James et al. 2008, 2013). This contrasts with the rest of the southern hemisphere, the Neotropical and the Australasian regions, where Leptophlebiidae is the family with the highest diversity (Christidis 2005, Domínguez et al. 2006). The lower diversity of Afrotropical Leptophlebiidae may be ascribed to the lack of taxonomic research in the Afrotropics, not only on Leptophlebiidae but on Ephemeroptera as a whole (Elouard 2001, Barber-James and Gattolliat 2012).

The first extensive taxonomical research on South African mayflies was by Barnard (1932, 1940), later complemented by Crass (1947b), both of whom, innovatively, included descriptions of the nymphs. Until now, their work was the most well-regarded taxonomic reference for South African Leptophlebiidae, greatly improving on the earlier research by Eaton (1871, 1881), Esben-Petersen (1913, 1920) and Lestage (1924). The systematics of South African Leptophlebiidae has shown little improvement since Barnard and Crass (Agnew 1961, 1962, Peters and Edmunds 1964, 1970, Demoulin 1970, Peters 1997).

## 1.2 Afrotropical Leptophlebiidae: past to present

Species of Leptophlebiidae were first grouped by Eaton (1884) as "Section 5 of *Leptophlebia*", and the earliest usages of the family name Leptophlebiidae were by Klapálek (1909) and Ulmer (1920). Authorship, however, is given to Banks (1900) who introduced the family-group name as the tribe Leptophlebiini. Taxonomical research on Afrotropical Leptophlebiidae dates back to 1860, when a British researcher named the first species, *Ephemera dislocans* (= *Adenophlebia dislocans* (Walker, 1860)), from South Africa (Walker 1860). A summary of the Afrotropical members of Leptophlebiidae with known species descriptions is compiled in Table 1.1, with the *Aprionyx* Barnard, 1940 species listed as they were in 2023.

Originally, Leptophlebiidae was divided into two subfamilies, with all of the Afrotropical leptophlebiids classified under Atalophlebiinae Peters, 1980 (Peters 1980a). Since then, new subfamilies have been proposed, such as Castanophlebiinae Kluge, 2009 (Kluge 2009, Godunko et al. 2015). The latest classification by Monjardim et al. (2020) restricts the Atalophlebiinae subfamily to the amphinotic taxa of the Australian and Neotropical regions. In the latter study, Choroterpinae Kluge, 2012 was raised from Choroterpini to subfamily status. Most of the Afrotropical genera are thus not assigned to any subfamily, and are kept in Atalophlebiinae s.l. until their relationships can be resolved. The relationship of some genera, such as *Aprionyx* and *Adenophlebia* Eaton, 1881, could not be resolved at a higher level. A few studies, however, argue that *Aprionyx* is perhaps closely related to the Neotropical subfamily, Terpidinae Kluge, 2009 (Kluge 2012, Monjardim et al. 2020).

Leptophlebiids have been described from three Afrotropical islands, Madagascar, Comoros and Seychelles, and include only species endemic to each island. Ulmer (1909, 1920) and Eaton (1913) pioneered the taxonomy of Leptophlebiidae from the Comoros and Seychelles. Subsequent research included some generic changes, and the descriptions of nymphs and an additional species (Peters et al. 1964, Peters and Edmunds 1966, 1970, Peters 1980). Each archipelago has only two described species: *Euthraulus starmuehlneri* (Peters, 1980) and *Thraulus turbinatus* (Ulmer, 1909) from the Comoros; and *Hagenulodes braueri* Ulmer, 1920 and *Maheathraulus scotti* (Eaton, 1913) from the Seychelles. The two genera from the Seychelles are monotypic and consequently endemic to this island, while the Comoros species are in two widely-distributed genera.

Contrary to the Comoros and Seychelles species, the Malagasy Leptophlebiidae remained unknown until after 1950, when Demoulin was the first taxonomist to venture into the Malagasy leptophlebiids (Demoulin 1955a, 1966, 1973, Peters and Edmunds 1964, Akers et al. 2003). Mayflies of Madagascar received increased interest after 1990 owing to the intriguing, diverse and endemic fauna on the island. As it stands, Baetidae is by far the most diverse mayfly family on the island (Elouard et al. 2003, Kaltenbach and Gattolliat 2021). Based on recent collections, it was estimated that Leptophlebiidae would be much more diverse once the undescribed species have been studied (Sartori et al. 2000, Elouard et al. 2003). The high endemism of Malagasy fauna is also portrayed in Leptophlebiidae by the five genera and seven species described thus far. *Nesophlebia* Peters & Edmunds, 1964, *Polythelais* Demoulin, 1973 and *Radima* Akers, Peters & Peters, 2003 are monotypic genera, whereas *Petersophlebia* Demoulin, 1973 and *Ulmerophlebia* Demoulin, 1955 consist of two species each (Table 1.1). *Ulmerophlebia* is an Australian genus and, thus, also the only genus that is not endemic to the island. Demoulin (1966) was uncertain about the placement of his two species, *U. succinea* Demoulin, 1966 and *U. variegata* Demoulin, 1966, in this genus, and they could represent a new genus.

The continental Afrotropical Leptophlebiidae have been largely neglected, and revisionary studies are long overdue (Elouard 2001, Barber-James and Gattolliat 2012). The South African species are generally better known than those from the rest of the continent. A total of 52 species in 17 genera are currently recorded from the Afrotropics, and nearly 40%, 20 species in seven genera, have been recorded from South Africa (Table 1.1; Barber-James et al. 2008, 2013).

The history of the taxonomy of South African Leptophlebiidae began with *Adenophlebia dislocans*, a female specimen collected from the "Cape of Good Hope" (perhaps in the vicinity of Cape Town, but at that time, modern Western, Northern and Eastern Cape provinces formed the region known by the same name) and named *Ephemera dislocans* (Walker 1860). The description was brief, unillustrated, based mainly on pigmentation and insufficient to recognise specimens, although it was clear that this species has distinctive markings on its wings. In 1871, Eaton described a male specimen, *Leptophlebia auriculata* (= *Adenophlebia auriculata* (Eaton, 1871)) and changed *Ephemera dislocans* to *Leptophlebia dislocans* (Eaton 1871). Later, Eaton established the new genus, *Adenophlebia*, for the two *Leptophlebia* Westwood, 1840 species from South Africa, but synonymised *A. auriculata* and

*A. dislocans* (Eaton 1881, 1884). This was based on the fact that *A. auriculata* was known only as a male without wing pigmentation and *A. dislocans* only as a female with wing pigmentation, and since both were from South Africa's Cape region, Eaton assumed this must be a case of sexual dimorphism. Esben-Petersen (1913) first discovered the male of *A. dislocans* but with the male of *A. dislocans* presumed known, named his material *A. westermanni* Esben-Petersen, 1913 and mentioned its uncertain placement in *Adenophlebia*. Lestage agreed and later created a new genus, *Esbenophlebia* Lestage, 1924, for this species (Lestage 1918, 1924). This decision was based only on the differences found between the respective drawings of the forewings by Esben-Petersen and Eaton, but Eaton's drawing is evidently *A. auriculata*, which explains the discrepancies. Lestage also described *Adenophlebia peringueyella* Lestage, 1924, based on both male and female specimens collected from Paarl, but the specimens examined were dry (Lestage 1924). Barnard (1932) was the first to discover the nymphs of *A. dislocans* and *A. peringueyella* with adult-nymph association from reared material. With this knowledge, he also re-established *A. auriculata* and synonymised *A. westermanni* with *A. dislocans*. It was only in 1947 that Crass (1947b) reported on the nymphs of *A. auriculata* and described a new species, *Adenophlebia sylvatica* Crass, 1947. According to him, the only way to distinguish between *A. auriculata*, *A. sylvatica* and *A. peringueyella* nymphs is by comparing the inner lamella of the gills.

The contributions by Barnard (1932) to the taxonomy of South African Leptophlebiidae not only include the first nymphal descriptions, but he also created three new genera, *Aprionyx*, *Castanophlebia* Barnard, 1932 and *Euthraulus* Barnard, 1932. Unlike *Aprionyx,* which was created for established species of *Atalophlebia* Eaton, 1881, *Castanophlebia* and *Euthraulus* were established for new species. *Castanophlebia calida* Barnard, 1932 was argued to belong to a distinct genus based on the claws, wings and genitalia of the imagos and the claws and gills of the nymphs. A second species, *C. albicauda* Barnard, 1940, with very distinctive maxillary palpi, was later discovered (Barnard 1940).

The genus *Euthraulus* was established by Barnard (1932) for *E. elegans* Barnard, 1932. He reasoned that the genus displays similarities to *Thraulus* Eaton, 1881 but is separated based on the imaginal wings and the nymphal gills. *Choroterpes* Eaton, 1881 was not known from Africa until Barnard (1932) described *C. nigrescens* Barnard, 1932 as the first species of this genus from South Africa. The only other South African species was later described by Agnew (1962) as *C. ndebele* Agnew, 1962. Since then, these are the only members of *Choroterpes* in

the Afrotropical region apart from *C. pacis* Sartori, 1991 described from the Arabian Peninsula. Peters and Edmunds (1964) reasoned the similarities between these two genera justify their decision to synonymise *Euthraulus* with *Choroterpes*, but they also suggested keeping the two groups as distinct subgenera, *Choroterpes* (*Choroterpes*) and *Choroterpes* (*Euthraulus*). McCafferty and de Moor (1995) re-established the two groups as genera, but because they did not justify their decision, it has not been widely accepted. In southern Africa, at least, *Choroterpes* and *Euthraulus* are distinctly different from each other, therefore, this manuscript will follow McCafferty and de Moor's (1995) decision.

*Adenophlebiodes* Ulmer, 1924 is an Afrotropical genus created by Ulmer (1924) for *A. ornatus* (Ulmer, 1916) previously placed in *Adenophlebia*. Crass (1947b) created *Euphlebia* Crass, 1947 for a South African species, *A. bicolor* (Crass, 1947) and Edmunds (1953) synonymised this genus with Ulmer's genus. Based on various characters of the wings, Demoulin (1955b) proposed to split *Adenophlebiodes* into two subgenera: *Adenophlebiodes* (*Adenophlebiodes*) and *Adenophlebiodes* (*Hyalophlebia*). Two additional species were described from South Africa: *A. masonella* Agnew, 1961 and *A. patriciae* Agnew, 1962. *Adenophlebiodes masonella* was described in the subgenus *Adenophlebiodes,* and *A. patriciae* in the subgenus *Hyalophlebia* Demoulin, 1955 (Agnew 1961, 1962). Peters and Edmunds (1964) argued that wing pigmentation is the only reliable character that separates the adults, whereas the nymphs are undoubtedly distinguishable, but they did not accept the subgenus status nor did they separate them into genera. Demoulin (1970) separated the groups again into the subgenera that he established earlier. As with *Choroterpes* and *Euthraulus*, McCafferty and de Moor (1995) restored *Adenophlebiodes* and *Hyalophlebia* as two distinct genera without any justification. Until a formal revision, their status remains controversial, but here, the generic status, as suggested by McCafferty and de Moor (1995), will be accepted.

*Aprionyx tabularis* (Eaton, 1884) made its first appearance in Eaton's *Monograph* as the only *Atalophlebia* species from South Africa (Eaton 1884). Esben-Petersen reported a subimago from M'fongosi as possibly belonging to *Atalophlebia* (though it seems that it was assumed to be *A. tabularis* since it was the only species known from the region at the time) and later described three males from Ceres as *A. tabularis* (Esben-Petersen 1913, 1920). The descriptions given by Esben-Petersen were based on a subimago and dried material, yet Lestage (1924) created a new species, *Atalophlebia phoeocera* Lestage, 1924, because of a few inconsistencies with Eaton's description. This decision was based on questionable literature

without consulting the type material, so Barnard (1932) argued it to be a synonym of *A. tabularis*. The status of *A. phoeocera* needs to be revised because, even though Lestage's arguments were rejected by Barnard, Esben-Petersen's description does not agree with Eaton's description. Barnard rightfully rejected Lestage's arguments for a new species and discussed the similarities of his specimens to Esben-Petersen's type material, but his arguments for synonymising *A. phoeocera* need to be re-analysed. Lestage (1924) also described *A. peterseni* (Lestage, 1924) in *Atalophlebia*, which Barnard (1932) later placed in a new genus that he created for all of the South African *Atalophlebia* species.

*Aprionyx natalicus* (Lestage, 1924) was originally described as *Atalophlebia natalica* (Lestage 1924). Barnard (1932) discussed the possibility of the subimago from M'fongosi being this species because it was the only species known from that area, but he could not locate the type specimen to examine it. This conjecture is plausible, but since then, two more species have been discovered in the region, and without the specimen to refer to, it is impossible to identify it with confidence. It was not until 1947 that Crass reported on the nymphs of *A. natalica* and added very little to distinguish it from similar species (Crass 1947b). The name was later grammatically corrected to *A. natalicus* by Demoulin (1970).

*Aprionyx pellucidulus* (Esben-Petersen, 1920) was discovered by Esben-Petersen (1920) and named *Atalophlebia pellucidula*, but the nymph was described only in 1940 (Barnard 1940). When Barnard (1932) moved the species to *Aprionyx*, he also changed the spelling to *A. pellucidulus* to agree with the grammatical gender of the genus. The adults of *Aprionyx argus* Barnard, 1940 were described by Barnard (1940), and Crass (1947b) discovered nymphs from the same locality that he placed in this species. This placement should be accepted with caution because it is justified only by the locality. Barnard (1932) described the different stages of *Aprionyx intermedius* Barnard, 1932, but his description is brief and has some concerns, i.e., the description of the male was incomplete and incorrectly indicated as the female; after the short description of the male, he continued describing the female imago, but in the "remarks" section he stated that only a male imago and subimagos were collected (clearly the species description was largely based on the female subimago). A closer examination of this species is needed to determine its synonymy to *A. tabularis* (*sensu* Eaton, 1884; *nec A. tabularis* Barnard, 1932). Only the adults of *Aprionyx rubicundus* Barnard, 1932 were discovered by Barnard (1932), and he listed a specimen recorded by Lestage (1924) as

potentially this species. *Aprionyx tricuspidatus* Crass, 1947 was the last addition to the genus, with both the adults and nymphs described by Crass (1947b).

## 1.3 Ecology and distribution of *Aprionyx* within southern Africa

Members of *Aprionyx* are classified as paleo-endemic, cold stenothermal species because of their distribution and habitat preferences (Harrison and Agnew 1962, Harrison 1965b, 1965a, 1978, Pescador and Peters 1980). Like previous authors, this study found that *Aprionyx* species have a preference for the cooler mountain streams and foothill river reaches with permanent flow (Barnard 1932, Crass 1947b). Physical environmental data gathered by the author also showed that *Aprionyx* species are generally collected from rivers with lower water temperatures, ranging between 12 and 24°C and Dallas et al. (2015) also reported that *Aprionyx* has a lower upper thermal limit than other leptophlebiids. In these rivers, they favour the slow-flowing marginal areas, backwaters and pools. The nymphs are usually found on stones (Figure 1.1A), but *A. rubicundus* was collected mainly from decomposing plant matter and vegetation (Figure 1.1B). *Aprionyx phoeocera* (= *A. tabularis sensu* Barnard, 1932) seems to prefer deeper waters (roughly 0.5 m) with large stones and boulders where nymphs can find refugium from strong water currents (Figure 1.1C). This species is, therefore, expected in deeper foothill rivers and is believed to be a useful indicator species due to its sensitivity to a deteriorating state of the river, such as from water abstraction (Harrison 1949). Barnard (1932, 1940) and Harrison (1949) found that *A. tabularis* (= *A. intermedius*), *A. pellucidulus* and *A. rubicundus* are fairly uncommon, but their efforts were mainly restricted to trout rivers and the trout fishing seasons. Harrison (1949), therefore, believed that these species might be more common and possibly restricted to the higher mountain streams. *Aprionyx peterseni* appears to be more common than the other Cape species and is often found in large numbers in, for example, the upper Berg River, Western Cape (Figure 1.1D). The ecology and distribution of *A. argus*, *A. natalicus* and *A. tricuspidatus* are not as well known. According to Crass (1947b), they are all mountain stream species, but *A. tricuspidatus* favours shaded streams and *A. natalicus* streams with boulders as the dominant substrate.

*Aprionyx* are thus far known from three South African provinces, Western Cape, Eastern Cape and KwaZulu-Natal, and although no species were apparently recorded from Lesotho, it is possible that some species, such as *A. argus*, *A. natalicus* and *A. tricuspidatus*,

also occur in that country. Five species (*A. phoeocera*, *A. pellucidulus*, *A. peterseni*, *A. rubicundus* and *A. tabularis*) are endemic to the acidic streams of the southern and Western Cape and were collected from streams with a pH between 3.5 and 6.5, corroborating the findings of Harrison (1958) and Harrison and Agnew (1962). The remaining three species (*A. argus*, *A. natalicus* and *A. tricuspidatus*) occur in alkaline rivers, for example, the Tugela River, with a pH above 7 (Oliff 1960).

There is still much to learn about the seasonality of *Aprionyx* species in South Africa, but several species (such as *A. peterseni* and *A. rubicundus*) emerge during spring and summer, whereas *A. phoeocera* adults can be found in autumn (Barnard 1932, Harrison 1949). Early research shows that *A. tabularis* adults emerge between August and February and have been encountered more frequently in the winter and spring months (Barnard 1932, 1940, Harrison 1949, Demoulin 1970). The data thus far suggests that *Aprionyx* are not confined to a single season but probably emerge regularly in small numbers or have multiple emergence events in large numbers during most of the year. Emergence generally peaks in spring, whereas some species emerge during winter. This event generally occurs at dawn or dusk, rarely at night (*A. tabularis*), and the imagos form swarms in late morning to late afternoon, depending on the species (Barnard 1932, Crass 1947b, Harrison 1949).

## 1.4 Abbreviations and symbols

Institution codens follow Evenhuis (2023):

| | |
|---|---|
| AMGS | Albany Museum, Makhanda (formerly Grahamstown), Eastern Cape, South Africa |
| NHMUK | Natural History Museum, London, United Kingdom (formerly the BMNH; British Museum, Natural History) |
| NMSA | KwaZulu-Natal Museum, Pietermaritzburg, KwaZulu-Natal, South Africa |
| SAMC | Iziko Museum, Cape Town, South Africa (formerly the South African Museum) |
| SMNS | State Museum of Natural History, Stuttgart, Germany |

In the descriptions, the following symbols are used to distinguish between objective and subjective synonyms:

≡ Objective synonyms

= Subjective synonyms

The following abbreviations were used in the manuscript to distinguish between *Aprionyx* and *Atalophlebia*:

*A*. *Aprionyx*

*At.* *Atalophlebia*

## 1.5 Scope and aims

Ephemeroptera are well-known as bioindicators in river quality assessments (Dickens and Graham 2002) because of their diverse morphology and adaptations, which result in a range of tolerances to environmental disturbances (Brittain 1982, Bauernfeind and Moog 2000, Beketov 2004). The biodiversity of Leptophlebiidae has been underestimated as a result of its poor research status, evident from the absence of recent taxonomic research and limited taxonomic works after Barnard (1932, 1940) and Crass (1947b). *Aprionyx* is the most diverse leptophlebiid genus in South Africa and is endemic to the southern, southwestern and southeastern parts of the region, with eight taxa currently known (Barber-James 2015). More than half of these species are found in the Greater Cape Floristic Region of the southern and Western Cape. Their sensitivity to environmental changes could be useful as indicators of disturbances in, for example, biomonitoring studies. The revision of the systematics will substantially improve the current taxonomic state of *Aprionyx* and give a clearer view of its species diversity, which can then contribute to decision-making in conservation management. The new knowledge can also be used to establish the conservation status of the species since no South African mayflies are currently assessed for the IUCN Red List (IUCN 2022).

The scope of this study was limited to the genus *Aprionyx* because it has the highest diversity of all Leptophlebiidae in South Africa, and it is endemic to the region. Ultimately, the aim of this thesis is to conduct a revisional study to improve the systematics of the endemic South African genus *Aprionyx* using both molecular and morphological analysis.

The main objectives are to:

- Review the validity of all currently known *Aprionyx* species and report potentially new species using molecular analyses, i.e., phylogenetics and species delimitation methods.
- Provide revised descriptions of the eight established species of *Aprionyx*, assign prospective lectotypes and review nomenclatural queries.
- Generate a dichotomous key to the nymphs and imagos of *Aprionyx*.
- Investigate the relationship between *Aprionyx* species by studying the molecular and morphological phylogenies.
- Compare the molecular and morphological phylogenies for topological congruency, i.e., inspect for agreements on the interspecies relationships and main clades.
- Estimate the age of *Aprionyx* and its species to investigate the biogeography of the genus.

The first step (Chapter 2) of this study will use a molecular approach to identify different lineages in *Aprionyx* by including data on currently described species and potentially new species, which will better define the known species on a molecular level. The next step (Chapter 3) will be to revise the taxonomy of the currently described species and then use the amended taxonomic knowledge in a morphological approach to identify *Aprionyx* lineages. Lastly, Chapter 4 will compare the molecular and morphological relationships side by side to see if the two approaches are in agreement with each other. With the newly acquired molecular and morphological data, this chapter will also take a biogeographical approach to estimate the divergence age of *Aprionyx*.

**Table 1.1 A summary of Leptophlebiidae known from the Afrotropical region (including the southern Arabian Peninsula; Crosskey 1980) before the changes to *Aprionyx* in this study. The valid species names are listed under each genus, followed by the subordinate names if present. The known distribution for each genus is given in square brackets. The statuses of the subordinate names are given in parentheses: combination (comb.), homonym (hom.), original (orig.), renamed (renam.), spelling (spell.) or synonym (syn.). The author(s) and published date of each name, valid or not, are also cited. The type species of each genus is marked with an asterisk (*) if that species occurs in the Afrotropical region.**

**Family** Leptophlebiidae Banks 1900

1. **Genus** *Adenophlebia* Eaton, 1881 [= *Esbenophlebia* Lestage, 1924]
    i. *Adenophlebia auriculata* (Eaton, 1871) [South Africa]
        - *Leptophlebia auriculata* Eaton, 1871 (orig.)
        - *Adenophlebia dislocans* Eaton, 1881 (*nec* Walker, 1860) (syn.)
        - *Adenophlebia dislocans* Eaton, 1884 (*partim* ♂; *nec* Walker, 1860) (syn.)
        - *Adenophlebia dislocans* Esben-Petersen, 1913 (*nec* Walker, 1860) (syn.)
        - *Adenophlebia auriculata* Barnard, 1932 (comb.)
    ii. *Adenophlebia burgeoni* Navás, 1929 [Congo, Uganda, Tanganyika]
        - *Adenophlebia Burgeoni* Navás, 1929 (orig.)
        - *Adenophlebia elegantula* Navás, 1931 (syn.)
        - *Adenophlebia burgeoni* Demoulin, 1955 (spell.)
    iii. **Adenophlebia dislocans* (Walker, 1860) [South Africa]
        - *Ephemera dislocans* Walker, 1860 (orig.)
        - *Leptophlebia dislocans* Eaton, 1871 (comb.)
        - *Adenophlebia dislocans* Eaton, 1881 (comb.)
        - *Adenophlebia westermanni* Esben-Petersen, 1913 (syn.)
        - *Esbenophlebia westermanni* Lestage, 1924 (comb., syn.)
    iv. *Adenophlebia infuscata* Navás, 1936 [Congo]
    v. *Adenophlebia peringueyella* Lestage 1924 [South Africa]
    vi. *Adenophlebia sylvatica* Crass 1947 [South Africa]
2. **Genus** *Adenophlebiodes* Ulmer, 1924 [= *Euphlebia* Crass, 1947]
    i. *Adenophlebiodes adrieni* Elouard-Hideux & Elouard, 1991 [West Africa]

ii. *Adenophlebiodes bicolor* (Crass, 1947) [South Africa]
   - *Euphlebia bicolor* Crass, 1947 (orig.)
   - *Habrophlebia delamarei* Verrier, 1951 (*partim* ♀) (syn.) - Elouard-Hideux & Elouard (1991) explain why they disagree.
   - *Adenophlebiodes bicolor* Edmunds, 1953 (comb.)

iii. *Adenophlebiodes callasae* Elouard-Hideux & Elouard, 1991 [Guinea]

iv. *Adenophlebiodes decoratus* (Navás, 1931) [Central and East Africa]
   - *Adenophlebia decorata* Navás, 1931 (orig.)
   - *Adenophlebia decora* Navás, 1931 (syn.)
   - *Adenophlebiodes decorata* Demoulin, 1955 (comb.)
   - *Adenophlebiodes decoratus* Elouard-Hideux & Elouard, 1991 (*nec* Kimmins, 1956) (spell.)

v. *Adenophlebiodes masonella* Agnew, 1961 [South Africa]

vi. *Adenophlebiodes massirius* Elouard-Hideux & Elouard, 1991 [West Africa]

vii. **Adenophlebiodes ornatus* (Ulmer, 1916) [East, West and Central Africa]
   - *Adenophlebia ornata* Ulmer, 1916 (orig.)
   - *Adenophlebiodes ornata* Ulmer, 1924 (comb.)
   - *Habrophlebia Delamarei* Verrier, 1951 (partim ♂; syn.)
   - *Adenophlebiodes ornatus* Kimmins, 1960 (spell.)

viii. *Adenophlebiodes rubeus* Elouard-Hideux & Elouard, 1991 [West Africa]

3. **Genus** *Aprionyx* Barnard, 1940 [= Barnard, 1932 *nomen nudum*]

i. *Aprionyx argus* Barnard, 1940 [South Africa]

ii. *Aprionyx intermedius* Barnard, 1932 [South Africa]

iii. *Aprionyx natalicus* (Lestage, 1924) [South Africa]
   - *Atalophlebia natalica* Lestage, 1924 (orig.)
   - *Aprionyx natalica* Barnard, 1932 (comb.)
   - *Aprionyx natalicus* Demoulin, 1970 (spell.)

iv. *Aprionyx pellucidulus* (Esben-Petersen, 1920) [South Africa]
   - *Atalophlebia pellucidula* Esben-Petersen, 1920 (orig.)
   - *Aprionyx pellucidulus* Barnard, 1932 (comb.)

v. *Aprionyx peterseni* (Lestage, 1924) [South Africa]
   - *Atalophlebia peterseni* Lestage, 1924 (orig.)
   - *Aprionyx peterseni* Barnard, 1932 (comb.)

vi. *Aprionyx rubicundus* Barnard, 1932 [South Africa]

vii. **Aprionyx tabularis* (Eaton, 1884) [South Africa]
- *Atalophlebia tabularis* Eaton, 1884 (orig.)
- *Atalophlebia tabularis* Esben-Petersen, 1920 (syn.)
- *Atalophlebia phoeocera* Lestage, 1924 (syn.)
- *Aprionyx tabularis* Barnard, 1932 (comb.)

viii. *Aprionyx tricuspidatus* Crass, 1947 [South Africa]

4. **Genus** *Castanophlebia* Barnard, 1932
   i. *Castanophlebia albicauda* Barnard, 1940 [South Africa]
   ii. **Castanophlebia calida* Barnard, 1932 [South Africa]
5. **Genus** *Choroterpes* Eaton, 1881
   i. *Choroterpes ndebele* Agnew, 1962 [South Africa]
   ii. *Choroterpes nigrescens* Barnard, 1932 [South Africa]
   iii. *Choroterpes pacis* Sartori 1991 [Arabian Peninsula]
6. **Genus** *Euthraulus* Barnard, 1932
   i. *Euthraulus bugandensis* Kimmins, 1956 [Uganda, Tanzania]
      - *Euthraulus bugandensis* Kimmins, 1956 (orig.)
      - *Choroterpes bugandensis* Peters & Edmunds, 1964 (comb.)
      - *Euthraulus bugandensis* McCafferty & de Moor, 1995 (comb.)
   ii. *Euthraulus curtus* Kimmins, 1956 [Uganda, Tanzania]
      - *Euthraulus curtus* Kimmins, 1956 (orig.)
      - *Choroterpes curtus* Peters & Edmunds, 1964 (comb.)
      - *Euthraulus curtus* McCafferty & de Moor, 1995 (comb.)
   iii. **Euthraulus elegans* Barnard, 1932 [South Africa]
      - *Euthraulus elegans* Barnard, 1932 (orig.)
      - *Choroterpes elegans* Peters & Edmunds, 1964 (comb.)
      - *Euthraulus elegans* McCafferty & de Moor, 1995 (comb.)
   iv. *Euthraulus magnaculeata* (Kopelke, 1980) [DRC]
      - *Choroterpes magnaculeata* Kopelke, 1980 (orig.)
      - *Euthraulus magnaculeata* McCafferty & de Moor, 1995 (comb.)
   v. *Euthraulus starmuehlneri* (Peters, 1980) [Comoros]
      - *Choroterpes starmuehlneri* Peters, 1980 (orig.)
      - *Euthraulus starmuehlneri* McCafferty & de Moor, 1995 (comb.)
   vi. *Euthraulus tropicalis* Gillies, 1957 [Tanzania]
      - *Euthraulus tropicalis* Gillies, 1957 (orig.)

- *Choroterpes tropicalis* Peters & Edmunds, 1964 (comb.)
- *Euthraulus tropicalis* McCafferty & de Moor, 1995 (comb.)

vii. *Euthraulus usambarae* Gillies, 1957 [Tanzania]

- *Euthraulus usambarae* Gillies, 1957
- *Choroterpes usambarae* Peters & Edmunds, 1964 (comb.)
- *Euthraulus usambarae* McCafferty & de Moor, 1995 (comb.)

7. **Genus** *Fulleta* Navás, 1930 [Congo]
   i. **Fulleta dentata* Navás, 1930
8. **Genus** *Fulletomimus* Demoulin, 1956 [Congo]
   i. **Fulletomimus marlieri* Demoulin, 1956
9. **Genus** *Hagenulodes* Ulmer, 1920 [Seychelles]
   i. **Hagenulodes braueri* Ulmer, 1920 [Seychelles]
      - *Hagenulodes Braueri* Ulmer, 1920 (orig.)
      - *Hagenulodes braueri* Peters & Edmunds, 1964 (spell.)
10. **Genus** *Hyalophlebia* Demoulin, 1955
    i. *Hyalophlebia demoulini* (Kimmins, 1960) [Uganda]
       - *Adenophlebiodes decoratus* Kimmins, 1956 (*nec* Navás, 1931) (hom. renam.)
       - *Adenophlebiodes demoulini* Kimmins, 1960 (orig.)
       - *Hyalophlebia demoulini* McCafferty & de Moor, 1995 (comb.)
    ii. **Hyalophlebia dentifera* (Navás, 1930) [Congo]
       - *Atalophlebia dentifera* Navás, 1930 (orig.)
       - *Adenophlebiodes dentifera* Demoulin, 1955 (comb.)
       - *Hyalophlebia dentifera* McCafferty & de Moor, 1995 (comb.)
    iii. *Hyalophlebia patriciae* (Agnew, 1962) [South Africa]
       - *Adenophlebiodes patriciae* Agnew, 1962 (orig.)
       - *Hyalophlebia patriciae* McCafferty & de Moor, 1995 (comb.)
    iv. *Hyalophlebia seydeli* (Navás, 1930) [Congo]
       - *Adenophlebia Seydeli* Navás, 1930 (orig.)
       - *Adenophlebiodes seydeli* Demoulin, 1955 (comb.)
       - *Hyalophlebia seydeli* McCafferty & de Moor, 1995 (comb.)
11. **Genus** *Maheathraulus* Peters, Gillies & Edmunds, 1964
    i. **Maheathraulus scotti* (Eaton, 1913) [Seychelles]
       - *Hagenulus scotti* Eaton, 1913 (orig.)
       - *Maheathraulus scotti* Peters, Gillies & Edmunds, 1964 (comb.)

12. **Genus** *Nesophlebia* Peters & Edmunds, 1964
    i. **Nesophlebia adusta* Peters & Edmunds, 1964 [Madagascar]
13. **Genus** *Petersophlebia* Demoulin, 1973
    i. *Petersophlebia inaequalis* (Demoulin, 1955) [Madagascar]
        - *Atalophlebiodes inequalis* Demoulin, 1955 (orig.)
        - *Atalophlebiodes inaequalis* Demoulin, 1970 (spell.)
        - *Petersophlebia inaequalis* Demoulin, 1973 (comb.)
    ii. **Petersophlebia insularis* Demoulin, 1973 [Madagascar]
14. **Genus** *Polythelais* Demoulin, 1973
    i. **Polythelais digitata* Demoulin, 1973 [Madagascar]
15. **Genus** *Radima* Akers, Peters & Peters, 2003
    i. **Radima edmundsorum* Akers, Peters & Peters, 2003 [Madagascar]
16. **Genus** *Thraulus* Eaton, 1881 [= *Masharikella* Peters, Gillies & Edmunds, 1964]
    i. *Thraulus fasciatus* (Kimmins, 1956) [Uganda, Tanzania]
        - *Hagenulus fasciatus* Kimmins, 1956 (orig.)
        - *Masharikella fasciata* Peters, Gillies & Edmunds, 1964 (comb.)
        - *Thraulus fasciatus* Peters & Edmunds, 1970 (comb.)
    ii. *Thraulus torrentis* (Gillies, 1964) [Tanzania]
        - *Masharikella torrentis* Gillies, 1964 (orig.)
        - *Thraulus torrentis* Peters & Edmunds, 1970 (comb.)
    iii. *Thraulus turbinatus* (Ulmer, 1909) [Comoros]
        - *Hagenulus turbinatus* Ulmer, 1909 (orig.)
        - *Masharikella turbinata* Peters, Gillies & Edmunds, 1964 (comb.)
        - *Thraulus turbinatus* Peters & Edmunds, 1970 (comb.)
17. **Genus** *Ulmerophlebia* Demoulin, 1955 (Generic placement uncertain)
    i. *Ulmerophlebia succinea* Demoulin, 1966 [Madagascar]
    ii. *Ulmerophlebia variegata* Demoulin, 1966 [Madagascar]


**Figure 1.1 Specific habitat types where *Aprionyx* nymphs were collected: A) *A. peterseni* nymphs were usually collected from stones, under which they prefer to retreat; B) *A. rubicundus* was particularly collected at pools with decomposed plant material and vegetation, such as in the Swartboskloof stream at Jonkershoek Nature Reserve, Stellenbosch; C) *A. phoeocera* was collected from sites with a higher water depth (about 50 cm or more) under larger stones and boulders, such as from the Wemmershoek River at La Ferme, Franschhoek; D) *A. peterseni* can occur in abundance at favourable sites, like the upper Berg River above the Berg River dam. Plentiful nymphs and adults were collected here, and the stones had many nymphal exuviae from recent emergence events, which indicated an ideal site for this species to flourish. © IS Ferreira.**

# Chapter 2: Molecular phylogeny of *Aprionyx*

## 2.1 Introduction

Molecular approaches have become markedly more prevalent in mayfly systematics compared to a few decades ago, yet their application remains limited in South African mayfly research. The prong-gills (Leptophlebiidae), for example, are one family that has not been studied on a molecular scale in this region but has become more popular in the Australian and Neotropical regions (Baker et al. 2004, O'Donnell and Jockusch 2008, Monjardim et al. 2020, Salles et al. 2020, Gatti et al. 2021, 2022, Hrivniak et al. 2023, and more).

*Aprionyx* has long been suggested to have stronger affinities with the Australian, New Zealand and southern South American Leptophlebiidae than with the northern hemisphere taxa (Peters and Edmunds 1964). Morphological phylogenetic studies have previously been used to study the possible relationships within the Southern Hemisphere Leptophlebiidae, of which only a few included the South African genus *Aprionyx* (Pescador and Peters 1980, Finlay and Bae 2008). Peters and Edmunds (1970) also presented a plausible morphological phylogeny of the Eastern Hemisphere Leptophlebiidae but excluded Australian and New Zealand genera. Recently, more extensive molecular phylogenetics on Leptophlebiidae included genera from across the world (including *Aprionyx*) to advance our understanding of the relationships within the family (O'Donnell and Jockusch 2008, Monjardim et al. 2020). Only selected South African genera (from museum donations or sequences on GenBank) have been represented in these studies, and they were not able to resolve the relationship of *Aprionyx* within Atalophlebiinae.

The evolutionary relationships within *Aprionyx* have not been studied until now. The aim of this chapter is, therefore, to reveal the species composition of the genus by applying molecular approaches and investigating the potential relationships between those species. *Aprionyx* is the most diverse genus of the South African leptophlebiids, and their endemicity (majority to the southern and Western Cape region) suggests that the diversity of its species will surely increase because they have been understudied. A revision of the genus will help to validate the current species and identify any potentially new species by implementing various species delimitation methods.

## 2.2 Materials and methods

### *2.2.1 Selection of taxa and preparation of material*

A total of 89 *Aprionyx*, *Choroterpes* and *Euthraulus* specimens were selected for DNA extraction (Appendix A). Samples were collected as per Chapter 3 and supplemented from the AMGS collection. The *Aprionyx* specimens selected from these samples represented five (*A. natalicus*, *A. pellucidulus*, *A. peterseni*, *A. rubicundus*, and *A. phoeocera*) of the eight recognized species from 30 sites across the Western Cape, Eastern Cape and KwaZulu-Natal. The remaining three species (*A. argus*, *A. tabularis* and *A. tricuspidatus*) were not identified among the specimens. Various other *Aprionyx* species that could not be identified (*Aprionyx* sp. 1 to *Aprionyx* sp. 5 in Appendix A) and specimens identified with uncertainty, i.e., *A.* cf. *tricuspidatus*, were also included.

All specimens selected for DNA extraction were photographed before being dissected. Images of at least a dorsal or lateral habitus were captured with a Nikon 7200 DSRL with a 105 mm micro lens. This was connected to a computer with Helicon Remote v3.9.10W software (www.heliconsoft.com/heliconsoft-products/helicon-remote) for remote imaging and then stacked with Helicon Focus v7.7.4 software (www.heliconsoft.com/heliconsoft-products/helicon-focus) to create images with a greater depth of focus. The tissue from at least three legs was used to extract DNA. In rare circumstances, up to six legs were used for very small specimens; when all the legs were broken off and missing, tissue from the thorax was used. The rest of the specimen was set aside for further morphological examination or for future research. In the morphological analyses (Chapter 3), these specimens were examined in addition to other material, such as the type specimens and recently collected material. Dissection equipment was sterilized with heat from a flame between working on each specimen to prevent cross-contamination.

### *2.2.2 DNA extraction, amplification and sequencing*

The DNeasy Blood and Tissue Kit (QIAGEN) was used to successfully extract DNA from 89 specimens using the non-destructive spin-column protocol for animal tissues provided with the kit. Samples were lysed overnight for about 17 hours at 56°C (70 swivel speed) and eluted with 100 µl elution buffer. The concentration (ng/µl) and absorbance ratio (A260/A280) were measured with a Thermo Scientific NanoDrop 2000 Spectrophotometer to determine the DNA quality.

Three gene regions were targeted for amplification and sequencing: the mitochondrial genes cytochrome oxidase subunit I (COI) and 16S rRNA (16S) and the nuclear gene 28S rRNA (28S), with the primers specified in Table 2.1. Polymerase chain reaction (PCR) was performed in 25 µl of a solution containing DNA template (1-5 µl), relevant forward and reverse primers (initially 3 µl, later decreased to 0.5 µl each), KAPA Taq ReadyMix (12,5 µl) and PCR-grade water. The PCR conditions were optimized for each primer pair, as listed in Table 2.2.

DNA amplifications were confirmed by standard agarose gel electrophoresis using a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The successfully amplified PCR products were sent for purification and sequencing to Macrogen (Netherlands) or Stellenbosch University Central Analytical Facilities (CAF; South Africa). Extraction and PCR protocols were conducted in the molecular laboratory at the South African Institute for Aquatic Biodiversity (SAIAB), managed by Ms Taryn Bodill. Sequences submitted to GenBank are recorded in Appendix A with accession numbers.

### *2.2.3 Additional Aprionyx COI sequences*

Dr Arnold Staniczek (State Museum of Natural History, Stuttgart, Germany; SMNS) provided additional forward and reverse COI sequences for producing consensus sequences of unidentified *Aprionyx* specimens collected from the Western and Eastern Cape provinces. Sixteen sequences (*Aprionyx* sp. 6 to *Aprionyx* sp. 11 in Appendix A), representative of each site, were selected for this study. The SMNS also photographed the habitus of specimens representative of any morphospecies identified.

### *2.2.4 Outgroups*

*Choroterpes* and *Euthraulus* were included as outgroup taxa from southern Africa; the former mainly came from the Western Cape and the latter from Western Cape, KwaZulu-Natal, Kruger National Park, and Angola. Additional outgroup taxa from other countries were downloaded from GenBank (Benson et al. 2013). The Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) was used to find sequences highly similar to *Aprionyx* (using one representative *Aprionyx* sequence). In other words, the outgroups were selected based on sequence similarity by considering the top results from the BLAST searches of each gene and not literature-based searches. Four Leptophlebiidae genera (*Austrophlebioides* Campbell & Suter, 1988, *Atalophlebia*, *Penaphlebia* Peters & Edmunds, 1972 and *Thraulodes* Ulmer, 1920)

were selected from the BLAST results as the outgroup taxa, but they are not represented by all three genes (Appendix A). listed among the most similar sequences for each of the genes,

### *2.2.5 Preparation and alignment of sequences and phylogenetic reconstruction*

Successful sequences were trimmed and cleaned (i.e., base calling; any ambiguous bases were indicated as such using 'N') in Chromas v2.6.6 (Technelysium), and then all sequences of each gene were concatenated using BioEdit v7.0.5.3 (Hall 1999). Consensus sequences of the additional COI sequences from the SMNS were created with BioEdit before being added to the COI dataset. Sequence alignment was completed with the multiple sequence alignment online server, MAFFT v7 (Katoh et al. 2019). The congruence between the three genes was determined with the Incongruence Length Difference (ILD) test (Farris et al. 1994) by performing a partition homogeneity test in PAUP* 4.0a169 (Swofford 2002) with 1000 replicates.

Phylogenetic inference analysis was performed with the COI, 16S and 28S datasets individually and COI/16S/28S-combined (i.e., the three genes concatenated into one dataset). Maximum likelihood (ML) inference trees were created with the IQ-TREE online web server, CIBIV, Austria (Nguyen et al. 2015). IQ-TREE used ModelFinder (Kalyaanamoorthy et al. 2017) to find the best evolutionary models (Table 2.3) and the edge-linked partition model (Chernomor et al. 2016) to allow for different evolutionary rates between various partitions in multi-gene and codon-position analysis. Branch support was evaluated in IQ-TREE with the ultrafast bootstrap (UFBoot) approximation (Hoang et al. 2018) and 1000 bootstrap replicates. The recommended branch support for accepting the reliability of a clade is 95% or above for UFBoot values (Minh et al. 2013), consequently the support values will be categorized as follows: strong (≥ 80%), moderate (70-80%), weak (50-70%) or no support (< 50%). Bayesian inference (BI) analyses were run in MrBayes v3.2.7a (Ronquist et al. 2012) through the CIPRES Science Gateway v3.3 (Miller et al. 2010). Different models were applied to each partition/gene based on the best model selection by IQ-TREE (Table 2.3). The Markov Chain Monte Carlo (MCMC) sampling was run with 10 million generations and a 0.25 burn-in fraction. The MCMC trace files were viewed on Tracer v1.7.2 (Rambaut et al. 2018) to validate the Effective Sample Size (ESS) >200. The branch support for accepting the reliability of a clade is accepted as 0.95 or above for probability values in BI analyses (Hillis and Bull 1993) and the support values were categorized as follows: strong (≥ 0.95), moderate (0.8-0.95), weak (0.5-0.8) or no support (< 0.5). Phylogenetic trees were viewed and rooted in FigTree v1.4.4

and annotated with the web-based Interactive Tree of Life (iTOL, http://itol.embl.de) and Inkscape v1.1 (https://inkscape.org) software.

### *2.2.6 Species delimitation*

Two species delimitation approaches were used to determine putative species boundaries: the Poisson Tree Processes (PTP) and Generalized Mixed Yule Coalescent (GMYC) models. The two single-locus methods were executed on COI and 16S, whereas 28S was considered too conserved to delineate species in this scenario (Appendix G). For the Bayesian PTP (bPTP) analysis (Zhang et al. 2013), the rooted BI trees were used as input to conduct calculations on the bPTP webserver (http://species.h-its.org/ptp/), with 200 000 MCMC generations, thinning of 100 and a 0.2 burn-in fraction. The trace files confirmed the MCMC chain convergence. The maximum likelihood and Bayesian solutions were in agreement; thus, a consensus bPTP delimitation outcome is provided.

For the GMYC (Pons et al. 2006) calculations, an ultrametric phylogenetic tree was obtained for each gene under a strict molecular clock. Parameters were specified in Bayesian Evolutionary Analysis Utility (BEAUti) v2.7.4 (Bouckaert et al. 2014, 2019) and executed in Bayesian Evolutionary Analysis Sampling Trees (BEAST) v2.7.4 (Drummond and Rambaut 2007, Suchard and Rambaut 2009, Bouckaert et al. 2014, 2019) on CIPRES Science Gateway to generate the trees. The substitution models were specified as per the BI models (Table 2.3), while the other settings were kept as default, including the Yule tree prior model and 10 million MCMC generations. The maximum clade credibility (MCC) tree was produced in TreeAnnotator v2.7.4 (Bouckaert et al. 2014, 2019) with a 25% burn-in and mean node heights. The MCC tree was used in the GMYC analysis, which was conducted in R v4.3.0 (R Core Team 2023) with the *splits* package (Ezard et al. 2021).

A third method, the multi-locus Bayesian Phylogenetics and Phylogeography (BPP) method, was also considered to cross-validate the species delimitations based on the ML tree but was later rejected for reasons mentioned below. The BPP v4.6.2 software was used to run the A10 analysis for species delimitation with the ML inferred tree as the user-specified tree (Yang and Rannala 2010, Rannala and Yang 2013). The reversible-jump MCMC (rjMCMC) algorithm 0 with $e = 2$ was used with the following parameters: *thetaprior* = 2, 0.001; *thauprior* = 2, 0.01; *burn-in* = 10 000; *sample frequency* = 2 and *number of samples* = 100 000. The *finetune* parameter was set to automatically adjust the step lengths, and the initial steps were specified as follows: 99, 0.00055, 0, 99, 0.085. The analysis was run three times to check that

the results between the runs concurred. The BPP analysis was in agreement with the molecular phylogeny, corresponding to the 15 species clades, and it was moderately supported (PP = 0.79-0.81) in all three runs with well-supported (PPs of 0.98-1.0) ancestral nodes. While the BPP method is considered a good choice for multi-locus analyses, the results were excluded here because of the unreliability when there are missing data, i.e., species data that do not overlap (Yang 2015, Flouri et al. 2018, Luo et al. 2018). In this case, the data consist of four species (*Aprionyx* sp. 7, 8, 10 and 11) with only COI sequences and four species (*Aprionyx* sp. 2, 3 and 5 and *A. natalicus*) with only 16S and 28S sequences, thus no overlap of data between the two groups. The decision was therefore made to exclude the multi-locus BPP method and only use the single-locus methods, bPTP and GMYC.

## 2.3 Results

### *2.3.1 Data characteristics*

The final DNA data included a total of 109 specimens (89 of this study, section 2.2.1; 16 of the SMNS, section 2.2.3; 4 outgroups from BLAST, section 2.2.4) with sequences for at least one gene region (Appendix A) and the trimmed sequence length of each gene was: COI = 639 bp; 16S = 486 bp; 28S = 593 bp. *Aprionyx* comprised 69 specimens, of which 41 had sequences for all three genes, whereas 16 (SMNS sequences) had only COI sequences, two had only 16S sequences, and one had only a 28S sequence. The COI/16S/28S-combined dataset contained 22 sequences (three in the outgroup) with more than 60% missing data and 28 sequences (19 in the outgroup) with 20-40% missing, attributable to absent sequences from one or two genes. The COI, 16S and 28S were not significantly incongruent according to the partition homogeneity test ($p = 0.839$) and were therefore analysed as a concatenated dataset. Protein-codon partitioning was applied to the COI gene region to compensate for different evolutionary rates between the first (COI $1^{st}$), second (COI $2^{nd}$) and third (COI $3^{rd}$) codon positions (CP). Each CP was tested separately to find the best model for each (Table 2.3). The COI gene had the highest percentage of parsimony-informative sites (37%) compared to 16S and 28S. Codon partitioning revealed that the COI $2^{nd}$ CP was not parsimony-informative (0%), and in the COI $3^{rd}$ CP, 92% of its sites were parsimony-informative. In other words, the COI phylogeny was mostly the result of COI $3^{rd}$ CP, while COI $2^{nd}$ CP had no effect on the outcome.

The 28S gene contained the lowest proportion of parsimony-informative sites (10%) of the three genes (Table 2.3).

### *2.3.2 Phylogenetic reconstruction*

The phylogenetic reconstruction of *Aprionyx* recovered 15 species clades: five named species (*A. natalicus*, *A. pellucidulus*, *A. peterseni*, *A. phoeocera* and *A. rubicundus*), and an additional ten species, including *A.* cf. *tricuspidatus* and nine unknown *Aprionyx* species, i.e., sp. 1-5, 7, 8, 10 and 11 (Figure 2.1). The identities of two unidentified taxa (additional sequences from SMNS) were resolved through the phylogenetic analysis, i.e., *Aprionyx* sp. 6 as *A. peterseni* and *Aprionyx* sp. 9 as *A. rubicundus*. *Aprionyx* spp. 2-5 and *Aprionyx* sp. 8 were recovered as singleton species (i.e., represented by a single specimen). All of the other species were resolved as monophyletic clades and were mostly well supported by the total evidence analyses (the majority with UFBoot = 98-100% and PP = 0.96-1.00). The exceptions were *Aprionyx* sp. 11, with slightly lower support at UFBoot = 91% and PP = 0.92, and *A. natalicus*, with weak support in the BI analysis (PP = 0.53). The phylogenetic results grouped the *Aprionyx* species into two distinct clades: Clade A includes specimens distributed in the Western Cape and western Eastern Cape, hence it will be termed the western *Aprionyx* group; and Clade B, with specimens distributed in the eastern Eastern Cape and KwaZulu-Natal will be termed the eastern *Aprionyx* group (Figure 2.2A).

The topologies of the ML and BI trees in each data set (COI, 16S, 28S and COI/16S/28S-combined) are generally similar (Figure 2.1; Appendix E-Appendix H). The differences were mostly related to the BI analyses that were often unable to resolve the relationships between some of the clades, resulting in polytomies. The differences in topology are not as subtle between each scenario. While COI, 16S and 28S each yielded 11 species, the composition thereof in COI varies from the other two genes. In the COI analyses, the most striking discrepancies are that (1) *Aprionyx* sp. 7 was not recovered in the *rubicundus* group but as sister to the *A. pellucidulus* group and (2) *A. phoeocera* was not recovered in the *pellucidulus* group (Appendix E). The 16S phylogenies also did not recover *A. phoeocera* in the *pellucidulus* group, and *A. peterseni* was recovered in the *rubicundus* group (Appendix F). The 28S Bayesian and likelihood inferences deviated the most because 28S supplied little resolution to species but contributed to some of the ancestral nodes in the species trees (COI/16S/28S-combined). In both of the 28S phylogenies, the western *Aprionyx* group consisted of two polyphyletic groups: the first consisting of *A. rubicundus*, *Aprionyx* sp. 3,

*Aprionyx* sp. 4 and *Aprionyx* sp. 5; the second included *A. peterseni*, *A. pellucidulus*, *A. phoeocera*, *Aprionyx* sp. 1 and *Aprionyx* sp. 2 (Appendix G). The topologies of the resulting gene trees are, however, still generally comparable to each other and the species trees, with three distinct clades discernable in all scenarios: the (1) *rubicundus* group, (2) *pellucidulus* group and (3) eastern *Aprionyx* group (Clade B). The species composition of the *rubicundus* and *pellucidulus* groups is not always consistent, but in all scenarios, these two groups were recovered in the western *Aprionyx* clade (Clade A).

*Aprionyx* was recovered as monophyletic by the total evidence analyses (ML and BI of COI/16S/28S-combined) and was particularly well supported by the BI analysis with PP = 0.97 (Figure 2.1; Appendix H). For the ML inference, support was lower than the recommended 95% (Minh et al. 2013) but still relatively high with UFBoot = 87%. However, when 28S, 16S and COI were analysed separately, it did not always recover *Aprionyx* as monophyletic, and its monophyly was well supported by 16S only (ML: UFBoot = 98%; BI: PP = 1.00; Appendix F). Neither ML nor BI analyses of COI recovered *Aprionyx* as monophyletic but a polyphyletic clade with weak support (UFBoot = 47%; PP = 0.54), which includes *Thraulodes* as sister to Clade B (Appendix E). The ML inference of 28S agrees with the monophyly of *Aprionyx*, though support is weak (UFBoot = 58%), whereas the BI phylogeny recovered *Aprionyx* in a polytomy with the outgroups (Appendix G).

The western *Aprionyx* clade (Clade A) was recovered in all eight analyses, except that in the COI BI phylogeny *A. phoeocera* was not in this clade but in a polytomy with Clade A and Clade B. The clade was well supported by the total evidence BI (PP = 1.00) and ML inference (UFBoot = 94%) analyses and consists of six clades and five singletons. *Aprionyx phoeocera*, *A. pellucidulus*, *A. peterseni*, *A. rubicundus*, *Aprionyx* sp. 1 and *Aprionyx* sp. 7 were strongly supported as species clades by all analyses except by 28S (UFBoot > 97%; PP > 0.96). The total evidence analyses recovered five unknown species (*Aprionyx* sp. 3, 4, 5, 7 and 8) in a clade with *A. rubicundus*, the *rubicundus* group (Figure 2.1), which is well supported by the BI analysis (PP = 0.99) but slightly lower in the ML analysis (UFBoot = 91%). The rest of the western *Aprionyx* species (*Aprionyx* sp. 1, *Aprionyx* sp. 2, *A. pellucidulus*, *A. peterseni* and *A. phoeocera*) were recovered in a moderately supported clade (UFBoot = 78%; PP = 0.88), the *pellucidulus* group (Figure 2.1). The interspecies relationships in these two clades were not always well supported.

The eastern *Aprionyx* clade (Clade B) was highly supported as monophyletic by all analyses (UFBoot ≥ 99% and PP = 1.0) except by 28S ML and BI, but species relationships within the clade were not resolved. This clade consists of four putative species, i.e., *A. natalicus*, *A.* cf. *tricuspidatus*, *Aprionyx* sp. 10 and *Aprionyx* sp. 11. *Aprionyx* cf. *tricuspidatus* and *Aprionyx* sp. 10 were highly supported with UFBoot > 98% and PP > 0.95. Support for *Aprionyx* sp. 11 was lower at 91% UFBoot and 0.92 PP, but *A. natalicus* received high support only in the ML reconstruction (UFBoot = 99%; PP = 0.53; Figure 2.1). Two taxa, *Aprionyx* sp. 10 and *Aprionyx* sp. 11, have only COI sequences, and *A. natalicus* was represented only by 28S and one 16S sequences. This suggests that one of the unnamed species might be *A. natalicus* because the sequences of *Aprionyx* sp. 10 and *Aprionyx* sp. 11 do not overlap with *A. natalicus*. The ML species tree showed that *A. natalicus* is closer to *Aprionyx* sp. 11 than *Aprionyx* sp. 10; therefore, the possibility of *Aprionyx* sp. 11 belonging to *A. natalicus* cannot be eliminated (Figure 2.1). The BI phylogeny recovered all four species in a polytomy because the relationships between them could not be established due to the lack of data in this clade (Appendix H).

### *2.3.3 Species delimitation*

The species delimitation results for the bPTP and GMYC methods are presented in Figure 2.1. The bPTP and GMYC analyses of 16S both identified 12 putative species. Eleven corresponded to the species identified in the molecular phylogeny, but in both analyses, *A. peterseni* consisted of two species. The bPTP analysis recovered a single specimen (LEP014 collected from the Hugos River near Paarl, Western Cape) as a different species entity within *A. peterseni*, while the GMYC analysis identified three specimens (LEP073, LEP074 and LEP075) as a separate species entity of the *A. peterseni* lineage. The additional species entity of the GMYC analyses are specimens collected from two sites in the Huis River at Barrydale, Western Cape (Figure 2.2C). This Barrydale population is also fairly well supported in the total evidence phylogeny (UFBoot = 96%; PP = 0.91; Figure 2.1). The COI bPTP and GMYC analyses identified 11 putative species, with no deviation from the respective molecular species, as identified by the species tree.

## 2.4 Discussion

The species trees (Figure 2.1; Appendix H) support *Aprionyx* as a genus, recovering *Aprionyx* as a monophyletic clade. The COI gene trees did not support monophyly, but in both phylogenies (ML and BI), the multi-genus (*Aprionyx* and *Thraulodes*) clade was poorly supported. This suggests that *Thraulodes* is not an ideal outgroup taxon for the COI region because the species trees recovered *Thraulodes* as distinctly different from *Aprionyx*. The main problem is likely the variability of COI at higher taxonomic levels and the saturation caused by homoplasies found between taxa that are more distantly related (Deagle et al. 2014). In other words, COI phylogenies can result in misleading evolutionary relationships between genera with homoplasies. To address this, more outgroups should be included in an effort to better resolve the deeper relationships between *Aprionyx* and other taxa. The 28S gene trees did not agree on the monophyly of *Aprionyx* or were poorly supported. The disagreements between the gene trees were overcome in the species tree that combines all of the data, but the likelihood of *Aprionyx* being non-monophyletic should not be ignored, because two distinctly different clusters are recognised by the molecular phylogenies. Unlike the monophyly of *Aprionyx*, the two major lineages (eastern and western *Aprionyx* groups) are generally better supported by the species and gene trees. In other words, it supports the probability of two separate genera, which are also distinct in their geographical distribution (Figure 2.2A). The next chapter (Chapter 3) also discusses the morphology distinguishing the western and eastern *Aprionyx* groups.

The molecular analyses were able to distinguish 15 *Aprionyx* species, but only five of them were among the eight presently described species. *Aprionyx tabularis*, *A. argus* and *A. tricuspidatus* were not identified among the analysed specimens, but the potential of being one of the unknown species should not be disregarded. Accurately identifying the latter two species with the insufficient data provided by the current descriptions is particularly difficult, and therefore, they remain problematic to distinguish (Chapter 3 for the morphological revision). Ten unnamed species were also identified by the molecular analyses, but for different reasons, these species could not be confidently identified as one of the known species and were consequently excluded from the morphological examination. Several were represented by nymphs only and, in some cases, were too immature for the morphological analyses. The descriptions of *A. argus* and *A. natalicus*, adults and nymphs, make it practically impossible to confidently distinguish them and *A. tricuspidatus* from each other (Barnard 1940, Crass 1947b;

Chapter 3), nonetheless, adults of *A. natalicus* were positively identified. Nymphs collected at Kranskop, KwaZulu-Natal (LEP 024 and LEP 025) could not be identified with certainty but resemble *A. tricuspidatus* and *A. natalicus*. The type locality for *A. natalicus* is at Kranskop, but the molecular analyses recovered this species and *A. natalicus* as two species entities. For these reasons, the Kranskop specimens were named *A.* cf. *tricuspidatus*, as they are more likely to belong to *A. tricuspidatus* if they are not in fact a new species.

As explained in the results (section 2.3.2), *Aprionyx* sp. 10 or *Aprionyx* sp. 11 (more likely the latter) could be *A. natalicus,* but data is insufficient to draw certain conclusions. One of these two species could also be *A. tricuspidatus* if it turns out that *A.* cf. *tricuspidatus* is a new species because *A. tricuspidatus* is distributed in the same locality (Figure 2.2I-J). The *A.* cf. *tricuspidatus* nymphs are not *A. argus* because the gills (Figure 2.3J) are different from Crass' (1947b) description. The current range of the distribution of *A. argus* is still unknown since it has only been recorded from the Drakensberg (Figure 2.2G). Therefore, the possibility that *Aprionyx* sp. 10 or *Aprionyx* sp. 11 are *A. argus* remains. Only photographic evidence was obtained (from the SMNS) for morphological investigations of *Aprionyx* sp. 10 and *Aprionyx* sp. 11 (Figure 2.3H-I). Close inspection of these photographs suggests that *Aprionyx* sp. 10 is more likely a new species because the gills are unlike the other eastern *Aprionyx* species, with the lateral filamentous protrusions much shorter than the median protrusions (Figure 2.3H). Taking this into account, the eastern *Aprionyx* group is expected to have at least one new species but could have up to three new species, bringing the total eastern *Aprionyx* species to between four and six species.

*Aprionyx* sp. 1 and *A. pellucidulus* are closely related according to the molecular analyses. The general appearance of *Aprionyx* sp. 1, at least its nymphs, also resembles *A. pellucidulus* in pigmentation, but the gills rather resemble *A. peterseni* and *A. rubicundus*. Only nymphs were collected from the Hottentots Holland mountains (Figure 2.2J and 2.3A), one of the regions where Barnard (1940) recorded *A. pellucidulus*. Immature nymphs collected from the Klein Berg River catchment area were identified as a potentially new species that is closely related to *A. peterseni*, i.e., *Aprionyx* sp. 2 (Figure 2.3B), but this relationship was not well supported by the molecular evidence. Due to their immaturity, the pigmentation is not as clear, though they do somewhat resemble *A. peterseni*.

*Aprionyx* sp. 3, 4, 5, 7 and 8 (Figure 2.2J) are all related to *A. rubicundus*, but the molecular analyses were generally unable to resolve their relationships. *Aprionyx* sp. 8 is very

well supported as sister species to *A. rubicundus*. The specimen is immature and largely unpigmented, but it resembles *A. rubicundus* in general appearance, especially the gills (Figure 2.3G). *Aprionyx* sp. 7 was collected in the Tsitsikamma region, and although there are some resemblances to *A. rubicundus* in abdominal pigmentation and gill shape, closer inspection showed that these characters could tentatively distinguish them: the non-pigmented spots on the abdomen are generally larger, and the filamentous processes of the gills are generally shorter than in *A. rubicundus* (Figure 2.3F). The nymphs are also not nearly as dark as in *A. rubicundus*, but pigmentation is not a reliable character on its own. Barnard's (1932) descriptions, for example, relied heavily on pigmentation patterns, which could be misleading for inexperienced researchers who might rely on the distinctive pigmentation patterns depicted by his work. From personal experience, after examining many *Aprionyx* specimens, variability in their pigmentation is commonly encountered, especially the nymphs, but it is generally a good starting point. A more extensive investigation is needed to identify reliable salient characters for morphological species delimitation. *Aprionyx* sp. 3, 4 and 5 (Figure 2.3C-E) bear some resemblance to *A. rubicundus* and *A. tabularis*, and are all potential candidates for *A. tabularis*. *Aprionyx* sp. 4 seems to be the most likely candidate owing to the gill shape, abdominal posterior marginal denticles and abdominal posterolateral spines. However, *Aprionyx* sp. 3 and 5 are more immature specimens and can, therefore, not be disregarded. These results suggest that there are at least 11 species in the western *Aprionyx* group.

The bPTP and GMYC species delimitations are largely in agreement with one another and correspond with the phylogenetic species clades, with minor deviations only in the 16S analyses. The results of the bPTP approach were largely satisfactory, with only one singleton identified within the *A. peterseni* group from the Hugos River. This singleton was not supported by any of the other analyses. The GMYC method identified the Barrydale population as significantly different from the rest of the *A. peterseni* group and was also well supported by the species trees and 16S phylogenies, but these specimens were not represented by COI sequences. Various studies have demonstrated that the GMYC approach can be used to successfully detect potential cryptic species (Lucentini et al. 2011, Pereira-da-Conceicoa et al. 2012, Talavera et al. 2013, Tyagi et al. 2017). Barrydale, more specifically the Tradouw River, is well known for its endemic redfin fish, *Pseudobarbus burchelli* Smith, 1841, which was identified as a lineage separate from the Breede River lineage (Swartz et al. 2014). It is, for these reasons, recommended to study *A. peterseni* across its distribution to determine whether

it is actually a cryptic species complex, for example, like *Baetis harrisoni* Barnard, 1932 (Pereira-da-Conceicoa et al. 2012).

## 2.5 Summary

The molecular analyses supported *Aprionyx* as a monophyletic genus but have shown greater support for the two main clades identified within the genus, named the western and eastern *Aprionyx* groups. Based on these results, the western and eastern *Aprionyx* groups could be considered subgenera or genera due to their distinctive molecular and geographical nature. However, it is crucial to also examine them morphologically to determine whether the two phylogenies concur. Having strong statistical evidence from both the molecular and morphological analyses will strengthen the justification for splitting the genus, and their distinct geographical occurrences only increase the support. The results also indicated that the relationships within each cluster, especially the eastern *Aprionyx* group, should be investigated further. Including more specimens from a wider distribution range can lead to a better representation of all of the species and could contribute to increased phylogenetic support throughout the molecular phylogeny. In other words, future studies should aim to collect more samples and extend the sampling range to get a more adequate representation of the *Aprionyx* species.

At the start of this study, eight species were listed for *Aprionyx*. This work identified *A. natalicus*, *A. pellucidulus*, *A. peterseni*, *A. rubicundus* and *A. phoeocera* as monophyletic species clades, but the other three species are still excluded from molecular studies. The results also identified several potentially new species, but because of insufficient data, they cannot be confirmed as such. This study has shown that the biodiversity of *Aprionyx* has been understudied since the species count is potentially double that previously recorded, with seven to ten potentially new species. The Greater Cape Floristic Region remains the richest in diversity and is especially affected by these results in terms of prioritising ecosystems for conservation.

**Table 2.1 Primer sequences for each of the gene regions: cytochrome c oxidase subunit I (COI), 16S rRNA (16S) and 28S rRNA (28S).**

| Gene | Primer | Primer sequence | Reference |
|---|---|---|---|
| **COI** | LCO1490 | 5'-GGTCAACAAATCATAAAGATATTGG-3' | (Folmer et al. 1994) |
| | HCO2198 | 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' | |
| **16S** | 16Sa | 5'-GCCTGTTTATCAAAAACAT-3' | (Ogden and Whiting 2005) |
| | 16Sb | 5'-CTCCGGTTTGAACTCAGATCA-3' | |
| **28S** | EP4a | 5'-CGTCTTGAAACACGGACCAA-3' | (Ogden and Whiting 2005) |
| | EP5b | 5'-TCCTGCTGTCTTAAGCAACC-3' | |

**Table 2.2 Polymerase chain reaction (PCR) conditions set for each gene: cytochrome c oxidase subunit I (COI), 16S rRNA (16S) and 28S rRNA (28S).**

| Gene | Initial denaturation | Cycles of denaturation | Annealing | Extension | Final extension |
|---|---|---|---|---|---|
| **COI** | 95°C / 120 s | 35-38 @ 95°C / 60 s | 40°C / 60 s | 72°C / 90 s | 72°C / 7 min |
| **16S** | 94°C / 300 s | 35 @ 94°C / 30 s | 48°C / 45 s | 72°C / 90 s | 72°C / 10 min |
| **28S** | 94°C / 300 s | 35 @ 94°C / 60 s | 52°C / 90 s | 72°C / 60 s | 72°C / 5 min |

**Table 2.3 Data characteristics of each gene (cytochrome c oxidase subunit I (COI), 16S rRNA (16S) and 28S rRNA (28S)) and codon position (CP) of the partitioned COI gene (COI 1st CP, COI 2nd CP, COI 3rd CP), showing the numbers of parsimony-informative sites and models selected for the maximum likelihood (ML) and Bayesian inference (BI) analyses.**

| Partition | Total sites | Parsimony-informative | | ML Model | BI Model |
|---|---|---|---|---|---|
| **COI** | 639 | 234 | (37%) | GTR+F+I+G4 | - |
| **COI 1st CP** | 213 | 37 | (17%) | TIM2e+I | GTR+I |
| **COI 2nd CP** | 213 | 0 | (0%) | F81+F | JC69 |
| **COI 3rd CP** | 213 | 197 | (92%) | TN+F+G4 | GTR +G |
| **16S** | 486 | 161 | (33%) | TPM3u+F+I+G4 | GTR +I+G |
| **28S** | 593 | 60 | (10%) | TN+F+I | GTR +I |

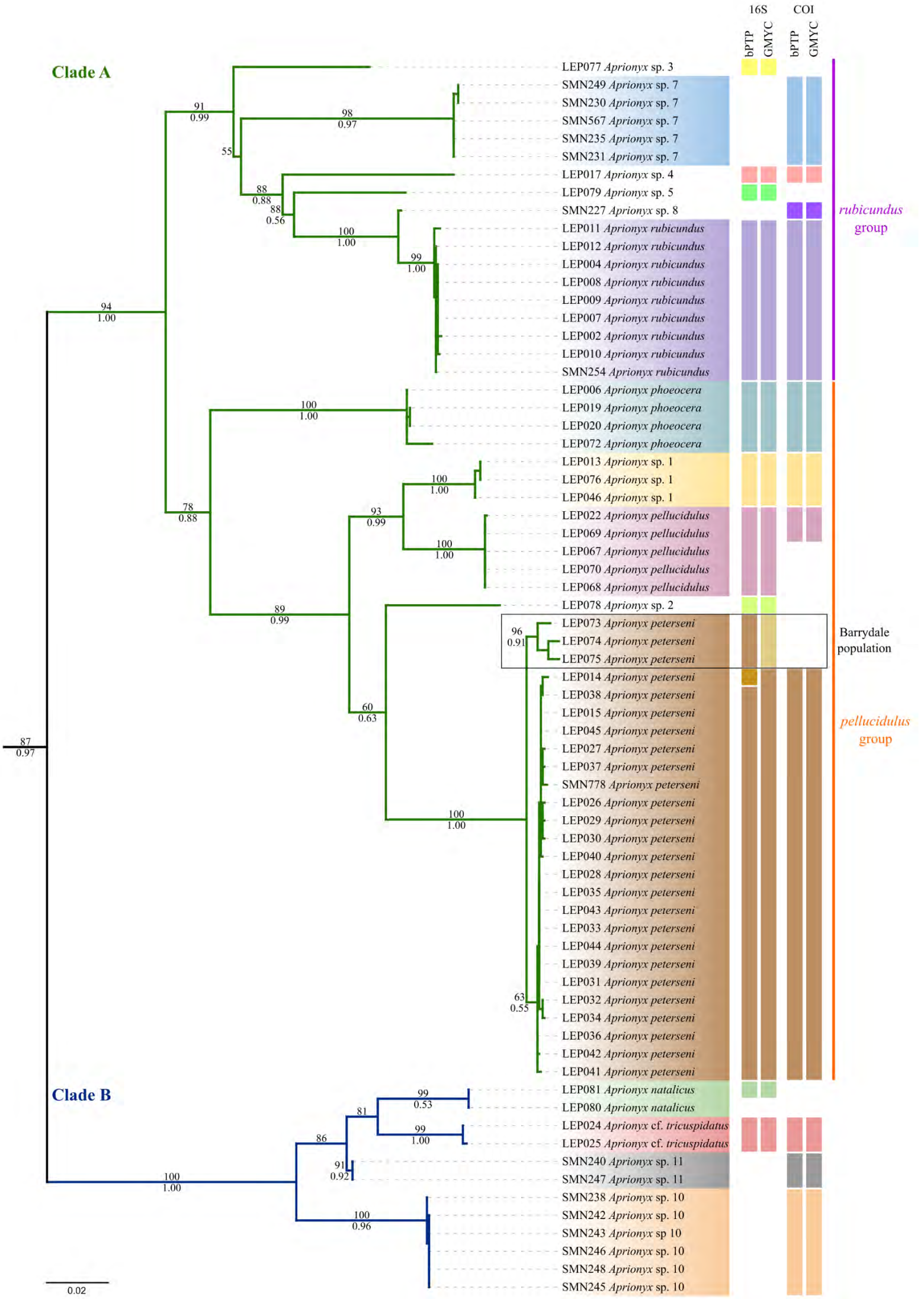

16S
COI
bPTP
GMYC
bPTP
GMYC
Clade A
LEP077 Aprionyx sp. 3
SMN249 Aprionyx sp. 7
SMN230 Aprionyx sp. 7
SMN567 Aprionyx sp. 7
SMN235 Aprionyx sp. 7
SMN231 Aprionyx sp. 7
LEP017 Aprionyx sp. 4
LEP079 Aprionyx sp. 5
SMN227 Aprionyx sp. 8
LEP011 Aprionyx rubicundus
LEP012 Aprionyx rubicundus
LEP004 Aprionyx rubicundus
LEP008 Aprionyx rubicundus
LEP009 Aprionyx rubicundus
LEP007 Aprionyx rubicundus
LEP002 Aprionyx rubicundus
LEP010 Aprionyx rubicundus
SMN254 Aprionyx rubicundus
LEP006 Aprionyx phoeocera
LEP019 Aprionyx phoeocera
LEP020 Aprionyx phoeocera
LEP072 Aprionyx phoeocera
LEP013 Aprionyx sp. 1
LEP076 Aprionyx sp. 1
LEP046 Aprionyx sp. 1
LEP022 Aprionyx pellucidulus
LEP069 Aprionyx pellucidulus
LEP067 Aprionyx pellucidulus
LEP070 Aprionyx pellucidulus
LEP068 Aprionyx pellucidulus
LEP078 Aprionyx sp. 2
LEP073 Aprionyx peterseni
LEP074 Aprionyx peterseni
LEP075 Aprionyx peterseni
LEP014 Aprionyx peterseni
LEP038 Aprionyx peterseni
LEP015 Aprionyx peterseni
LEP045 Aprionyx peterseni
LEP027 Aprionyx peterseni
LEP037 Aprionyx peterseni
SMN778 Aprionyx peterseni
LEP026 Aprionyx peterseni
LEP029 Aprionyx peterseni
LEP030 Aprionyx peterseni
LEP040 Aprionyx peterseni
LEP028 Aprionyx peterseni
LEP035 Aprionyx peterseni
LEP043 Aprionyx peterseni
LEP033 Aprionyx peterseni
LEP044 Aprionyx peterseni
LEP039 Aprionyx peterseni
LEP031 Aprionyx peterseni
LEP032 Aprionyx peterseni
LEP034 Aprionyx peterseni
LEP036 Aprionyx peterseni
LEP042 Aprionyx peterseni
LEP041 Aprionyx peterseni
Clade B
LEP081 Aprionyx natalicus
LEP080 Aprionyx natalicus
LEP024 Aprionyx cf. tricuspidatus
LEP025 Aprionyx cf. tricuspidatus
SMN240 Aprionyx sp. 11
SMN247 Aprionyx sp. 11
SMN238 Aprionyx sp. 10
SMN242 Aprionyx sp. 10
SMN243 Aprionyx sp 10
SMN246 Aprionyx sp. 10
SMN248 Aprionyx sp. 10
SMN245 Aprionyx sp. 10
rubicundus group
pellucidulus group
Barrydale population
91 0.99
98 0.97
55
88 0.88
88 0.56
100 1.00
99 1.00
94 1.00
100 1.00
78 0.88
100 1.00
93 0.99
100 1.00
89 0.99
96 0.91
60 0.63
87 0.97
100 1.00
63 0.55
99 0.53
81
99 1.00
86
91 0.92
100 1.00
100 0.96
0.02

**Figure 2.1 The maximum likelihood inference tree of COI/16S/28S-combined for *Aprionyx*. Branch labels indicate the UFBoot (ultrafast bootstrap) percentage above and Bayesian posterior probabilities (rounded to two decimals) of supported branches below the branch. The green (Clade A: western *Aprionyx*) and blue (Clade B: eastern *Aprionyx*) branches indicate the two main clades identified in *Aprionyx*. The two groups within the western *Aprionyx* clade, *rubicundus* (purple) and *pellucidulus* (orange), are indicated on the right. The species delimitation results are presented by the bars to the right, as determined by the PTP (Poisson tree processes) and GMYC (Generalized Mixed Yule Coalescent) methods, each for 16S and COI. The congruences of the different methods are highlighted by the multi-coloured bars, where each colour represents a different putative species and is left blank for specimens not included in that analysis.**

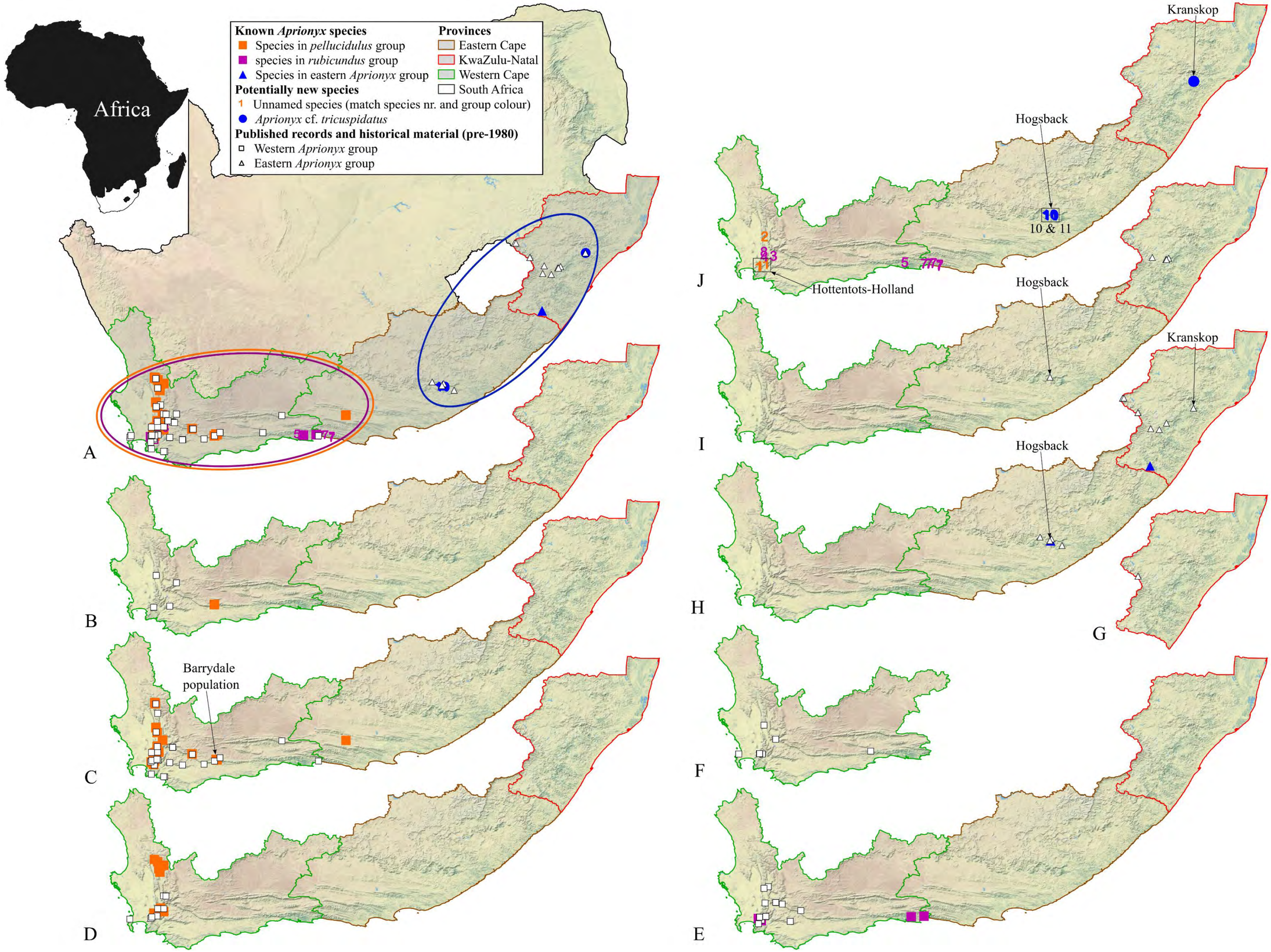

Africa
Known Aprionyx species
Species in pellucidulus group
species in rubicundus group
Species in eastern Aprionyx group
Potentially new species
1 Unnamed species (match species nr. and group colour)
Aprionyx cf. tricuspidatus
Published records and historical material (pre-1980)
Western Aprionyx group
Eastern Aprionyx group
Provinces
Eastern Cape
KwaZulu-Natal
Western Cape
South Africa
A
B
C
D
E
F
G
H
I
J
Barrydale population
Hottentots-Holland
Hogsback
Kranskop
10 & 11

**Figure 2.2 Distribution of *Aprionyx* in South Africa: A) an overview of all species, indicating the separation between the western *Aprionyx* species (enclosed in orange for the *pellucidulus* clade and purple for the *rubicundus* clade) and eastern *Aprionyx* species (enclosed in blue). B-I) The distribution of each species: B) *A. pellucidulus*, C) *A. peterseni*, D) *A. phoeocera*, E) *A. rubicundus*, F) *A. tabularis*, G) *A. argus*, H) *A. natalicus*, I) *A. tricuspidatus*. J) The distribution of all the unnamed species. The extent of published records and historical material are presented with white-filled shapes.**


A
B
C
D
E
F
G
H
I
J

**Figure 2.3 Nymph habitus of all unnamed *Aprionyx* species: A) *Aprionyx* sp. 1, B) *Aprionyx* sp. 2, C) *Aprionyx* sp. 3, D) *Aprionyx* sp. 4, E) *Aprionyx* sp. 5, F) *Aprionyx* sp. 7, G) *Aprionyx* sp. 8, H) *Aprionyx* sp. 10 with gills enlarged, I) *Aprionyx* sp. 11, and J) *A.* cf. *tricuspidatus* with gills enlarged. Scale = 2 mm. Photographs A-E = © IS Ferreira and F-I = © SMNS (State Museum of Natural History, Stuttgart, Germany).**

# Chapter 3: Morphological revision and phylogeny of *Aprionyx*

## 3.1 Introduction

The earliest *Aprionyx* species was described as *Atalophlebia tabularis*, based on a single adult male specimen that resembled *Atalophlebia* (Eaton 1884). The genus *Aprionyx* was first erected by Barnard (1932) after discovering the nymphs of the South African *Atalophlebia* species, which revealed the nymphs were without denticles on the claws (hence 'a-prionyx'). This evidence was enough to justify the new genus, because no other *Atalophlebia* nymphs had this unique characteristic. Prior to Barnard's work, only four species were known as adults (Eaton 1884, Esben-Petersen 1920, Lestage 1924). Barnard (1932, 1940) mainly dealt with species in the western region and he was able to add three species as well as descriptions of most of the nymphs that were still unknown. In the eastern region, Crass (1947b) discovered another species and the nymphs of the three species of that region. So far, only eight species have been described, but the nymphs of only seven of the *Aprionyx* species have been described.

Uncertainties arose from Barnard's (1932) descriptions of *A. tabularis* and *A. intermedius*. The nymph of *A. rubicundus* is still unknown, and Crass (1947b) was unsure about his placement of the Drakensberg nymphs as *A. argus*. Several species are also rather difficult to distinguish, either as nymphs or adults, or both, and therefore need to be redescribed so that they can be better defined.

The morphological relationships between members of the *Aprionyx* genus have not been investigated until now. On a generic level, Peters and Edmunds (1964, 1970) concluded that the morphological evidence suggests a close resemblance between *Aprionyx*, *Adenophlebia* and *Atalophlebioides*, a morphologically conservative lineage expressing many plesiomorphic states and related to southern hemisphere genera. Pescador and Peters (1980) classified *Aprionyx* in the *Hapsiphlebia* lineage with cool-adapted taxa from Australia, New Zealand and South America, which agrees with Peters and Edmunds (1964, 1970). This lineage includes *Acanthophlebia* Towns, 1983 from New Zealand, a taxon that presents some resemblance to the unique labial palpi of *Aprionyx* (Towns and Peters 1996). Based on the presence of the distal dentiseta in *Aprionyx* and Terpides/fg1 (Kluge 2012), *Aprionyx* could be

closely affiliated with the Terpidinae group. A more recent molecular study recovered *Aprionyx* in a clade that is sister to Terpidinae, but this was not supported by the analysis (Monjardim et al. 2020), leaving the relationships of *Aprionyx* within Leptophlebiidae unresolved. Other morphological studies focused on the tentorium, thorax and terga, but were not useful at the species level (Hudson 1951, Tsui and Peters 1972, 1975).

The main objective of this chapter is to provide a revised morphological analysis of *Aprionyx*, relating to the taxonomy and phylogeny. The taxonomic component provides revised descriptions for each of the known *Aprionyx* species. With this, a character list was compiled for further morphological analyses. This data are based on nymphs and imagos, and was used to investigate the interspecies relationships of *Aprionyx* from a morphological perspective. The chapter concludes with a dichotomous key for the adults and nymphs of *Aprionyx*. Lestage (1924) was the only author who produced an identification key, but because of the limited knowledge of *Aprionyx* at that time it was based only on the adults and needed to be revised.

## 3.2 Materials and methods

### *3.2.1 Type material*

Species of *Aprionyx* were described by Eaton (1884), Esben-Petersen (1920), Lestage (1924), Barnard (1932, 1940) and Crass (1947b) and their material was presumably deposited in NMSA and SAMC. Type specimens of *Aprionyx* were sought in the AMGS, NHMUK and SAMC.

Barnard's (1932, 1940) material in the wet collection of the SAMC is currently in the AMGS on permanent loan and was recorded in the SAM catalogue. *Aprionyx* in the pinned collection of SAMC is still in the Iziko Museum and includes material from Esben-Petersen (1920), Lestage (1924), and Barnard (1932, 1940). Mayflies of the NMSA were donated to the AMGS and included Crass' (1947b) *Aprionyx*, recorded in the RSC catalogue. The NHMUK has a small collection (wet and pinned) of South African Leptophlebiidae (presumably sent there by K.H. Barnard), which includes *Aprionyx* specimens of Barnard (1932, 1940).

All samples published in the original descriptions are syntypes by default, except if specified otherwise by the author (Article 72.4, ICZN 2000). A few specimens or samples were labelled as type material, though most lacked an explicit indication of being type material. The

samples in the SAMC, NHMUK and AMGS were, therefore, linked to published material to positively identify the syntypes (Table 3.1). Material listed in Eaton (1884), Esben-Petersen (1920), Lestage (1924), Barnard (1932, 1940), Crass (1947b, 1947a) and Demoulin (1970) were considered during the linking process, and much of this published material was not in any of the three museum collections (Appendix B).

The syntypes were considered to assign prospective lectotypes for each species (Table 3.1; Figure 3.2). Where more than one syntype was identified, and none labelled as the type, a male imago was selected from the syntypes as the prospective lectotype. The remaining syntypes then become paralectotypes (Article 74.1.3, ICZN 2000). The type material and other historical material are mostly degraded and faded (in ethanol) or darker and broken (pinned specimens), so it was used primarily for taxonomic verification when fresh material was available.

### *3.2.2 Additional specimens*

Fresh *Aprionyx* specimens were systematically collected (Permits: Cape Nature, Western Cape, no. 0056-AAA041-00177; Environmental Affairs, Eastern Cape, no. CRO 69/17CR; Ezemvelo KZN Wildlife, KwaZulu-Natal, no. 565/2017) at 36 sites in Western Cape, Eastern Cape and KwaZulu-Natal from 2017 to 2019 (Appendix C). Sites were coordinated to be in the general region of the type localities, and also include additional sites visited during opportunistic collecting. In most cases the type localities were not very specific, so feasible sites were identified in or near the mountain range, town, river, district or other place names specified in the descriptions. Five *Aprionyx* species (*A. pellucidulus*, *A. peterseni*, *A. phoeocera*, *A. rubicundus* and *A. natalicus*) were collected, but only one species (*A. peterseni*) was arguably collected near its type locality.

The aim was to collect both winged (imago and subimago) and wingless (nymph) stages in the best possible condition, as Leptophlebiidae are very fragile, and their gills, legs and caudal filaments detach especially easily. Nymphs were collected by handpicking from loose stones or with an aquatic sampling net (mesh ≤ 1 mm) in vegetation and detritus (when present). Adults flying between dusk and dawn were collected overnight with a pan light trap fitted with either 6 V or 12 V battery-powered actinic UV lights (Figure 3.1A-B), and later also a bespoke LED UV light source powered by 1.2 V AA rechargeable batteries (Figure 3.1C). Several South African Leptophlebiidae species, including some *Aprionyx* species, have been observed to fly

during the day (Crass 1947b). These adults were collected by searching for swarming groups or individuals resting on vegetation or other substrates and catching them with a net or by hand.

Specimens were preserved in 80-90% ethanol. Captured mature nymphs, and live subimagos captured during the day, were kept alive and reared to imagos before they were preserved with their exuviae.

### *3.2.3 Morphological analysis*

*Aprionyx* specimens were examined with an Olympus SZ51 stereo microscope at 8x to 40x magnification. A Nikon D7200 DSRL camera with a 105 mm micro lens was used to take the photographs of whole specimens for focus stacking using Helicon Remote v3.9.10W software (www.heliconsoft.com/heliconsoft-products/helicon-remote). The ZEISS Axiocam ERc 5s camera mounted on a ZEISS Stemi 508 stereo microscope was used for photographing specimens at higher magnification using ZEN 3.0 (ZEN lite) software. The mouthparts, legs, abdominal segments and gills of the nymphs and the wings, legs, male genitalia and female subanal plate were excised and mounted on permanent microscope slides using Euparal. These slides where then examined and photographed using a ZEISS Axiocam 208c camera on a Leica DM 1000 compound microscope using ZEN 3.0 (ZEN lite) software. All focus Z-stacks, including those taken with either of the microscope setups, were stacked with Helicon Focus v7.7.4 software (www.heliconsoft.com/heliconsoft-products/helicon-focus).

Characters and character states were compiled with the DELTA (DEscription Language for TAxonomy) software package to create descriptions and a taxonomic key (Dallwitz 1974, 1980, Dallwitz et al. 2013, 2020). The general morphological terminology used in this study follows Barber-James and Lugo-Ortiz (2003) to retain the familiarity of existing South African terminology and Edmunds et al. (1976) mainly for adult terminology not covered by the aforementioned work. Segments and legs are numbered in Roman numerals, with I situated proximally or anteriorly. The wing venation follows that used by Kluge (2004), except $iCu_1$, $iCu_2$, etc., are used instead of x, y and z for the intercalaries in the cubital field (Appendix J). Measurements were taken as proposed by Hubbard (1995).

### *3.2.4 Morphological phylogenetics*

The character matrix was created with the DELTA software package and included a total of 58 characters, with 24 characters of the male imago and 34 of the nymphal stages (Appendix D). Morphological characters were derived from the eyes, wings, claws, abdomen,

genitalia and caudal filaments of the male imago, and the mouthparts, legs, abdomen, gills and caudal filaments of the nymph. Characters were selected based on what has been used for Leptophlebiidae in the past (Domínguez 1995, 1999, Domínguez et al. 2001, 2019, Christidis 2005, Salles and Domínguez 2012, Salles et al. 2020), though each was adapted to fit *Aprionyx* and the selected outgroups. Several newly introduced characters based on *Aprionyx* features were also added. Continuous characters were categorised into discrete states defined by data ranges that are distinctly different between taxa. Discrete characters were compiled as neomorphic or transformational characters, and by coding the absent character state as inapplicable in transformational characters (Sereno 2007). Missing data was assigned as '?' and '-' for inapplicable data. The characters and character states are defined in section 3.5.

Characters were coded for each of the eight *Aprionyx* species by examining fresh material, but when this was not available, the historic material was used. For the nymphs of *A. argus* and *A. natalicus*, only the little information given in their respective descriptions was used, since no specimens were found in any of the collections.

Three genera were selected as outgroup taxa, i.e., *Acanthophlebia*, *Austrophlebioides* and *Choroterpes*. Two cool-adapted taxa from New Zealand and Australia, *Acanthophlebia* and *Austrophlebioides*, were selected as close relatives of *Aprionyx*. Pescador and Peters (1980) classified *Acanthophlebia* in the *Hapsiphlebia* lineage, which also includes *Aprionyx*, and *Austrophlebioides* in the more derived lineage, *Meridialaris* Peters & Edmunds, 1972. *Choroterpes* was included as one of the South African genera included in the molecular analysis (Chapter 2). The characters for each genus were largely based on representative species (*Acanthophlebia cruentata* (Hudson, 1904), *Austrophlebioides pusillus* (Harker, 1954) and *C. nigrescens*), but the information from generic descriptions was often used to be more inclusive of the genus, rather than focused on one species (except for *Acanthophlebia* which is monotypic). The option to root trees with the outgroup as a monophyletic sister group to the ingroup where selected as the rooting method in PAUP. Monjardim et al. (2020), recovered the three selected outgroups in a sister clade to the clade containing the *Aprionyx* species. It was for this reason that all three outgroups were set as the root of the trees instead of only *Choroterpes*.

Cladistics were carried out in PAUP* v. 4.0a169 under maximum parsimony, with gaps treated as a new state and all characters as unordered. Analysis was performed under equal weighting (EW) and implied weighting (IW; K = 4) to reduce the effect of homoplasious

characters on the tree length (Goloboff et al. 2006, 2008). The heuristic search option was run with random sequence addition, 1000 replications and TBR (tree bisection reconnection) branch swapping. The branch support was assessed using bootstrap analysis with 100 replications and the full heuristic search option. The branch support for accepting the reliability of a clade is accepted as 70% or above for bootstrap values (Hillis and Bull 1993) and the support values were categorized as follows: strong (≥ 70%), moderate (60-70%), weak (50-60%) or no support (< 50%). The heuristic search analysis was also performed on only the imaginal characters (all taxa) and on only the nymphal characters (nine taxa) under IW. *Aprionyx argus* and *A. natalicus* were excluded from the analysis of the nymphal characters because of the high proportion of missing data. The ACCTRAN (accelerated transformation) character optimization was implemented in PAUP to generate the list of character changes, and the unambiguous changes were mapped on all of the trees. The trees were viewed in FigTree v1.4.4 and annotated using the web-based Interactive Tree of Life (http://itol.embl.de) and Inkscape v1.1 (https://inkscape.org) software. The same IW analyses were run using the full character matrix (nymphs and imagos) but with different values for K (K = 2, 4, 7, 10 and 15). All the analyses resulted in exactly the same number of trees, topology, tree length, CI, RI and HI. Consequently, K = 4 were selected for all IW analyses.

## 3.3 Descriptions

***Aprionyx* Barnard, 1940**

*GBIF taxon ID: 4683642*

*Vernacular name: Smooth-clawed prong-gill* (Barber-James 2015)

*Aprionyx* Barnard, 1940 Ann. S. Afr. Mus. 32(6): 616

≡ *Aprionyx* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 233–234 [*nomen nudum*]

*Type species*: *Aprionyx tabularis* (Eaton, 1884) [orig. design.].

*Etymology*: The name combines the Greek words *prion* (saw) and *onyx* (claw) with the prefix *a-* (not) (α-πριων-όνυξ / *a-priōn-ónyx*), referring to the characteristic edentate claws of the nymphs that distinguish the South African species from the other species in *Atalophlebia* at the time (Barnard 1932). *Aprionyx* is grammatically masculine.

*Nomenclatural notes*: Barnard (1932) originally created the genus *Aprionyx* in 1932 without explicitly designating a type species for the genus as required by Article 68 (Article 68.2, ICZN 2000). Instead, he assumed the species described first (i.e., the oldest of the species allocated to the genus) is automatically the type species. Article 13 requires that, if published after 1930, a type species must be nominated correctly in order for a genus name to be considered available by the Code (Article 13.3, ICZN 2000). Due to Barnard's failure to designate a type species in his original description of the genus in 1932, the name is considered a *nomen nudum* (i.e., unavailable) until he officially designated *A. tabularis* as the type species for *Aprionyx* in 1940 (Barnard 1940). This is why the authorship falls to Barnard, 1940 and not Barnard, 1932.

*Diagnosis*: The imagos differ from other South African Leptophlebiidae genera by the following combination of characters: Cubital field of the forewing usually with one pair of long intercalaries, parallel to each other but not to CuA (Figure 3.5A, 3.7A, 3.9A, 3.11A, 3.13A, 3.15A, 3.16A and 3.17A); costal projection of the hind wing not well developed, obtuse and rounded (Figure 3.5B, 3.7B, 3.9B, 3.11B, 3.13B, 3.15B, 3.16B and 3.17B); claws alike and hooked; and penes fused with shallow cleft (Figure 3.5D, 3.7D, 3.9D, 3.11D, 3.13D and 3.16D), or separated apically (less than one third; Figure 3.15D and 3.17D).

The nymphs are distinguished from other South African genera by the following characters: Labium (Figure 3.6G, 3.8G, 3.10G, 3.12G, 3.14G and 3.18G) with stout palpi; segment II with outer margin rounded, convex and longer than inner margin; inner margin straight; segment III very short, wider than long and subtriangular; claws without denticles (Figure 3.6I, 3.8I, 3.10I, 3.12I, 3.14I and 3.18I); and gills obovate, with at least a median filamentous process (Figure 3.21E).

***Imago:*** *Dimensions*. ♂: body 6.5–13 mm; forewing 6.8–13 mm; hind wing 1.2–3.2 mm; caudal filaments 13–30 mm (smallest to largest species).

♀: body 6.5–14 mm (with subimagos of up to 18.4 mm); forewing 6.8–15 mm (up to 16.9 mm in subimagos); hind wing 1.3–2.4 mm; caudal filaments 13–29 mm (smallest to largest species).

***Male:*** *Eyes*. Divided into dorsal and ventral portions; dorsal portion unstalked. *Wings*. Fore- and hind wings hyaline. Forewing with four bullae. Cubital field usually with one pair of long intercalary veins parallel to each other but not to CuA (e.g. *A. rubicundus* can be with only one as illustrated by Barnard (1932, fig. 28a)); vein $iCu_1$ free basally or attached at base

to CuA, but never attached to CuP. Hind wing costal projection not well developed, obtuse and rounded; vein MP forked. *Legs*. Tarsus of leg II and III with four free segments; first tarsal segment fused with tibia. Claws both hooked. *Genitalia*. Styliger plate entire. Forceps segment I distinctly longer than segments II and III together; basally widened about half or less than half its length; narrowing abruptly; basal broadening quadrangular. Segment II subequal to, or longer than, segment III. Penes fused with shallow cleft, or separated in apical third. *Caudal filaments*. Dark annulations present.

***Nymph:*** *Dimensions*. Maximum body length 7.5–17 mm (smallest to largest species).

*Head*. Prognathous head; antennae more than twice head length. *Mouthparts*. Labrum narrower than clypeus. Anterior medial emargination shallow; denticulate. Outer margin of left mandible with distomedial tuft of long setae. Hypopharynx with lateral processes well developed. Maxilla with inner margin with a patch of long hairs distally, and proximal to that, a row of longer hairs increasing in length. Maxillary palp 3-segmented; segment III triangular, longer than wide; slightly more than half as long as segment II. Labial palp stout; 3-segmented. Segment II with outer margin rounded, convex and longer than inner margin; inner margin straight. Segment III very short; subtriangular; wider than long. *Thorax*. Prothorax with anterolateral patch of setae. *Legs*. Patella-tibial suture present on legs II and III only. Lengths of tarsi I>III>II. Claws edentate. *Gills*. Present on segments I-VII; bilamellate; obovate; gill I bilamellate and narrower than the other gills. Median filamentous process always present, lateral processes absent or variable.

**Western *Aprionyx* group**

***Aprionyx tabularis* (Eaton, 1884)**

(Figure 3.2A, Figure 3.5-Figure 3.6)

*GBIF taxon ID: 6934103*

*Vernacular name: Intermediate* (Barnard 1932)

*Atalophlebia tabularis* Eaton, 1884 Trans. Linn. Soc. London 3: 91 Pl. x, fig. 16h (♂ imago) **senior hom**. (*nec* Esben-Petersen, 1920 and Barnard, 1932)

= *Aprionyx intermedius* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 238 **n. syn.**

*Type locality*: SOUTH AFRICA: Western Cape. Table Mountain, Platteklip streamlet, 33.958S 18.416E.

*Material*: **Holotype:** SOUTH AFRICA: Western Cape. 1 ♂ imago, Table Mountain, Platteklip streamlet, [33.958S 18.416E], 1874, AE Eaton [ethanol, NHMUK; material missing]; **iconotype:** The original description with drawings of the holotype specimen by Eaton (1884). **Prospective lectotype (*A. intermedius*):** SOUTH AFRICA: Western Cape. 1 ♂ imago, Groot Drakenstein, no date, AC Harrison [ethanol, AMGS].

**Paralectotypes (*A. intermedius*):** SOUTH AFRICA: Western Cape. 1 ♀ subimago, 1 nymph, Groot Drakenstein, no date, AC Harrison [ethanol, AMGS]; 2 ♀ subimagos, same data [ethanol, NHMUK].

**Non-type material examined:** SOUTH AFRICA: Western Cape. 1 ♀ imago, Groot Drakenstein, 12.ix.1932, AC Harrison [ethanol, AMGS]; 1 ♀ subimago, Outeniqua Mountain Range, Robinson Pass, Ruiterbosch, ii.1932, KH Barnard [ethanol, AMGS]; 3 ♂ imagos, 10 ♀ imagos, 1 ♂ subimago, 2 ♀ subimagos, 3 nymphal exuviae, Table Mountain, 28.xi.1934, KH Barnard [ethanol, NHMUK].

*Etymology*: This was not stated by Eaton (1884), but was probably derived from the Latin *tabularis* (flat), alluding to the flat top of the type locality, Table Mountain.

*Nomenclatural notes*: The holotype of *A. tabularis* is accepted to be missing or destroyed because Kimmins (1960, 1971) was unable to locate it when he dealt with A.E. Eaton's type material while employed at the British Museum of Natural History (1925–1970, Principal Scientific Officer since 1958). Eaton's (1884) original description of *At. tabularis* is limited, and some of his figures are inaccurate, leading to misinterpretation and disagreement amongst taxonomists (Esben-Petersen 1920, Lestage 1924, Barnard 1932). The description was adequate to recognise the species and the drawings provide an iconotype (Article 73.1.4, ICZN 2000). Table 3.2 and Figure 3.19 compare the relevant characteristics to justify the proposed taxonomic changes.

Esben-Petersen (1920) described three male imagos (Ceres, iv.1913, Lightfoot, SAMC) as *At. tabularis*. He was ambivalent about this identification and noted that the specimens he described were probably much darker than in reality because they were dried (pinned). This led to some equivocal characteristics, i.e., the colour of the eyes and caudal filaments (Table 3.2).

Lestage (1924) created *At. phoeocera* Lestage, 1924 based on the description by Esben-Petersen (1920), without examining any of the material. By comparing the descriptions by Eaton (1884) and Esben-Petersen (1920), he explained why he considered Esben-Petersen's specimens a new species. His argument was founded mainly on two characters, i.e., the presence of dark markings on the caudal filaments and legs. He noted that Eaton's specimen could have discoloured from being preserved in ethanol for a long time but dismissed this because it did not explain the appearance of annulations (on the caudal filaments) and median bands (on the femora). Lestage (1924) failed to address the consequences of drying a pinned specimen (i.e., much darker colouration), which should have discouraged him from creating *At. phoeocera*. Ultimately, Lestage (1924) was correct in believing that Esben-Petersen's (1920) specimens are not *At. tabularis* Eaton, 1884, but failed to identify the true distinguishing characteristics (Table 3.2 summarises supporting evidence).

Barnard (1932) disagreed with Lestage (1924) and synonymised *At. phoeocera* with *At. tabularis* Eaton, 1884 when he redescribed *A. tabularis* and placed it in *Aprionyx*. The description was based on fresh material, but he also examined Esben-Petersen's (1920) material. Barnard (1932) confirmed that in both his fresh material and the pinned material of Esben-Petersen, all caudal filaments had narrow dark annulations, and the femora were banded medially and distally (as in Eaton's description). He also reasoned that in the respective figures by Eaton and Esben-Petersen, the outline of the penis matched (evidence in Table 3.2 and Figure 3.19 disagrees), and the number of crossveins in the costal area is the same (Table 3.2 indicates that this character is uninformative). Barnard (1932) accepted Eaton's (1884) figure of the penis and not Esben-Petersen's (1920) figure because the latter is based on a dry specimen and not described in the text, while the former is based on material in ethanol, which Barnard considered more reliable.

Barnard (1932) examined Esben-Petersen's (1920) material to confirm that his fresh material was conspecific. The original material of both authors was examined for the current study, and it was concluded that *A. tabularis sensu* Barnard, 1932 is indeed synonymous with *At. tabularis sensu* Esben-Petersen, 1920. Barnard did, however, fail to recognise any of the differences from Eaton's (1884) description, possibly because he was fixated on the ambiguous arguments of Lestage (1924).

Barnard's (1932) description and material of *A. intermedius* are a better match to Eaton's (1884) description and figures of *At. tabularis* than the match between *A. tabularis sensu* Barnard, 1932 and *At. tabularis sensu* Eaton, 1884. The description of *A. intermedius* was based mainly on the female subimago, and the little information given about the male

genitalia was incorrectly indicated as the "♀" imago (Barnard 1932). The illustrations of *A. intermedius* also only included the female subimago. After examining the type material from Groot Drakenstein (AMGS), the male specimen was clearly similar to what Eaton (1884) described as *At. tabularis*. The following two characters of the male imago were a good match to what was figured by Eaton (1884): 1) the penis is very broad and bluntly pointed; 2) the eyes are narrowly separated on the meson. These are two integral characters that Barnard (1932) did not describe, and they are important in separating the imagos of *A. intermedius* and *At. phoeocera* (Table 3.2, Figure 3.19).

From these arguments, it was deduced that *At. tabularis sensu* Eaton, 1884 and *At. tabularis sensu* Esben-Petersen, 1920 are recognized as homonyms (Article 52, ICZN 2000). As the senior homonym, *At. tabularis sensu* Eaton, 1884 is the valid name and *A. intermedius* Barnard, 1932 (**n. syn**.) is synonymous to it.

*Atalophlebia phoeocera* Lestage, 1924 (**stat. rev.**) regains taxonomic validity to replace the junior homonym, *At. tabularis sensu* Esben-Petersen, 1920, with *A. tabularis* sensu Barnard, 1932 its new synonym (the species list was adapted as indicated in Table 3.3). See also the description of *A. phoeocera*.

*Diagnosis*: The imagos are separated from other *Aprionyx* species by the following combination of characters: dorsal portion of the eyes (Figure 3.5C, F) in males is large, largest of all *Aprionyx* species (ventral portion less than 0.63 times the length of the dorsal portion), and narrowly separated on meson; and penes (Figure 3.5D-E) broad basally and medially, tapering in apical half to an obtusely angular apex, and laterally projecting downward about midway between base and apex.

The nymphs are distinguished by: labrum (Figure 3.6A) widest in distal third; anteromedial emargination (Figure 3.6B) with teeth flattened (at least the outside teeth) and poorly defined; maxilla with apical-ventral row of about 15 pectinate setae; tarsus of leg I with a ventral fringe of spinelike setae; claws (Figure 3.6I) slender and not hooked; abdominal posterolateral projections present on segments VI to IX; and tergites with well-developed posterior marginal denticles on all segments.

***Imago:*** (Figure 3.2A) *Dimensions*. ♂: body 10 mm; forewing 8.9–9 mm; hind wing 2.3 mm.

♀: body 8.2 mm; forewing 8.8 mm; hind wing 2.4 mm.

***Male:*** *Eyes*. Dorsal portion clove brown; large; narrowly separated on the meson of the head (Figure 3.5C). Ventral portion ca. 0.62 x dorsal portion (Figure 3.5F). *Wings*. Forewing (Figure 3.5A) crossveins (excluding pterostigma) barely shaded. Crossveins in costal area prior to pterostigma well developed; 8–10 crossveins before bulla. Pterostigmal crossveins rarely anastomosing, and then not extensively. Vein RS forked at 0.34 x wing length; MA fork closer to wing margin than to RS-MA fork; $MP_2$ attached to $MP_1$ 0.15 to 0.25 the distance from RS-MA fork to RS fork. Hind wing (Figure 3.5B) width 0.65 x length; length 0.26 x forewing; vein Sc 0.85 x wing length; subcostal area with 6–7 crossveins. *Legs*. Femur with dark bands medially and distally. *Abdomen*. Posterolateral projections on segment IX biacuminate, but not as prominent as in nymphs. *Genitalia*. Penes (Figure 3.5D-E) fused with shallow cleft; broad; tapering in apical half; apex obtusely angular; laterally projecting downward medially. Gonopores with recurved apical flap-like projections. *Caudal filaments*. Dark annulations narrow, almost indistinct.

***Female***: Similar to male. Subanal plate with shallow indentation.

***Nymph:*** *Dimensions*. Maximum body length 12 mm.

*Mouthparts*. Labrum (Figure 3.6A) length about 0.50 x width; widest in distal third; lateral margin rounded; dorsal surface proximally with broad row of scattered long setae interrupted medially, distally with narrow row of denser long setae. Anterior medial emargination (Figure 3.6B) with five teeth; teeth small, flattened (at least the outside teeth) and poorly defined. Outer margin of left mandible (Figure 3.6D) curved distally, more or less straight proximally; median margin proximal to prostheca with shallow concavity. Prostheca as illustrated in Figure 3.6E (left) and Figure 3.6F (right). Maxilla (Figure 3.6C) with apical-ventral row of ca. 15 pectinate setae. Maxillary palp segment II 1.10 x segment I; with distal end about 1.5 times the proximal end. Segment III covered in long hairs on outer margin and short hairs on inner margin; apical hairs extending beyond apex about equal to segment III. Labial palp (Figure 3.6G) segment I width 0.58 x length. Segment II 1.07 x segment I; distally about 1.5 times as wide as proximally. *Legs* (Figure 3.6H). Femur with dark bands similar to imagos. Lengths of femurs III>I>II; lengths of tibias III>I>II. Leg I with length of segments 1.14:1(1.39):0.64. Ventral margin of tibia I with fringe of bipectinate setae; without hairlike setae. Tarsus I ventral margin with fringe of spinelike setae. Claws (Figure 3.6I) slender, not hooked. *Abdomen*. Posterolateral projections present on segments VI to IX; biacuminate on segments VIII and IX. Posterior marginal denticles well developed on all segments. *Gills*.

Median apical filamentous process present; lamella narrow (Peters and Edmunds 1964, fig. 126); filamentous processes long, more than half the length of lamella (Peters and Edmunds 1964, fig. 126). *Caudal filaments*. Primary swimming setae absent.

***Aprionyx pellucidulus* (Esben-Petersen, 1920)**

(Figure 3.2B, Figure 3.3A, Figure 3.4A, Figure 3.7-Figure 3.8, Figure 3.20A, I, P)

*GBIF taxon ID: 7746564*

*Atalophlebia pellucidula* Esben-Petersen, 1920 Ann. S. Afr. Mus. 17(6): 499–500 Fig. 1–2 (♂ imago)

≡ *Aprionyx pellucidulus* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 239–240

*Type locality*: SOUTH AFRICA: Western Cape. Great Winterhoek, Tulbagh, 4300 ft (ca. 1311 m), 33.17S 19.11E.

*Material*: **Prospective lectotype:** SOUTH AFRICA: Western Cape. 1 ♂ imago, Great Winterhoek Mountains, Tulbagh District, 4300 ft (ca. 1311 m), [33.17S 19.11E], xi.1916, RM Lightfoot [pinned, SAMC].

**Paralectotypes:** SOUTH AFRICA: Western Cape. 2 ♀ subimagos, Great Winterhoek Mountains, Tulbagh District, 4300 ft (ca. 1311 m), [33.17S 19.11E], xi.1916, RM Lightfoot [pinned, SAMC].

**Non-type material examined:** SOUTH AFRICA: Western Cape. 3 ♂ imagos, Ceres, north slopes of Matroosberg, 4000 ft (ca. 1219 m), xi.1917, RM Lightfoot [pinned, SAMC]; 4 ♂ imagos, Hottentots Holland Mountains, 4000 ft (ca. 1219 m), i.1933, KH Barnard and HG Wood [pinned, SAMC]; 3 ♂ imagos, same data [pinned, NHMUK]; 12 ♂ imagos, 1 ♀ subimago, same data [ethanol, NHMUK]; 13 ♂ imagos, 1 ♂ subimago, same data [ethanol, AMGS]; 1 ♂ imago, 2 ♂ subimagos, 1 ♀ subimago, Riviersonderend Mountains, 3500 ft (ca. 1067 m), i.1933, HG Wood [ethanol, AMGS]; 4 ♂ imagos, 1 ♀ imago 2 ♀ subimagos, same locality, i.1934, KH Barnard [pinned, SAMC]; 10 ♂ imagos, Great Winterhoek Mountains, Tulbagh district, 4000 ft (ca. 1219 m), xi.1932, KH Barnard and HG Wood [pinned, SAMC]; 5 ♂ imagos, same data [pinned, NHMUK]; 9 ♂ imagos, 1 ♀ imago, 2 ♀ subimagos, 3 nymphs, same data [ethanol, NHMUK]; 9 ♂ imagos, 1 ♀ imago, 6 ♂ subimagos, 2 nymphs, same data [ethanol, AMGS]; 3 nymphs, Tradouw River tributary, Piekniekbos, 33.9651S 20.7046E,

20.xi.2017, IS Ferreira, HM Barber-James, AJ Holland and FC de Moor [ethanol, AMGS]; 6 nymphs, same locality, 21.xi.2017, IS Ferreira, HM Barber-James, AJ Holland and FC de Moor [ethanol, AMGS]; 1 nymph, same locality, 24.xi.2017, IS Ferreira, HM Barber-James, AJ Holland and FC de Moor [ethanol, AMGS]; 17 nymphs, 2 nymphal exuviae, 2 ♂ nymphs reared to imagos, 1 ♀ nymph reared to imago, same locality, 25.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS].

*Etymology*: The species' name is derived from the Latin *pellucida* (translucent), alluding to the male imago's transparent abdomen described by Esben-Petersen (1920), and changed by Barnard (1932) to the masculine *pellucidulus* to preserve agreement with the grammatical gender of the genus.

*Diagnosis*: Imagos are distinguished as follows: males with the dorsal portion of the eyes touching to narrowly separated on the meson of the head (Figure 3.7C); forewing (Figure 3.7A and 3.20A) crossveins dark but barely shaded beyond the vein, six to nine crossveins before bulla; $MP_2$ attached to $MP_1$ 0.15 to 0.25 the distance from RS-MA fork to RS fork; penes (Figure 3.7D-E) gradually tapering towards apex, apically with a rounded tip, base swollen ventrally; and caudal filaments with narrow dark annulations (Figure 3.20P).

Nymphs are separated by the following characters: labrum (Figure 3.8A) widest at about halfway its length; maxilla with about 18 pectinate setae in apical-ventral row; lengths of tibias I>III>II; tarsus I ventrally with a row of fine hairlike setae (shorter than the scattered hairs); abdominal segments with posterolateral projections on segments VI to IX; and caudal filaments with short, sparse primary swimming setae.

***Imago:*** (Figure 3.2B and Figure 3.3A) *Dimensions*. ♂: body 10–12.2 mm; forewing 10.1–13 mm; hind wing 2–2.1 mm; caudal filaments 23–29 mm.

♀: body 12–13 mm; forewing 13–14 mm; caudal filaments 23–29 mm.

***Male:*** *Eyes*. Dorsal portion brown; medium; touching to narrowly separated on the meson of the head (Figure 3.7C). Ventral portion 0.71 x dorsal portion (Figure 3.7F). *Wings*. Forewing (Figure 3.7A and Figure 3.20A) crossveins barely shaded beyond the vein. Crossveins in costal area prior to pterostigma well developed; 6–9 crossveins before bulla. Pterostigmal crossveins not anastomosing. Vein RS forked at 0.26–0.28 x wing length; MA fork closer to wing margin than to RS-MA fork; $MP_2$ attached to $MP_1$ 0.15 to 0.25 the distance from RS-MA fork to RS fork. Hind wing (Figure 3.7B) width 0.58–0.62 x length; length 0.20

x forewing; vein Sc 0.83–0.85 x wing length; subcostal area with 6–7 crossveins. *Legs*. Femur with dark bands medially and distally. Leg I with length of segments 0.76:1(4.02):0.05:0.31:0.41:0.29:0.09; total length 11.65 mm; length of femur less than 0.80 of tibia. *Abdomen*. Posterolateral projections on segment IX biacuminate, but not as prominent as in nymphs (Figure 3.20I). *Genitalia*. Penes (Figure 3.7D-E) fused with shallow cleft; gradually tapering towards apex; apex rounded; base swollen ventrally. Gonopores with recurved apical flap-like projections. *Caudal filaments*. Dark annulations narrow, almost indistinct (Figure 3.20P).

***Female***: Similar to male. Pregenital plate present. Subanal plate with shallow indentation.

***Nymph:*** (Figure 3.4A) *Dimensions*. Maximum body length 11.2 mm.

*Mouthparts*. Labrum (Figure 3.8A) length about 0.50 x width; widest at about halfway; lateral margin rounded; dorsal surface with row of scattered long setae, medially expanding towards distal margin. Anterior medial emargination (Figure 3.8B) with five teeth; teeth small, rounded/bluntly pointed and well defined. Outer margin of left mandible (Figure 3.8D) curved distally, more or less straight proximally; median margin proximal to prostheca with shallow concavity. Prostheca as illustrated in Figure 3.8E (left) and Figure 3.8F (right). Maxilla (Figure 3.8C) with apical-ventral row of ca. 18 pectinate setae. Maxillary palp segment II 1.17 x segment I; with distal end about 1.5 times the proximal end. Segment III covered in long hairs on outer margin and short hairs on inner margin; apical hairs extending beyond apex about equal to segment III. Labial palp (Figure 3.8G) segment I width 0.60 x length. Segment II 0.88 x segment I; distally about 1.5 times as wide as proximally. *Legs* (Figure 3.8H). Femur with dark bands similar to imagos. Lengths of femurs III>I>II; lengths of tibias I>III>II. Leg I with length of segments 1.02:1(2.18):0.37. Ventral margin of tibia I with fringe of bipectinate setae; with hairlike setae short, longer distally. Tarsus I ventral margin with row of fine hairlike setae (shorter than scattered hairs). Claws (Figure 3.8I) hooked. *Abdomen*. Posterolateral projections present on segments VI to IX; biacuminate on segments VIII and IX. Posterior marginal denticles well developed on segments IX and X. *Gills*. Median apical filamentous process present; lamella narrow; filamentous processes about half the length of lamella. *Caudal filaments*. Primary swimming setae short, sparse.

***Aprionyx peterseni* (Lestage, 1924)**

(Figure 3.2C, Figure 3.3B, Figure 3.4B, Figure 3.9-Figure 3.10, Figure 3.20C, G, N, Figure 3.21B, J)

*GBIF taxon ID: 6934112*

*Vernacular name: Pied dun* (Barnard 1932)

*Atalophlebia peterseni* Lestage, 1924 Rev. Zool. Afr. 12(3): 328–330

≡ *Aprionyx peterseni* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 236–238

*Type locality*: SOUTH AFRICA: Western Cape. Paarl, 33.73S 18.98E.

*Material*: **Prospective lectotype:** SOUTH AFRICA: Western Cape. 1 ♂ imago, Paarl, [33.73S 18.98E], x.1919, Rev. G Hawke [pinned, SAMC].

**Paralectotypes:** SOUTH AFRICA: Western Cape. 4 ♂ imagos, 1 ♀ imago, Paarl, [33.73S 18.98E], x.1919, Rev. G Hawke [pinned, SAMC].

**Non-type material examined:** SOUTH AFRICA: Western Cape. 2 ♂ imagos, 1 ♂ subimago, Groot Drakenstein, 29.iii.1931, AC Harrison [pinned, SAMC]; 2 ♂ imagos, 1 ♀ imago, 9 nymphs, same data [ethanol, NHMUK]; 1 ♂ nymph reared to imago, same data [ethanol, AMGS]; 1 ♀ imago, 1 ♂ subimago, 1 ♀ subimago, 3 nymphal exuviae, 1 subimaginal exuvium, Stellenbosch, Jonkershoek, no date, AC Harrison [ethanol, NHMUK]; 6 ♂ imagos, 1 ♀ subimago, Franschhoek, iv.1931, KH Barnard [ethanol, NHMUK]; 4 ♂ imagos, same data [ethanol, AMGS]; 1 ♂ imago, Montagu, x.1919, KH Barnard [pinned, SAMC]; 1 ♀ subimago, Witte River, Wellington Mountains, 1500 ft (ca. 457 m), xi.1922, KH Barnard [pinned, SAMC]; 1 ♂ imago, Riviersonderend Mountains, 1500 ft (ca. 457 m), xi.1928, KH Barnard [pinned, SAMC]; 11 ♂ imagos, 4 ♂ subimagos, 1 ♀ subimago, 4 nymphs, 3 subimaginal exuviae, 1 nymphal exuvium, Worcester Mountains, Keeromsberg, Boskloof, i.1930, KH Barnard [ethanol, AMGS]; 2 ♂ imagos, 1 ♀ imago, 3 ♀ subimagos, 2 nymphal exuviae, Clanwilliam, Cederberg, i.1930, KH Barnard [ethanol, AMGS]; 3 ♂ imagos, Langeberg Mountains, Heidelberg, Lemoenshoek, xi.1927, KH Barnard [pinned, SAMC]; 5 ♂ imagos, 9 ♂ subimagos, 1 ♀ subimago, Franschhoek Pass, xii.1932, KH Barnard [ethanol, NHMUK]; 6 ♂ imagos, 2 ♀ imagos, 1 ♂ subimago, 5 ♀ subimagos, Riviersonderend Mountains, Oudebosch, 1500 ft (ca. 457 m), i.1933, HG Wood [ethanol, NHMUK]; 1 ♂ imago, 2 ♀ imagos, 6 ♂ subimagos, 5 ♀ subimagos, same data [ethanol, AMGS]; 2 ♂ imagos, 1 ♂ subimago, Riviersonderend Mountains, Oudebosch, 1500 ft (ca. 457 m), i.1934, KH Barnard [pinned, SAMC]; 1 ♂ subimago, Swellendam, 17.xii.1931–18.i.1932, RE Turner [pinned, NHMUK];

21 nymphs, Huis River, Hiking trail waterfall, ca. 500 m, 33.931S 20.7769E, 22.xi.2017, IS Ferreira, HM Barber-James, AJ Holland and FC de Moor [ethanol, AMGS]; 1 nymph, 1 ♂ nymph reared to imago, Huis River, Huis River at canal, ca. 390 m, 33.9199S 20.7527E, 23.xi.2017, IS Ferreira, HM Barber-James, AJ Holland and FC de Moor [ethanol, AMGS]; 7 nymphs, 4 nymphal exuviae, Jonkershoek River, Jonkershoek 1, 33.995S 18.9793E, 17.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 3 nymphs, Jonkershoek River tributary, Tributary crossing to Eerstewaterval, 33.9971S 18.984E, 17.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 1 ♀ subimago, 15 nymphs, 4 nymphal exuviae, Jonkershoek River tributary, Second waterfall, 34.0042S 18.9938E, 17.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 9 nymphs, 5 nymphal exuviae, 2 ♀ nymphs reared to imagos, Bakkerskloof River, Bakkerskloof weir, 33.8222S 19.0462E, 18.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 2 nymphs, Kasteelskloof River, Kasteelskloof weir, 33.8222S 19.0584E, 18.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 13 nymphs, Klein Berg tributary, Secret Falls - Site 1 (stream), 33.1846S 19.1361E, 24.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 1 nymph, Hottentots Holland Mountains, Riviersonderendkloof, 34.0419S 19.0363E, 7.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 24 nymphs, Hottentots Holland Mountains, Palmiet River, 34.0548S 19.0363E, 8.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 3 ♂ imagos, 1 ♀ imago, 110 ♂ subimagos, 69 ♀ subimagos, Berg River, upstream of dam, 33.9618S 19.069E, 9.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 25 ♂ imagos, 4 ♂ subimagos, 2 ♀ subimagos, 33 nymphs, 2 nymphal exuviae, Berg River tributary, Wolwekloof, upstream of weir, 33.9441S 19.0244E, 9.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 2 ♂ subimagos, 2 ♀ subimagos, 26 nymphs, Jonkershoek River, Jonkershoek 2, 33.9958S 18.9815E, 11.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 4 nymphs, Hugos River, Element Adventures, 33.7407S 19.0561E, 15.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 29 nymphs, Groot Winterhoek, Klein Kliphuis River, 33.0468S 19.1006E, 17.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; Ceres, Breede River tributary, Tolhuis, 33.3911S 19.2852E, 20.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 3 ♂ subimagos, 20 nymphs, 2 nymphal exuviae, Wit River tributary, Wolwekloof, Tweede Tol, 33.568S 19.1366E, 20.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 2 nymphs, 4 nymphal exuviae, Wit River, 33.5702S 19.1394E, 20.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 2 ♂ imagos, 1 ♀ imago, 11 ♂ subimagos, 11 ♀ subimagos, 53 nymphs, 2 nymphal exuviae, 1 ♂ nymph reared to imago, 2 ♀ nymphs to imagos, Krom River, Du Toit's Kloof, 33.7262S 19.1109E, 22.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 16 nymphs, Klip River,

Du Toit's Kloof, 33.7241S 19.183E, 22.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS], 1 ♂ subimago, 8 nymphs, 1 ♂ nymph reared to imago, Keisie River tributary, Silver Stream Waterfall, 33.7712S 20.0907E, 24.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS].

*Etymology*: The species is dedicated to P. Esben-Petersen in gratitude for his services (Lestage 1924).

*Diagnosis*: Imagos are characterized by: the dorsal portion of the eyes of males touching to narrowly separated on the meson of the head (Figure 3.9C); forewing crossveins (excluding pterostigma) with heavy oval-shaped shading in costal area (give a spotted appearance; Figure 3.9A and 3.20C), four to six crossveins before bulla; vein $MP_2$ attached to $MP_1$ at RS-MA fork (Figure 3.20G); penes (Figure 3.9D-E) gradually tapering towards apex, apically with a rounded tip, base swollen ventrally; and caudal filaments with broad dark annulations (Figure 3.20N).

Nymphs are distinguished as follows: labrum (Figure 3.10A) widest at about halfway its length; maxilla with apical-ventral row of ca. 18 pectinate setae; tarsus I ventrally with a row of fine hairlike setae (shorter than the scattered hairs; Figure 3.21B); abdominal segments with posterolateral projections on segments IV or V to IX; and caudal filaments with primary swimming setae short and sparse (Figure 3.21J).

***Imago:*** (Figure 3.2C and Figure 3.3B) *Dimensions*. ♂: body 8–10.5 mm; forewing 8.5–11 mm; hind wing 2.3–2.4 mm; caudal filaments 15–20 mm.

♀: body 8–10.5 mm; forewing 10–11 mm; caudal filaments 13–15 mm.

***Male:*** *Eyes*. Dorsal portion orange-brown; medium; touching to narrowly separated on the meson of the head (Figure 3.9C). Ventral portion 0.72–0.73 x dorsal portion (Figure 3.9F). *Wings*. Forewing (Figure 3.9A and Figure 3.20C) crossveins (excluding pterostigma) with heavy oval-shaped shading in costal area; narrower in subcostal area. Crossveins in costal area prior to pterostigma well developed; 4–6 crossveins before bulla. Pterostigmal crossveins not anastomosing. Vein RS forked at 0.26–0.27 x wing length; MA fork closer to wing margin than to RS-MA fork; $MP_2$ attached to $MP_1$ at RS-MA fork (less than 0.10 the distance from RS-MA fork to RS fork; Figure 3.20G). Hind wing (Figure 3.9B) width 0.61 x length; length 0.24–0.25 x forewing; vein Sc 0.81–0.85 x wing length; subcostal area with 6–7 crossveins. *Legs*. Femur with dark bands medially and distally. Leg I with length of segments 0.87:1(3.64):0.06:0.34:0.39:0.36:0.14; total length 11.51 mm; length of femur 0.80 or more of

tibia. *Abdomen*. Posterolateral projections on segment IX biacuminate, but not as prominent as in nymphs. *Genitalia*. Penes (Figure 3.9D-E) fused with shallow cleft; gradually tapering towards apex; apex rounded; base swollen ventrally. Gonopores with recurved apical flap-like projections. *Caudal filaments*. Dark annulations broad (Figure 3.20N).

***Female***: Similar to male.

***Nymph:*** (Figure 3.4B) *Dimensions*. Maximum body length 12 mm; caudal filaments 15 mm.

*Mouthparts*. Labrum (Figure 3.10A) length about 0.49 x width; widest at about halfway; lateral margin rounded; dorsal surface with broad row of scattered long setae, slightly denser distally. Anterior medial emargination (Figure 3.10B) with five teeth; teeth small, rounded/bluntly pointed and well defined. Outer margin of left mandible (Figure 3.10D) curved distally, more or less straight proximally; median margin proximal to prostheca with shallow concavity. Prostheca as illustrated in Figure 3.10E (left) and Figure 3.10F (right). Maxilla (Figure 3.10C) with apical-ventral row of ca. 18 pectinate setae. Maxillary palp segment II 1.29 x segment I; with distal end about 1.5 times the proximal end. Segment III covered in long hairs on outer margin and short hairs on inner margin; apical hairs extending beyond apex about equal to segment III. Labial palp (Figure 3.10G) segment I width 0.62 x length. Segment II 0.98 x segment I; distally about 1.75 times as wide as proximally. *Legs* (Figure 3.10H). Femur with dark bands similar to imagos. Lengths of femurs III>I>II; lengths of tibias III>I>II. Leg I with length of segments 1.09:1(1.58):0.48. Ventral margin of tibia I with fringe of bipectinate setae; with hairlike setae short, longer distally. Tarsus I ventral margin with row of fine hairlike setae (shorter than scattered hairs; Figure 3.21B). Claws hooked (Figure 3.10I). *Abdomen*. Posterolateral projections present on segments IV or V to IX; biacuminate on segments VIII and IX. Posterior marginal denticles well developed on segments IX and X. *Gills*. Median apical filamentous process present; lamella narrow; filamentous processes about half the length of lamella. *Caudal filaments*. Primary swimming setae short, sparse (Figure 3.21J).

***Aprionyx phoeocera* (Lestage, 1924) stat. rev.**

(Figure 3.2D, Figure 3.3C, Figure 3.4C, Figure 3.11-Figure 3.12, Figure 3.20, E, Figure 3.21L, O)

*Vernacular name: April dun* (Barnard 1932)

≡ *Atalophlebia tabularis* Esben-Petersen, 1920 Ann. S. Afr. Mus. 17(6): 500–501 Fig. 3–4 (♂ imago) **junior hom.** (*nec* Eaton, 1884)

≡ *Atalophlebia phoeocera* Lestage, 1924 Rev. Zool. Afr.12: 325 **stat. rev.**

= *Aprionyx tabularis* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 234–236. **n. syn.** (*nec* Eaton, 1884)

*Type locality*: SOUTH AFRICA: Western Cape. Ceres, 33.37S 19.31E.

*Material*: **Prospective lectotype:** SOUTH AFRICA: Western Cape. 1 ♂ imago, Ceres, [33.37S 19.31E], iv.1913, RM Lightfoot [pinned, SAMC].

**Paralectotypes:** SOUTH AFRICA: Western Cape. 2 ♂ imagos, Ceres, [33.37S 19.31E], iv.1913, RM Lightfoot [pinned, SAMC].

**Non-type material examined:** SOUTH AFRICA: Western Cape. 1 ♂ imago, 1 ♂ subimago, 2 ♀ subimagos, 6 nymphs, 1 nymphal exuvium, Groot Drakenstein, no date, AC Harrison [ethanol, AMGS]; 1 ♂ imago, 2 ♀ subimagos, 3 nymphs, 1 subimaginal exuvium, 2 nymphal exuviae, same data [ethanol, NHMUK]; 21 nymphs, Wemmershoek River, La Ferme, 33.849S 19.0498E, 18.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 1 nymph, Molenaars River, 33.7036S 19.232E, 22.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 7 nymphs, Holsloot River, Dwarsberg (camp 4), 33.7994S 19.3139E, 23.iii.2018, IS Ferreira and HM Barber-James [ethanol, AMGS]; 3 nymphs, Cederberg Mountains, Krom River, site R-R 3, 32.5331S 19.3167E, 23.i.2017, Terrence Bellingan [ethanol, AMGS]; 3 nymphs, Cederberg Mountains, Hex River, Upper site, 32.7213S 19.2115E, 12.iv.2005, FC de Moor, R Bills and IJ de Moor [ethanol, AMGS]; 2 ♀ subimagos, Cederberg Mountains, Tentskloof, Suurvlei River, 32.6289S 19.1821E, 12.iv.2005, FC de Moor, R Bills and IJ de Moor [ethanol, AMGS]; 1 nymph, Cederberg Mountains, Rondegat River, 32.3734S 19.0602E, 18.iv.2005, FC de Moor, R Bills and IJ de Moor [ethanol, AMGS]; 1 ♂ imago, 6 ♀ subimagos, Cederberg Mountains, Driehoeks River, 32.4314S 19.1492E, 17.iv.2005, FC de Moor, R Bills and IJ de Moor [ethanol, AMGS].

*Etymology*: Lestage (1924) named the species after Esben-Petersen's description of the caudal filaments as brown by combining *φαιός (faiós)*/brown and *χέρας (chéras)*/horn (cerci).

*Nomenclatural notes*: The description given by Esben-Petersen (1920) was based on three male specimens that are dried and thus darker in appearance. He identified them with

uncertainty as *At. tabularis*. Lestage (1924) noticed inconsistencies between the descriptions of Eaton (1884) and Esben-Petersen (1920) and, therefore, renamed the junior homonym (*At. tabularis sensu* Esben-Petersen, 1920) as *At. phoeocera* without seeing any of the specimens. The main reasons given by Lestage (1924) were questionable because they could be attributed to the fact that the specimens were dry. Barnard (1932) rejected Lestage's (1924) distinguishing characters but synonymised *At. phoeocera* and *At. tabularis sensu* Eaton, 1884 when he placed it in *Aprionyx*. Barnard (1932) was probably too concerned with Lestage's mistakes and explored only the apparent similarities, rather than trying to identify any true differences. The homonymy identified by Lestage (1924) is accepted, with the eyes and genitalia as the distinguishing characters (Table 3.2, Figure 3.19). *Atalophlebia phoeocera* **(stat. rev.)** is therefore reinstated as the replacement of the junior homonym (Article 52, ICZN 2000) with *A. tabularis sensu* Barnard, 1932 (**n. syn.**) as its new synonym (See also section on *A. tabularis*).

*Diagnosis*: Imagos are recognised by the following characters: dorsal portion of the male's eyes is widely separated on the meson of the head (Figure 3.11C); forewing (Figure 3.11A) with costal crossveins prior to pterostigma weak; anastomosis of pterostigmal crossveins always present (often extensive; Figure 3.20E); hind wing with vein Sc 0.72–0.75 x wing length (Figure 3.11B); femur banded dark distally only, broadly suffused medially; and penes (Figure 3.11D-E) basally and medially broad, tapering in apical half to a narrow, acute tip and dorsoventrally flattened.

Nymphs are distinguished by the following characters: labrum (Figure 3.12A) length about 0.56 x width, widest in distal quarter, with lateral margin more or less straight; left mandible with median margin proximal of prostheca with deep concavity (Figure 3.12D); apical-ventral row of maxilla with ca. 16 pectinate setae; lengths of femurs I>III>II, and lengths of tibias I>III>II; leg I (Figure 3.12H) with tibia longer than femur, ventral margin of tibia I with long, dense hairlike setae, tarsus I with long hairlike setae concentrated along anterior face and ventrally with a row of small spinelike setae; abdominal segments with posterolateral projections on segments IV or V to IX; gills with broad lamella (about half the length) and short filamentous processes, generally less than a third of lamella; and caudal filaments with long, dense primary swimming setae (Figure 3.21O).

***Imago:*** (Figure 3.2D and 3.3C) *Dimensions*. ♂: body 9–13 mm; forewing 10–12 mm; hind wing 2.7–3.2 mm; caudal filaments 17 mm.

♀: body 9.8–18.4 mm (subimagos); forewing 13–16.9 mm (subimagos).

***Male:*** *Eyes*. Dorsal portion pale pinkish brown; medium; widely separated on the meson of the head (Figure 3.11C). Ventral portion 0.70 x dorsal portion (Figure 3.11F). *Wings*. Forewing (Figure 3.11A) crossveins (excluding pterostigma) barely shaded. Crossveins in costal area prior to pterostigma weak; 9–11 crossveins before bulla. Pterostigmal crossveins always anastomosing, often extensively (Figure 3.11A and Figure 3.20E). Vein RS forked at 0.31 x wing length; MA fork closer to wing margin than to RS-MA fork; $MP_2$ attached to $MP_1$ 0.15 to 0.25 the distance from RS-MA fork to RS fork. Hind wing (Figure 3.11B) width 0.60–0.70 x length; length 0.25–0.30 x forewing; vein Sc 0.72–0.75 x wing length; subcostal area with 9–13 crossveins. *Legs*. Femur with dark bands distally only, broadly suffused medially. Leg I with length of segments 0.96:1(3.66):0.08:0.31:0.29:0.25: 0.14; total length 11.07 mm; length of femur 0.80 or more of tibia. *Abdomen*. Posterolateral projections on segment IX biacuminate, but not as prominent as in nymphs. *Genitalia*. Penes (Figure 3.11D-E) fused with shallow cleft; broad; tapering in apical half; apex narrow, acute; dorsoventrally flattened. Gonopores with recurved apical flap-like projections. *Caudal filaments*. Dark annulations narrow, almost indistinct.

***Female***: Similar to male. Subanal plate with shallow indentation.

***Nymph:*** (Figure 3.4C) *Dimensions*. Maximum body length 16.2 mm; caudal filaments 20 mm.

*Mouthparts*. Labrum (Figure 3.12A) length about 0.56 x width; widest in distal quarter; lateral margin more or less straight; dorsal surface proximally with scattered shorter setae, distally with narrow convex row of long setae which widens laterally. Anterior medial emargination (Figure 3.12B) with five teeth; teeth small, rounded/bluntly pointed and well defined. Outer margin of left mandible (Figure 3.12D) curved distally and proximally, flattened medially; median margin proximal to prostheca with deep concavity. Prostheca as illustrated in Figure 3.12E (left) and Figure 3.12F (right). Maxilla (Figure 3.12C) with apical-ventral row of ca. 16 pectinate setae. Maxillary palp segment II 1.10 x segment I; with distal end about 1.5 times the proximal end. Segment III covered in long hairs on outer margin and short hairs on inner margin; apical hairs extending beyond apex about equal to segment III. Labial palp (Figure 3.12G) segment I width 0.55 x length. Segment II 0.91 x segment I; distally about 1.75 times as wide as proximally. *Legs* (Figure 3.12H). Femur with dark bands similar to imagos. Lengths of femurs I>III>II; lengths of tibias I>III>II. Leg I with length of segments 0.91:1(4.36):0.23; tibia longer than femur. Ventral margin of tibia I with bipectinate setae

reduced to only a few distally (Figure 3.21L); with hairlike setae very long, dense. Tarsus I with long hairlike setae concentrated along anterior face; ventral margin with row of small, spinelike setae. Claws hooked (Figure 3.12I). *Abdomen*. Posterolateral projections present on segments IV or V to IX; biacuminate on segments VIII and IX. Posterior marginal denticles well developed on segments IX and X (can have a few also on segments VII and VIII). *Gills*. Median apical filamentous process present; lamella broad (nearly half the length); filamentous processes short, generally less than a third of lamella. *Caudal filaments*. Primary swimming setae long, dense (Figure 3.21O).

### ***Aprionyx rubicundus* Barnard, 1932**

(Figure 3.2E, Figure 3.3D, Figure 3.4D, Figure 3.13-Figure 3.14, Figure 3.20B, H, K, Figure 3.21A, C, K)

*GBIF taxon ID: 6934108*

*Aprionyx rubicundus* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 239 Fig. 28 (♂ imago)

*Type locality*: SOUTH AFRICA: Western Cape. Montagu, 33.78S 20.12E.

*Material*: **Prospective lectotype:** SOUTH AFRICA: Western Cape. 1 ♂ imago, Montagu, [33.78S 20.12E], x.1919, KH Barnard [pinned, SAMC].

**Paralectotypes:** SOUTH AFRICA: Western Cape. 1 ♂ imago, 1 ♀ imago, Montagu, [33.78S 20.12E], x.1919, KH Barnard; 3 ♂ imagos, same data [ethanol, NHMUK].

**Non-type material examined:** SOUTH AFRICA: Western Cape. 4 ♂ imagos, Oudebosch, Riviersonderend Mountains, 1500 ft (ca. 457 m), xii.1931, HG Wood [ethanol, AMGS]; 7 ♂ imagos, Oudebosch, Riviersonderend Mountains, 1500 ft (ca. 457 m), ix.1933, HG Wood [ethanol, NHMUK]; 3 ♀ imagos, 3 ♂ subimagos, 1 ♀ subimago, Worcester, Fairy Glen, 12.viii.1932, KH Barnard [ethanol, AMGS]; 3 ♂ imagos, Hottentots Holland Mountains, Steenbras, 23.xi.1932, KH Barnard & HG Wood [ethanol, AMGS]; 9 ♂ imagos, 2 ♀ imagos, Great Winterhoek Mountains, Tulbagh, xi.1932, KH Barnard and HG Wood [ethanol, AMGS]; 4 ♂ imagos, 4 ♀ imagos, Franschhoek Pass, 1.x.1933, KH Barnard [ethanol, AMGS]; 6 ♂ imagos, 9 ♀ imagos, 1 ♂ subimago, same data [ethanol, NHMUK]; 1 ♂ subimago, 3 nymphal exuviae, Hottentots Holland Mountains, Riviersonderendkloof, 34.0419S 19.0363E, 7.xi.2018,

IS Ferreira and AJ Holland [ethanol, AMGS]; 8 ♂ imagos, 1 ♀ imago, 2 ♀ subimagos, 16 nymphs, 3 nymphal exuviae, 1 ♂ nymph reared to imago, Jonkershoek, Swartboskloof, 33.9968S 18.9580E, 11.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 3 ♀ imagos, 1 ♂ subimago, 1 nymph, 3 nymphal exuviae, Jonkershoek, Eerste Waterval, 33.9987S 18.9832E, 12.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 3 nymphal exuviae, Hottentots Holland Mountains, Palmiet River, 34.0548S 19.0363E, 8.xi.2018, IS Ferreira and AJ Holland [ethanol, AMGS]; 1 ♂ imago, 4 nymphal exuviae, Salt River, Fern Farm (Sout 11), 33.9321S 23.4924E, 11.viii.2000, HM Barber-James and N Kohly [ethanol, AMGS]; 3 ♂ imagos, Outeniqua, Diepwalle River, 33.9474S 23.141E, 5.xi.2012, HM Barber-James and LL Pereira-da-Conceicoa [ethanol, AMGS].

*Etymology*: The name is probably derived from the Latin *rubicundus*, meaning ruddy or red, because of its "distinctive maroon mesothorax" mentioned by Barnard (1932).

*Nomenclatural notes*: The female imago was recorded only later (Barnard 1940), and until now, the nymph has not been described.

*Diagnosis*: Imagos are characterised as follows: males with the dorsal portion of the eyes narrowly separated on the meson of the head (Figure 3.13C); forewing (Figure 3.13A) narrow (≤0.32 the length), with the posterior margin truncated proximally; crossveins (excluding pterostigma) with narrow shading in costal area, heavier in subcostal area (Figure 3.20B); vein Sc of hind wing 0.87–0.91 x wing length (Figure 3.13B); and penes basally and medially broad, tapering in apical half to an obtusely angular (almost truncated) apex and laterally projecting downward medially (Figure 3.13D-E and Figure 3.20K).

The nymphs are recognised by the following combination of characters: labrum widest in distal third (Figure 3.14A); anteromedial emargination with teeth small, flattened (at least the outside teeth) and poorly defined (Figure 3.14B); maxilla with about 16 pectinate setae in apical-ventral row; segment I of labial palp wide, about 0.69 x length; leg I tarsus with ventral fringe of spinelike setae (Figure 3.21C); claws slender, not hooked (Figure 3.14I); abdominal segments with posterolateral projections on segment VII to IX; and gills with lamella narrow and filamentous processes long, more than half the length of lamella.

***Imago:*** (Figure 3.2E and Figure 3.3D) *Dimensions*. ♂: body 6.5–9.7 mm; forewing 6.8–8.7 mm; hind wing 1.2–1.8 mm; caudal filaments 13–15 mm.

♀: body 6.5–7.8 mm; forewing 6.8–10 mm; hind wing 1.3 mm.

***Male:*** *Eyes*. Dorsal portion orange-brown; medium; narrowly separated on the meson of the head (Figure 3.13C). Ventral portion 0.64–0.71 x dorsal portion (Figure 3.13F). *Wings*. Forewing (Figure 3.13A) crossveins (excluding pterostigma) with narrow shading in costal area; heavier in subcostal area (Figure 3.20B). Crossveins in costal area prior to pterostigma well developed; 6–10 crossveins before bulla. Pterostigmal crossveins not anastomosing. Vein RS forked at 0.29–0.32 x wing length; MA fork closer to wing margin than to RS-MA fork (0.55–0.58); $MP_2$ attached to $MP_1$ 0.30 to 0.80 the distance from RS-MA fork to RS fork. Forewing narrow, ≤0.32 the length; posterior margin proximally truncated. Hind wing (Figure 3.13B) width 0.56–0.68 x length; length 0.11–0.21 x forewing; vein Sc 0.87–0.91 x wing length; subcostal area with 5–7 crossveins. *Legs*. Femur with dark bands medially and distally. Leg I with length of segments 0.93:1(1.12):0.08:0.31:0.34:0.27:0.13; total length 3.43 mm; length of femur 0.80 or more of tibia. *Abdomen*. Posterolateral projections on segment IX biacuminate, but not as prominent as in nymphs (Figure 3.20H). *Genitalia*. Penes (Figure 3.13D-E and Figure 3.20K) fused with shallow cleft; tapering in apical half; apex obtusely angular; laterally projecting downward medially. Gonopores with recurved apical flap-like projections. *Caudal filaments*. Dark annulations narrow, almost indistinct.

***Female***: Similar to male. Subanal plate with shallow indentation.

***Nymph (previously undescribed):*** (Figure 3.4D) *Dimensions*. Maximum body length 7.5 mm; caudal filaments 9.5–12 mm.

*Mouthparts*. Labrum (Figure 3.14A) length about 0.53 x width; widest in distal third; lateral margin rounded; dorsal surface proximally with broad row of scattered long setae interrupted medially, distally with narrow row of denser long setae. Anterior medial emargination (Figure 3.14B) with five teeth; teeth small, flattened (at least the outside teeth) and poorly defined. Outer margin of left mandible (Figure 3.14D) curved distally, more or less straight proximally; median margin proximal to prostheca with shallow concavity. Prostheca as illustrated in Figure 3.14E (left) and Figure 3.14F (right). Maxilla (Figure 3.14C) with apical-ventral row of ca. 16 pectinate setae. Maxillary palp segment II 1.30 x segment I; with distal end about 1.5 times the proximal end. Segment III covered in long hairs on outer margin and short hairs on inner margin; apical hairs extending beyond apex about equal to segment III. Labial palp (Figure 3.14G) segment I width 0.69 x length. Segment II 1.12 x segment I; distally about 1.5 times as wide as proximally. *Legs* (Figure 3.14H). Femur with dark bands similar to imagos. Lengths of femurs III>I>II; lengths of tibias III>I>II. Leg I with length of segments

1.13:1(0.99):0.66. Ventral margin of tibia I with fringe of bipectinate setae; without hairlike setae. Tarsus I ventral margin with fringe of spinelike setae (Figure 3.21C). Claws slender, not hooked (Figure 3.14I). *Abdomen*. Posterolateral projections present on segments VII to IX; biacuminate on segments VIII and IX. Posterior marginal denticles well developed on segments IX and X. *Gills*. Median apical filamentous process present; lamella narrow; filamentous processes long, more than half the length of lamella. *Caudal filaments*. Primary swimming setae absent (Figure 3.21K).

**Eastern *Aprionyx* group**

***Aprionyx argus* Barnard, 1940**

(Figure 3.2F, Figure 3.15)

*GBIF taxon ID: 6934110*

*Aprionyx argus* Barnard, 1940 Ann. S. Afr. Mus. 32(6): 628–630 Fig. 6d-f (♂ imago)

= *Aprionyx argus* Crass, 1947 Ann. Natal Mus. 11(1): 96.32 (nymph)

*Type locality*: SOUTH AFRICA: KwaZulu-Natal. Drakensberg, Cathkin Peak, 29.08S 29.35E.

*Material*: **Prospective lectotype:** SOUTH AFRICA: KwaZulu-Natal. 1 ♂ imago (originally preserved in ethanol, found desiccated May 2020, rehydrated for examination), Drakensberg, Cathkin Peak, 6000 ft (ca. 1829 m), [29.08S 29.35E], i.1938, RF Lawrence [ethanol, AMGS].

**Paralectotypes:** SOUTH AFRICA: KwaZulu-Natal. 2 ♂ imagos, 1 ♀ imago, 1 ♀ subimago (♂ imagos originally preserved in ethanol, found desiccated May 2020), Drakensberg, Cathkin Peak, 6000 ft (ca. 1829 m), [29.08S 29.35E], i.1938, RF Lawrence [ethanol, AMGS].

*Etymology*: *Argus* is the Greek name (ἀργός) for a mythological person who was fabled to have had a hundred eyes, and by extension, indicates a very vigilant person, watcher or guardian. Barnard (1940) did not explain why it is appropriate, but one possibility is that the abdominal pigmentation with the pale spots reminded him of many eyes.

*Nomenclatural notes*: Crass (1947b) believed that two nymphs collected from Cathkin Peak could be *A. argus* but without any kind of association between nymphs and adults. He based his opinion mainly on the facts that 1) it was collected in the same general locality and 2) it is different from the other two species of this region in terms of the number of filamentous protrusions on the gills and the length of maxillary palp segment II. The two nymphs collected by Crass (1947b) could not be found and are thus presumed missing. For these reasons, the nymph of this species is of doubtful placement.

*Diagnosis*: At present the imagos are recognised by the following characteristics: males with the dorsal portion of the eyes small (ventral portion 0.82 x dorsal portion) and widely separated on the meson of the head (Figure 3.15C); forewing crossveins (excluding pterostigma) with narrow shading in costal area, heavier in subcostal area, and somewhat oval-shaped in distal anterior radial area (shading slightly heavier than in *A. tricuspidatus*); hind wing with vein Sc 0.91 x wing length (Figure 3.15B); femur with dark bands distally only; and penes separated in apical third and gonopores with curled processes apically (Figure 3.15D).

The nymphs are currently differentiated only by their gills, specifically the presence of the median apical filamentous process and lateral bulges (Crass 1947b).

***Imago:*** (Figure 3.2F) *Dimensions*. ♂: body 10–10.6 mm; forewing 10–11 mm; hind wing 2.1 mm.

♀: body 12–12.2 mm; forewing 11.8–12 mm; hind wing 2.2 mm.

***Male:*** *Eyes*. Dorsal portion small; widely separated on the meson of the head (Figure 3.15C). Ventral portion 0.82 x dorsal portion (Figure 3.15F). *Wings*. Forewing (Figure 3.15A) crossveins (excluding pterostigma) with narrow shading in costal area; heavier in subcostal area; oval-shaped in distal anterior radial area. Crossveins in costal area prior to pterostigma well developed; 7–10 crossveins before bulla. Pterostigmal crossveins not anastomosing. Vein RS forked at 0.25 x wing length; MA fork closer to wing margin than to RS-MA fork; $MP_2$ attached to $MP_1$ at RS-MA fork (less than 0.10 the distance from RS-MA fork to RS fork). Hind wing (Figure 3.15B) width 0.62 x length; length 0.20 x forewing; vein Sc 0.91 x wing length; subcostal area with 4–7 crossveins. *Legs*. Femur with dark bands distally only, broadly suffused medially. Leg I with length of segments 0.83:1(3.89):0.06:0.30:0.31:0.26:0.11; total length 11.12 mm; length of femur 0.80 or more of tibia. *Abdomen*. Posterolateral projections on segment IX acuminate and blunt. *Genitalia*. Penes (Figure 3.15D-E) separated in apical third; oblong shaped; dorsoventrally flattened. Gonopores with curled processes apically.

***Female***: Similar to male. Pregenital plate present. Subanal plate with shallow indentation.

***Nymph (Identification unconfirmed):*** *Dimensions*. Maximum body length not available.

*Gills*. Median apical filamentous process and lateral bulges present; filamentous processes about half the length of lamella (vis. illustration in Crass (1947b, fig. 32b)).

***Aprionyx natalicus* (Lestage, 1924)**

(Figure 3.2G, Figure 3.16, Figure 3.20D, F, J, L, O)

*GBIF taxon ID: 8268117*
*Atalophlebia natalica* Lestage, 1924 Rev. Zool. Afr. 12(3): 325–328
≡ *Aprionyx natalica* Barnard, 1932 Trans. R. Soc. S. Afr. 20(3): 240
≡ *Aprionyx natalicus* Demoulin, 1970 South African Animal Life 14: 101

*Type locality*: SOUTH AFRICA: KwaZulu-Natal. Kranskop, 28.96S 30.87E.

*Material*: **Prospective lectotype.** SOUTH AFRICA: KwaZulu-Natal. 1 ♂ imago, Kranskop, [28.96S 30.87E], xi.1917, KH Barnard [pinned, SAMC].

**Non-type material examined:** SOUTH AFRICA: Eastern Cape. 4 ♂ imagos, 1 ♂ subimago, Hogsback, Tyume source stream, Lower site (H1), 32.5944S 26.9562E, 2.xii.2017, IS Ferreira, B Price and LL Pereira-da-Conceicoa [ethanol, AMGS]; 2 ♂ imagos, Hogsback, Tyume source stream, Middle site (H2), 32.5931S 26.9581E, 2.xii.2017, IS Ferreira, B Price and LL Pereira-da-Conceicoa [ethanol, AMGS]. KwaZulu-Natal. 7 ♂ imagos, 1 ♀ imago, 3 ♂ subimagos, Cathkin Peak, 6000 ft (ca. 1829 m), no date [ethanol, AMGS]; 1 ♂ imago, Ngele Forest, Mackton farm, 30.542S 29.682E, FC de Moor and WRJ de Moor [ethanol, AMGS].

*Etymology*: The species is, beyond doubt, named after the old provincial name, Natal, where the type was collected. Demoulin (1970) corrected the grammatical gender to be masculine.

*Nomenclatural notes*: Esben-Petersen (1913) identified a specimen (1 subimago, KwaZulu-Natal, M'fongosi, ix.1911, WE Jones, SAMC) as *Atalophlebia* and Lestage (1918)

accepted it as *At. tabularis* (*sensu* Esben-Petersen, 1920). When he described *At. tabularis* (*sensu* Esben-Petersen, 1920) as the new species, *At. phoeocera* Lestage, 1924, he included this subimago. It was, without a doubt, a misidentification because it was collected in KwaZulu-Natal, and therefore, it must be one of the species from that locality. The specimen is accepted as missing, since Barnard (1932) stated that it was not in the SAMC collection where it was expected to be. He then decided to place it under *A. natalicus* (with uncertainty) because that was the only species known from that region at the time. Since then, two more species have been described, i.e., *A. argus* and *A. tricuspidatus*. The specimen is missing and undescribed, and it, therefore, cannot be identified and should be disregarded for now.

*Diagnosis*: Imagos: dorsal portion of the male's eyes small (ventral portion 0.80 x dorsal portion) and widely separated on the meson of the head (Figure 3.16C and F); forewing crossveins (excluding pterostigma) with narrow shading in costal area, heavier in subcostal area and heavier but not oval-shaped in distal anterior radial area (Figure 3.16A and Figure 3.20D); hind wing with vein Sc 0.93–0.95 x wing length (Figure 3.16B); femur with dark bands distally only; and penes fused with shallow cleft and gonopores with curled processes apically (Figure 3.16D-E and Figure 3.20L).

The nymphs are known only from the short description by Crass (1947b) that separated them as follows: gills usually with median and lateral apical filamentous processes present, but irregular in size and number (more irregular than *A. tricuspidatus*; Crass 1947b).

***Imago:*** (Figure 3.2G) *Dimensions*. ♂: body 10.6–12.6 mm; forewing 10–12 mm; hind wing 1.7 mm.

♀: body 11–12 mm; forewing 13–14 mm.

***Male:*** *Eyes*. Dorsal portion very dark brown to black; small; widely separated on the meson of the head (Figure 3.16C). Ventral portion 0.80 x dorsal portion (Figure 3.16F). *Wings*. Forewing (Figure 3.16A) crossveins (excluding pterostigma) with narrow shading in costal area; heavier in subcostal area; heavier but not oval-shaped in distal anterior radial area (Figure 3.20D). Crossveins in costal area prior to pterostigma well developed; 6–8 crossveins before bulla. Pterostigmal crossveins not anastomosing. Vein RS forked at 0.29 x wing length; MA fork closer to wing margin than to RS-MA fork; $MP_2$ attached to $MP_1$ at RS-MA fork (Figure 3.20F). Hind wing width 0.65 x length; length 0.17 x forewing; vein Sc 0.93–0.95 x wing length; subcostal area with 5–6 crossveins. *Legs*. Femur with dark bands distally only, broadly suffused medially. Leg I with length of segments 0.80:1(4.01):0.05:0.31:0.32:0.25: 0.11; total

length 11.41 mm; length of femur 0.80 or more of tibia. *Abdomen*. Posterolateral projections on segment IX acuminate and blunt (Figure 3.20J). *Genitalia*. Penes (Figure 3.16D-E and Figure 3.20L) fused with shallow cleft; oblong shaped; dorsoventrally flattened. Gonopores with curled processes apically. *Caudal filaments*. Dark annulations broad (Figure 3.20O).

***Female***: Similar to male. Pregenital plate present. Subanal plate with shallow indentation.

***Nymph:*** *Dimensions*. Maximum body length not available.

*Gills*. Usually median and lateral apical filamentous processes present; filamentous processes about half the length of lamella (but irregular in size and number; more irregular than *A. tricuspidatus*; (Crass 1947b)).

***Aprionyx tricuspidatus* Crass, 1947**

(Figure 3.2H, Figure 3.17-Figure 3.18, Figure 3.20M, Figure 3.21D, F, N)

*GBIF taxon ID: 6934111*

*Aprionyx tricuspidatus* Crass, 1947 Ann. Natal Mus. 11(1): 97–100 Fig. 33–34 (♂ imago; nymph)

*Type locality*: SOUTH AFRICA: KwaZulu-Natal. Curry's Post, 29.37S 30.13E.

*Material*: **Prospective lectotype:** SOUTH AFRICA: KwaZulu-Natal. 1 ♂ imago (originally preserved in ethanol, found desiccated May 2020, rehydrated for examination), Curry's Post district, [29.37S 30.13E], i.1946, RS Crass [ethanol, AMGS].

**Paralectotypes:** SOUTH AFRICA: KwaZulu-Natal. 5 ♂ imagos, 1 ♀ imago, 3 nymphs, (♂ imagos originally preserved in ethanol, found desiccated May 2020), Curry's Post district, [29.37S 30.13E], i.1946, RS Crass [ethanol, AMGS]; 3 ♂ imagos, 1 ♀ imago, 1 nymph, Newstead Balgowan, Curry's Post district, 10.xi.1945, RS Crass [ethanol, NHMUK].

*Etymology*: This name is possibly a combination of the Latin *tria* (three) and *cuspidatus* (pointed), most likely alluding to the three apical projections of the gill lamella.

*Diagnosis*: The imagos are characterised as follows: eyes of the males with the dorsal portion small (ventral portion 0.82 x dorsal portion) and widely separated on the meson of the

head (Figure 3.17C and F); forewing crossveins (excluding pterostigma) with narrow shading in costal area, heavier in subcostal area and oval-shaped in distal anterior radial area (Crass 1947b); fork of vein MA closer to RS-MA fork than to wing margin; hind wing vein Sc 0.90 x wing length (Figure 3.17B); femur with dark bands distally only; male leg I with length of femur less than 0.80 of tibia; and penes separated in apical third and gonopores with curled processes apically (Figure 3.17D and Figure 3.20M).

The nymphs are distinguished primarily from all of the western species here because *A. argus* and *A. natalicus* are largely unknown: labrum length about 0.43 x width (Figure 3.18A); anterior medial emargination with six large, acute and well-defined teeth (triangular; Figure 3.18B); left mandible with median margin proximal to prostheca without concavity (Figure 3.18D); maxilla (Figure 3.18C) with apical-ventral row of ca. 12 pectinate setae; maxillary palp segment II with distal end about 2.5 times the proximal end; segment III covered in long hairs, and apical hairs extending beyond apex markedly longer than segment III (at least 1.33); labial palp segment II distally about twice as wide as proximally (Figure 3.18G); abdominal segments with posterolateral projections present on segments VIII and IX and acuminate (Figure 3.21N); posterior marginal denticles on tergites well developed on segment X; and gills with median and lateral apical filamentous processes usually present, but irregular in size and number (Figure 3.21F).

***Imago:*** (Figure 3.2H) *Dimensions*. ♂: body 9.5–12.5 mm; forewing 10–13 mm; hind wing 1.6 mm; caudal filaments 23–30 mm.

♀: body 11–14 mm; forewing 12–15 mm; hind wing 2 mm; caudal filaments 20–25 mm.

***Male:*** *Eyes*. Dorsal portion very dark brown to black; small; widely separated on the meson of the head (Figure 3.17C). Ventral portion 0.82 x dorsal portion (Figure 3.17F). *Wings*. Forewing (Figure 3.17A) crossveins (excluding pterostigma) with narrow shading in costal area; heavier in subcostal area; oval-shaped in distal anterior radial area. Crossveins in costal area prior to pterostigma well developed; 6–10 crossveins before bulla. Pterostigmal crossveins not anastomosing. Vein RS forked at 0.28 x wing length; MA fork closer to RS-MA fork than to wing margin; $MP_2$ attached to $MP_1$ at RS-MA fork. Hind wing (Figure 3.17B) width 0.61 x length; length 0.15 x forewing; vein Sc 0.90 x wing length; subcostal area with 5–6 crossveins. *Legs*. Femur with dark bands distally only, broadly suffused medially. Leg I with length of segments 0.77:1(5.22):0.04:0.33:0.33:0.28:0.10; total length 14.86 mm; length of femur less

than 0.8 of tibia. *Abdomen*. Posterolateral projections on segment IX acuminate and blunt. *Genitalia*. Penes (Figure 3.17D-E and Figure 3.20M) separated in apical third; oblong shaped; dorsoventrally flattened. Gonopores with curled processes apically. *Caudal filaments*. Dark annulations broad.

***Female***: Similar to male. Pregenital plate present. Subanal plate with shallow indentation.

***Nymph:*** *Dimensions*. Maximum body length 17 mm; caudal filaments 24 mm.

*Mouthparts*. Labrum (Figure 3.18A) length about 0.43 x width; widest in distal third; lateral margin rounded; dorsal surface with row of scattered long setae, medially expanding towards distal margin. Anterior medial emargination (Figure 3.18B) with six teeth; teeth large, acute and well-defined (triangular). Outer margin of left mandible (Figure 3.18D) curved distally and proximally, flattened medially; median margin proximal to prostheca without concavity. Prostheca as illustrated in Figure 3.18E (left) and Figure 3.18F (right). Maxilla (Figure 3.18C) with apical-ventral row of ca. 12 pectinate setae. Maxillary palp segment II 1.2 x segment I; with distal end about 2.5 times the proximal end. Segment III covered in long hairs; apical hairs extending beyond apex distinctly longer than segment III (at least 1.33). Labial palp (Figure 3.18G) segment I width 0.53 x length. Segment II 0.86 x segment I; distally about twice as wide as proximally. *Legs* (Figure 3.18H). Femur with dark bands similar to imagos. Leg I with length of segments 1.09:1(2.05):0.54. Ventral margin of tibia I with fringe of bipectinate setae; without hairlike setae. Tarsus I ventral margin with row of small, spinelike setae (Figure 3.21D). Claws hooked (Figure 3.18I). *Abdomen*. Posterolateral projections present on segments VIII and IX; all acuminate (Figure 3.21N). Posterior marginal denticles well developed on segment X. *Gills*. Usually median and lateral apical filamentous processes present; filamentous processes about half the length of lamella (but irregular in size and number; Figure 3.21F). *Caudal filaments*. Primary swimming setae absent.

## 3.4 Taxonomic discussion

*Aprionyx* is easily distinguished from other South African genera by the wings, claws, and genitalia of the imagos, as well as the labium, claws, and gills of the nymphs. The forewings most resemble those of *Adenophlebia* and *Adenophlebiodes* (Barnard 1932, Crass

1947b). However, in *Aprionyx*, the intercalaries in the cubital area are not parallel to CuA, while *Adenophlebia* and *Adenophlebiodes* have at least one vein parallel (at least more or less) to CuA. The hind wings are easily recognised for having a poorly developed, obtuse and rounded costal process, with a long Sc. The penes are at least almost completely fused, like *Adenophlebia*, but not with the recurved spinelike processes. *Aprionyx* is the only South African taxon with edentate nymphal claws, and the labial palps are unique among South African leptophlebiid species. The labial palps of the New Zealand species, *Acanthophlebia cruentata*, are somewhat similar, but in this species the third segment is not as short as in *Aprionyx*, the claws are denticulate and the gills are *Adenophlebia*-like (Phillips 1930). *Euthraulus* is the only South African genus with gills that resemble some *Aprionyx* nymphs in having three filamentous processes, but the gills are oval, and the first gill is filamentous (Barnard 1932).

The previous chapter (Chapter 2) identified two distinct groups within *Aprionyx*, the eastern and western groups. With the revised description in this chapter, the imagos of these two groups can be distinguished based on several characters. In the males of all of the eastern species, the dorsal portion of the eyes is very dark brownish-black (about as dark as the ventral portion), widely separated and small (ventral portion >0.79 of the dorsal portion). In the forewing, vein $MP_2$ is always attached to $MP_1$ at the RS-MA fork and vein Sc of the hind wing reaches about 0.90 to 0.95 of the wing length. The femurs are dark-banded distally and broadly suffused medially, often much darker on the first legs. The abdominal posterolateral projections on segment IX are acuminate and blunt. The penes are oblong with rounded processes at the gonopores. In the western species, the dorsal portion of the eyes varies in colour between males but is always a lighter tone compared to the ventral portion, and the size varies from medium to large (ventral portion <0.74 the dorsal portion). The point of attachment of vein $MP_2$ to $MP_1$ varies, and *A. peterseni* is the only species in this group where it is attached at the RS-MA fork, though it is not quite as close as in the eastern species. The length of vein Sc in the hind wing also varies and *A. rubicundus* is the only species that can have an Sc vein as long as some eastern species. The femurs are dark-banded distally and medially in all western species except for *A. phoeocera*, which is similar to the eastern species but with the broad suffusion generally darker. The abdominal posterolateral projections on segment IX are biacuminate, but not as prominent as in the nymphs. The penes are broad basally, tapering towards the apex with recurved apical flap-like projections covering the gonopores.

The imagos of the three eastern species are very similar to each other, with only a few characters separating them. The shading of the crossveins in the forewings is similar to each other and differs only in the degree of shading. The shading is heavy, much like *A. peterseni*, but oval-shaped in the distal anterior radial area instead of the costal area. The crossveins of *A. tricuspidatus* is with slightly less shading than in *A. argus* (Barnard 1940, Crass 1947b). *Aprionyx natalicus* is not as heavily shaded and is more similar to *A. rubicundus*. The penes of *A. natalicus* are completely fused, with only a shallow cleft apically, as opposed to *A. argus* and *A. tricuspidatus* where they are separated apically for less than a third. *Aprionyx tricuspidatus* was the only *Aprionyx* species with vein MA of the forewing fork closer to the point of attachment to RS than to the wing margin, and in leg I the tibia is longer relative to the femur than in *A. argus* and *A. natalicus*.

The morphological revision of the nymphs was unable to determine the salient characters responsible for distinguishing the eastern from the western *Aprionyx* species. However, the two groups are separable by their abdominal pigmentation, which is generally similar in the imagos and nymphs. The abdominal pigmentation of the eastern species is nearly inseparable from each other (Crass 1947b) but is unmistakenly different from all of the western species, which show some variation (Barnard 1932, 1940). *Aprionyx tricuspidatus* is the only nymph of the three eastern species that could be studied since no nymphal material was available for the other two. Several characters distinguish *A. tricuspidatus* from all of the western species, and are expected to be among the distinctive eastern *Aprionyx* characters identified in future research. The labrum is wider relative to the length compared to any of the western species and the medial emargination has six acute teeth. The maxilla has distinctly fewer pectinate setae (less than 13) in the apical-ventral row. Segment II of the maxillary palps has a wider distal end compared to the proximal end than in any of the western species, while segment III is covered only in long hairs and the apical hairs are distinctly longer than segment III (extends beyond the apex about 1.33 x segment III). Segment II of the labial palps is distally about twice the width of proximally. The abdominal posterolateral projections are present on segments VIII and IX only, and are acuminate, and the posterior margin has well-developed denticles on segment X only.

With the nymphs of *A. argus* and *A. natalicus* still largely undescribed, it remains difficult to distinguish the three eastern species from each other. With the meagre descriptions currently available for *A. argus* and *A. natalicus*, nymphs of the three eastern species are at the

moment distinguished only by the gills. The lateral processes are merely bulges in *A. argus*, while in *A. natalicus* and *A. tricuspidatus*, they usually have filamentous processes but are rather irregularly only bulges on some gills (Figure 3.21F). According to Crass (1947b) this anomaly of the gills is more irregular in *A. natalicus* than in *A. tricuspidatus*. Crass (1947b) also defines *A. natalicus* as having segment II of the maxillary palp with a slightly larger relative length than in *A. tricuspidatus*, and different to *A. argus*, but without measurements for comparison.

The imagos of *A. tabularis* most resemble those of *A. rubicundus* but can also be confused with *A. phoeocera*. All three species are generally darker, and the pigmentation patterns of the abdomen can cause these species to be mistaken for each other, especially by inexperienced observers. The penes of all three species are basally and medially broad, but in *A. phoeocera* they taper to a narrow, acute tip, and in *A. tabularis* they are slightly wider than in *A. rubicundus*. In *A. rubicundus* the tip is also more obtuse than in *A. tabularis*, appearing almost truncated. The margin is also more angular where it starts tapering in *A. tabularis*. The forewings are broad, as in *A. phoeocera*, while *A. rubicundus* has narrow forewings, posteriorly truncated proximally. The males have large eyes, narrowly separated mesally, whereas in *A. rubicundus* the eyes are also narrowly separated but slightly further apart and smaller; and widely separated in *A. phoeocera*. The nymphs of *A. tabularis* are nearly indistinguishable from *A. rubicundus* in terms of the mouthparts and legs; both have slender claws, not hooked, and caudal filaments without swimming setae. Unlike *A. rubicundus*, the nymphs of *A. tabularis* have posterolateral projections on segments VI to IX of the abdomen, with posterior marginal denticles well developed on all segments. According to Barnard (1932), *A. tabularis* nymphs are also very similar to *A. peterseni*, but it is now clear that they can easily be distinguished by close examination of the mouthparts, legs and abdomen.

*Aprionyx tabularis* is not as well-known as the other species and appears to be amongst the rarer ones. This species is probably restricted to higher mountain streams (Harrison 1949) or just more common in these types of streams. It is also possible that sampling efforts did not include their preferred habitat, but *A. tabularis* will be presumed to be an uncommon species until more information can be gathered. As a result, only limited material was examined and it had degraded markedly due to age. The current description had to rely on earlier descriptions, which were incomplete or inaccurate (Eaton 1884, Barnard 1932, Peters and Edmunds 1964). From Barnard's (1932: A. intermedius) description, it appears as though the wings have weak

crossveins and are barely shaded, as in *A. phoeocera*. The degraded wings made it difficult to see the venation clearly, but the costal crossveins before the pterostigma do not seem weaker than the other crossveins. The crossveins in the pterostigma have been described as rarely anastomosing (Eaton 1884, Barnard 1932), yet anastomosis was absent in the only male imago examined. It is likely to be more common in females, since Barnard (1932, fig. 27a) illustrated a female subimago wing with few anastomosed veins, and for this study, the condition was observed in another female imago. It also seems that anastomosis is never as extensive as in *A. phoeocera*. In Barnard's (1932) type material (Table 3.1), there is only one nymphal specimen to examine. All of the gills of this nymph were lost, so the descriptions of the gills relied on the figures by Peters and Edmunds (1964, fig. 126). Caudal filaments of the *A. tabularis* nymph examined are broken off and missing, but Barnard (1932) described them as being "sparsely setose" compared to those of *A. peterseni*. Primary swimming setae are present but rather short and sparse in *A. peterseni*; hence, they are described here as absent in *A. tabularis*. These discrepancies can only be resolved once substantial fresh material has been collected and studied.

*Aprionyx pellucidulus* resembles *A. peterseni* the most, and the nymphs are especially alike. The imagos can usually be distinguished by their pigmentation since those of *A. peterseni* are typically identified by wings that have a spotted appearance and caudal filaments with broad annulations. *Aprionyx pellucidulus* are overall darker in pigmentation, whereas *A. peterseni* are recognised by the contrasting dark and light pigmentation, giving it a somewhat spotted appearance. Identification is more difficult when the pigmentation has faded, as the genitalia is very similar. The wing venation (generally less costal crossveins in *A. peterseni* and in *A. peterseni* vein $MP_2$ is attached to $MP_1$ nearly at the RS-MA fork) and caudal filaments (narrow annulations in *A. pellucidulus*) can then be used to distinguish these two species. The nymphs are more difficult to separate, but the abdominal posterior projections (present only on segments VI to IX in *A. pellucidulus*) and caudal filaments (primary swimming setae present in *A. peterseni*) are convenient characters.

## 3.5 Characters

Imaginal characters in the following list and the identification key (section 3.7) were primarily drawn from male specimens, since most females are still undescribed and have fewer

distinctive morphological characters, mainly because of the reproductive structures of the males (Edmunds et al. 1976). Although females are typically similar to males in overall appearance except, for example, the eyes and reproductive organs, small variations in wing venation seem likely. Therefore, where gender is not specified, characters apply to males but can be used with caution to identify females. This, however, should generally only be attempted by experienced specialists. For the same reason, the characters and identification key should be used cautiously for subimagos and only by people experienced in the differences between subimagos and imagos, such as the size and development of the caudal filaments, forelegs, eyes and genitalia (Edmunds and McCafferty 1988).

### *3.5.1 Imagos*

MALE EYES

1. Dorsal portion, shape: (0) unstalked; (1) stalked

Male imagos in Leptophlebiidae typically have the eyes divided into dorsal and ventral portions, rarely undivided (Kluge 2004, Domínguez et al. 2006). In South African Leptophlebiidae, the males' eyes are always divided and distinctly larger compared to the females' eyes. When divided, the dorsal portion may be stalked or unstalked (Edmunds et al. 1976, Savage and Peters 1982). According to Savage and Peters (1982) and Kluge (2004) unstalked and divided eyes are the plesiomorphic state; hence, the primitive state is expressed in *Aprionyx* (e.g. Figure 3.5F). Among the outgroup taxa, only *Choroterpes* have eyes stalked, though a very short stalk. This character was derived from previous studies by Savage and Peters (1982: no character number), Domínguez et al. (2001: character 42) and Domínguez et al. (2019: character 48).

2. Dorsal portion, distance between eyes relative to width of eye: (0) touching to narrowly separated, <0.33; (1) widely separated, >0.40

The separation between the dorsal portions of the eyes in male imagos has been useful to discern species, for example, the species of *Askola* Peters, 1969 (Campos et al. 2019). Two discrete states, expressed as the distance between the dorsal portion of the eyes relative to the dorsal width of an eye, were recognised amongst the *Aprionyx* species: <0.33, i.e., touching to narrowly separated (state 0; Figure 3.5C, Figure 3.7C, Figure 3.9C and Figure 3.13C) or, >0.40,

i.e., widely separated (state 1; Figure 3.11C, Figure 3.15C, Figure 3.16C and Figure 3.17C). The eyes of *A. pellucidulus* and *A. peterseni* typically meet (or at least nearly meet) dorsally on the meson, whereas in *A. rubicundus* and *A. tabularis,* the eyes are narrowly separated, particularly in *A. tabularis* (state 0). In the remainder of the *Aprionyx* species, the eyes are widely separated. In all three of the outgroup taxa, the eyes meet or nearly meet on the meson. This character was adapted from Campos et al. (2019: character 1) for the states identified in the *Aprionyx* species.

3. Ventral portion, length relative to dorsal portion: (0) <0.63, large dorsally; (1) 0.64 to 0.78, medium dorsally; (2) >0.79, small dorsally

The eyes of male imagos differ in size, and can be classified into three groups by the relative length of the ventral to dorsal portions, measured on the lateral face (Appendix J). In *Aprionyx* the upper portion was always longer than the lower portion, but longest in *A. tabularis* (<0.63; state 0; Figure 3.5F) and smallest in the three eastern species (>0.79; state 2; Figure 3.15F, Figure 3.16F and Figure 3.17F). In the rest of the species the dorsal portions are medium in size (0.64-0.78; state 1; Figure 3.7F, Figure 3.9F, Figure 3.11F and Figure 3.13F). Taxa in the outgroup either had large or medium-sized dorsal portions. This character was introduced to define the size differences presented in the eyes of *Aprionyx* species.

WINGS

4. Forewing, crossveins in costal area (excluding pterostigma), extent of shading: (0) barely extends beyond vein; (1) narrow; (2) heavy, oval-shaped

The forewings of *Aprionyx* generally have clearly visible veins, with dark shading on the crossveins, sometimes referred to as maculae (Domínguez et al. 2006). The extent of this shading in the costal area is variable between species. In some species the crossveins are barely shaded, i.e., any dark shading present is confined to the vein, as in *A. pellucidulus* (state 0; Figure 3.7A and Figure 3.20A), whereas in others the shading is distinctly thicker than the veins, e.g., in *A. rubicundus* (state 1; Figure 3.13A and Figure 3.20B). In *A. peterseni* the shading is even heavier and somewhat oval, giving it a spotted appearance (state 2; Figure 3.9A and Figure 3.20C). The outgroup taxa are variable, with *Acanthophlebia* similar to *A. rubicundus* while *Austrophlebioides* and *Choroterpes* have darker veins but without shading that is distinctly thicker. This is a revised character from one used by Domínguez (1995:

character 5), Salles and Domínguez (2012: character 4) and Salles et al. (2020: character 4) for the *Aprionyx* species, and character 5 is an extention to conform to the eastern species.

5. Forewing, crossveins in distal anterior radial area, shape of shading: (0) not oval-shaped; (1) oval-shaped

In the eastern *Aprionyx* species, the shading on the distal crossveins in the anterior radial area is distinctly heavier than in the costal and subcostal areas, and can be somewhat oval-shaped (state 1). According to the original drawings, this is most pronounced in *A. argus* (Barnard 1940) and less so in *A. tricuspidatus* (Crass 1947b), but in *A. natalicus* the shading is not oval-shaped (state 0; Figure 3.20D). This character is not applicable to the western *Aprionyx* species or the outgroup taxa because it applies only to species that has heavier shading in the radial area compared to the costal and subcostal areas. This character was introduced as an extended version of character 4 to accommodate the specific states of the eastern species.

6. Forewing, crossveins in costal area before bulla, number: (0) 0 to 3; (1) 4 or more

The number of crossveins before the bulla is not a distinctive character between the *Aprionyx* species, except for *A. peterseni* which has the least and *A. phoeocera* the most. In the outgroup taxa, however, *Choroterpes* is distinctly different from *Aprionyx* with 3 or fewer crossveins before the bulla. This character was modified for this scenario from Salles and Domínguez (2012: character 3) and Salles et al. (2020: character 3).

7. Forewing, pterostigmal crossveins: (0) not anastomosing; (1) anastomosing

In most *Aprionyx* species anastomosis is absent from the stigmatic crossveins (state 0), but it is always present and rather extensive in *A. phoeocera* (state 1; Figure 3.11A and Figure 3.20E). The presence of anastomosing stigmatic crossveins was inconsistent in the few *A. tabularis* specimens studied. According to Eaton (1884) anastomoses are rare and when present, not extensive, but he obtained only one male *A. tabularis* specimen and did not supply illustrations of the wings. Since it is not clear what he meant by ‘rare’ and with the holotype specimen missing, *A. tabularis* is coded as missing (i.e. uncertain if state 0, 1 or a third state), as both the absence and presence of anastomosis were identified in the specimens examined. This character was introduced as a salient character that can distinguish *Aprionyx* species, such as *A. phoeocera*.

8. Forewing, MA fork, location: (0) closer to RS-MA fork than to wing margin; (1) closer to wing margin than to RS-MA fork

In all *Aprionyx* species, vein MA of the forewing is forked near the middle of its length. The total vein length was measured from the RS-MA fork to the wing margin midway between $MA_1$ and $MA_2$, and compared to the distance of the MA fork from the RS-MA fork (Appendix J). Nearly all *Aprionyx* species have the fork of vein MA slightly (at about 0.52-0.58 of total length) closer to the wing margin (state 1), but in *A. tricuspidatus* vein MA forks slightly (at about 0.48 of total length) closer to the RS-MA fork (state 0). One taxon in the outgroup, *Austrophlebioides*, also has the MA fork positioned closer to the RS-MA fork. This character was adapted from Ogden et al. (2009b: character 13).

9. Forewing, vein $MP_2$ attached to $MP_1$, location relative to the distance from RS-MA fork to RS fork: (0) at RS-MA fork (less than 0.10); (1) 0.15 to 0.25; (2) 0.30 to 0.80

In the forewing of *Aprionyx* vein $MP_2$ is attached to $MP_1$ between the RS-MA fork and the RS fork. The location, however, varies and was used to categorise the species into three groups. In the three eastern *Aprionyx* species vein $MP_2$ is attached to $MP_1$ at the RS-MA fork and in *A. peterseni* nearly at the RS-MA fork (state 0; Figure 3.20F-G). *Aprionyx tabularis*, *A. pellucidulus* and *A. phoeocera* have the vein attached between 0.15 and 0.25 of the distance from the RS-MA fork to the RS fork (state 1; Figure 3.5A, Figure 3.7A and Figure 3.11A). The point of attachment is further away from the RS-MA fork and more variable in *A. rubicundus*, i.e., between 0.37 and 0.72 the RS-MA fork to RS fork distance (state 2; Figure 3.13A). All taxa in the outgroup are characterised by state 2. This character was introduced as a beneficial character for *Aprionyx* species.

10. Forewing, vein $iCu_1$, attachment of vein base: (0) free basally or attached to CuA, but never attached to CuP; (1) attached to CuA and CuP

The cubital area in *Aprionyx* has two long intercalaries, and the first intercalary, $iCu_1$, is free or attached at the base to CuA, but it is never attached to CuP (state 0). *Austrophlebioides* is the only outgroup taxa with the $iCu_1$ attached to CuA and CuP, or almost so, i.e., the vein is typically longer (state 1). This character was adapted from characters in studies by Domínguez (1995: character 2), Domínguez (1999: character 13), Domínguez et al. (2001: character 46) and Domínguez et al. (2019: character 52).

11. Forewing, posterior margin, proximally: (0) not truncated, wing broad; (1) truncated, wing narrow.

Nearly all *Aprionyx* species have broad forewings (>0.32 of length), with the posterior margin not truncated proximally, and a clear tornus (state 0; e.g., Figure 3.5A). *Aprionyx rubicundus*, however, has relatively narrow forewings (≤0.32 of length) and the posterior margin truncated proximally (state 1; Figure 3.13A). This character was introduced to accommodate the narrow wings identified in *A. rubicundus*.

12. Hind wing, vein Sc, length relative to wing length: (0) less than 0.69; (1) 0.70 to 0.79; (2) 0.80 to 0.85; (3) more than 0.86

Distinct differences in the length of vein Sc of the hind wing were found between *Aprionyx* species. The length of Sc was calibrated in relation to the hind wing length (Appendix J). *Aprionyx phoeocera* consists of a Sc vein proportionately shorter than any of the other *Aprionyx* species (state 1; Figure 3.11B). In *A. tabularis*, *A. pellucidulus* and *A. peterseni* this vein is between 0.80 and 0.85 the length of the hind wing (state 2; Figure 3.5B, Figure 3.7B and Figure 3.9B). *Aprionyx rubicundus* and the three eastern *Aprionyx* species have a long Sc vein (>0.86 wing length) nearly reaching the wing apex (state 3; Figure 3.13B, Figure 3.15B, Figure 3.16B and Figure 3.17B). Vein Sc in the outgroup taxa is either very short (state 0), i.e., *Choroterpes*, or long, i.e., *Acanthophlebia* and *Austrophlebioides* (state 3). This character was altered accordingly for the *Aprionyx* species, from a relatively common charater in Domínguez (1995: character 8), Christidis (2005: character 35), Salles and Domínguez (2012: character 8) and Salles et al. (2020: character 8).

13. Hind wing, crossveins in subcostal area, number: (0) 4 to 8; (1) more than 8

Like the number of crossveins found before the bullae in the forewing, the cross-vein count in the subcostal area of the hind wing is not very diagnostic in *Aprionyx* species. Only *A. phoeocera* has markedly more crossveins (up to 13) in the subcostal area (state 1), whilst the rest of the *Aprionyx* species have 4 to 8 crossveins (state 0). Of the outgroup taxa, only *Austrophlebioides* has more than 8 crossveins in the subcostal region. This character was introduced as a modified version of the character used in studies such as Domínguez (1995: character 9), Christidis (2005: character 36), Salles and Domínguez (2012: character 9) and Salles et al. (2020: character 9).

LEGS

14. Claws, type: (0) dissimilar, one hooked and one pad-like; (1) both hooked

The type of claws found in adult mayflies is characteristically a genus-level character (Domínguez et al. 2006). In *Aprionyx* and *Acanthophlebia* both claws are hooked (state 1), but *Austrophlebioides* and *Choroterpes* have dissimilar claws, with one hooked and one pad-like (state 0). This character was derived from studies such as Domínguez et al. (2001: character 50), Christidis (2005: character 37) and Domínguez et al. (2019: character 56).

ABDOMEN

15. Posterolateral projections on segment IX, form: (0) acuminate; (1) biacuminate (often appearing truncated)

Posterolateral projections are present only on the nineth abdominal segment of imagos. This projection is somewhat similar to that of the nymphs, but not as prominent. The western *Aprionyx* species are characterised by a rather subtle biacuminate projection, which can look like a truncated acuminate projection (state 1; Figure 3.20H-I). In the eastern *Aprionyx* species this projection is acuminate, as seen in the nymphs, but usually smaller and sometimes more rounded (state 0; Figure 3.20J). *Choroterpes* has very small acuminate projections, and the status of the other two taxa in the outgroup is unknown. This character was introduced as a distinguishing character between the eastern and western *Aprionyx* species.

MALE GENITALIA

16. Forceps, basal broadening: (0) with inner corner rounded; (1) quadrangular

Dominguez (1995: character 10) used this character to describe the basal section of the forceps, but only two of the four states are applicable in this scenario, as for example character 5 in Domínguez (1999). The taxa included in the morphological analysis have a broadened section at the base. The shape of this broadening is the same in all *Aprionyx* species and can be described as quadrangular. Of the outgroup taxa, only *Choroterpes* has rounded inner corners, the rest being quadrangular like *Aprionyx*.

17. Penis lobes, division: (0) separated except at base; (1) separated in apical half to apical third; (2) fused, typically with shallow cleft

The penis in ephemeropterans is bilobed, but the degree to which it is fused differs between taxa (Edmunds et al. 1976, Domínguez 1999, Domínguez et al. 2006). In most *Aprionyx* species the lobes are completely fused and typically have a shallow cleft (state 2; Figure 3.5D and Figure 3.20K-L). In *A. argus* and *A. tricuspidatus* the lobes are fused for the most part (state 1; Figure 3.15D and Figure 3.20M), similar to *Acanthophlebia* and *Austrophlebioides*. The penis lobes of *Choroterpes* are almost entirely separated (state 0). This character was modified from Domínguez (1999: character 6), Domínguez et al. (2001: character 52), Christidis (2005: character 41) and Domínguez et al. (2019: character 59) for the states relevant to the species used in this study.

18. Penis, width: (0) narrow; (1) broad

In most taxa included for analysis, the penis is relatively narrow (state 0), though in *A. tabularis* and *A. phoeocera*, the penis is predominantly wider than (or at least as wide as) the broad inner margin of the forceps (state 1; Figure 3.5D and Figure 3.11D). All outgroup taxa have a narrow penis. This character was introduced to characterise the penis width.

19. Penis, shape: (0) oblong shaped; (1) gradually tapering towards apex; (2) tapering in apical half

In the three eastern *Aprionyx* species the penis is oblong (state 0; Figure 3.15D, Figure 3.16D and Figure 3.17D). The penis is tapering gradually towards the apex in *A. pellucidulus* and *A. peterseni* (state 1; Figure 3.7D and Figure 3.9D), but in the other three western *Aprionyx* species it tapers only in the apical half (state 2; Figure 3.5D, Figure 3.11D and Figure 3.13D). *Acanthophlebia* is similar to the eastern *Aprionyx* species, and in *Austrophlebioides* the penis usually tapers gradually towards the apex. The character is not applicable to *Choroterpes* because structurally, different characters and character states apply. This character was introduced to characterise the penis shape.

20. Penis, apex, shape: (0) rounded; (1) obtusely angular; (2) narrow, acute

This character applies only to the five western *Aprionyx* species with a penis tapering to a pointed apex and separates them based on the shape of the penis apex, i.e., rounded (state 0; Figure 3.7D and Figure 3.9D), obtusely angular (state 1; Figure 3.5D and Figure 3.13D) or acute and narrow (state 2; Figure 3.11D). This character was inspired by Domínguez (1995:

character 12), Domínguez (1999: character 7), Salles and Domínguez (2012: character 12) and Salles et al. (2020: character 12).

21. Penis, ventral, shape: (0) dorsoventrally flattened; (1) laterally projecting downward medially; (2) base swollen

The penis of some *Aprionyx* species present with a projection or swelling about midway between the base and apex, which is visible from the lateral view of the penis. *Aprionyx pellucidulus* and *A. peterseni* have the same basal swelling that gives the penis a more angular appearance (state 2; Figure 3.7E and Figure 3.9E). Both *A. tabularis* and *A. rubicundus*, have paired projections, situated laterally on the penis and protruding ventrally (state 1; Figure 3.5D-E and Figure 3.13D-E), but it is more subtle in *A. rubicundus* than in *A. tabularis*. The rest of the taxa do not present with such projections, but are dorsoventrally flattened (state 0; Figure 3.11E, Figure 3.15E, Figure 3.16E and Figure 3.17E). The character is not applicable to *Choroterpes* because structurally, different characters and character states apply. This character was introduced to further characterise the penis shape in terms of projections.

22. Penis, dorsolateral spines: (0) absent; (1) present on each lobe

*Aprionyx* species do not have any spines on the penis, but *Austrophlebioides* species have at least a dorsolateral spine on each lobe. The character is not applicable to *Choroterpes* because structurally, different characters and character states apply. This character was inspired by Domínguez (1999: character 11), Christidis (2005: character 42), Salles and Domínguez (2012: character 13) and Salles et al. (2020: character 13).

23. Gonopores, apical processes at opening, type: (0) curled; (1) recurved flap-like

In *Aprionyx*, each gonopore opens apically at a projection. In the western species the gonopores have flap-like projections that are recurved and usually flush with the outline of the penis (state 1; Figure 3.5D and Figure 3.20K). The eastern species lack these flap-like structures and the gonopores open at curled processes (state 0; Figure 3.20L-M). Though it is clear that the outgroup taxa lack these structures, the type of processes in *Acanthophlebia* and *Austrophlebioides* is unknown. The character is not applicable to *Choroterpes* because structurally, different characters and character states apply. This character was introduced to further characterise the penis in terms of projections at the gonopores.

CAUDAL FILAMENTS

24. Caudal filaments, dark annulations, size: (0) narrow; (1) broad

The caudal filaments in all of the included taxa have dark annulations. *Aprionyx peterseni* have very distinct broad annulations (Figure 3.20N), while those in *A. natalicus* and *A. tricuspidatus* are also broad but not as distinctive (Figure 3.20O). The state of *A. argus* is unknown at this stage. The remainder of the *Aprionyx* species has narrow and often indistinct annulations (state 0; Figure 3.20P). Only *Choroterpes* in the outgroup has narrow annulations. This character was introduced to define the annulated caudal filaments.

### *3.5.2 Nymphs*

MOUTHPARTS

25. Labrum, width relative to clypeus: (0) narrower; (1) wider

The width of the labrum relative to the clypeus has been recognized as informative at a generic level by various studies (Domínguez et al. 2001, 2019, Christidis 2005), and in this case it was coded as either narrower or wider. In *Aprionyx,* the labrum is always narrower than the clypeus (state 0). The labrum of *Choroterpes* and *Austrophlebioides* is distinctly wider than the clypeus, but in *Acanthophlebia* it is similar to *Aprionyx*. This character was simplified from Domínguez et al. (2001, 2019: character 1) and Christidis (2005: character 1).

26. Labrum, length relative to width: (0) less than 0.45; (1) 0.46 to 0.54; (2) more than 0.55

The maximum length relative to the width of the labrum is useful in delimiting species of *Aprionyx*. For most *Aprionyx* species, the length:width ratio of the labrum is approximately 0.5:1, with *A. rubicundus* slightly narrower (state 1; Figure 3.14A). The labrum of *A. phoeocera* is relatively longer (state 2; Figure 3.12A), and in *A. tricuspidatus* it is evidently wider (state 0; Figure 3.18A). All three of the outgroup taxa have a labrum that is markedly wider than long. This character was introduced to define the width of the labrum.

27. Labrum, widest section along its length, position: (0) about halfway; (1) distal third; (2) distal quarter

The lateral margins of the labrum in *Aprionyx* are divergent up to the widest part. The widest part is situated at different positions along the length, which is distinctive in some *Aprionyx* species. In *A. tabularis*, *A. rubicundus* and *A. tricuspidatus,* the labrum is widest in the distal third (state 1), while in *A. pellucidulus* and *A. peterseni,* the widest part is about halfway (state 0). *Aprionyx phoeocera* is the only species with the widest part in the distal quarter (state 2). The outgroup taxa correspond with either state 0 or state 1. This character was adapted from Domínguez (1999: character 18) and Domínguez et al. (2001, 2019: character 3).

28. Labrum, lateral margin, shape: (0) rounded; (1) more or less straight

Apart from being divergent, the lateral margin of the labrum is usually rounded (state 0), but in *A. phoeocera,* the margins are more or less straight and diverging slightly (state 1). The margins are rounded in all of the outgroup taxa. This character was derived from Domínguez et al. (2001, 2019: character 2).

29. Labrum, anteromedial emargination, teeth: (0) absent; (1) present

The anteromedial emargination is denticulate in all of the taxa included in this study, except for *Choroterpes*, which is edentate. This character was modified from Domínguez (1995: character 13), Domínguez et al. (2001, 2019: character 4), Christidis (2005: character 4), Salles and Domínguez (2012: character 14) and Salles et al. (2020: character 16).

30. Labrum, anteromedial emargination, teeth, number: (0) five or less; (1) six; (2) seven

The anteromedial emargination in the five western *Aprionyx* species, usually has five teeth (state 1), though *A. rubicundus*, for example, can have as few as three (Figure 3.21A). *Aprionyx tricuspidatus* has six teeth in the emargination (state 2), and seven teeth are present in *Austrophlebioides* (state 3). The anteromedial emargination of *Choroterpes* is edentate and therefore inapplicable, and in *Acanthophlebia* it has five teeth. This character was introduced as an extention of character 29 to accommodate the specific amount of teeth if present.

31. Labrum, anteromedial emargination, teeth, shape: (0) flattened (at least the outside teeth); (1) rounded/bluntly pointed; (2) sharply pointed; (3) triangular

The shape of the teeth in the anteromedial emargination was also distinct in *Aprionyx* species. In *A. rubicundus* and *A. tabularis* the teeth are rather flattened and not well defined, with the outside teeth often wider (state 0; Figure 3.14B and Figure 3.6B). In *A. pellucidulus*

(Figure 3.8B), *A. peterseni* (Figure 3.10B) and *A. phoeocera* (Figure 3.12B), the teeth are rounded or bluntly pointed, well-defined and usually more uniform in size (state 1). The teeth in *A. tricuspidatus*, however, are rather triangular-shaped and larger (state 3; Figure 3.18B). In *Acanthophlebia,* the teeth are small, sharply pointed and well-defined (state 2), whereas in *Austrophlebioides* they are also flattened. This character was introduced to describe the shape of the teeth in more detail and was inspired by characters of Christidis (2005: character 5) and Salles et al. (2020: character 17).

32. Left mandible, median margin proximal of prostheca, shape: (0) flat; (1) shallowly concave; (2) deeply concave

The median margin proximal of the prostheca is shallowly concave in the majority of *Aprionyx* species (state 1; Figure 3.6D, Figure 3.8D, Figure 3.10D and Figure 3.14D). In *A. phoeocera,* this concavity is deeper (state 2; Figure 3.12D), whereas, in *A. tricuspidatus* and two of the outgroup taxa, this margin is flat (state 0; Figure 3.18D). This character was introduced to define the shape of the median margin of the mandible of *Aprionyx* species.

33. Maxilla, apical-ventral row of pectinate setae, number: (0) less than 13; (1) 14 to 20; (2) more than 20

The number of pectinate setae in the apical-ventral row of the maxilla differs between *Aprionyx* species. *Aprionyx tricuspidatus* has the least (state 0), and all of the western *Aprionyx* species have between 14 and 20 pectinate setae (state 1). Except for *Acanthophlebia*, the outgroup taxa have more than 20 pectinate setae. This character was adapted from Christidis (2005: character 18), to conform to *Aprionyx* species and the specified outgroups.

34. Maxillary palp, segment II, distal end relative to proximal end: (0) about 1.5; (1) about 2.5

The second segment of the maxillary palp in *Aprionyx* is always thicker distally compared to the proximal end. Usually, this is around 1.5 times thicker (state 0; Figure 3.6C), but in *A. tricuspidatus,* the distal end is distinctly thicker at about 2.5 times the proximal end (state 1; Figure 3.18C). The illustrations of Phillips (1930, fig. 14) and Towns (1983, fig. 129) suggest that *Acanthophlebia* is similar to *A. tricuspidatus*. This character was introduced as a useful character of the maxillary palps of *Aprionyx* species.

35. Maxillary palp, segment III, long hairlike setae, location (0) apical tuft; (1) outer margin; (2) inner and outer margin

The third segment of the maxillary palp in *Aprionyx* is covered in hairlike setae. In the western *Aprionyx* species, these hairs are long on the outer margin but shorter on the inner margin (state 1; Figure 3.6C, Figure 3.8C, Figure 3.10C, Figure 3.12C and Figure 3.14C), while in *A. tricuspidatus,* the third segment of the maxillary palp is covered in only long hairlike setae (state 2; Figure 3.18C). In both these cases, the apical tuft is not as distinct as in state 0. *Acanthophlebia* is again similar to *A. tricuspidatus,* while in *Austrophlebioides* and *Choroterpes,* this segment is covered with scattered hairs but has a distinct apical tuft of dense, long hairs (state 0). This character was introduced as a useful character of the maxillary palps of *Aprionyx* species.

36. Maxillary palp, segment III, apical hairs extending beyond apex, length relative to segment III: (0) shorter; (1) about equal; (2) longer (at least 1.33)

The tuft of long hairs on the third maxillary palp segment extends beyond the apex and the length varies when compared to the length of the third segment. In *Aprionyx,* the tuft is approximately equal to (state 1) or longer (at least 1.33) than segment III (state 2). In the outgroup taxa, it is either shorter than segment III (state 0) or about equal. This character was introduced as a useful character of the maxillary palps of *Aprionyx* species.

37. Labial palp, segment II, width distally relative to proximal width: (0) about equal; (1) about 1.5; (2) about 1.75; (3) about 2

The stout labial palps are characteristic of *Aprionyx,* and the ratio of the distal to proximal width of the second segment varies among species. The distal end of segment II is widest in *A. tricuspidatus* (state 3), whereas in *A. tabularis*, *A. pellucidulus* and *A. rubicundus,* the distal end is only about 1.5 times the proximal end (state 1). In *A. peterseni* and *A. phoeocera* the width of segment II distally equates to about 1.75 the proximal width (state 2). In *Austrophlebioides* and *Choroterpes*, the labial palpi are slender with the distal and proximal ends about equal in width (state 0). This character was introduced as a useful character of the labial palps of *Aprionyx* species.

38. Labial palp, segment III, length relative to width: (0) more than twice; (1) less than twice; (2) wider than long (short)

The third segment of the labial palp is very short and distinctly wider than long in *Aprionyx* (state 2). This segment is longer than wide in the outgroup taxa, though in *Austrophlebioides* and *Choroterpes* it is longer than twice the width (state 0) and shorter than twice the width in *Acanthophlebia* (state 1). This character originated from Domínguez et al. (2001: character 34), Christidis (2005: character 23) and Domínguez et al. (2019: character 39).

LEGS

39. Femur, relative lengths of femur I to III: (0) III>II>I; (1) III>I>II; (2) I>III>II

In the western *Aprionyx* species, the femur of leg II is always the shortest and usually the hind femur is the longest (state 1), but in *A. phoeocera* the front femur is the longest (state 2). In *Choroterpes,* the hind femur is the longest and the front femur the shortest (state 0). This is unknown for all of the eastern *Aprionyx* species and two of the outgroup taxa. This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

40. Tibia, relative lengths of tibia I to III: (0) III>I>II; (1) I>III>II

Similar to the femur, the tibia of leg II is always the shortest in the western species, but femur III is the longest in *A. tabularis*, *A. peterseni* and *A. rubicundus* (state 0), and in *A. phoeocera* and *A. pellucidulus* tibia I is the longest (state 1). The tibia ratios in *Choroterpes* corresponds to state 0. The state of all of the eastern *Aprionyx* species and two of the outgroup taxa is unknown. This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

41. Leg I, tibia, length relative to femur: (0) shorter (or subequal); (1) longer

On the front leg of *A. phoeocera*, the tibia is longer than the femur (state 1), whereas, in the other taxa, it is shorter (state 0). In the outgroup taxa the tibia is shorter than or subequal to the femur. This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

42. Leg I, tibia, ventral margin, bipectinate setae, extent: (0) fringe; (1) reduced to only a few distally

The examined *Aprionyx* species have bipectinate setae on the ventral margin of the front tibia, usually a fringe of abundant setae (state 0; Figure 3.21G). In *A. phoeocera*, only a few

bipectinate setae are present distally (state 1; Figure 3.21L). *Choroterpes* also has a fringe of bipectinate setae ventrally and according to Towns (1983) both fine and coarse bipectinate setae are present in *Acanthophlebia*. *Austrophlebioides* was coded as missing because Parnrong and Campbell (1997) describe *Au. marchanti* Parnrong & Campbell, 1997 as having bipectinate setae distally, but do not say what type of setae are present on the rest of the ventral margin, thus the extent is unknown. This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

43. Leg I, tibia, ventral margin, fringe of dense long hairlike setae: (0) absent; (1) present

The front legs of *A. phoeocera* are uniquely evolved with the presence of a dense fringe of very long hairlike setae (state 1; Figure 3.12H). This is not present in any of the other taxa included in this study. This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

44. Leg I, tarsus, anterior face, concentrated long hairlike setae: (0) absent; (1) present

In *Aprionyx*, the front tarsus usually has scattered hairlike setae on the entire surface (state 0), but in *A. phoeocera* the tibial fringe of dense long hairlike setae continues along the anterior face of the tarsus, only less dense (state 1; Figure 3.12H). This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

45. Leg I, tarsus, ventral margin, setae, type: (0) spinelike; (1) fine hairlike setae

Spinelike setae are absent from the front tarsi in *A. pellucidulus* and *A. peterseni*, though with careful inspection a row of fine hairlike setae is discernible, shorter than the scattered hairs (state 1; Figure 3.21B). This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

46. Leg I, tarsus, ventral margin, spinelike setae, extent: (0) small, few in row; (1) fringe

A fringe of spinelike setae is present on the ventral margin of the front tarsus in *A. tabularis* and *A. rubicundus* (state 1; Figure 3.21C). *Aprionyx phoeocera* and *A. tricuspidatus* only have a row of small spinelike setae, similar to that in *Austrophlebioides* and *Choroterpes* (state 0; Figure 3.21D). This character was introduced as a distinguishing character of the legs of *Aprionyx* species.

47. Claws, denticles: (0) present; (1) absent

*Aprionyx* nymphs are the only South African Leptophlebiidae species characterised by smooth claws, lacking denticles (state 1; Figure 3.6I). All of the outgroup taxa have denticulate claws (state 0). This character was derived from Christidis (2005: character 26), and character 48 is an extention to conform to the *Aprionyx* species.

48. Claws, shape: (0) hooked; (1) slender, not hooked

The nymphal claws on all legs are similar in *Aprionyx* and usually hooked as seen in *A. peterseni* (state 0; Figure 3.10I), but in *A. tabularis* and *A. rubicundus* the claws are rather slender and not hooked (state 1; Figure 3.6I and Figure 3.14I). All of the outgroup taxa have claws that are hooked (state 0). This character was introduced as an extended form of character 47 to define the claw shape of *Aprionyx* species.

ABDOMEN

49. Posterolateral projections, located on segment numbers: (0) II to IX; (1) IV or V to IX; (2) VI to IX; (3) VII to IX; (4) VIII and IX

*Aprionyx* species have spinelike projections on the posterolateral corners of the distal segments, but the number of segments with spines is species-specific. The proximal posterolateral projections are often rather small and it are always absent from the first segment. *Aprionyx rubicundus*, for example, has posterolateral projections only on segments VII to IX, while *A. phoeocera* and *A. peterseni* have projections from the fourth or fifth segment, as seen in *Choroterpes*. In *Acanthophlebia* and *Austrophlebioides* they are present on all segments except on the first segment. This character was modified from Domínguez et al. (2001: character 38), Christidis (2005: character 28), Salles and Domínguez (2012: character 28), Domínguez et al. (2019: character 44) and Salles et al. (2020: character 32).

50. Segments VIII and IX, biacuminate posterolateral projections: (0) absent; (1) present

Posterolateral projections are acuminate or biacuminate, and biacuminate projections are found on segments VIII and IX, if present. Biacuminate projections are present in the five western *Aprionyx* species and *Acanthophlebia* (state 1; Figure 3.21M). In *A. tricuspidatus* the posterolateral projections on segments VIII and IX are acuminate and rather large (state 0; Figure 3.21N). If the projections in the imagos (character 16) are indeed remnants to the

projections in the nymphs, the projections on segments VIII and IX in *A. argus* and *A. natalicus* will also be acuminate, but for now they are coded as missing until material is found to confirm. This character was introduced as an extended form of character 49 to distinguish the eastern and western *Aprionyx* species.

51. Posterior margin, tergites, well-developed denticles, located on segment numbers: (0) all; (1) IX and X; (2) X only

The posterior margin of the terga is denticulate. Tsui and Peters (1975) identified two types of denticles, i.e., single or grouped, but they did not consider that both types are present in a species. This character defines the segments with well-developed denticles (Figure 3.21H) which is homologous to Tsui and Peters' (1975) single spines. Segments without well-developed denticles have groups of minute denticles (Figure 3.21I) corresponding to the spines in groups of Tsui and Peters (1975). *Aprionyx* generally has well-developed denticles on segments IX and X (state 1), but in *A. tabularis* all of the segments (state 0), and in *A. tricuspidatus* only segment X (state 2) has well developed denticles. This character was introduced to describe the terga of *Aprionyx* species.

GILLS

52. Gill I, type: (0) single lamella; (1) bilamellate

The first gill in Leptophlebiidae is often different from the rest of the gills, for example single, lanceolate and narrower as seen in *Choroterpes* (state 0). In *Aprionyx* gill I is similar but narrower, thus bilamellate (state 1). This character originated from Ogden et al. (2009b: character 83).

53. Gills, shape: (0) lanceolate; (1) obovate

*Aprionyx* species have bilamellate gills which are obovate (state 1) and all of the outgroup taxa have lanceolate gills (state 0). *Choroterpes*, however, has lanceolate gills with apical indentations, which is why it was also coded as lanceolate. This character was inspired by Christidis (2005: character 31).

54. Apical filamentous processes: (0) absent; (1) present

Apical filamentous processes are present at least medially, on the gills of all *Aprionyx* species (state 1), but absent in all of the outgroup taxa (state 0). This character was introduced as a distinguishing character of the gills of *Aprionyx* species.

55. Lateral apical processes: (0) present; (1) absent

The western *Aprionyx* species do not have lateral apical processes on their gills (state 1; Figure 3.21E), but in the eastern *Aprionyx* species two types (character 56) of lateral apical processes can be present (state 0). This character was introduced as a distinguishing character of the gills of *Aprionyx* species.

56. Lateral apical processes, type: (0) filamentous; (1) bulges

According to Crass (1947b, fig. 32b), the gills of *A. argus* has lateral bulges (state 1), whereas *A. natalicus* and *A. tricuspidatus* usually have lateral filamentous processes (state 0; Figure 2.3J). The filamentous processes are often variable on the latter two species, more so in *A. natalicus* (Crass 1947b). In other words, on a single specimen the number of filamentous processes is irregular and can, therefore, have gills with extra processes or have mere bulges instead of filamentous processes (Figure 3.21F). This character was introduced as a distinctive character of the gills of *Aprionyx* species.

CAUDAL FILAMENTS

57. Primary swimming setae: (0) absent; (1) present

Primary swimming setae are present on the inner margins of the cerci and lateral margins of the medial caudal filament (Kluge 2004). Swimming setae are usually absent in *Aprionyx* species (state 0; Figure 3.21K), but present in *A. peterseni* and *A. phoeocera* (state 1). This character was introduced as a distinguishing character of the caudal filaments of *Aprionyx* species, and was inspired by Ogden et al. (2009b: character 99).

58. Primary swimming setae, extent: (0) short, sparse; (1) long, dense

Swimming setae are either sparse and short as seen in *A. peterseni* (state 0; Figure 3.21J) or dense and long as seen in *A. phoeocera* (state 1; Figure 3.21O). This character was introduced to define the caudal filaments of *Aprionyx* species, and was inspired by Ogden et al. (2009b: character 99).

## 3.6 Morphological phylogeny

### *3.6.1 Results*

Phylogenetic analysis of the full morphological data matrix yielded nine trees under equal weighting (EW) and three most-parsimonious trees under implied weighting (IW). In each scenario, the trees were used to generate a 50% majority rule consensus tree. The EW and IW consensus trees (Figure 3.22) both have a tree length of 121, a consistency index (CI) of 0.79, and a retention index (RI) of 0.71. The two consensus trees are topologically nearly the same, the only difference being that the EW tree did not recover *A. tabularis* in a monophyletic clade with *A. rubicundus*, but as a sister taxon to *A. phoeocera*, *A. pellucidulus* and *A. peterseni*. The EW analysis revealed ambiguity amongst the taxa as a result of homoplasious characters. The homoplasy was, however, arguably low (both CI and RI are relatively high), but the impact was visible in the number of trees generated to accommodate the uncertainty in the relationships between taxa. A strict consensus tree left the species in all clades as unresolved polytomies because of these uncertainties, so a 50% majority rule consensus tree was computed. To reduce the impact of homoplasy, the analysis was repeated with IW and, even though the tree length stayed the same, it produced fewer trees with identical topologies in the 50% majority rule and strict consensus trees. In other words, it reduced the ambiguity in species relations caused by homoplasious characters but not the length of the tree.

The imago and nymph data were analysed separately to further inspect the cause of the ambiguity within *Aprionyx*, but *A. argus* and *A. natalicus* were excluded from the nymphal data because they are still largely unknown (Figure 3.23). The imago-only analysis generated eight trees, but the majority of the trees could not be rooted on all three outgroup taxa as a monophyletic sister group to *Aprionyx*. This resulted in a consensus tree that suggests the western *Aprionyx*, eastern *Aprionyx*, *Acanthophlebia* and *Austrophlebioides* + *Choroterpes* clades to be a polytomy, because the relations between these four groups could not be established. As a result, the tree was rooted on *Austrophlebioides* and *Choroterpes*. The nymph-only analysis produced two trees, which resulted in a consensus tree nearly identical to the trees with all of the characters. Analysing the two data sets separately demonstrates the importance of nymphal characters, which resulted in less ambiguity, whereas the imago tree deviated most from the total evidence trees.

The EW, IW and nymph-only trees recovered *Aprionyx* as monophyletic and moderately to well supported by the bootstrap analysis (61%, 62% and 74%, respectively). Three nymphal synapomorphies define the monophyly of *Aprionyx*: the claws edentate [47: 1] and the obovate gills [53: 1] with filamentous apical processes [54: 1]. Two distinct clades were recovered within the genus by all four parsimony analyses: Clade A, consisting of the western *Aprionyx* group (*A. tabularis*, *A. pellucidulus*, *A. peterseni*, *A. phoeocera* and *A. rubicundus*), and Clade B, the eastern *Aprionyx* group (*A. argus*, *A. natalicus* and *A. tricuspidatus*). Unlike the *Aprionyx* clade, both of these clades are supported by synapomorphic nymphal and imaginal characters.

The western *Aprionyx* lineage was recovered as monophyletic with particularly good bootstrap support in the EW, IW and nymph-only analyses (98%, 97% and 94%, respectively) but weak in the imago-only analysis (52%). The same character changes are generally responsible for this lineage in all of the analyses. Four nymphal apomorphies support this lineage in the EW, IW and nymph-only consensus trees. Three synapomorphies: the labrum 0.46 to 0.54 x as long as wide [26: 1]; the maxilla with 14 to 20 apical-ventral pectinate setae [33: 1]; and the maxillary palp with long hairlike setae on the outer margin of segment III [35: 1]; and one homoplasy shared with *Austrophlebioides* [32: 1]. In the EW, IW and imago-only consensus trees, five imaginal character changes define this lineage. Three synapomorphies: biacuminate posterolateral projections on segment IX [15: 1]; the penis tapering in its apical half [19: 2]; and the apex of penis obtusely angular [20: 1] (ambiguous character change in IW tree); and two homoplasies: one is shared with *A. natalicus* [17: 2] and one with an outgroup taxon, *Choroterpes* [24: 0].

The eastern *Aprionyx* lineage was recovered as a polytomy in all but the nymph-only analysis, which included only one of the eastern *Aprionyx* species, *A. tricuspidatus*. The clade is well supported by bootstrap analyses in the EW (95%), IW (78%) and imago-only (72%) trees. The node is based on four unambiguous imaginal character changes, i.e., two synapomorphies: the ventral portion of the male's eyes more than 0.79 the dorsal portion [3: 2], and the dark shading in the distal anterior radial area heavier and oval-shaped (though, heavier but not oval-shaped in *A. natalicus*) [5: 1]; and two homoplasies: the widely separated eyes of the male imago [2: 1] shared with *A. phoeocera*; and the forewing with vein $MP_2$ attached to $MP_1$ at the RS-MA fork [9: 0] shared with *A. peterseni* (not in IW tree). In the EW and IW consensus trees, the node is defined by a single nymphal synapomorphy: the gills with

lateral apical processes filamentous (but reduced to bulges in *A. argus*) [56: 0]. In the nymph-only consensus tree, the clade is represented by a single taxon, *A. tricuspidatus*, defined by five apomorphies: [30: 1]; [31: 3]; [36: 2]; [51: 2] and [56: 0]. The first four are seemingly unique characters, but since the other species of this lineage are not represented in the nymphal data, any one or all of these autapomorphies have the potential to be characters shared between two or all species in the eastern *Aprionyx* clade. While most of the ambiguity in the nymphal characters was caused by missing data from two of the three species, the ambiguity in imaginal characters was more likely caused by the higher homoplasy and relatively low informativeness of characters. The missing nymphal data, therefore, might explain why the relationships between the three species in this lineage remain unresolved because the imago characters alone are unable to resolve it. In other words, with the missing nymphal data, it is more likely that their relation to each other would be resolved.

The western *Aprionyx* group consists of two lineages, named Clade a and Clade b. The topology of the IW and nymph-only trees are nearly similar for the western *Aprionyx* species, where Clade a comprises *A. rubicundus* and *A. tabularis*, while *A. phoeocera*, *A. pellucidulus* and *A. peterseni* are included in Clade b. The EW and imago-only trees resulted in fairly similar topologies but suggested different species compositions compared to the IW and nymph-only trees for these two clades. *Aprionyx tabularis* is suggested to be in Clade b as a sister species either to the rest of the clade (EW tree) or to *A. phoeocera* (imago-only tree), while *A. rubicundus* is the sole representative in Clade a and sister species to Clade b.

Clade a is well supported by the bootstrap analysis only in the nymphal tree (82%) and is defined by one imago synapomorphy in the IW tree only: the penis shape, laterally projecting downwards medially [21: 1]; and one nymphal synapomorphy in the IW and nymph-only trees: the nymphal claws being slender and not hooked [48: 1]. This clade is also supported in the IW analysis by a homoplasy shared with *Acanthophlebia*, i.e., tarsus I with a ventral fringe of spinelike setae [46: 1].

In the IW and nymph-only trees, Clade b is defined by two and three nymphal synapomorphies, respectively: anteromedial emargination of labrum with bluntly pointed teeth [31: 1], primary swimming setae present [57: 1] and short and sparse, which change to long and dense in *A. phoeocera* [58: 0] (ambiguous character change in IW tree). Different apomorphies are responsible for Clade b in the EW and imago-only trees, all being imago characters: i.e., two synapomorphies [9: 1; 12: 2] and one homoplasy [4: 0]. Clade b is well

supported by the IW (76%) and nymph-only (96%) bootstrap analysis, but has weak support in the EW analysis (53%) and no support in the adult-only analysis (< 50%). *Aprionyx pellucidulus* and *A. peterseni* are supported as sister species to each other in the EW, IW and imago-only trees, though it is rather weak to moderate (Bootstrap value of 57%, 64% and 62%, respectively), whereas in the nymph-only tree, these taxa were recovered as a polytomy with *A. phoeocera*.

The homoplasies responsible for the topology in the two total evidence trees (IW and EW) are reasonably low and most are imaginal characters. In contrast, nearly all of the terminal taxa in *Aprionyx* are characterised by at least one autapomorphy, the exceptions being *A. pellucidulus* and *A. tricuspidatus*, though *A. tricuspidatus* is supported by a homoplasy [8: 0] shared with *Austrophlebioides*. The rest of the species are defined by either a nymphal or imaginal autapomorphic character, whereas *A. phoeocera* is defined by numerous nymphal and one adult (only in the EW tree) autapomorphies.

### *3.6.2 Discussion*

*Aprionyx* was formerly classified under *Atalophlebia*, founded on adults only (Eaton 1884, Esben-Petersen 1920, Lestage 1924). After discovering the nymphs of some South African *Atalophlebia* species, Barnard (1932) established *Aprionyx* as the only Leptophlebiidae genus in South Africa with edentate nymphal claws. The current hypothesis supports *Aprionyx* as a monophyletic genus and strengthens Barnard's argument; therefore, naming the genus after this synapomorphy is especially fitting. The *Aprionyx* clade is supported with bootstrap analysis, but this is weak in the total evidence and relatively good in the nymph-only data. The three nymphal synapomorphies supporting this clade (edentate claws and general structure of the gills) are all non-unique apomorphies which *Aprionyx* shares with *Atalomicria* Harker, 1954, an Australian genus in Pescador and Peters' (1980) *Hapsiphlebia* lineage. The edentate claws are also a synapomorphy shared by all of the species of *Atalomicria*, whereas the gills, especially in *Atalomicria chessmani* Campbell & Peters, 1993, are rather similar to the western *Aprionyx* group by having only a median filament, but variable in width between species (Campbell and Peters 1993). Denticulate claws are more common in Leptophlebiidae nymphs, but the loss of denticles evolved independently in several taxa (Kluge 2004). This character seems particularly common in Australian Leptophlebiidae adapted to the still water conditions found in the pools of rivers, for example, *Loamaggalangta pedderensis* Dean, Forteath &

Osborn, 1999 from Tasmania (Dean et al. 1999). The majority of taxa in the *Hapsiphlebia* lineage are from the Australian region, and claws with small denticles (e.g., *Hapsiphlebia* Peters & Edmunds, 1972) or complete loss of denticles (e.g., *Aprionyx*) are common amongst these taxa. The *Hapsiphlebia* lineage is believed to be the primitive stem of cool-adapted leptophlebiids, including *Aprionyx* as the only cool-adapted taxon in South Africa (Pescador and Peters 1980). The low diversity of cool-adapted taxa agrees with the concept that Africa was one of the first landmasses being isolated from the other Gondwanan countries (Sanmartín and Ronquist 2004, Seton et al. 2012), especially the southern parts (i.e. lower geneflow compared to the other southern countries that were connected for longer), whereas the homoplasious characteristics (edentate claws, obovate gills and presence of apical filament) suggests that the genus is more likely to share a common ancestor with Australian cool-adapted taxa such as *Atalomicria*, but currently there is no other evidence to support this.

The imago tree is the only tree that does not support the monophyly of *Aprionyx* with the current data. The western and eastern *Aprionyx* groups are, however, supported as evidently distinct clades within the genus, even in the high ambiguity scenario displayed by the imago characters. This leads to reasonable doubt in the monophyly of *Aprionyx* and rather proposes the possibility of the western and eastern clades being two separate genera, as suggested in Chapter 2. Since adults have a very short lifespan, their evolution is rather limited and portrays fewer and largely primitive characters, which could explain the higher number of homoplasies among the imago characters (Brittain 1982). One can, therefore, argue that the imaginal apomorphies rather support a theory in which the western and eastern *Aprionyx* groups are two distinct genera.

## 3.7 Identification key to the male imagos and nymphs of *Aprionyx* species

### *3.7.1 Imagos*

1. Posterolateral projections on segment IX biacuminate (sometimes appear truncated), but not as prominent as in nymphs (Figure 3.20H-I). Penis tapering apically (Figure 3.5D, Figure 3.7D, Figure 3.9D, Figure 3.11D and Figure 3.13D); gonopores with recurved apical flap-like processes (Figure 3.5D and Figure 3.20K).. 2 (Western *Aprionyx* clade)

Posterolateral projections on segment IX acuminate and blunt (Figure 3.20J). Penis oblong shaped (Figure 3.15D, Figure 3.16D and Figure 3.17D); gonopores with curled processes apically (Figure 3.20L-M)..... 6 (Eastern *Aprionyx* clade)

2(1). Dorsal portion of the male's eyes is widely separated on the meson of the head (Figure 3.11C). Pterostigmal crossveins always anastomosing, often extensively (Figure 3.11A and Figure 3.20E). Hind wing subcostal area with more than eight crossveins; vein Sc less than 0.8 x wing length (Figure 3.11B). Penes with narrow, acute tip and dorsoventrally flattened (Figure 3.11D-E) .....***Aprionyx phoeocera*** **(Lestage, 1924)**

Dorsal portion of the male's eyes is touching to narrowly separated on the meson of the head (Figure 3.5C, Figure 3.7C, Figure 3.9C and Figure 3.13C). Pterostigmal crossveins not anastomosing (or with only a few veins anastomosing). Hind wing subcostal area with less than eight crossveins; vein Sc more than 0.8 x wing length (Figure 3.5B, Figure 3.7B, Figure 3.9B and Figure 3.13B). Penes variable but with the apex not narrow and acute..... 3

3(2). Penes gradually tapering towards apex; with a rounded tip; base swollen ventrally (Figure 3.7D-E and Figure 3.9D-E) ..... 4

Penes tapering in apical half; with obtusely angular tip; laterally projecting downward medially (Figure 3.5D-E and Figure 3.13D-E) ..... 5

4(3). Forewing crossveins (excluding pterostigma) with heavy oval-shaped shading in costal area (Figure 3.9A and Figure 3.20C); vein $MP_2$ attached to $MP_1$ at RS-MA fork (<0.10 the distance from RS-MA fork to RS fork; Figure 3.20G). Caudal filaments with dark annulations broad (Figure 3.20N).....***Aprionyx peterseni*** **(Lestage, 1924)**

Forewing crossveins dark but barely shaded beyond the vein (Figure 3.7A and Figure 3.20A); vein $MP_2$ attached to $MP_1$ 0.15 to 0.25 the distance from RS-MA fork to RS fork (Figure 3.7A). Caudal filaments with dark annulations narrow, almost indistinct (Figure 3.20P) .....***Aprionyx pellucidulus*** **(Esben-Petersen, 1920)**

5(3). Forewing narrow (≤0.32 the length), with posterior margin proximally truncated (Figure 3.13A); crossveins (excluding pterostigma) with narrow shading in costal area (Figure

3.20B); vein $MP_2$ attached to $MP_1$ 0.30 to 0.80 the distance from RS-MA fork to RS fork (Figure 3.13A) ..... ***Aprionyx rubicundus* Barnard, 1932**

Forewing broad (>0.32 the length), with posterior margin not truncated proximally; crossveins (excluding pterostigma) barely shaded in costal area; vein $MP_2$ attached to $MP_1$ 0.15 to 0.25 the distance from RS-MA fork to RS fork (Figure 3.5A) ..... ..... ***Aprionyx tabularis* (Eaton, 1884)**

6(1). Penes entirely fused (Figure 3.20L). Forewing crossveins (excluding pterostigma) shaded heavier but not oval-shaped in distal anterior radial area (Figure 3.16A and Figure 3.20D) ..... ***Aprionyx natalicus* (Lestage, 1924)**

Penes separated in apical third (Figure 3.20M). Forewing crossveins (excluding pterostigma) with oval-shaped shading in distal anterior radial area ..... 7

7(6). Forewing with vein MA fork closer to RS-MA fork than to wing margin (Figure 3.17A). Leg I with length of femur less than 0.8 of tibia .... ***Aprionyx tricuspidatus* Crass, 1947**

Forewing with vein MA fork closer to wing margin than to RS-MA fork (Figure 3.15A). Leg I with length of femur 0.8 or more of tibia..... ***Aprionyx argus* Barnard, 1940**

### *3.7.2 Nymphs*

1. Posterolateral projections acuminate on segments VIII and IX (Figure 3.21N). Anterior medial emargination of labrum with six teeth; teeth large and acute (Figure 3.18B). Maxilla with about 12 pectinate setae in the apical-ventral row. Labial palp with segment II distally about twice as wide as proximally (Figure 3.18G) ..... ..... ***Aprionyx tricuspidatus* Crass, 1947**

Posterolateral projections biacuminate on segments VIII and IX (Figure 3.21M). Anterior medial emargination of labrum with five or less teeth; teeth small and not acute. Maxilla with more than 14 pectinate setae in the apical-ventral row. Labial palp with segment II distally about 1.5 to 1.75 times as wide as proximally..... 2 (Western *Aprionyx* clade)

2(1). Ventral margin of tibia I with hairlike setae very long, dense (Figure 3.12H). Tarsus I with long hairlike setae concentrated along anterior face (Figure 3.12H). Lengths of femurs I>III>II. Leg I with tibia longer than femur. Labrum narrow, with the length more

than 0.55 x width and the lateral margin more or less straight (Figure 3.12A)..... ..... ***Aprionyx phoeocera*** **(Lestage, 1924)**

Ventral margin of tibia I with or without hairlike setae, but never as long as in *A. phoeocera*. Tarsus I without long hairlike setae concentrated along the anterior face. Lengths of femurs III>I>II. Leg I with femur longer than tibia. Labrum length about 0.46 to 0.54 x width and the lateral margin rounded..... 3

3(2). Claws hooked (Figure 3.8I, Figure 3.10I and Figure 3.12I). Primary swimming setae short and sparse (Figure 3.21J). Labrum widest at about halfway its length (Figure 3.8A and Figure 3.10A). Tarsus I ventral margin with a row of fine hairlike setae, shorter than the scattered hairs (Figure 3.21B) ..... 4

Claws slender, not hooked (Figure 3.6I and Figure 3.14I). Primary swimming setae absent (Figure 3.21K). Labrum widest in distal third (Figure 3.6A and Figure 3.14A). Tarsus I ventral margin with fringe of spinelike setae (Figure 3.21C) ..... 5

4(3). Posterolateral projections present on segments IV or V to IX. Lengths of tibias III>I>II .....***Aprionyx peterseni*** **(Lestage, 1924)**

Posterolateral projections absent on segments IV and V. Lengths of tibias I>III>II..... .....***Aprionyx pellucidulus*** **(Esben-Petersen, 1920)**

5(3). Posterior marginal denticles well developed on all segments. Posterolateral projections present on segments VI to IX..... ***Aprionyx tabularis*** **(Eaton, 1884)**

Posterior marginal denticles well developed only on segments IX and X. Posterolateral projections present on segments VII to IX, absent on VI..... .....***Aprionyx rubicundus*** **Barnard, 1932**

## 3.8 Summary

*Aprionyx* species are unique and easily distinguished from other South African Leptophlebiidae. The results of this study increased our knowledge about their morphological characteristics, which contributes to our ability to distinguish species from each other. The morphological revision of *Aprionyx* was the first comprehensive study after Barnard (1932)

and Crass (1947b) and identified *A. tabularis* as a homonym, with the original *A. tabularis sensu* Eaton, 1884 the senior homonym and *A. tabularis sensu* Esben-Petersen, 1920 the junior homonym. The following taxonomic changes were also justified with this discovery: *A. intermedius* n. syn. to the senior homonym; *A. phoeocera* stat. rev. was re-established as a valid species name for the junior homonym; and *A. tabularis* n. syn. (*sensu* Barnard, 1932) to *A. phoeocera*. The revision made substantial progress in terms of the morphological characters to generate the first dichotomous key to members of the *Aprionyx* genus that includes both adults and nymphs, instead of only adults. Furthermore, the revision also includes the first description of the nymph of *A. rubicundus* after it was identified among samples from rivers of the Hottentots Holland Mountains, Western Cape.

The morphological phylogeny showed a distinct separation of the genus into the western and eastern *Aprionyx* groups. The analysis included all the currently described species, but the eastern *Aprionyx* group is still poorly known compared to the western *Aprionyx* group. Even with the insufficient data of some species (i.e. nymphs of *A. argus* and *A. natalicus*), the findings of the morphological analyses agreed with the results of the molecular analyses. *Aprionyx* was recovered as monophyletic, but the two main lineages received higher support. The results also suggest that nymphs have more distinctive characters than adults and are, therefore, especially important for distinguishing species.

The morphological revision revealed the knowledge gaps that still need to be addressed. A major problem identified here is the fact that the eastern *Aprionyx* species are still poorly known, highlighting the need for more comprehensive collecting efforts in the eastern region (eastern parts of the Eastern Cape and KwaZulu-Natal). Increasing the collecting efforts could also lead to the discovery of additional new species, besides the advancement of knowledge on the recognized species. This will also strengthen the outcome of the morphological phylogeny because missing data can negatively influence the phylogenetic outcome in cases like this, where some nymphs are almost entirely unknown (Salles et al. 2014). The phylogenetic results can be strengthened by a more complete data set. In other words, by minimising the missing data and also by including new species, the phylogeny could be better resolved and produce clades with higher support.

**Table 3.1 Type material indicating their prospective status and the facility. This table is explicitly disclaimed as a nomenclatural work for purposes of zoological nomenclature, and is not published within the meaning of the International Code of Zoological Nomenclature, in line with Article 8 ("What constitutes published work") of that Code (ICZN 2000).**

| Catalogue # | Species | Institute | Type status | Material | Date | Site name | Collector | Material published |
|---|---|---|---|---|---|---|---|---|
| SAM2NA | *A. tabularis* | AMGS | Lectotype | 1 male imago | No date | Groot Drakenstein | AC Harrison | Barnard, 1932 |
| SAM2NB | *A. tabularis* | AMGS | Paralectotype | 1 female subimago | No date | Groot Drakenstein | AC Harrison | Barnard, 1932 |
| SAM2NC | *A. tabularis* | AMGS | Paralectotype | 1 nymph | No date | Groot Drakenstein | AC Harrison | Barnard, 1932 |
| NHMUK013707484 | *A. tabularis* | NHMUK | Paralectotype | 2 subimagos | No date | Groot Drakenstein | AC Harrison | Barnard, 1932 |
| SAM-EPH-A000294 | *A. peterseni* | SAMC | Lectotype | 1 male imago | 10/1919 | Paarl | Rev. G Hawke | Lestage, 1924 |
| SAM-EPH-A000294 | *A. peterseni* | SAMC | Paralectotype | 4 male, 1 female imago | 10/1919 | Paarl | Rev. G Hawke | Lestage, 1924 |
| SAM-EPH-A000286 | *A. pellucidulus* | SAMC | Lectotype | 1 male imago | 11/1916 | Groot Winterhoek, Tulbagh | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000286 | *A. pellucidulus* | SAMC | Paralectotype | 1 female subimago | 11/1916 | Groot Winterhoek, Tulbagh | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000287 | *A. pellucidulus* | SAMC | Paralectotype | 1 male imago | 11/1916 | Groot Winterhoek, Tulbagh | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000288 | *A. pellucidulus* | SAMC | Paralectotype | 1 male imago, 1 female subimago | 11/1916 | Groot Winterhoek, Tulbagh | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000291 | *A. pellucidulus* | SAMC | Paralectotype | 1 male imago | 11/1916 | Groot Winterhoek, Tulbagh | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000302 | *A. phoeocera* | SAMC | Lectotype | 1 male imagos | 04/1913 | Ceres | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000302 | *A. phoeocera* | SAMC | Paralectotype | 2 male imagos | 04/1913 | Ceres | RM Lightfoot | Esben-Petersen, 1920 |
| SAM-EPH-A000303 | *A. rubicundus* | SAMC | Lectotype | 1 male imago | 10/1919 | Montagu | KH Barnard | Barnard, 1932 |
| SAM-EPH-A000304 | *A. rubicundus* | SAMC | Paralectotype | 1 male, 1 female imagos | 10/1919 | Montagu | KH Barnard | Barnard, 1932 |
| NHMUK013707492 | *A. rubicundus* | NHMUK | Paralectotype | 3 male imagos | 10/1919 | Montagu | KH Barnard | Barnard, 1932 |
| SAM2WA | *A. argus* | AMGS | Lectotype | 1 male imago | 01/1938 | Cathkin Peak | RF Lawrence | Barnard, 1940 |
| SAM2WA | *A. argus* | AMGS | Paralectotype | 2 male imagos | 01/1938 | Cathkin Peak | RF Lawrence | Barnard, 1940 |
| SAM2WB | *A. argus* | AMGS | Paralectotype | 1 female imago | 01/1938 | Cathkin Peak | RF Lawrence | Barnard, 1940 |
| SAM2WC | *A. argus* | AMGS | Paralectotype | 1 female subimago | 01/1938 | Cathkin Peak | RF Lawrence | Barnard, 1940 |
| SAM-EPH-A000305 | *A. natalicus* | SAMC | Lectotype | 1 male imago | 11/1917 | Kranskop | KH Barnard | Lestage, 1924 |
| RSC5A | *A. tricuspidatus* | AMGS | Lectotype | 1 male imago | 1/1946 | Curry's Post | RS Crass | Crass, 1947 |
| RSC5A | *A. tricuspidatus* | AMGS | Paralectotype | 5 male imagos | 1/1946 | Curry's Post | RS Crass | Crass, 1947 |
| RSC5B | *A. tricuspidatus* | AMGS | Paralectotype | 1 female imago | 1/1946 | Curry's Post | RS Crass | Crass, 1947 |
| RSC5C | *A. tricuspidatus* | AMGS | Paralectotype | 3 nymphs | 1/1946 | Curry's Post | RS Crass | Crass, 1947 |
| NHMUK013707613 | *A. tricuspidatus* | NHMUK | Paralectotype | 3 male, 1 female imagos, 1 nymph | 10/11/1945 | Newstead Balgowan | RS Crass | Crass, 1947 |

**Table 3.2 A comparison of the original descriptions and original material, explaining the proposed nomenclatural changes. *Aprionyx tabularis*: *Atalophlebia tabularis sensu* Eaton, 1884 (senior hom.) and *A. intermedius* Barnard, 1932 (n. syn.). *A. phoeocera* stat. rev.: *At. tabularis sensu* Esben-Petersen, 1920 (junior hom.), *At. phoeocera* Lestage, 1924 and *A. tabularis sensu* Barnard, 1932 (n. syn.).**

| | ***Aprionyx tabularis*** | | ***Aprionyx phoeocera* stat. rev.** | | | **Comments** |
|---|---|---|---|---|---|---|
| | ***Atalophlebia tabularis sensu* Eaton, 1884 senior hom.** | ***Aprionyx intermedius* Barnard, 1932 n. syn.** | ***Atalophlebia tabularis sensu* Esben-Petersen, 1920 junior hom.** | ***Atalophlebia phoeocera* Lestage, 1924 stat. rev.** | ***Aprionyx tabularis sensu* Barnard, 1932 n. syn.** | |
| **Dorsal portion of male eyes: colour** | Clove-brown. | Not described. Faded in available syntype material. | Black.<br>This is based on dry specimens which are darker, therefore it should not be used for comparison. | | Light-pinkish. | The eye colours given by Eaton (1884) and Barnard (1932) are considered as valid. Eye colour is variable to some extent and change over time in ethanol or when dried, thus it is not a determining factor. The difference between synonymies is thus not noteworthy. |
| **Dorsal portion of male eyes** | Large<br>Nearly meet on meson (Figure 3.19A). | Large<br>Nearly meet on meson (Figure 3.19C). | Medium<br>Not meeting on meson, widely separated (Figure 3.19E). | | Medium<br>Not meeting on meson, widely separated (Figure 3.19H and Figure 3.11C). | Both candidates from the *A. tabularis* n. syn. have narrowly separated eyes on the meson and in all the *A. phoeocera* n. syn. the eyes are widely separated. |
| **Wings: pterostigma** | Rarely anastomosed (and then only very sparsely). | Rarely anastomosed. | Anastomosed. | * | Anastomosed extensively.<br>Barnard (1932) found considerable variation, with less anastomosis in smaller specimens, but always present. | *A. tabularis* n. syn. is characterized by only rarely and sparsely occurring anastomoses in the pterostigma, while in *A. phoeocera* n. syn. anastomoses are always present and often more extensive. |
| **Wings: crossveins in costal area** | Before bulla 10; after bulla 6; in pterostigma 13. | Before bulla 8-9; in pterostigma 12-14. | Before bulla 10; after bulla 6; in pterostigma 13. | * | Before bulla 10; in pterostigma 12-14. | There is no clear separation between the two groups in the number of costal crossveins, therefore it is not a determining factor. |
| **Legs: femora** | Banded black medially and distally. | Banded dark medially and distally. | Black distally.<br>Examining Esben-Petersen's (1920) material revealed that Barnard (1932) is correct. The median broad suffusion is not clear bands and could thus be seen as a result of the darkening effect after being dried. | Lestage accepted it as only black distally and not medially. | Suffused at apices and broadly across middle. Median band not as distinct as in other species, but the broad suffusion is more prominent than in e.g., *A. natalicus*. | The two synonymies can be separated by *A. tabularis* n. syn. having a distinct band medially and *A. phoeocera* n. syn. with a broad median dark suffusion (often not distinguishable, and perceived as the legs being darker). |
| **Genitalia: styliger plate** | Slight median projection.<br>Inaccurate. | | | | | All *Aprionyx* species lack a median projection. |

| | ***Aprionyx tabularis*** | | ***Aprionyx phoeocera* stat. rev.** | | | **Comments** |
|---|---|---|---|---|---|---|
| | ***Atalophlebia tabularis sensu* Eaton, 1884 senior hom.** | ***Aprionyx intermedius* Barnard, 1932 n. syn.** | ***Atalophlebia tabularis sensu* Esben-Petersen, 1920 junior hom.** | ***Atalophlebia phoeocera* Lestage, 1924 stat. rev.** | ***Aprionyx tabularis sensu* Barnard, 1932 n. syn.** | |
| **Genitalia: shape of penis** | Penis lobes flat and obliquely pointed (resembling nib of a pen flattened).<br>The figure by Eaton (1884) is inaccurate (Figure 3.19B), but it is rather shaped as in Barnard's material of *A. intermedius* (Figure 3.19D). | Much broader than any of the other species.<br>Barnard (1932) did not give a drawing, but the type material was examined for comparison (Figure 3.19D). | The drawing by Esben-Petersen (1920) is inaccurate (Figure 3.19F), but it does depict the apex as sharply pointed which can still be seen in his material (Figure 3.19G). | | Narrows rather abruptly towards apex.<br>Barnard's (1932) illustration is more accurate (Figure 3.19I). In both the drawing and the type material (Figure 3.19J), the apex is sharply pointed (Figure 3.11D-E). | All the drawings are rather inaccurate, with Barnard's (1932) being the most accurate. In the drawing by Eaton and Barnard's material of *A. intermedius*, the penis is broad with sharper corners medially before it narrows to form a bluntly pointed apex. *At. tabularis sensu* Esben-Petersen, 1920 (i.e., *At. phoeocera* Lestage, 1924) and *A. tabularis sensu* Barnard, 1932 have a broad penis with rounded corners medially, narrowing to an acute tip (i.e., sharply pointed). |
| **Caudal filaments** | Whitish, dark annulations on alternate joints. | Whitish or pale grey, narrow dark annulations. | Pale brown.<br>Based on dry specimens which is darker, making the narrow annulations more inconspicuous and difficult to notice. Inspecting his material confirmed the presence of narrow annulations. Barnard (1932) concurred. | Lestage mistakenly accepted pale brown, with annulations absent, and used this to justify and name his new species. | Ochraceous, articulations slightly darker (thus narrow annulations). | All are with narrow dark annulations, a character found in all Western Cape species except for *A. peterseni*, therefore it is not a distinguishing character in this case. |
| ♂ **body length** | - | [9-10 mm = ♀ subimago] | 13 mm | * | 9 mm | Barnard (1932) mentioned how variable the size of his *A. tabularis* specimens was. In this study subimagos of up to 18.4 mm were measured. Only female subimago measurements are given for *A. intermedius* by Barnard (1932). See also the descriptions for measurements. |
| ♂ **Forewing length** | 9 mm | [9.5-10 mm = ♀ subimago] | 12 mm | * | 10 mm | |
| ♂ **Caudal filaments length** | - | [10-11 mm = ♀ subimago] | 17 mm | * | - | |

***** Lestage did not notice that Esben-Petersen (1920) erroneously placed part of *At. tabularis'* description at the end of *At. pellucidula's description*, however, Barnard (1932) did recognise this mistake. This section includes these characteristics, omitted by Lestage (1924).

**Table 3.3 A table listing the taxonomic changes of *Aprionyx* species by comparing the original and revised species and their synonyms, i.e., homonymy (hom.), new synonymy (n. syn.) and revised status (stat. rev.).**

| Current | Revised |
|---|---|
| *Aprionyx tabularis* (Eaton, 1884) | *Aprionyx tabularis* (Eaton, 1884) |
| *Atalophlebia tabularis* Eaton, 1884 [Orig.] | *Atalophlebia tabularis* Eaton, 1884 [Orig.] **senior hom.** |
| = *Atalophlebia tabularis* Esben-Petersen, 1920 | = *Aprionyx intermedius* Barnard, 1932 **n. syn.** |
| = *Atalophlebia phoeocera* Lestage, 1924 | |
| = *Aprionyx tabularis* Barnard, 1932 | *Aprionyx phoeocera* (Lestage, 1924) **stat. rev.** |
| | ≡ *Atalophlebia tabularis* Esben-Petersen, 1920 **junior hom.** (*nec* Eaton, 1884) |
| *Aprionyx intermedius* Barnard, 1932 | ≡ *Atalophlebia phoeocera* Lestage, 1924 [Orig.] **stat. rev.** |
| *Aprionyx intermedius* Barnard, 1932 [Orig.] | = *Aprionyx tabularis* Barnard, 1932 **n. syn.** (*nec* Eaton, 1884) |


**Figure 3.1 The different UV light sources that was used with the pan light trap method for collecting adult mayflies: A) 6 V, B) 12 V, C) LED. © IS Ferreira.**


**Figure 3.2 Habitus of the lectotype specimens of *Aprionyx*: A) *A. tabularis*, B) *A. pellucidulus*, C) *A. peterseni*, D) *A. phoeocera*, E) *A. rubicundus*, F) *A. argus*, G) *A. natalicus*, H) *A. tricuspidatus*. Scale = 2 mm. © IS Ferreira.**


**Figure 3.3 Male imago habitus of *Aprionyx*: A) *A. pellucidulus*, B) *A. peterseni*, C) *A. phoeocera*, D) *A. rubicundus*. Scale = 2 mm. © IS Ferreira.**


**Figure 3.4 Nymph habitus of *Aprionyx*: A) *A. pellucidulus*, B) *A. peterseni*, C) *A. phoeocera*, D) *A. rubicundus*. Scale = 2 mm. © IS Ferreira.**

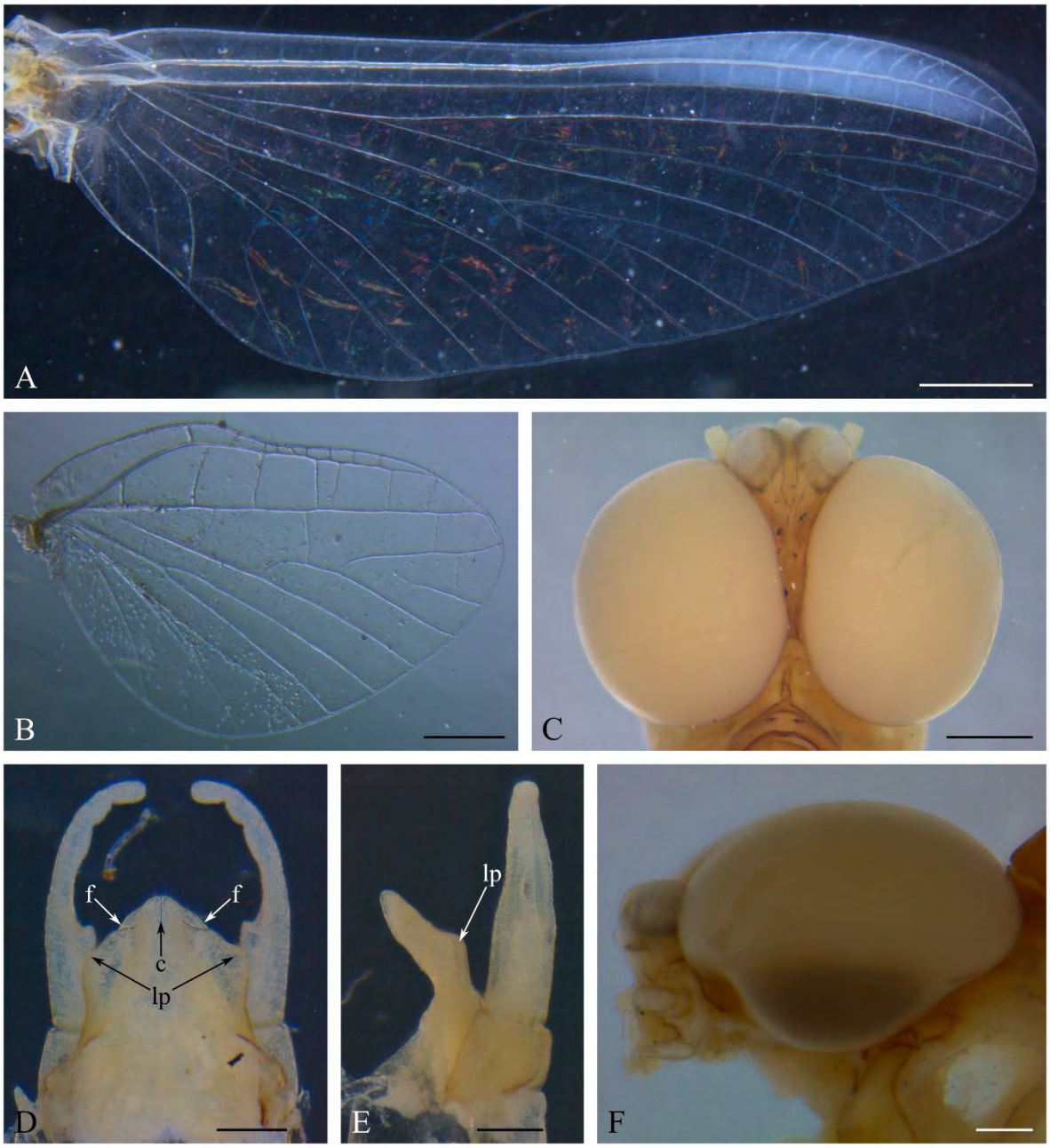



**Figure 3.5** ***Aprionyx tabularis*****, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) dorsal and E) lateral view. Abbreviations: c = cleft; f = flap-like projections; lp = lateral projections (projecting downwards laterally). Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

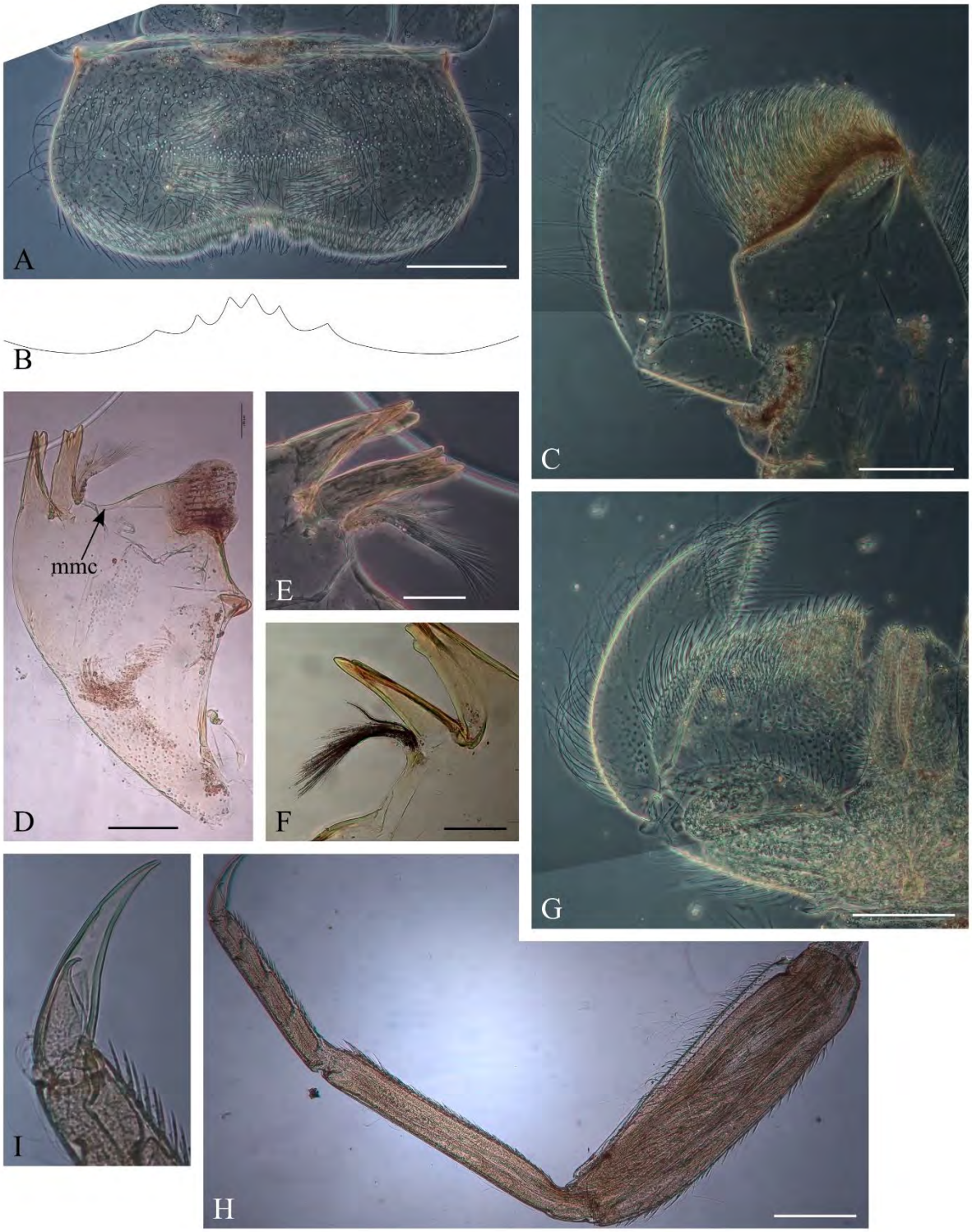



**Figure 3.6** ***Aprionyx tabularis*****, nymph: Mouthparts, A) labrum with B) the median emargination enlarged to show the denticles. Maxilla, C) distal section with the palpi; D) left mandible, and E) left and F) right prostheca; G) labium. H) Leg I, and I) claw enlarged. Abbreviation: mmc = median margin concavity. Scale: E-F = 0.1 mm; H = 0.4 mm; A, C, D and G = 0.2 mm. © IS Ferreira.**

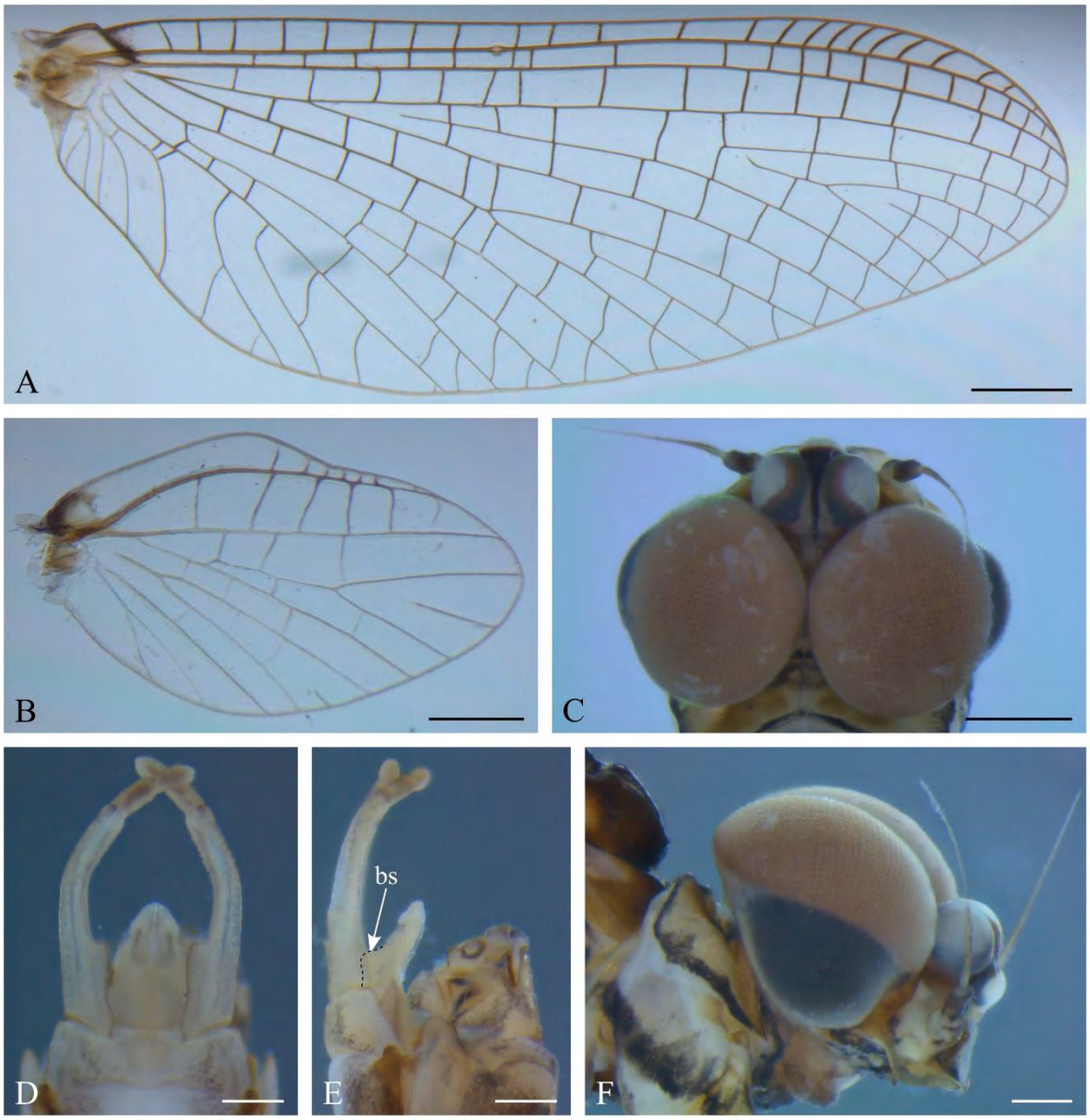



**Figure 3.7 *Aprionyx pellucidulus*, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviation: bs = basal swelling. Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

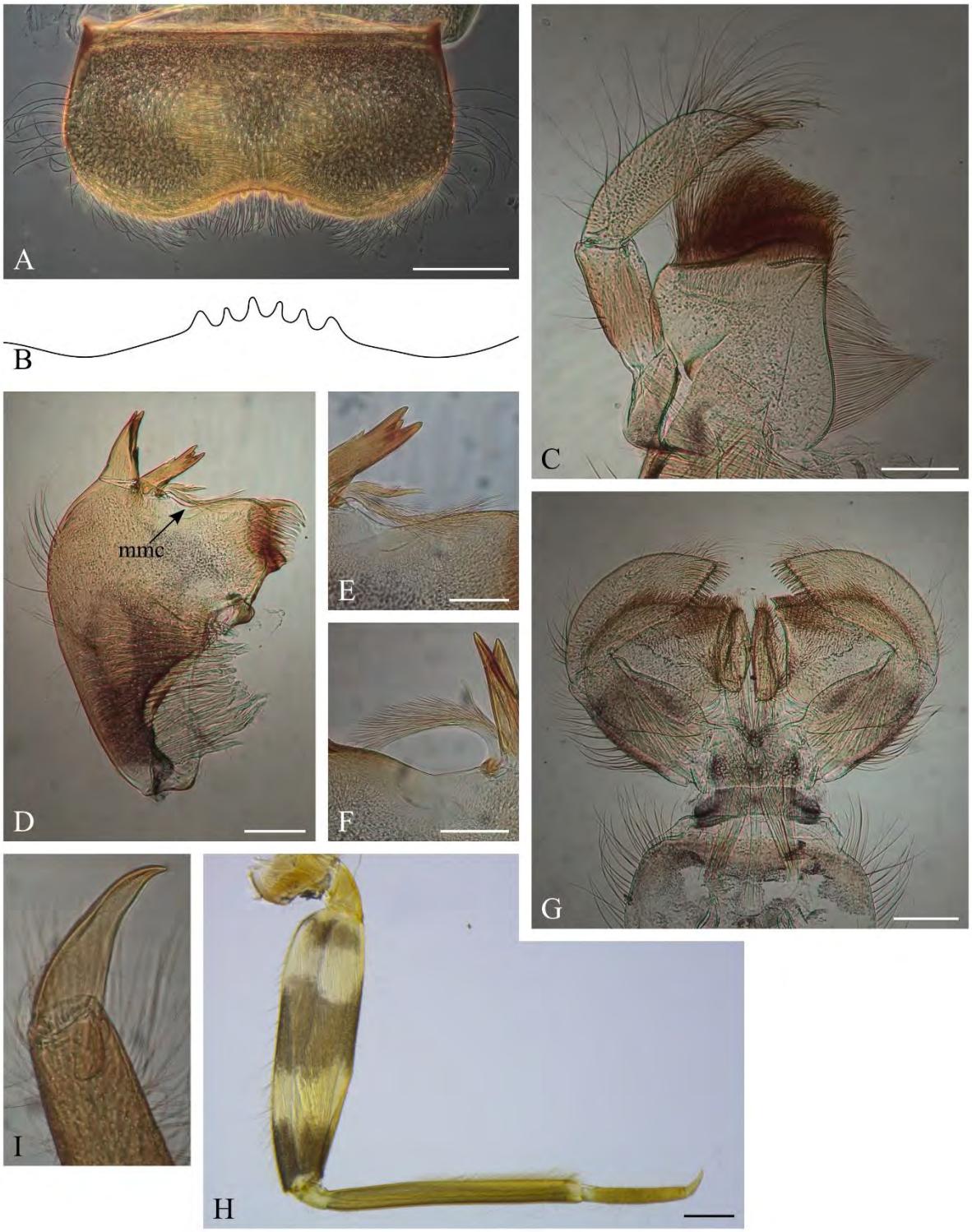



**Figure 3.8 *Aprionyx pellucidulus*, nymph: Mouthparts, A) labrum with B) the median emargination enlarged to show the denticles. Maxilla, C) distal section with the palpi; D) left mandible, and E) left and F) right prostheca; G) labium. H) Leg I, and I) claw enlarged. Abbreviation: mmc = median margin concavity. Scale: E-F = 0.1 mm; H = 0.4 mm; A, C, D and G = 0.2 mm. © IS Ferreira.**

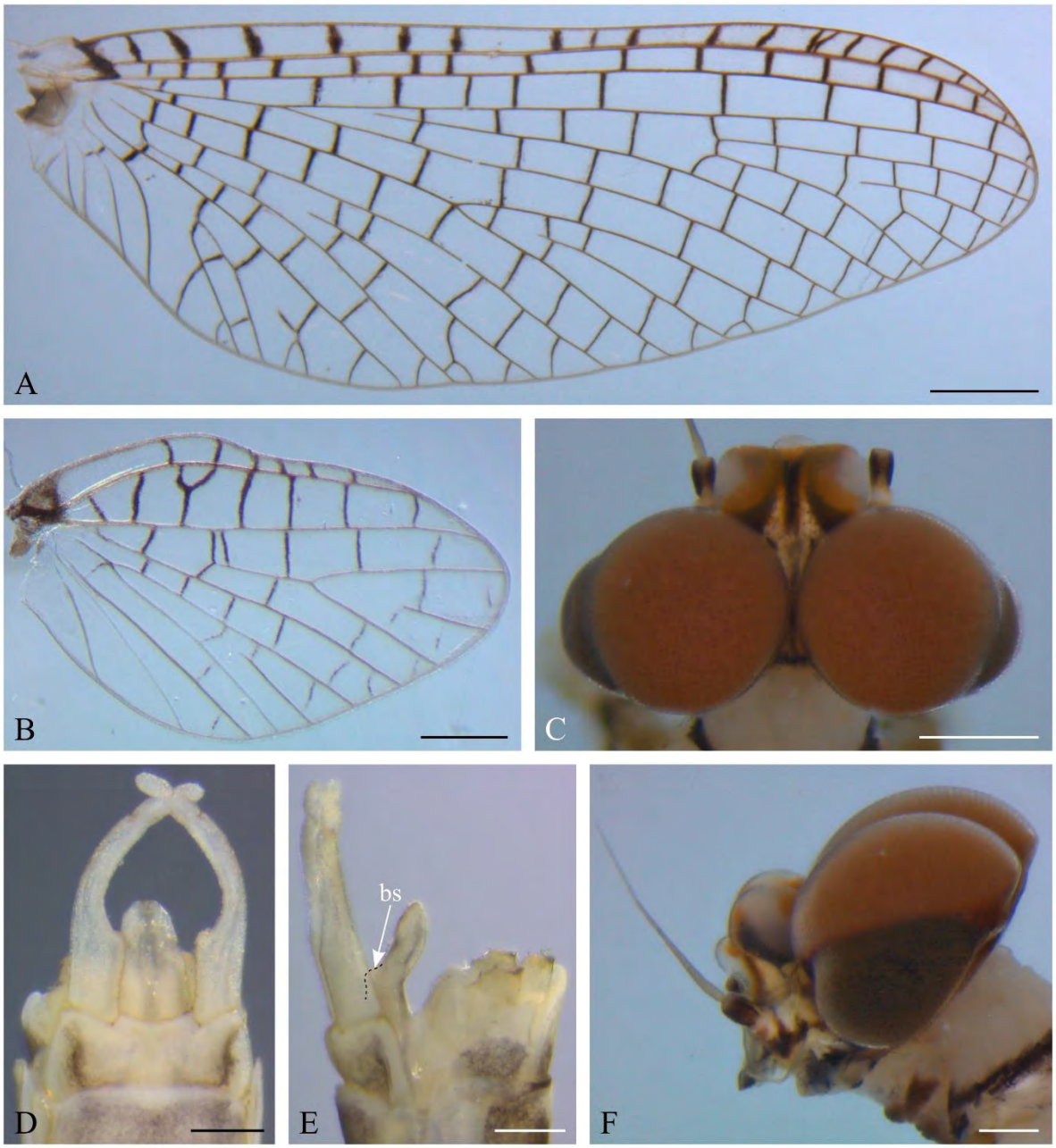



**Figure 3.9 *Aprionyx peterseni*, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviation: bs = basal swelling. Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

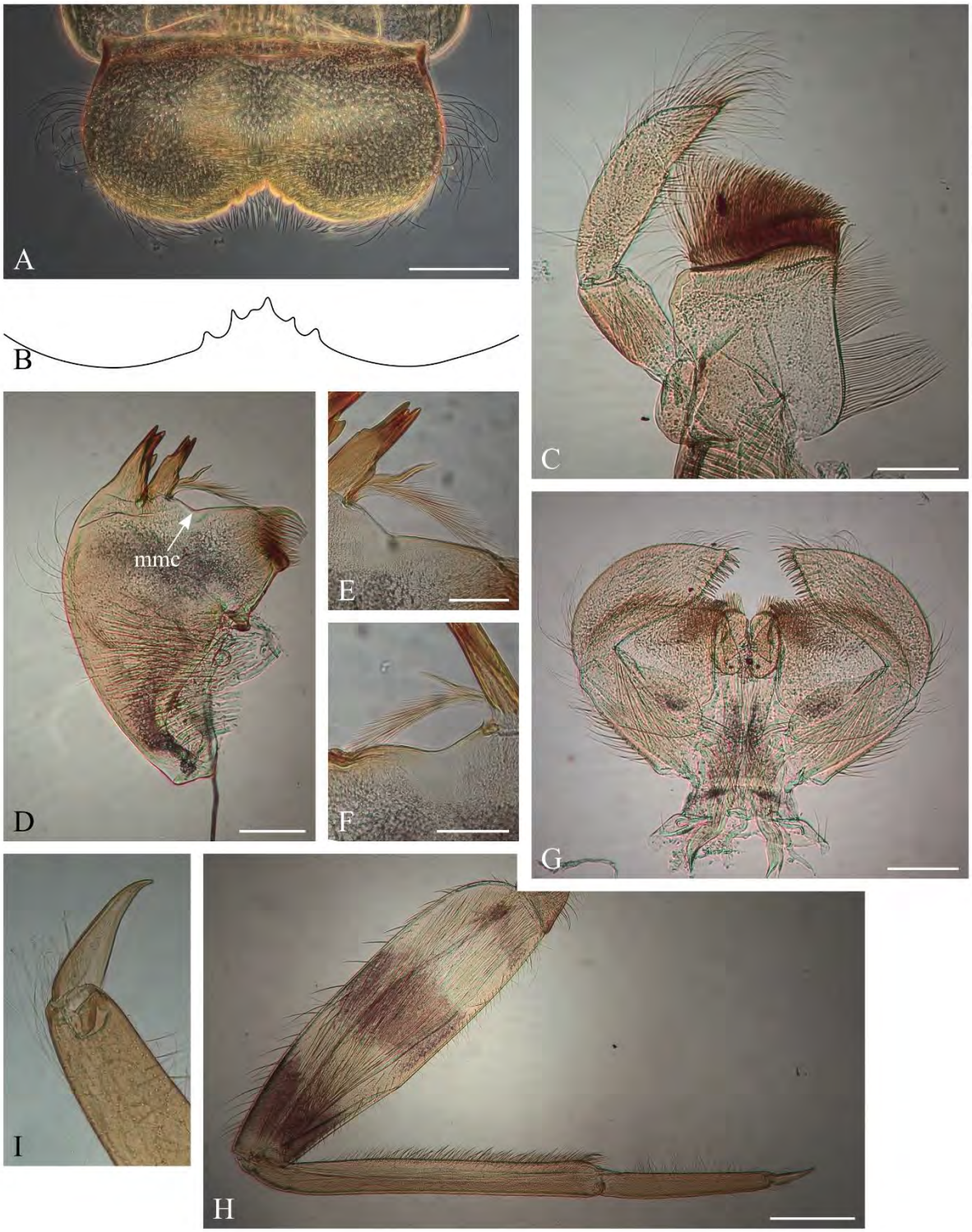



**Figure 3.10 *Aprionyx peterseni*, nymph: Mouthparts, A) labrum with B) the median emargination enlarged to show the denticles. Maxilla, C) distal section with the palpi; D) left mandible, and E) left and F) right prostheca; G) labium. H) Leg I, and I) claw enlarged. Abbreviation: mmc = median margin concavity. Scale: E-F = 0.1 mm; H = 0.4 mm; A, C, D and G = 0.2 mm. © IS Ferreira.**

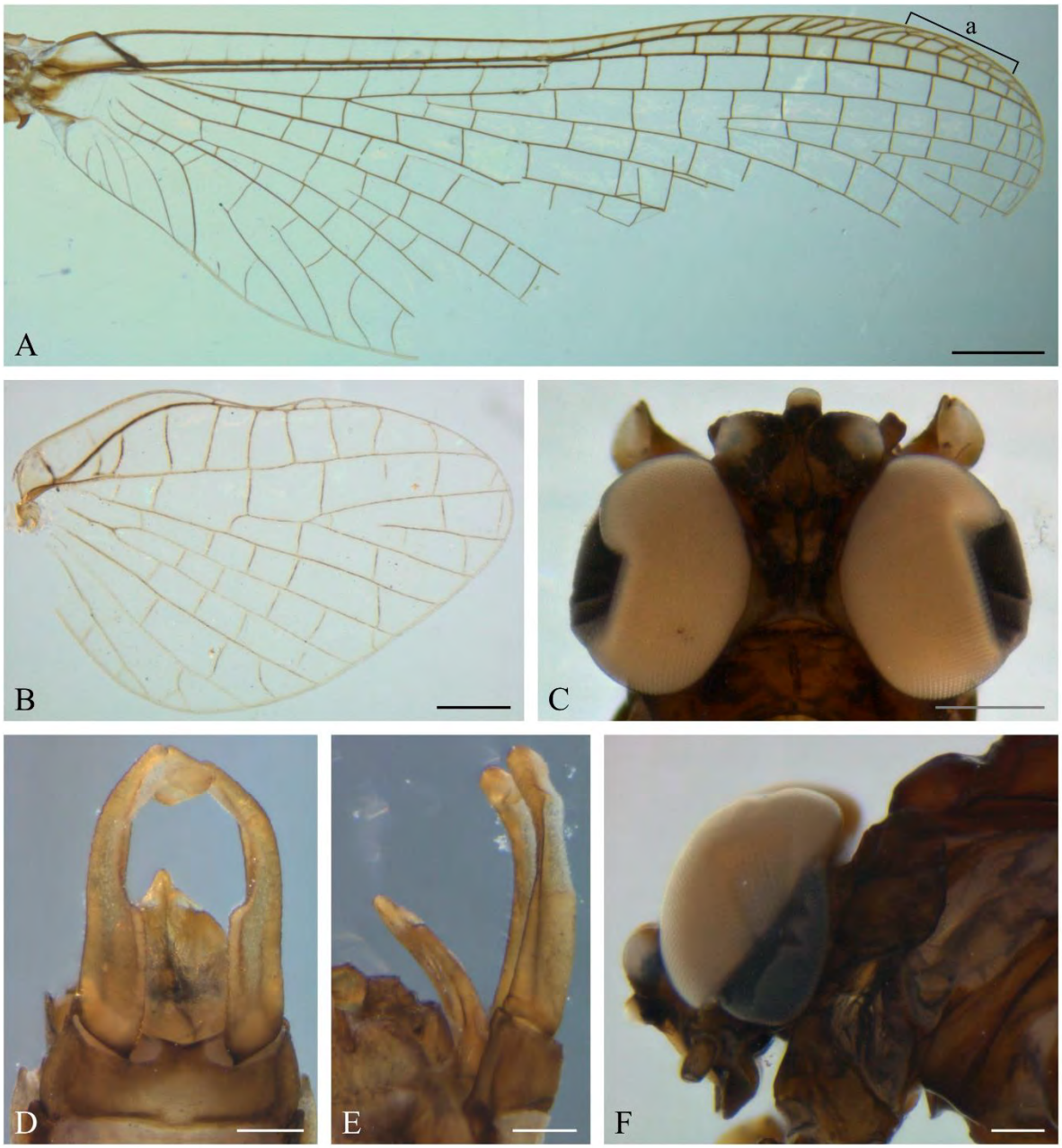



**Figure 3.11** ***Aprionyx phoeocera*****, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviation: a = anastomosed. Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

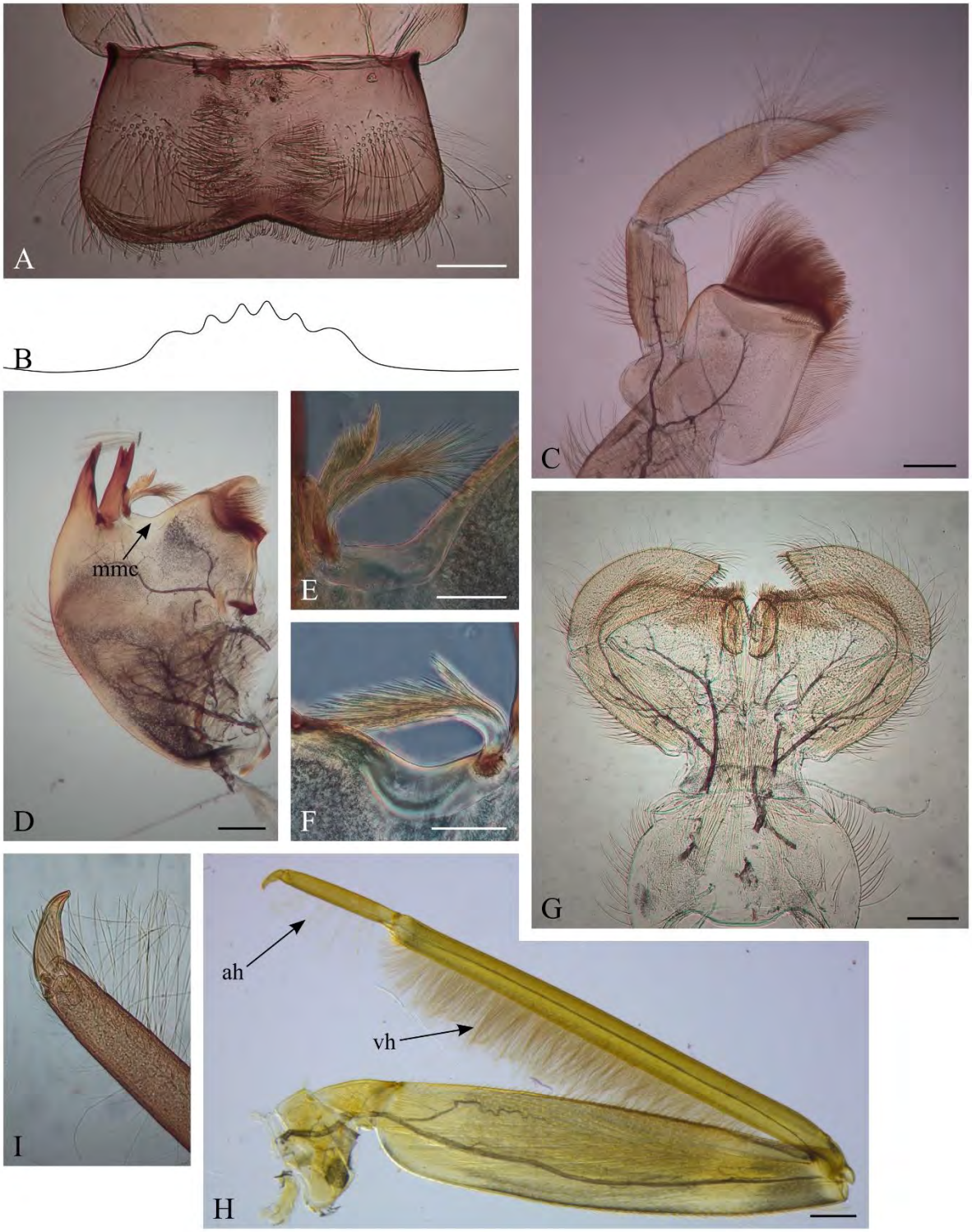



**Figure 3.12** ***Aprionyx phoeocera*****, nymph: Mouthparts, A) labrum with B) the median emargination enlarged to show the denticles. Maxilla, C) distal section with the palpi; D) left mandible, and E) left and F) right prostheca; G) labium. H) Leg I, and I) claw enlarged. Abbreviations: ah = anterior face with long hairlike setae; mmc = median margin concavity; vh = ventral long, dense hairlike setae. Scale: E-F = 0.1 mm; H = 0.4 mm; A, C, D and G = 0.2 mm. © IS Ferreira.**

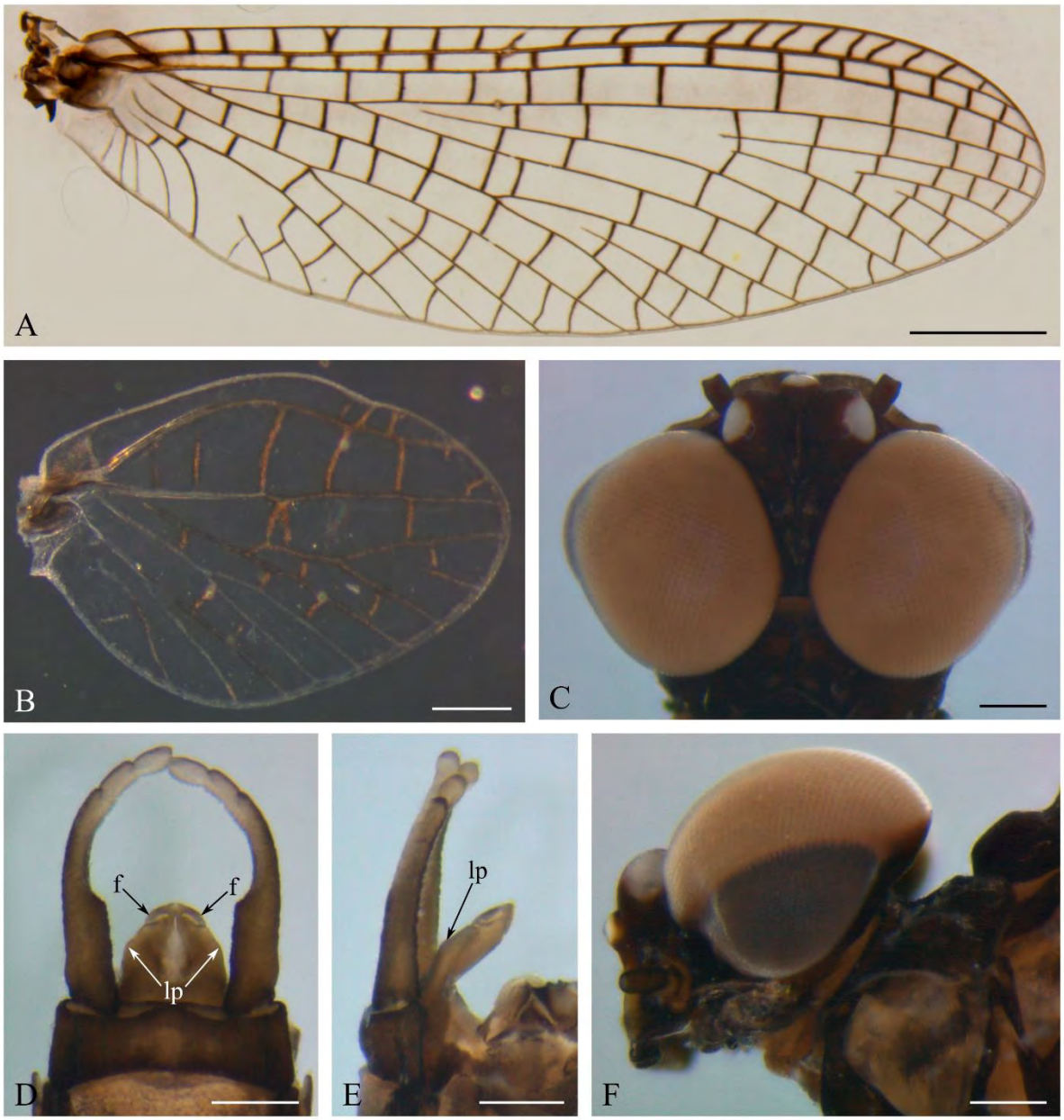



**Figure 3.13** ***Aprionyx rubicundus*****, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviations: f = flap-like projections; lp = lateral projections (projecting downwards laterally). Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

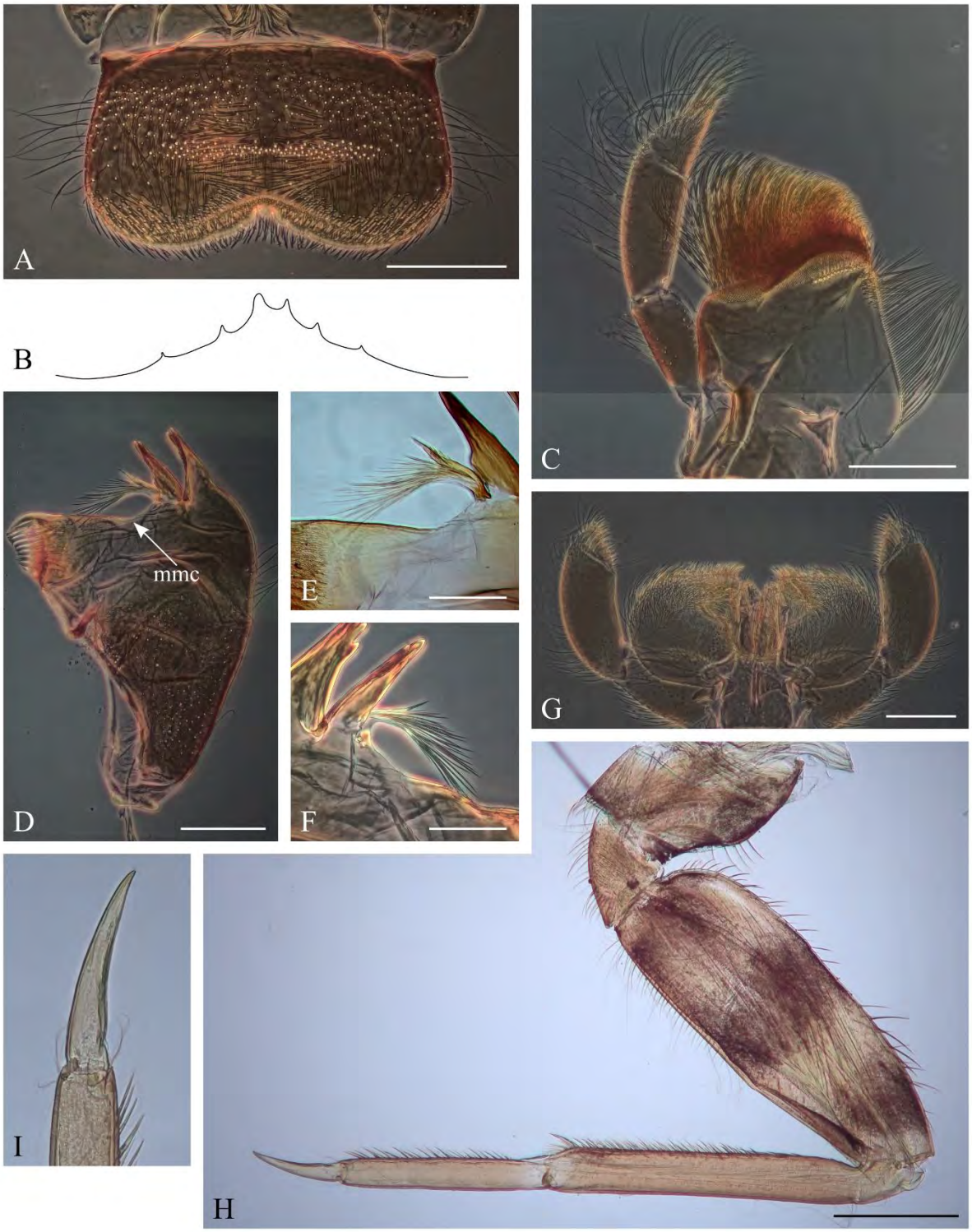



**Figure 3.14** ***Aprionyx rubicundus*****, nymph: Mouthparts, A) labrum with B) the median emargination enlarged to show the denticles. Maxilla, C) distal section with the palpi; D) left mandible, and E) left and F) right prostheca; G) labium. H) Leg I, and I) claw enlarged. Abbreviation: mmc = median margin concavity. Scale: E-F = 0.1 mm; H = 0.4 mm; A, C, D and G = 0.2 mm. © IS Ferreira.**

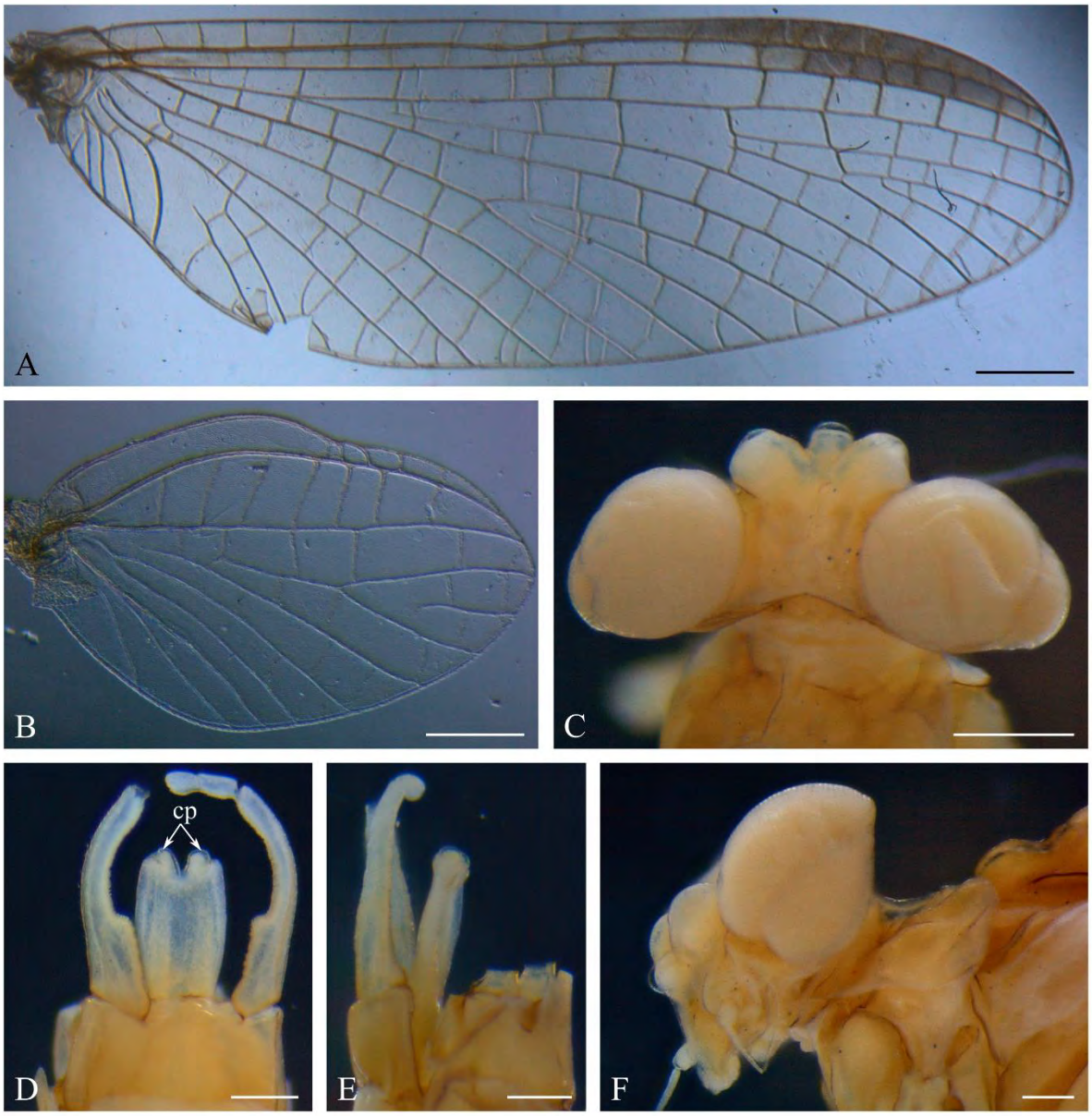



**Figure 3.15** ***Aprionyx argus*****, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviation: cp = curled projection. Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

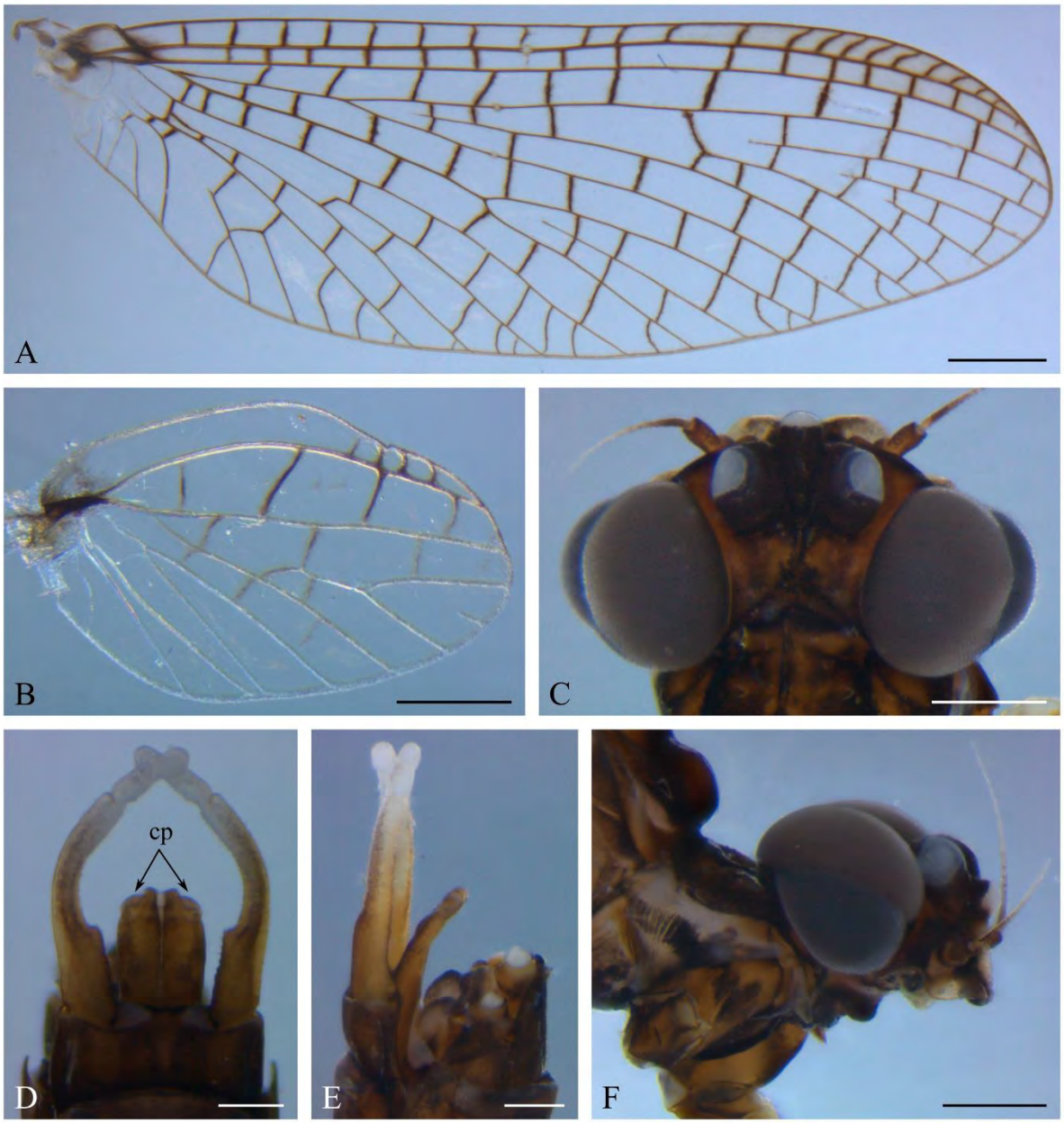



**Figure 3.16 *Aprionyx natalicus*, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviation: cp = curled projection. Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

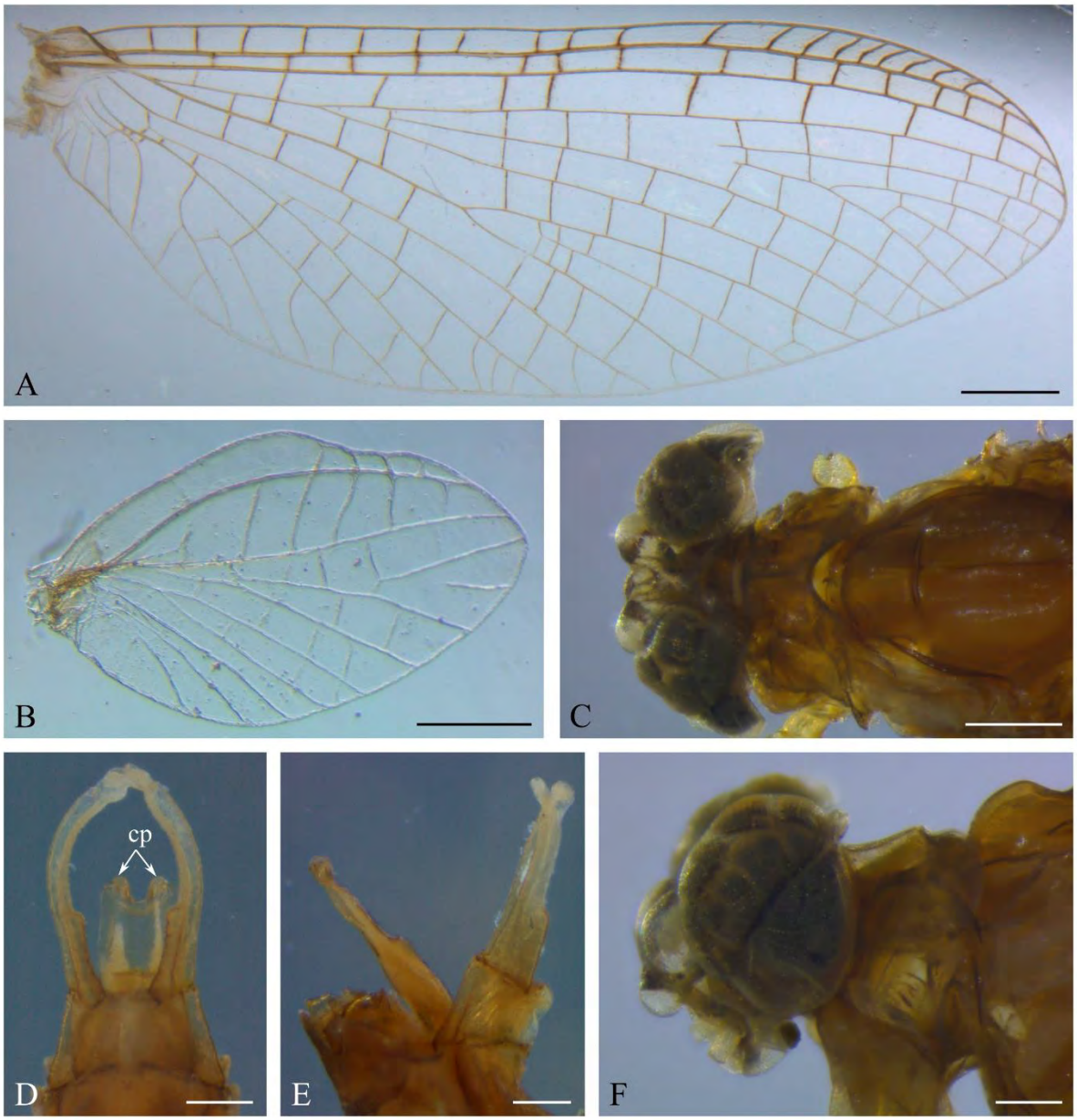



**Figure 3.17** ***Aprionyx tricuspidatus*****, male imago: Wings, A) forewing and B) hind wing. Eyes, C) dorsal and F) lateral view. Genitalia, D) ventral and E) lateral view. Abbreviation: cp = curled projection. Scale: A = 1 mm, B-C = 0.4 mm, D-F = 0.2 mm. © IS Ferreira.**

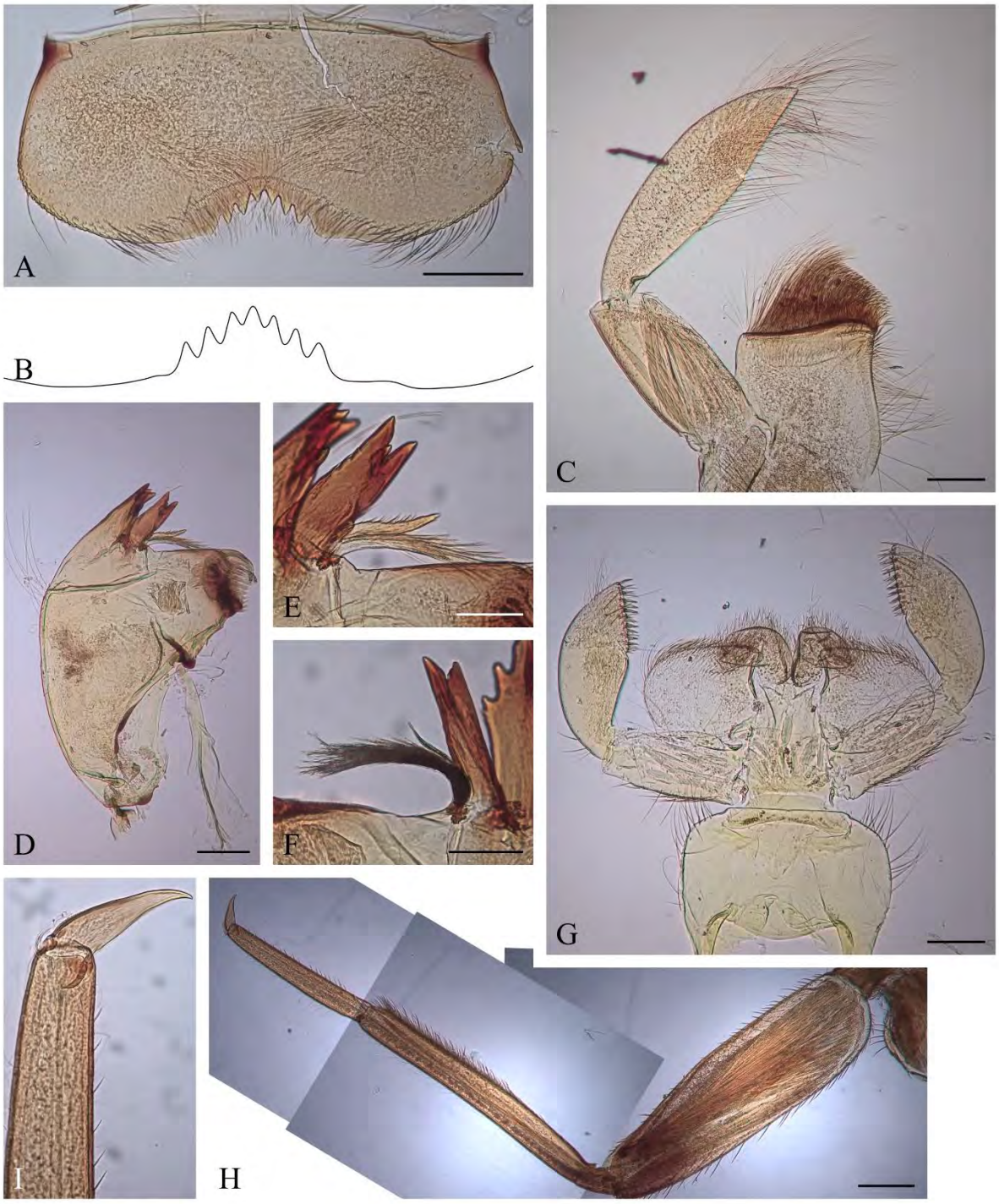
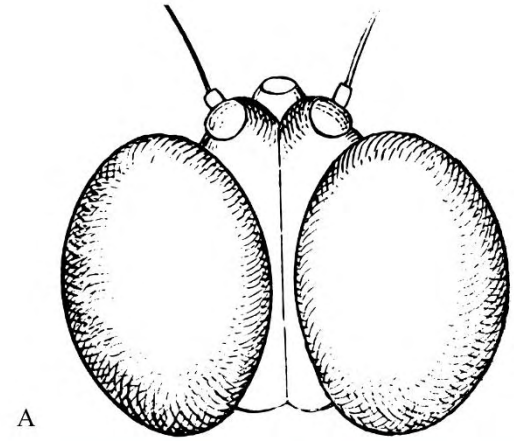



**Figure 3.18** ***Aprionyx tricuspidatus*****, nymph: Mouthparts, A) labrum with B) the median emargination enlarged to show the denticles. Maxilla, C) distal section with the palpi; D) left mandible, and E) left and F) right prostheca; G) labium. H) Leg I, and I) claw enlarged. Scale: E-F = 0.1 mm; H = 0.4 mm; A, C, D and G = 0.2 mm. © IS Ferreira.**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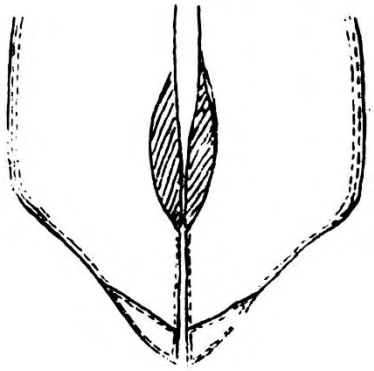
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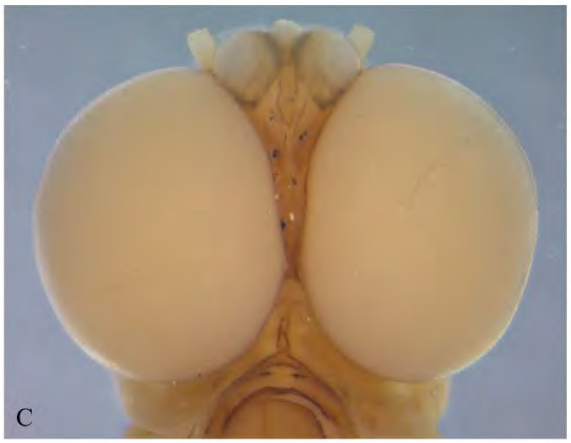
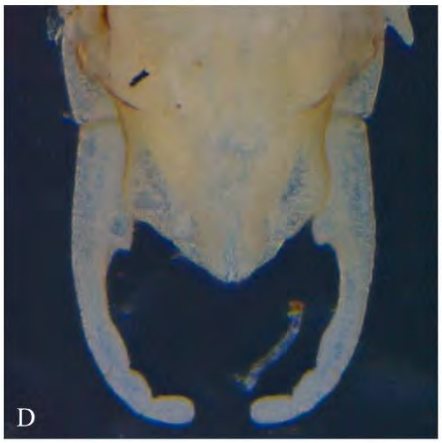

C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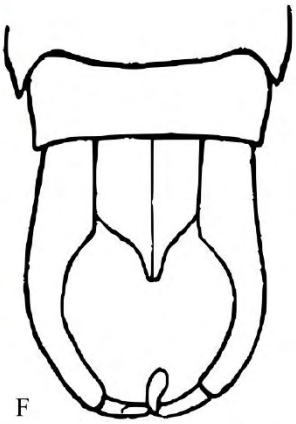
E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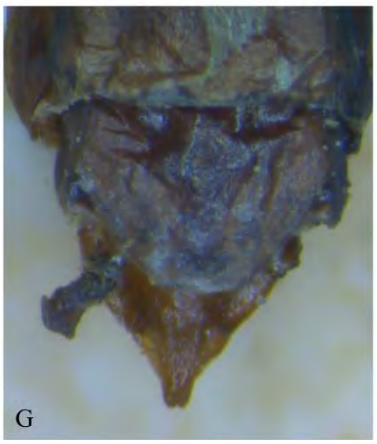
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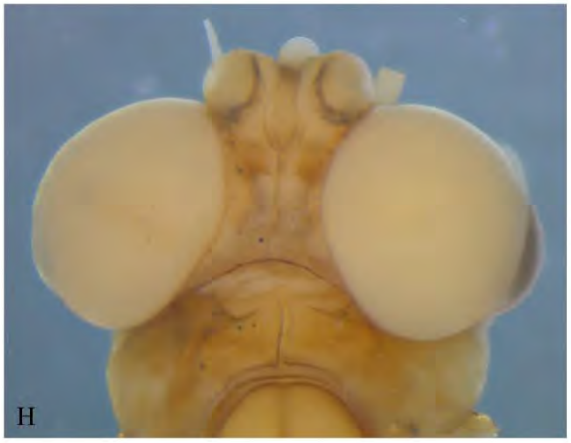
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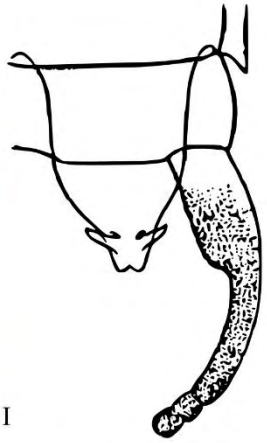
I

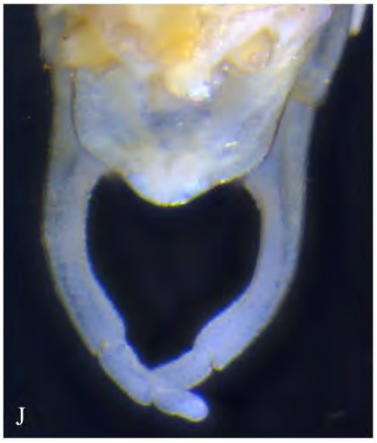
J

**Figure 3.19 A comparison of the original drawings and original material for the proposed nomenclatural changes of A-D) *Aprionyx tabularis* and E-J) *A. phoeocera* stat. rev. (as discussed in Table 3.2). *Atalophlebia tabularis* (iconotype, redrawn from Eaton 1884), A) eyes (dorsal) and B) penis; *A. intermedius* n. syn. type material of Barnard (1932), C) eyes (dorsal) and D) penis (dorsal); *At. tabularis* junior hom. material of Esben-Petersen (1920), E) eyes (dorsal), F) penis (ventral) redrawn from Esben-Petersen (1920) and G) penis (dorsal); *A. tabularis* n. syn. material of Barnard (1932), H) eyes (dorsal), I) penis (ventral) redrawn from Barnard (1932), and J) penis (dorsal view at slight angle; acute tip not visible from this angle). Photographs © IS Ferreira.**

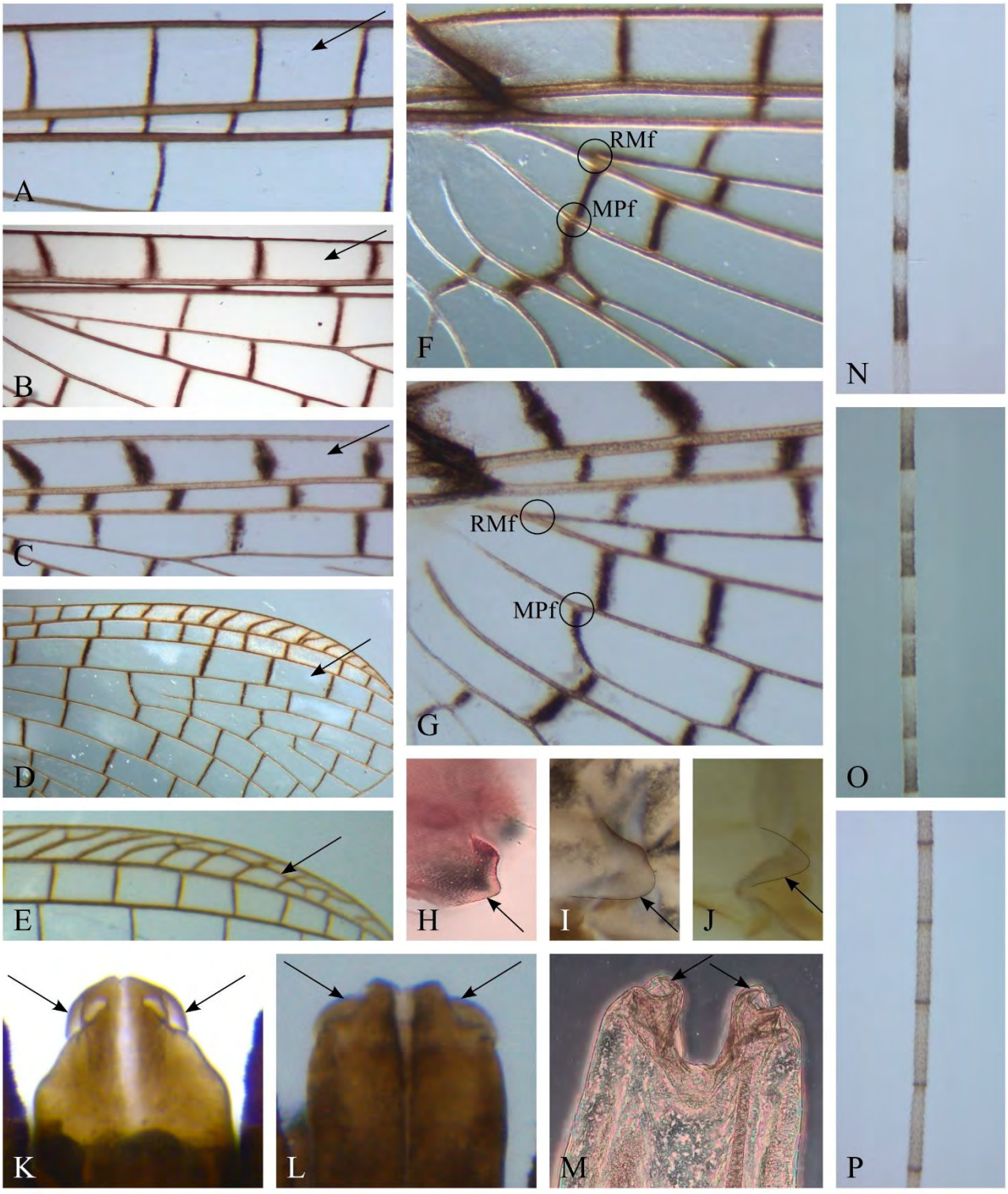


**Figure 3.20 Characters of the imagos of *Aprionyx*. Shading of crossveins in costal field (arrows indicate costal field) of A) *A. pellucidulus*, B) *A. rubicundus* and C) *A. peterseni*; D) shading of crossveins in anterior radial field of *A. natalicus* (arrow indicates anterior radial field); E) anastomosis in the pterostigma of *A. phoeocera* (anastomosis indicated by arrow); vein $MP_2$ attached to $MP_1$: F) at RS-MA fork, e.g. in *A. natalicus* and G) nearly at RS-MA fork in *A.***

***peterseni*; Segment IX with posterolateral projection (arrowed) biacuminate (appearing truncated) as in H) *A. rubicundus* and I) *A. pellucidulus*, or J) acuminate as in *A. natalicus*; penes enlarged to demonstrate the extent of fused penes and different types of processes (processes indicates with arrows): K) completely fused with shallow cleft and flap-like processes in *A. rubicundus*, L) completely fused with shallow cleft and curled processes in *A. natalicus* and M) apically separated with curled processes in *A.* tricuspidatus; caudal filaments with broad annulations as in N) *A. peterseni* and O) *A. natalicus* (different to *A. peterseni*) or P) with narrow annulations as in *A. pellucidulus*. RMf = RS-MA fork; MPf = $MP_2$ attached to $MP_1$. © IS Ferreira.**

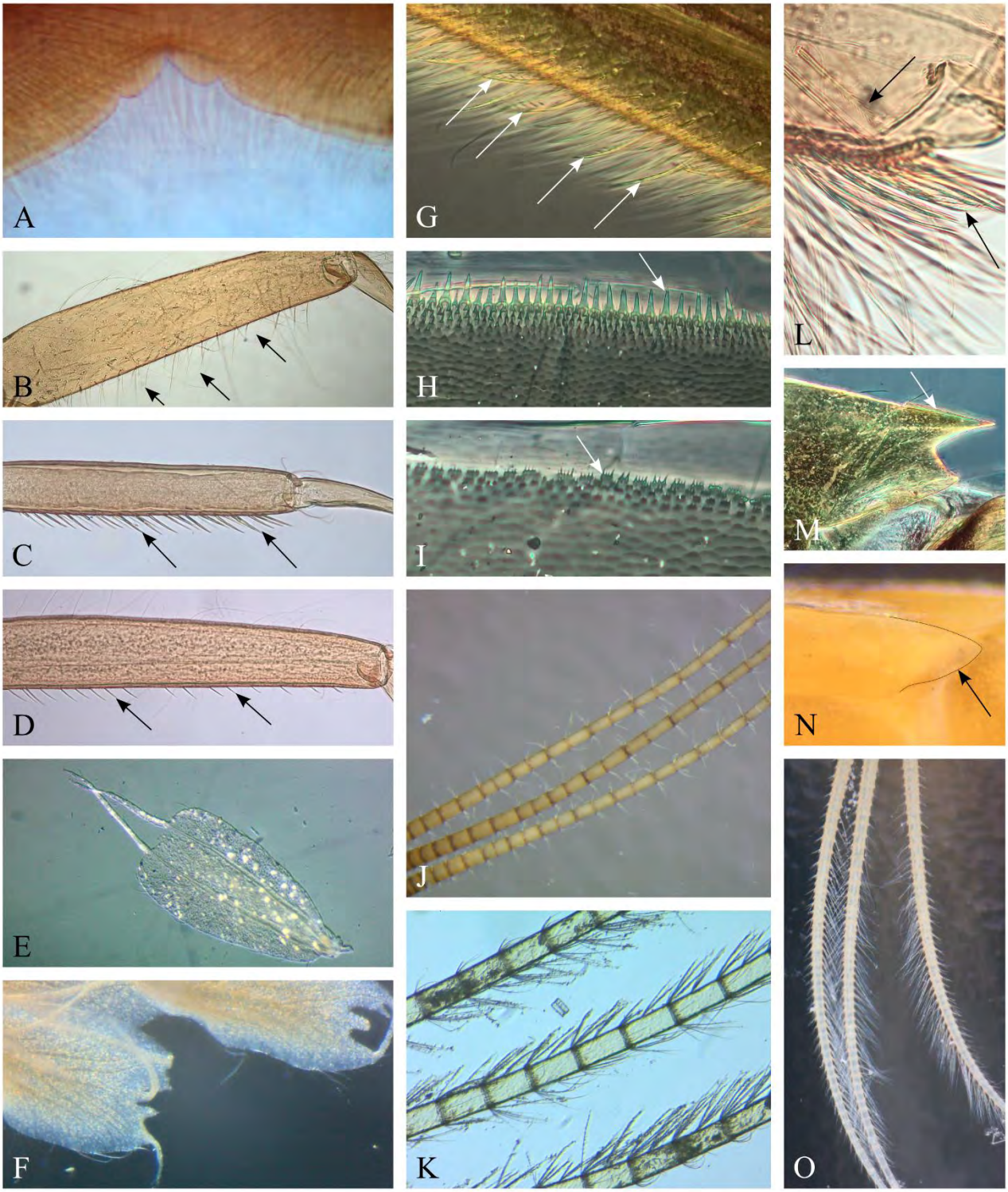


**Figure 3.21 Characters of the nymphs of *Aprionyx*. A) Labrum of *A. rubicundus* with only three teeth; setae (indicated by arrows) on the ventral margin of the tarsi are either a B) row of fine hairlike setae, C) fringe of spinelike setae, or D) row of small spinelike setae; E) gill with only the median filamentous process present; F) *A. tricuspidatus* gills showing the inconsistency of the filamentous processes; bipectinate setae present on ventral margin of tibia G) a fringe or L) only**

**a few distally; denticles on posterior margin of tergites (arrowed) H) well-developed or I) groups of minute denticles; caudal filaments with primary swimming setae J) absent, e.g. *A. rubicundus*, K) present and short, or O) present and long; abdominal posterolateral projections (arrowed) M) biacuminate or N) acuminate. © IS Ferreira.**

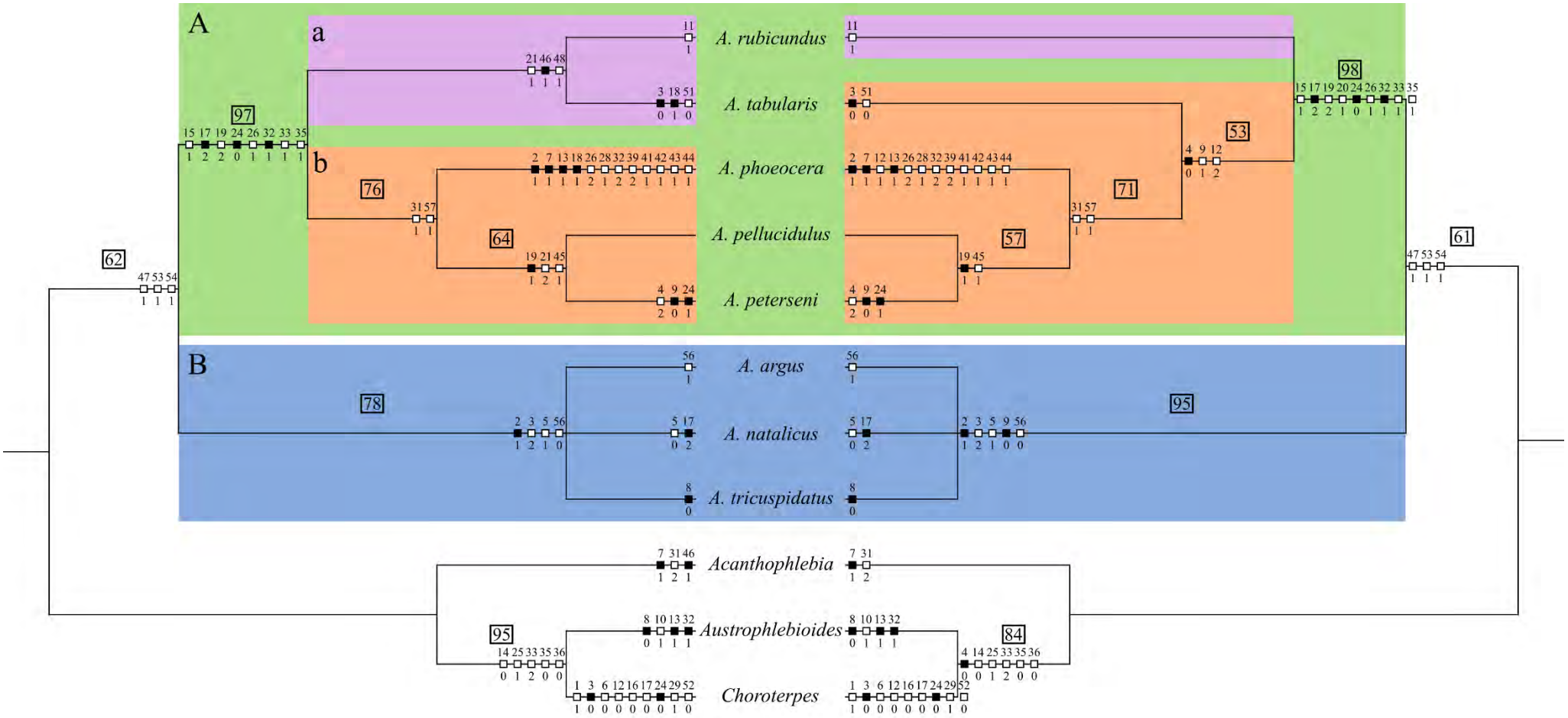


**Figure 3.22 Total evidence maximum parsimony consensus (50% majority) tree of heuristic search analyses under implied weighting (Goloboff criterion: K = 4) and equal weighting (on the left and right respectively). The two main clades are A) western *Aprionyx* (green) and B) eastern *Aprionyx* (blue). The western *Aprionyx* clade consists of two clades: a) highlighted purple and b) highlighted orange. The unambiguous character changes were mapped on the tree, with the squares indicating synapomorphies in white and homoplasies in black. Bootstrap values are shown above branches with support of > 50%.**

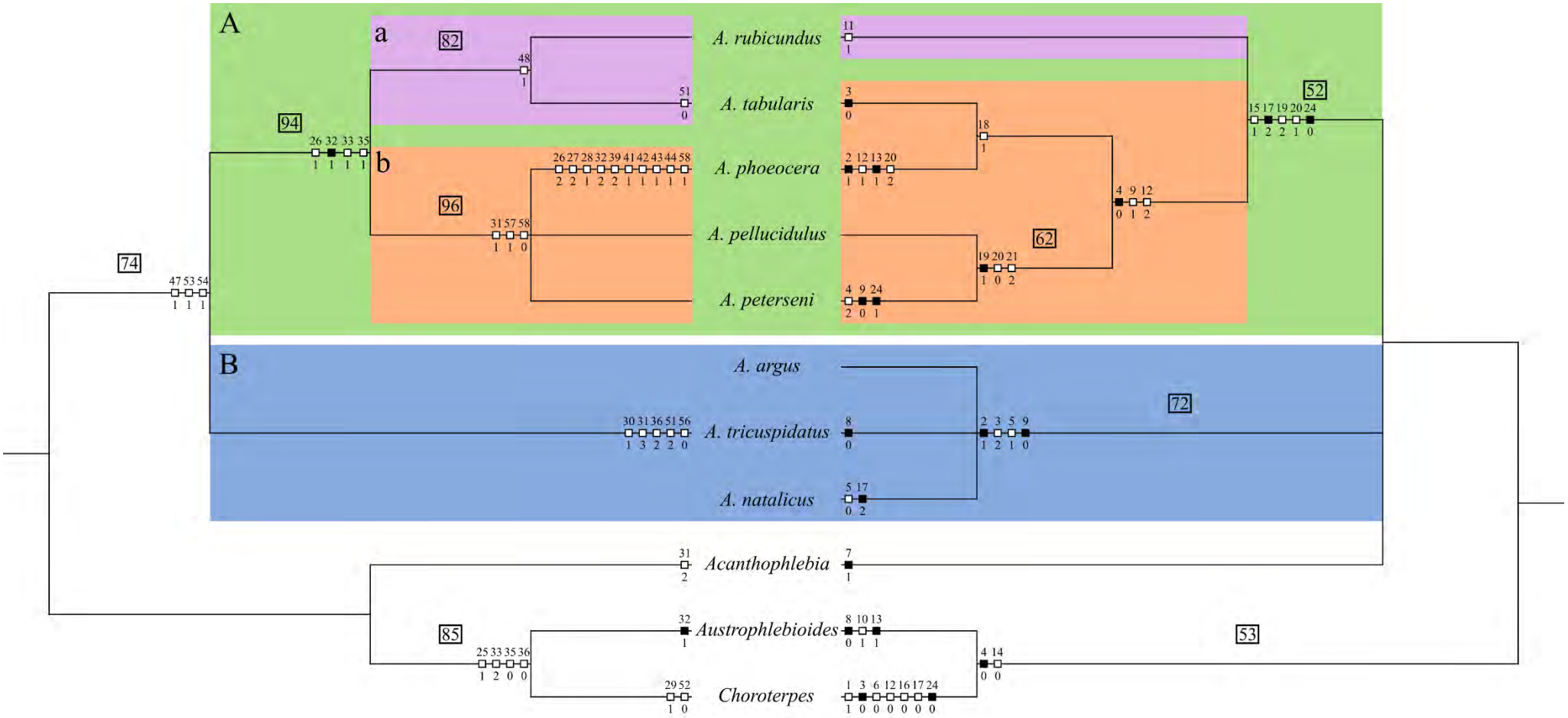


**Figure 3.23 Comparison of maximum parsimony consensus (50% majority) trees from heuristic search analyses under implied weighting (Goloboff criterion: K = 4) of the nymphal (left) and imago (right) characters. Nymphal data exclude *A. argus* and *A. natalicus*. The two main clades are A) western *Aprionyx* (green) and B) eastern *Aprionyx* (blue). The western *Aprionyx* clade consists of two clades: a) highlighted purple and b) highlighted orange. The unambiguous character changes were mapped on the tree, with the squares indicating synapomorphies in white and homoplasies in black. Bootstrap values are shown above branches with support of > 50%.**

# Chapter 4: Molecular and morphological phylogenetic congruence of *Aprionyx* species, with biogeographical notes

## 4.1 Introduction

The ancestral relationships of *Aprionyx* have not been formally studied, but some are of the opinion that its ancestry is of old Gondwanan decent, because of their affiliation to the amphinotic species in southern South America, Australia and New Zealand (Peters and Edmunds 1964, 1970, Pescador and Peters 1980). *Aprionyx* is also geographically restricted to the southern parts of South Africa, suggesting that species of this genus are decedents of the cool-adapted lineage present in the southern hemisphere (Harrison 1978). The relationship of *Aprionyx* in Leptophlebiidae is still indeterminate, but Pescador and Peters (1980) classified them in the *Hapsiphlebia* lineage, whereas Monjardim (2020) recovered *Aprionyx* as sister clade to the Terpidinae group, and together these two clades were recovered as a sister clade to Atalophlebiinae. The support for the relationship between *Aprionyx* and Terpidinae was too low for a reliable systematic placement of the *Aprionyx* genus, therefore its position within Leptophlebiidae is largely unresolved. Until further research resolves this, *Aprionyx* will keep its placement in the original Ataloplebiinae s.l. subfamily, as defined by Peters (1980a).

The traditional approach to resolve the evolutionary relationships of Ephemeroptera was based on morphological analysis (e.g. studies done by Pescador and Peters 1980, Christidis 2005, Campos et al. 2019). Today the use of molecular analyses is the preferred approach, but using both can be useful to increase the reliability of the results (Ogden et al. 2009b). However, the morphological results are often incongruent with the molecular analyses (Ogden et al. 2009a, Keating et al. 2023), and ideally congruency should be sought to increase the reliability of the results.

The previous chapters analysed the molecular and morphological aspects of *Aprionyx*. This chapter compares these results to discuss the topological congruency of the molecular and morphological phylogenies to see if they agree on the interspecies relationships. Further, this chapter uses the molecular data to get an estimated age for the genus and its species in order to

discuss the biogeography. This is the first study to attempt to understand the evolutionary history of the *Aprionyx* genus, and one of only a few on South African mayflies. Similar studies were conducted by Barber-James (2010) on Prosopistomatidae and Pereira da Conceicoa (2016) on Teloganodidae.

## 4.2 Congruence of molecular and morphological phylogenies

### *4.2.1 Materials and methods*

The molecular and morphological data are compared instead of merging the two datasets for a combined analysis because many of the species are not well represented in both data sets, e.g., *Aprionyx* sp. 7, *Aprionyx* sp. 8, *Aprionyx* sp. 10 and *Aprionyx* sp. 11 consist only of COI sequences while *A. argus* and *A. tricuspidatus* consist only of morphological data (mainly imago characters). Fifteen species are present in the molecular tree and eight species in the morphological tree, but only five species are in both.

The COI/16S/28S-combined maximum likelihood (ML) inference tree with Bayesian inference (BI) support added to applicable branches (Chapter 2; Figure 2.1) was used for the molecular phylogenetic tree and the total evidence maximum parsimony (MP) inference tree with implied weighting (Chapter 3; Figure 3.22) for the morphological phylogenetic tree. The thresholds of branch support for the reliability of a clade are specified in the molecular (section 2.2.5) and morphological (section 3.2.4) analyses. The trees were pruned to exclude the outgroups, and the species clades in the molecular tree, identified in Chapter 2, were collapsed to simplify the presentation of the results. The molecular and morphological phylogenies resulted in the same topology, therefore statistical analysis was deemed unnecessary.

### *4.2.2 Results*

A side-by-side comparison of the molecular and morphological phylogenies demonstrates the congruence between their topologies (Figure 4.1). Three distinct groupings are recognised within *Aprionyx*: the *rubicundus* group, the *pellucidulus* group and the eastern *Aprionyx* group. Collectively, the *rubicundus* and *pellucidulus* groups form the western *Aprionyx* group. The *rubicundus* group contains *A. rubicundus*, *A. tabularis* and five unnamed species (sp. 3-5, 7 and 8), whereas *A. pellucidulus*, *A. peterseni*, *A. phoeocera*, *Aprionyx* sp. 1 and *Aprionyx* sp. 2 are all in the *pellucidulus* group. The rest of the species, i.e., *A. argus*, *A.*

*natalicus*, *A*. *tricuspidatus*, *A*. cf. *tricuspidatus*, *Aprionyx* sp. 10 and *Aprionyx* sp. 11, are in the eastern *Aprionyx* group.

The *Aprionyx* clade and the various subordinate clades were generally not as well supported by morphology as by molecular analysis (Figure 4.1). The western *Aprionyx* and *pellucidulus* groups presented with similar support in both the morphological and molecular phylogenies: the western *Aprionyx* group was well supported (ML = 94%; BI = 1.00; MP = 97%) while support was lower for the *pellucidulus* group (ML = 78%; BI = 0.88; MP = 76%). The *rubicundus* and eastern *Aprionyx* groups were well supported by the molecular analysis, but the morphological analysis did not support the *rubicundus* clade (ML = 91%; BI = 0.99; MP < 50%) and was distinctly weaker for the eastern *Aprionyx* group (ML = 100%; BI = 1.00; MP = 78%). The support for the *pellucidulus* clade without *A*. *phoeocera* was higher in the molecular analysis and lower in the morphological analysis (ML = 89%; BI = 0.99; MP = 64%) compared with the clade including *A*. *phoeocera*. Also, the monophyly of *Aprionyx* was not as well supported by the morphological phylogeny as in the molecular phylogeny (ML = 87%; BI = 0.97; MP = 62%).

### *4.2.3 Discussion*

The *rubicundus* clade was supported by the molecular analysis, but not the morphological phylogeny (which excludes the four unnamed species). The question arises of whether *A*. *tabularis* belongs in the *rubicundus* clade or not. Two explanations suggest that it should be accepted as part of this group. Firstly, as explained in Chapter 2 (section 2.4), it is likely that *Aprionyx* sp. 4 is in fact *A*. *tabularis* based on a few salient characters, particularly the abdominal posterolateral spines, posterior marginal denticles and gills of the nymphs. Secondly, the general morphological appearance of *A*. *tabularis*, predominantly the nymphs, closely resembles *A*. *rubicundus*. In the imagos, the penis shape most closely resembles *A*. *rubicundus*, whereas in the nymphs, the claws and mouthparts, such as the labrum, are more similar to *A*. *rubicundus* than any of the other *Aprionyx* species. In the morphological phylogeny, this resemblance was not as distinct as it appeared to be in the morphological examination because of the low support and limited (only two) unambiguous synapomorphies identified in the morphological phylogeny: 1) ventral shape of the penis, projecting laterally downwards (character 21), and 2) nymphal claws slender and not hooked (character 48). The former conforms with the hypothesis that the genitalia of *A*. *tabularis* and the rest of the species in the *rubicundus* group are likely derived from the same ancestor with the penis tapering

apically, ventrally projecting laterally downwards and ending obtusely angular at the apex. The latter character appears to be unique to the *rubicundus* group since it was positively identified in *A. rubicundus*, *A. tabularis*, *Aprionyx* sp. 3, *Aprionyx* sp. 5 and *Aprionyx* sp. 7, whereas in *Aprionyx* sp. 4 and *Aprionyx* sp. 8 it is still questionable. This again supports a common ancestor for the proposed *rubicundus* group, including *A. tabularis*. While morphology supports the hypothesis of *A. tabularis* being part of the *rubicundus* group, it was not supported statistically. This could be ascribed to the more ambiguous results of the imago characters since the imago-only analysis could not resolve the relationship of *A. tabularis* with statistical support, whereas the nymph-only analysis strongly supports an *A. tabularis* and *A. rubicundus* affiliation (Chapter 3; Figure 3.23), therefore, added nymphal characters (for example, of the mouthparts) could strengthen the relationship between *A. tabularis* and *A. rubicundus*. More information on *A. tabularis* could give a more definitive answer, especially if *Aprionyx* sp. 4 (or any of the other unnamed species in the group) can be identified as *A. tabularis*, nonetheless the available data suggests that *A. tabularis* is part of the *rubicundus* group.

The *pellucidulus* group was fairly poorly supported by the molecular and morphological analyses. Closer inspection indicated that the relationships of *A. phoeocera* are questionable. The total evidence (COI/16S/28S-combined) species tree recovered *A. phoeocera* as sister species to the rest of the *pellucidulus* group, as did the morphology tree. The gene trees of COI and 16S, however, either recovered *A. phoeocera* as sister species to both western *Aprionyx* clades (*rubicundus* and *pellucidulus* clades) or were unable to resolve the relationship. The morphological analysis, on the other hand, mostly agrees with the hypothesis that *A. phoeocera* is a sister species to only the *pellucidulus* clade, suggesting that it is part of that group. The general morphology of *A. phoeocera* is unique, making it easier to distinguish, but the presence of all of the autapomorphies leads to little evidence to support relationships. Only two nymphal synapomorphies place *A. phoeocera* with the *pellucidulus* clade, i.e., the shape of the teeth in the anteromedial emargination of the labrum and the presence of primary swimming setae, while the imagos appear to be more closely related to *A. tabularis* (though this was not supported in the morphological analysis).

The eastern *Aprionyx* clade was highly supported by the molecular analysis, but the support was lower in the morphological analysis. The clade is mainly supported by imaginal characters in having small eyes with the ventral portion >0.79 times the dorsal portion, and heavy shading in the distal anterior radial area. The presence of lateral processes on the gills is

the only supportive character of the nymphs and those are usually filamentous processes. *Aprionyx argus* is so far the only known species with lateral bulges, whereas all four species in the molecular analysis present with filamentous processes. This raise the question of whether this species belongs in this clade, since *A*. *argus* was not included in the molecular analysis and the morphological analyses were unable to resolve its relationship to the other species. The synapomorphies and general morphological characters of the imagos are rather supportive of *A*. *argus* being part of the eastern *Aprionyx* clade, as it is especially difficult to tell them apart morphologically.

Both Barnard (1932) and Crass (1947b) observed an obvious distinction between the "Cape" (western *Aprionyx* group) and "Natal" (eastern *Aprionyx* group) *Aprionyx*. Here, both the molecular (Chapter 2) and morphological (Chapter 3) analyses recognised the two groups and were generally well supported. The species of the two *Aprionyx* groups are also clearly separated geographically, never occurring sympatrically (Figure 2.2A). The two lineages are distinct on a molecular, morphological and geographical level according to the results of this study, which could justify a split into two subgenera or genera if further research is able to increase the statistical support.

## 4.3 Estimating the time of divergence for *Aprionyx*

### *4.3.1 Materials and methods*

A molecular time-calibrated chronogram was produced with sequences of three molecular markers: COI, 16S and 28S (Chapter 2, section 2.2 for methods). The concatenated dataset included 66 sequences with a total length of 1714 bp. For the *Aprionyx* group, specimens were limited to those (from Chapter 2; Appendix A) with sequences for the 28S region, thus LEP072, LEP073 and all specimens from the SMNS data were excluded. Sequences for the outgroups were acquired from GenBank (Benson et al. 2013), except for the *Garinjuga* Campbell & Suter, 1988 28S sequences which were obtained from Gatti et al. (2021). Based on previous studies by Gatti et al. (2021, 2022), Monjardim et al. (2020) and O'Donnell and Jockusch (2008), 15 taxa, representative of the Atalophlebiinae (represented by Australian (*Atalomicria*, *Atalophlebia*, *Austrophlebioides* and *Garinjuga*) and South American (*Massartellopsis* Demoulin, 1955, *Meridialaris* and *Penaphlebia*) genera), Leptophlebiinae Banks, 1900 (represented by *Paraleptophlebia* Lestage, 1917) and Malagasy (represented by

*Radima*) groups, were selected as the outgroups (Table 4.1). The outgroups were increased and adjusted from the molecular analysis to improve the accuracy of the phylogeny (Rota-Stabelli and Telford 2008, Yu et al. 2021). The classification of these groups follows that of Monjardim et al. (2020), restricting Atalophlebiinae to amphinotic taxa, with *Aprionyx* unresolved and excluded from the Atalophlebiinae group.

To calibrate the tree, an approach similar to that used by Gatti et al. (2021, 2022) was followed and altered to fit this scenario. The analysis was run under the optimized relaxed clock model, which implements an uncorrelated, lognormal distribution for sampling substitution rates, and the Fossil Birth Death tree prior. The BI substitution models, as determined in Chapter 2 (Table 2.3), were applied for each partition, except for 28S and COI 3rd CP, the GTR model were replaced with the TN model. The monophyly of all calibrated clades was forced, by considering the results of Gatti et al. (2021, 2022), because the relationships of the outgroups in the BI tree were mostly unresolved and poorly supported (Appendix I).

Five nodes (i-v) were selected to calibrate the tree based either on secondary (n = 1), geological (n = 2) or palaeontological (n = 2) data (Table 4.2):

i. The age of Leptophlebiidae (in this case the root) was calibrated based on secondary information, at ca. 175 Ma (Kluge 1998, Grimaldi and Engel 2005, Gatti et al. 2021, 2022). All extant mayflies are classified under Euplectoptera, which likely started to diversify during the Early Jurassic about 200 Ma, suggesting Leptophlebiidae and several other families existed by the end of the Early Jurassic (Kluge 1998, 2004, Grimaldi and Engel 2005). The fossil records of Leptophlebiidae do not date back older than the Cretaceous, but two species have been placed in this family with certainty which suggest an initial diversification some time before the Cretaceous. *Ninadsa calyptrata* (Sinitshenkova, 1989) is a Leptophlebiidae from Mongolia (aged 125-139.8 Ma) and the younger *Kachinophlebia zhouchangfai* Chen & Zeng, 2022 (Leptophlebiinae) is a species from Myanmar (aged 99.6-93.5 Ma). The existence of Cretaceous Leptophlebiidae further justifies the calibration of the initial diversification of Leptophlebiidae at ca. 175 Ma, as applied by Gatti et al. (2021, 2022).

ii. The most recent common ancestor (MRCA) between *Aprionyx* and the Malagasy+Atalophlebiinae clade was calibrated on the time of separation between east (Madagascar, India, Australia and Antarctica) and west (Africa and South America)

Gondwana, at ca. 140 Ma (Seton et al. 2012, Gibbons et al. 2013, Mueller and Jokat 2019).

iii. The divergence between the Malagasy lineage and Atalophlebiinae was calibrated based on when Madagascar and India were separated from Australia and Antarctica, at about 100 Ma (Seton et al. 2012, Gibbons et al. 2013, White et al. 2013).

iv. *Paraleptophlebia prisca* (Pictet-Baraban & Hagen, 1856) was used for calibrating the node representing the MRCA of *Paraleptophlebia*.

v. *Atalophlebia culleni* (Etheridge & Olliff, 1890) was used as calibration for the divergence between *Atalophlebia* and *Atalomicria* (based on the findings of Gatti et al. (2021)).

Information for the fossils is accessible in the Paleobiology Database (https://paleobiodb.org).

Fossils are more reliable sources for node calibration and thus also more popular in calibrating time trees (Hipsley and Müller 2014), which is why two fossil calibrations were incorporated into the divergence time analysis. The only mayfly fossil known from South Africa, *Litophlebia optata* (Riek, 1976), dates back to the Late Triassic period (228-237 Ma) but is not a Leptophlebiidae. Based on its age *L. optata* pre-dates Leptophlebiidae and its wing morphology is not of the Leptophlebiidae type, therefore it is not a close relative of *Aprionyx*. McCafferty (1997) reasoned that the mayfly fossil, *Conovirilus poinari* McCafferty, 1997, from the Early Cretaceous (120-135 Ma), is more closely related to a clade consisting of *Aprionyx*, *Adenophlebia* (southern Africa) and *Atalophlebioides* (then known from Australia, Chile, Madagascar and New Zealand), based on the fused penes (Peters and Edmunds 1970). Peters and Peters (2000) disagreed with his classification, stating it is more likely related to the *Terpides* lineage. The uncertainty of its systematic position in Leptophlebiidae justified the omission of this fossil for this analysis. In the absence of *Aprionyx* or any South African Leptophlebiidae fossils, or close relatives, outgroups with known fossils were selected for the two fossil-calibrated nodes.

After specification of the parameters in BEAUti v2.7.5 (Bouckaert et al. 2014, 2019), the resulting input file was used to run BEAST2 v2.7.5 (Drummond and Rambaut 2007, Suchard and Rambaut 2009, Bouckaert et al. 2014, 2019) on the CIPRES Science Gateway (Miller et al. 2010) with a chain length of 200 million generations, sampling every 10 000 trees. The effective sample size (ESS > 200) was viewed in Tracer v1.7.2 (Rambaut et al. 2018). A maximum clade credibility tree, using a burn-in of 25% and median node heights, was

constructed in TreeAnnotator v2.7.5 (Bouckaert et al. 2014, 2019). The chronogram was viewed and annotated with FigTree v1.4.4 and Inkscape v1.1 software (https://inkscape.org).

### *4.3.2 Results*

In the Bayesian analysis of the divergence time estimation, *Aprionyx* was recovered as monophyletic and was strongly supported (>0.9; Appendix I), as in the BI analysis of the molecular phylogeny (Chapter 2 and section 4.2). However, the outgroups were mostly recovered as unresolved and their relationships were generally poorly supported. The reason for this was attributed to the data quality because many of the 28S sequences used from GenBank aligned with only about 50% of the length of the *Aprionyx* data. In other words, the amplified region of the 28S gene did not entirely correspond to the region amplified in this study. Since the three main outgroup lineages (Atalophlebiinae, Leptophlebiinae and Malagasy) were all represented in the studies by Gatti et al. (2021, 2022), the calibrated clades were forced as monophyletic based on their findings (in which the clades were strongly supported by the BI analysis). The BI analysis recovered the *Aprionyx* species within the same three clades (*rubicundus*, *pellucidulus* and eastern *Aprionyx* clades) recovered in the morphological and molecular comparison (section 4.2), but this was not the case in the divergence time tree. Instead, *A. phoeocera* was recovered as sister to the eastern *Aprionyx* clade, and together they share a common ancestor with the *pellucidulus* clade, whereas the *rubicundus* clade was recovered as sister to all other *Aprionyx* (Figure 4.2). Four lineages were therefore identified in the chronogram: the *rubicundus* lineage (*A. rubicundus*, *Aprionyx* sp. 3, 4 and 5), *pellucidulus* lineage (*A. pellucidulus*, *A. peterseni*, *Aprionyx* sp. 1 and 2), *phoeocera* lineage (*A. phoeocera*), and eastern *Aprionyx* lineage (*A. natalicus* and *A.* cf. *tricuspidatus*).

The chronogram suggests that the *Aprionyx* lineage originated during the Early Cretaceous period with initial diversification ca. 97.45 Ma (median age; 95% highest posterior density (95% HPD): 62.49-128.75; Table 4.3, Figure 4.2). The various *Aprionyx* crown-group species were estimated to be younger than 15 Ma (median age: 1-6.5 Ma; 95% HPD: 0-14 Ma), suggesting they originated between the middle Miocene to Pleistocene. *Aprionyx peterseni* was hypothesised to be the oldest known extant *Aprionyx* species with a median age of 6.47 Ma. The relationships of the eastern *Aprionyx* group and *A. phoeocera* to each other and to the *pellucidulus* clade were poorly supported (PP = 0.5 and 0.61; nodes 3 and 5; Figure 4.2), implying that the initial time of divergence for the main *Aprionyx* lineages may well be unreliable. Assuming there are some truths in the proposed diversification of *Aprionyx*, the

*phoeocera* lineage diverged from the eastern *Aprionyx* lineage (node 5) earlier than the divergence of the *pellucidulus* (node 4) and *rubicundus* (node 7) lineages, ca 56.9 Ma (median age; Table 4.3, Figure 4.2). It also suggests that the eastern *Aprionyx* lineage (node 6) is a relatively young lineage, with a median divergence time of 14.6 Ma (95% HPD: 3.77-32.59 Ma). The *pellucidulus* (node 4) and *rubicundus* (node 7) lineages diverged about the same period, with the *rubicundus* lineage slightly older.

The resulting ages of the major outgroup nodes were consistent with the findings of Gatti et al. (2021, 2022), with Leptophlebiidae ca. 160 Ma (node i), Atalophlebiidae and Malagasy divergence ca. 100 Ma (node iii), *Paraleptophlebia* ca. 45 Ma (node iv), *Atalophlebia-Atalomicria* divergence about 30 Ma (node v) and Atalophlebiinae about 90 Ma (node 1). The divergence between *Aprionyx* and the Atalophlebiinae-Malagasy group (node ii), which was calibrated based on the separation of the eastern and western Gondwana landmasses, was aged at about 130 Ma.

### *4.3.3 Biogeographical discussion*

The time-calibrated analysis suggests an ancestral lineage from the Early Cretaceous gave rise to the *Aprionyx* lineage (median age of initial diversification = 97.45 Ma), but with a relatively high range of uncertainty (95% HPD: 62.49-128.75 Ma). This implies a post-Gondwanan origin in the Cretaceous when southern Africa was separated from the other southern continents (Seton et al. 2012, Gibbons et al. 2013, Mueller and Jokat 2019). It also suggests that the *phoeocera* and eastern *Aprionyx* lineages are descendant from a common ancestor, and although it is poorly supported in the chronogram, morphologically these two lineages share a similar character of the male eyes, which are widely separated.

The ambiguity of the estimated ages is relatively high, with the 95% HPD range more than 60 Ma in some nodes. This ambiguity can be a reflection of the effect caused by the geological calibrations since it leads to higher variability in node ages (i.e., larger 95% HPD ranges) and is consequently less reliable (Ho et al. 2015). Calibrations based on geological events, such as the separation of landmasses, rely on two assumptions (Ho et al. 2015). This scenario, looking at node ii as an example, assumes that a Gondwana lineage diverged into east and west Gondwana lineages because of the separation into east and west Gondwana, thus the branching of the node was the effect of the geological event. It also assumes that the time of separation is known, thus an accepted timeframe for the event is available. When geological events are used to calibrate nodes, it is also important to remember that the formation of the

barrier is not immediate, but very slow, and the impact of the barrier on the branching of an ancestral species can be delayed (Ho et al. 2015).

Africa was positioned more to the south during the Early Jurassic when it formed part of Gondwana (Harrison 1978). Early African Leptophlebiidae lineages could have had a more northern distribution, with a north to south dispersal to stay in the cooler temperatures as Gondwana separated and Africa shifted in a northern direction, thus isolating the southern African lineages. This could explain the plausible affinity with Terpidinae from northern South America (Monjardim et al. 2020), which separated from Africa much later than the south. A pre-existing northern distribution suggests that ancestral *Aprionyx* from Early Cretaceous was distributed in the Orange River. Ancestral *Aprionyx* species must have dispersed from the Orange River to the western rivers of the Cape Fold belt (CFB) as erosion and uplift resulted in river capture. The Orange and Olifants Rivers were connected for some time during Late Cretaceous to Oligocene when the upper Orange River exited at the Olifants Mouth (McCarthy et al. 1985, Burke 1996).

In another scenario, ancestral *Aprionyx* could have had a widespread distribution in the cooler Gondwana climates, including Antarctica, Australia and southern South America (Balinsky 1962, Harrison 1978). As Africa moved northwards and separated from eastern Gondwana and southern South America, these ancestral *Aprionyx* retreated to the refugia of the cooler mountain rivers, often shaded, of the CFB as temperatures climbed.

The extant *Aprionyx* species are generally younger than 2 million years, i.e., from the Pleistocene. This relatively recent diversity is congruent with the glacial cycles during the Quaternary period, resulting in a higher extinction and speciation rate (Hewitt 2000). The dispersal and diversification can also be a result of transgression and regression of the sea, thus connecting river systems as well as the isolation and extinction of populations, similar other mayflies (Teloganodidae; Pereira da Conceicoa 2016) and some fish taxa (*Pseudobarbus* Smith, 1841; Swartz et al. 2007).

## 4.4 Summary

The molecular and morphological phylogenies were shown to be topologically congruent. Both of the analyses agreed on three distinctive lineages of *Aprionyx*: *rubicundus*,

*pellucidulus* and eastern *Aprionyx*. Even though the morphological phylogeny is not particularly well supported, the presence of the same three lineages in both analyses increase the reliability of the results. The results also suggest that *A. phoeocera* could be a separate lineage from the *pellucidulus* lineage, but this theory is currently poorly supported. This was raised again in the divergence time estimation analysis, where four lineages are recognized instead. Further research is needed to determine if *A. phoeocera* is a descendant from a fourth lineage and whether that lineage also gave rise to the eastern *Aprionyx* lineage.

The origin of *Aprionyx* was estimated to be during the Early Cretaceous period, with the initial divergence determined at about 100 million years ago. In other words, the *Aprionyx* genus is older than 100 million years. This study determined *A. peterseni* as the oldest of the *Aprionyx* species included here. The current hypothesis suggests that the eastern *Aprionyx* lineage diverged from an ancestor shared with the *phoeocera* lineage. The hypothesis also found that the eastern *Aprionyx* lineage is the youngest of the four identified lineages. This suggests that the genus could have originated in the Western Cape region and then dispersed to the eastern parts of their current distribution. On the other hand, the ancestral lineage could have existed in the region already and only after recent vicariant events, such as the glacial cycles, they diverged. Further research is needed to increase the accuracy of the divergence ages, and if additional and more closely related fossils are discovered, the results could also be improved.

**Table 4.1 List of outgroups selected for the estimated divergence time analysis with Genbank accession numbers.**

| Taxa | Country | 28S | 16S | COI |
|---|---|---|---|---|
| **Malagasy Leptophlebiidae** | | | | |
| ***Radima* sp. 1** | Madagascar | EU874473.1 | | |
| ***Radima* sp. 2** | Madagascar | EU874477.1 | | |
| ***Radima* sp. 3** | Madagascar | EU874478.1 | | |
| **Atalophlebiinae** | | | | |
| ***Atalomicria* sp.** | Australia | EU874483.1 | | JN289985.1 |
| ***Atalophlebia* sp.** | Australia | EU874484.1 | | KP697596.1 |
| ***Austrophlebioides* sp.** | Australia | AY749929.1 | MG764392.1 | KP697456.1 |
| ***Garinjuga* sp.** | Australia | (Gatti et al. 2021) | | JN290008.1 |
| ***Garinjuga* sp.** | Australia | (Gatti et al. 2021) | | JN290009.1 |
| ***Garinjuga* sp.** | Australia | (Gatti et al. 2021) | | JN289965.1 |
| ***Massartellopsis* sp.** | Chile, Argentina | EU874492.1 | | |
| ***Meridialaris diguillina*** | Chile, Argentina | EU874493.1 | | |
| ***Meridialaris laminata*** | Chile, Argentina | EU874494.1 | | |
| ***Penaphlebia* sp.** | Chile, Argentina | EU874500.1 | AY749782.1 | |
| **Leptophlebiinae** | | | | |
| ***Paraleptophlebia vaciva*** | Canada | AY749922.1 | AY749759.1 | JQ663110.1 |
| ***Paraleptophlebia submarginata*** | Europe | EU874514.1 | GQ118290.1 | HM421999.1 |

**Table 4.2 Calibration data for the five selected nodes, numbered on the tree in Figure 4.2 according to the first column. MRCA = Most recent common ancestor, mirs = mean in real space, M = Mean, SD = Standard deviation, O = Offset. ** Additional Leptophlebiidae fossils.**

| | | | | MRCA parameters | | | | |
|---|---|---|---|---|---|---|---|---|
| **Node #** | **Node name** | **Source type** | **Age (Ma)** | **Distribution type** | **M** | **SD** | **O** | **Source** |
| **i** | Leptophlebiidae origin (root) | Secondary | ca. 175 (Early Jurassic) | Normal | 175.0 | 25.0 | 0.0 | Kluge 1998, Grimaldi and Engel 2005, Gatti et al. 2021, 2022 |
| **ii** | East-West Gondwana split | Geological | ca. 140 (Early Cretaceous) | Lognormal (mirs) | 20.0 | 1.0 | 125.0 | Seton et al. 2012, Gibbons et al. 2013, Mueller and Jokat 2019 |
| **iii** | Madagascar-Australia split | Geological | ca. 100 (Late Cretaceous) | Lognormal (mirs) | 20.0 | 1.0 | 85.0 | Seton et al. 2012, Gibbons et al. 2013, White et al. 2013 |
| **iv** | *Paraleptophlebia prisca* | Fossil | 33.9 – 37.8 (Priabonian) | Lognormal (mirs) | 13.0 | 1.0 | 33.9 | Pictet-Baraban and Hagen 1856, https://paleobiodb.org |
| **v** | *Atalophlebia culleni* | Fossil | 2.6 – 5.3 (Pliocene) | Lognormal (mirs) | 20.0 | 1.0 | 2.6 | Etheridge and Olliff 1890, https://paleobiodb.org |
| ** | *Ninadsa calyptrata* | Fossil | 125-139.8 (Early Cretaceous) | | | | | Sinitshenkova 1989, Özdikmen 2008, https://paleobiodb.org |
| ** | *Kachinophlebia zhouchangfai* | Fossil | 93.5-99.6 (Cenomanian) | | | | | Chen and Zheng 2022, https://paleobiodb.org |

**Table 4.3 Results of the estimated time of divergence for *Aprionyx* and the outgroups based on the calibrated BEAST2 chronogram. Node numbers refer to Figure 4.2. HPD = highest posterior density; MRCA = most recent common ancestor.**

| Node nr | Clade | Median age | 95% HPD |
|---|---|---|---|
| **i** | Leptophlebiidae (All taxa) | 163.37 | 135.18-184.96 |
| **ii** | *Aprionyx* + (Atalophlebiinae + Malagasy) | 131.59 | 125.38-145.91 |
| **iii** | Atalophlebiinae + Malagasy | 102.56 | 86.06-129.12 |
| **iv** | Leptophlebiinae (= *Paraleptophlebia*) | 48.53 | 34.25-88.59 |
| **v** | *Atalophlebia* + *Atalomicria* | 31.25 | 9.65-60.75 |
| **1** | Atalophlebiinae | 91.21 | 63.07-122.62 |
| **2** | *Aprionyx* | 97.45 | 62.49-128.75 |
| **3** | *pellucidulus* group + (eastern *Aprionyx* group + *A. phoeocera*) | 75.77 | 45.06-106.36 |
| **4** | *pellucidulus* group (excl. *A. phoeocera*) | 40.43 | 20.60-65.31 |
| **5** | Eastern *Aprionyx* group + *A. phoeocera* | 56.90 | 27.86-91.24 |
| **6** | Eastern *Aprionyx* group | 14.60 | 3.77-32.59 |
| **7** | *rubicundus* group | 45.00 | 18.91-79.09 |
| **8** | *Aprionyx* sp. 1 | 1.66 | 0.27-4.66 |
| **9** | *A. pellucidulus* | 1.70 | 0.24-4.98 |
| **10** | *Aprionyx* sp. 2 (MRCA) | 26.73* | 11.38-46.70 |
| **11** | *A. peterseni* | 6.47 | 2.21-13.93 |
| **12** | *A. natalicus* | 1.33 | 0.00-7.23 |
| **13** | *A.* cf. *tricuspidatus* | 1.01 | 0.04-3.34 |
| **14** | *A. phoeocera* | 1.32 | 0.12-4.06 |
| **15** | *Aprionyx* sp. 4 (MRCA) | 30.37* | 12.15-54.38 |
| **16** | *Aprionyx* sp. 5 (MRCA) | 21.51* | 8.05-41.66 |
| **17** | *A. rubicundus* | 2.99 | 0.87-6.50 |
| **7** | *Aprionyx* sp. 3 (MRCA) | 45.00* | 18.91-79.09 |

* The age of the most recent common ancestor is given for *Aprionyx* sp. 2-5 and not the age of the species.

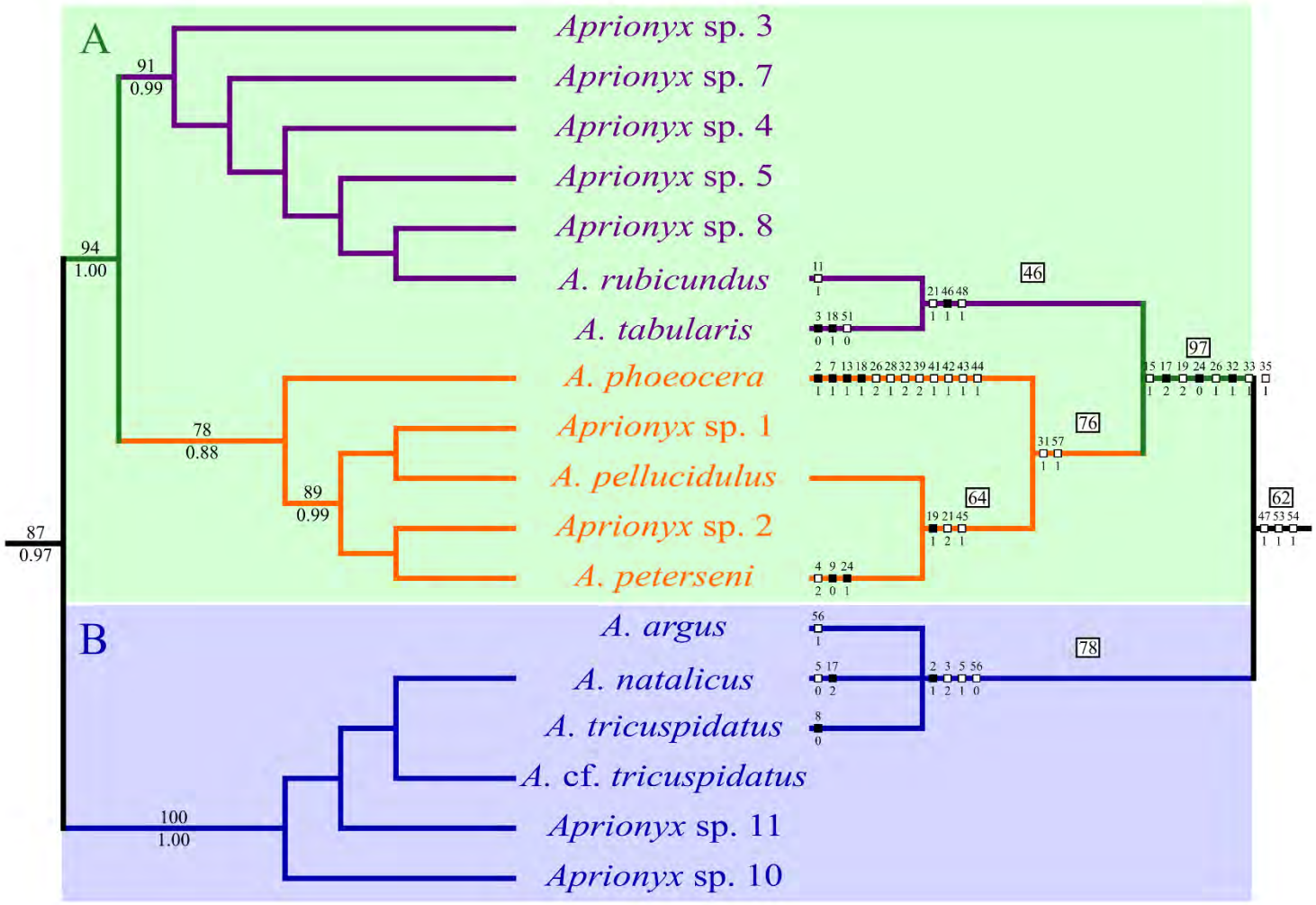


**Figure 4.1 A simplified presentation of the molecular (left) and morphological (right) inference trees for comparison between the respective topologies. The two main clades of *Aprionyx* are highlighted as Clade A (western *Aprionyx* group) in green and Clade B (eastern *Aprionyx* group) in blue; in Clade A the *rubicundus* group are indicated with purple, and the *pellucidulus* group with orange. For the molecular tree the ultrafast bootstrap and posterior probability support values are given above and below the branches, whereas the bootstrap support is shown above branches. The unambiguous character changes are indicated on the tree with white squares for synapomorphies and black for homoplasies.**

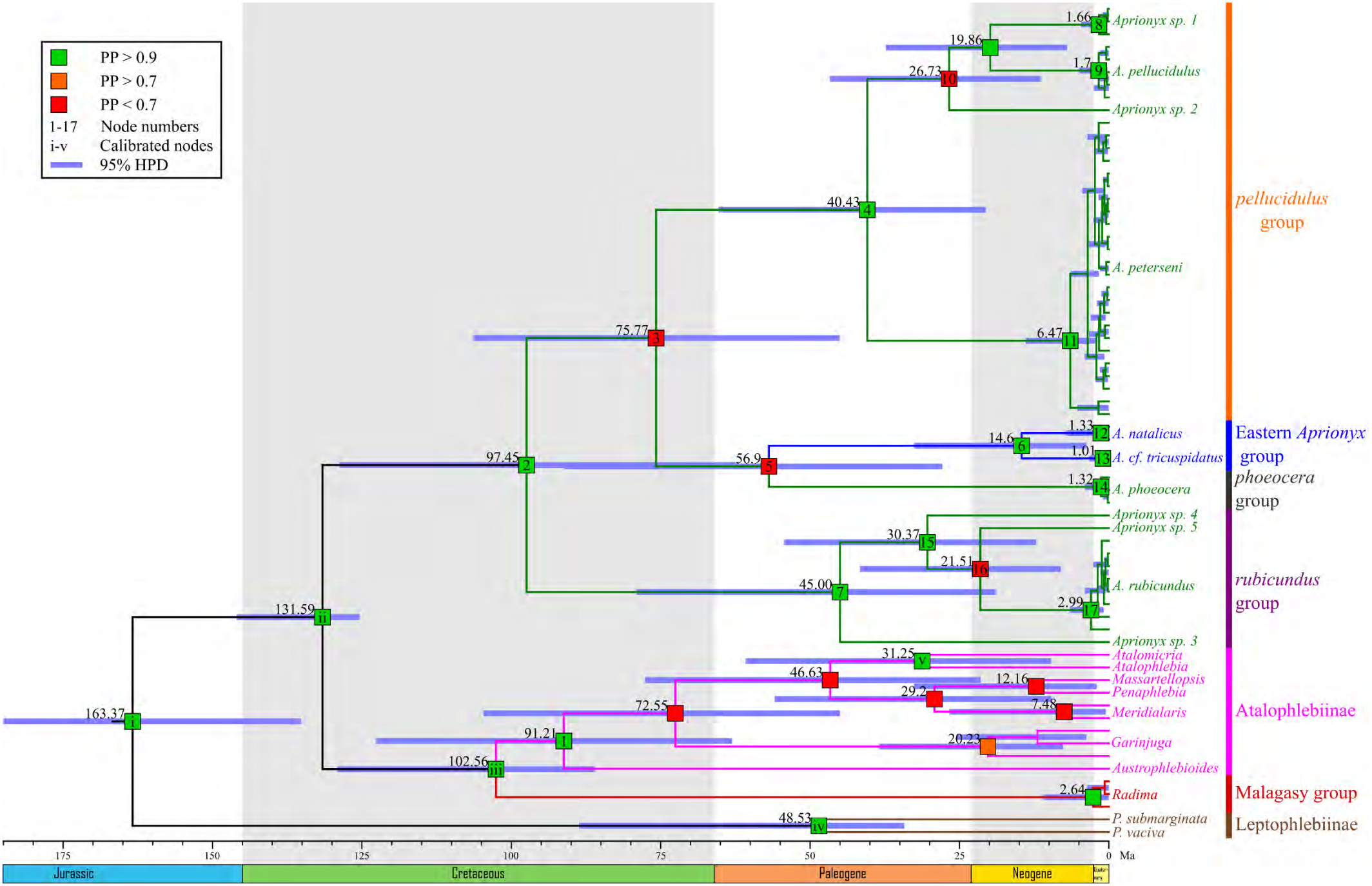
PP > 0.9
PP > 0.7
PP < 0.7
1-17 Node numbers
i-v Calibrated nodes
95% HPD
1.66
8
Aprionyx sp. 1
19.86
1.7
9
A. pellucidulus
26.73
10
Aprionyx sp. 2
40.43
4
A. peterseni
75.77
3
6.47
11
pellucidulus group
1.33
12
A. natalicus
14.6
6
1.01
13
A. cf. tricuspidatus
97.45
2
56.9
5
1.32
14
A. phoeocera
Eastern Aprionyx group
phoeocera group
Aprionyx sp. 4
Aprionyx sp. 5
30.37
15
21.51
16
45.00
7
A. rubicundus
2.99
17
rubicundus group
Aprionyx sp. 3
131.59
ii
31.25
v
Atalomicria
Atalophlebia
46.63
12.16
Massartellopsis
29.2
Penaphlebia
163.37
i
72.55
7.48
Meridialaris
91.21
1
20.23
Garinjuga
102.56
iii
Austrophlebioides
Atalophlebiinae
2.64
Radima
Malagasy group
48.53
iv
P. submarginata
P. vaciva
Leptophlebiinae
175
150
125
100
75
50
25
0 Ma
Jurassic
Cretaceous
Paleogene
Neogene

**Figure 4.2 Chronogram of *Aprionyx* with Atalophlebiinae, Malagasy group and Leptophlebiinae added as outgroups. The calibrated nodes (i-v) refer to the information given in Table 4.2 and nodes 1-17 correspond to the numbers in Table 4.3. The various lineages mentioned are given on the far right. HPD = highest posterior density.**

# Chapter 5: General conclusions and future research

## 5.1 General conclusions

The systematics of South African Leptophlebiidae was outdated and limited. *Aprionyx* is the largest Leptophlebiidae genus in South Africa and endemic in the southern provinces, but no extensive research had been done for well over half a century. The current work is the first substantial study of their taxonomy and diversity, as well as the first phylogenetic analyses on a species level. It also resulted in the creation of a preliminary key to the adults and nymphs of *Aprionyx* species, which will be adjusted as new information becomes available, for example, on the nymphs of *A. argus* and *A. natalicus* when fresh material is found, or when new species are described.

The current revision confirmed that the biodiversity of *Aprionyx* has been underestimated, as the molecular analysis identified at least seven new species. Various taxonomic uncertainties were also addressed, justifying a few nomenclatural changes. After identifying *A. tabularis* as a homonym, *A. phoeocera* stat. rev. was re-established as the species name for the junior homonym (*A. tabularis sensu* Esben-Petersen, 1920). *Aprionyx tabularis* n. syn. (*sensu* Barnard, 1932) was identified as synonymous to *A. phoeocera* and *A. intermedius* n. syn. to *A. tabularis* (*sensu* Eaton, 1884). The descriptions of the eight currently described species were substantially improved and the nymph of *A. rubicundus* was described for the first time. *Aprionyx argus* and *A. natalicus* are still poorly described, especially the nymphs, thus it remains difficult to distinguish the three described species (three eastern *Aprionyx* species) and identify new ones. The main obstacles that contributed to the limited progress on species knowledge is the absence of fresh specimens and missing historical specimens.

The molecular and morphological analyses were in agreement that *Aprionyx* is monophyletic and that it consists of three lineages: the *rubicundus*, *pellucidulus* and eastern *Aprionyx* lineages. The congruency between the analyses increases the reliability of the results, since this was not always well supported by the statistical evidence, and some details of the morphology are still inadequate, especially in that of the potentially new species identified through molecular analysis. A potential fourth lineage, the *phoeocera* lineage, was also

highlighted by the current work, because *A. phoeocera* was not well supported as a member of the *pellucidulus* lineage.

The current study also identified that there are two distinct clades in *Aprionyx*, the western and eastern *Aprionyx* groups, which can be separated molecularly, morphologically and geographically, suggesting that they are potentially two subgenera or genera. Unless further research on *Aprionyx* can positively, and with high support, identify it as non-monophyletic, the genus should remain one taxon.

The estimated age of *Aprionyx* suggests that initial divergence occurred in the Cretaceous and instead of the two distinct clades, four lineages were identified with the eastern *Aprionyx* clade nested in the western *Aprionyx* clade. The analysis suggests that the eastern *Aprionyx* and *phoeocera* lineage diverged from the same ancestral lineage, which is supportive of a potential fourth lineage.

In conclusion, the current study is the most extensive study on the systematics of *Aprionyx*, and is important foundational work for future research. As our knowledge on the taxonomy of mayflies, such as *Aprionyx*, improves, the ecological aspects, environmental requirements and distribution patterns can be studied more extensively. This will help to determine any unique biological and environmental preferences of each species and evaluate their sensitivity. Subsequently, this will aid in important decision-making relating to conservation and environmental management of freshwater systems.

## 5.2 Future research

This is the first multifaceted study of *Aprionyx* and it greatly advanced the current state of our knowledge on the genus, but there is still much to uncover. This work on the taxonomy of *Aprionyx* addressed important issues and improved the species descriptions, but taxonomic research needs to continue. Future work should especially be directed at the eastern *Aprionyx* species, which is undeniably not as well-known as the western *Aprionyx* species, even after this study. Species, such as *A. argus* and *A. natalicus*, are still poorly described because the historic material of the nymphs are missing and they are not well represented in museum collections. The western *Aprionyx* species should, however not be ignored in further research, since the descriptions can certainly be improved up on, especially *A. tabularis*. A number of possibly new species were identified in this study. These species need to be confirmed as new

and then they can also be described. The morphological knowledge can also be improved be examining the female imagos and their eggs, while introducing scanning electron microscopy into future research can lead to the discovery of additional distinguishing characters.

This study identified two distinct clades, named the eastern and western *Aprionyx* groups, and speculated around the possibility of splitting *Aprionyx* into two genera or subgenera. Future research should reconsider the separation of the two groups into two genera with improved morphological and molecular data. Future research should also be directed at a higher-level phylogenetic analysis to determine the position of *Aprionyx* within the family and the evolutionary relationships to other South African Leptophlebiidae. Another research option to consider is the geometric morphometrics approach to investigate the variation of, for example, wing shapes of *Aprionyx* species, which can also be used in advanced evolutionary phylogenetic analysis.

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# Appendices

**Appendix A. List of specimens with GenBank accession numbers for each gene region sequenced. Sequences that have not been submitted to GenBank are indicated under the corresponding gene by a '-'. References are provided for sequences from GenBank and the institute specified for new sequences. Trib. = tributary.**

| Taxa | DNA nr. | Catalogue nr. | Collection date | Country | River system | River name | Site name | Latitude | Longitude | COI | 16S rRNA | 28S rRNA | Institute/Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | **Ingroup** | | | | | | | |
| ***Aprionyx cf. tricuspidatus*** | LEP024 | ISF144B | 27.III.2019 | South Africa | Tugela | Mandleni trib. | Kranskop | -28,9567 | 30,8774 | OK413055 | OK412740 | OK412830 | AMGS |
| ***Aprionyx cf. tricuspidatus*** | LEP025 | ISF144B | 27.III.2019 | South Africa | Tugela | Mandleni trib. | Kranskop | -28,9567 | 30,8774 | OK413054 | OK412741 | OK412831 | AMGS |
| ***Aprionyx natalicus*** | LEP080 | ISF15A | 02.XII.2017 | South Africa | Keiskamma | Tyume | Lower site (H1) | -32,5944 | 26,9562 | | | OK412878 | AMGS |
| ***Aprionyx natalicus*** | LEP081 | ISF17A | 02.XII.2017 | South Africa | Keiskamma | Tyume | Middle site (H2) | -32,5931 | 26,9581 | | OK412790 | OK412879 | AMGS |
| ***Aprionyx pellucidulus*** | LEP022 | ISF46B | 25.III.2018 | South Africa | Breede | Tradouw trib. | Piekniekbos | -33,9651 | 20,7046 | OK413056 | OK412739 | OK412829 | AMGS |
| ***Aprionyx pellucidulus*** | LEP067 | ISF2D | 21.XI.2017 | South Africa | Breede | Tradouw trib. | Piekniekbos | -33,9651 | 20,7046 | | OK412778 | OK412868 | AMGS |
| ***Aprionyx pellucidulus*** | LEP068 | ISF46B | 25.III.2018 | South Africa | Breede | Tradouw trib. | Piekniekbos | -33,9651 | 20,7046 | | OK412779 | OK412869 | AMGS |
| ***Aprionyx pellucidulus*** | LEP069 | ISF46B | 25.III.2018 | South Africa | Breede | Tradouw trib. | Piekniekbos | -33,9651 | 20,7046 | OK413017 | OK412780 | OK412870 | AMGS |
| ***Aprionyx pellucidulus*** | LEP070 | ISF46J | 25.III.2018 | South Africa | Breede | Tradouw trib. | Piekniekbos | -33,9651 | 20,7046 | | OK412781 | OK412871 | AMGS |
| ***Aprionyx peterseni*** | LEP014 | ISF102B | 15.XI.2018 | South Africa | Berg | Hugos | Element Adventures | -33,7407 | 19,0561 | OK413061 | OK412734 | OK412824 | AMGS |
| ***Aprionyx peterseni*** | LEP015 | ISF44A | 24.III.2018 | South Africa | Berg | Klein Berg trib. | Secret Falls - Site 1 | -33,1846 | 19,1361 | OK413060 | OK412735 | OK412825 | AMGS |
| ***Aprionyx peterseni*** | LEP026 | ISF93D | 11.XI.2018 | South Africa | Eerste | Jonkershoek | Jonkershoek 2 | -33,9958 | 18,9815 | OK413053 | OK412742 | OK412832 | AMGS |
| ***Aprionyx peterseni*** | LEP027 | ISF93D | 11.XI.2018 | South Africa | Eerste | Jonkershoek | Jonkershoek 2 | -33,9958 | 18,9815 | OK413052 | OK412743 | OK412833 | AMGS |
| ***Aprionyx peterseni*** | LEP028 | ISF32A | 17.III.2018 | South Africa | Eerste | Jonkershoek trib. | Tweedewaterval | -34,0042 | 18,9938 | OK413051 | OK412744 | OK412834 | AMGS |
| ***Aprionyx peterseni*** | LEP029 | ISF59C | 08.XI.2018 | South Africa | Palmiet | Palmiet | Palmiet | -34,0548 | 19,0363 | OK413050 | OK412745 | OK412835 | AMGS |
| ***Aprionyx peterseni*** | LEP030 | ISF59C | 08.XI.2018 | South Africa | Palmiet | Palmiet | Palmiet | -34,0548 | 19,0363 | OK413049 | OK412746 | OK412836 | AMGS |
| ***Aprionyx peterseni*** | LEP031 | ISF66B | 09.XI.2018 | South Africa | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 | OK413048 | OK412747 | OK412837 | AMGS |
| ***Aprionyx peterseni*** | LEP032 | ISF66B | 09.XI.2018 | South Africa | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 | OK413047 | OK412748 | OK412838 | AMGS |
| ***Aprionyx peterseni*** | LEP033 | ISF72B | 09.XI.2018 | South Africa | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 | OK413046 | OK412749 | OK412839 | AMGS |
| ***Aprionyx peterseni*** | LEP034 | ISF72C | 09.XI.2018 | South Africa | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 | OK413045 | OK412750 | OK412840 | AMGS |
| ***Aprionyx peterseni*** | LEP035 | ISF77B | 09.XI.2018 | South Africa | Berg | Berg trib. | Wolwekloof (upstream of weir) | -33,9441 | 19,0244 | OK413044 | OK412751 | OK412841 | AMGS |
| ***Aprionyx peterseni*** | LEP036 | ISF82C | 09.XI.2018 | South Africa | Berg | Berg trib. | Wolwekloof (upstream of weir) | -33,9441 | 19,0244 | OK413043 | OK412752 | OK412842 | AMGS |
| ***Aprionyx peterseni*** | LEP037 | ISF34F | 18.III.2018 | South Africa | Berg | Bakkerskloof | Bakkerskloof weir | -33,8222 | 19,0462 | OK413042 | OK412753 | OK412843 | AMGS |

| Taxa | DNA nr. | Catalogue nr. | Collection date | Country | River system | River name | Site name | Latitude | Longitude | COI | 16S rRNA | 28S rRNA | Institute/Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Aprionyx peterseni*** | LEP038 | ISF105C | 17.XI.2018 | South Africa | Berg | Klein Kliphuis | Groot Winterhoek | -33,0468 | 19,1006 | OK413041 | OK412754 | OK412844 | AMGS |
| ***Aprionyx peterseni*** | LEP039 | ISF109C | 20.XI.2018 | South Africa | Breede | Breede trib. | Tolhuis (Ceres - Michell's Pass) | -33,3911 | 19,2852 | OK413040 | OK412755 | OK412845 | AMGS |
| ***Aprionyx peterseni*** | LEP040 | ISF109C | 20.XI.2018 | South Africa | Breede | Breede trib. | Tolhuis (Ceres - Michell's Pass) | -33,3911 | 19,2852 | OK413039 | OK412756 | OK412846 | AMGS |
| ***Aprionyx peterseni*** | LEP041 | ISF117B | 20.XI.2018 | South Africa | Breede | Breede trib. | Tolhuis (Ceres - Michell's Pass) | -33,5680 | 19,1366 | OK413038 | OK412757 | OK412847 | AMGS |
| ***Aprionyx peterseni*** | LEP042 | ISF132C | 22.XI.2018 | South Africa | Breede | Klip | Klip River (Du Toit's Kloof) | -33,7241 | 19,1830 | OK413037 | OK412758 | OK412848 | AMGS |
| ***Aprionyx peterseni*** | LEP043 | ISF126E | 22.XI.2018 | South Africa | Breede | Krom | Krom River (Du Toit's Kloof) | -33,7262 | 19,1109 | OK413036 | OK412759 | OK412849 | AMGS |
| ***Aprionyx peterseni*** | LEP044 | ISF126E | 22.XI.2018 | South Africa | Breede | Krom | Krom River (Du Toit's Kloof) | -33,7262 | 19,1109 | OK413035 | OK412760 | OK412850 | AMGS |
| ***Aprionyx peterseni*** | LEP045 | ISF134C | 24.XI.2018 | South Africa | Breede | Keisie trib. | Silver Stream Waterfall | -33,7712 | 20,0907 | OK413034 | OK412761 | OK412851 | AMGS |
| ***Aprionyx peterseni*** | LEP073 | ISF6A | 22.XI.2017 | South Africa | Breede | Huis | Hiking trail waterfall | -33,9310 | 20,7769 | | OK412783 | | AMGS |
| ***Aprionyx peterseni*** | LEP074 | ISF6A | 22.XI.2017 | South Africa | Breede | Huis | Hiking trail waterfall | -33,9310 | 20,7769 | | OK412784 | OK412872 | AMGS |
| ***Aprionyx peterseni*** | LEP075 | ISF10C | 23.XI.2017 | South Africa | Breede | Huis | Huis River at canal | -33,9199 | 20,7527 | | OK412785 | OK412873 | AMGS |
| ***Aprionyx phoeocera*** | LEP006 | ISF33A | 18.III.2018 | South Africa | Berg | Wemmershoek | La Ferme | -33,8490 | 19,0498 | OK413069 | OK412726 | OK412816 | AMGS |
| ***Aprionyx phoeocera*** | LEP019 | ISF33A | 18.III.2018 | South Africa | Berg | Wemmershoek | La Ferme | -33,8490 | 19,0498 | OK413058 | OK412737 | OK412827 | AMGS |
| ***Aprionyx phoeocera*** | LEP020 | ISF43A | 23.III.2018 | South Africa | Breede | Holsloot | Dwarsberg | -33,7994 | 19,3139 | OK413057 | OK412738 | OK412828 | AMGS |
| ***Aprionyx phoeocera*** | LEP072 | CED400B | 23.I.2017 | South Africa | Olifants | Krom | R-R 3 | -32,5331 | 19,3167 | | OK412782 | | AMGS |
| ***Aprionyx rubicundus*** | LEP002 | ISF87A | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413071 | OK412724 | OK412814 | AMGS |
| ***Aprionyx rubicundus*** | LEP004 | ISF88C | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413070 | OK412725 | OK412815 | AMGS |
| ***Aprionyx rubicundus*** | LEP007 | ISF87A | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413068 | OK412727 | OK412817 | AMGS |
| ***Aprionyx rubicundus*** | LEP008 | ISF88C | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413067 | OK412728 | OK412818 | AMGS |
| ***Aprionyx rubicundus*** | LEP009 | ISF89B | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413066 | OK412729 | OK412819 | AMGS |
| ***Aprionyx rubicundus*** | LEP010 | ISF88B | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413065 | OK412730 | OK412820 | AMGS |
| ***Aprionyx rubicundus*** | LEP011 | ISF98B | 12.XI.2018 | South Africa | Eerste | Jonkershoek trib. | Eerste waterval | -33,9987 | 18,9832 | OK413064 | OK412731 | OK412821 | AMGS |
| ***Aprionyx rubicundus*** | LEP012 | ISF56A | 07.XI.2018 | South Africa | Breede | Riviersonderend | Riviersonderendkloof | -34,0419 | 19,0363 | OK413063 | OK412732 | OK412822 | AMGS |
| ***Aprionyx sp. 1*** | LEP013 | ISF86B | 11.XI.2018 | South Africa | Eerste | Swartboskloof | Swartboskloof | -33,9968 | 18,9580 | OK413062 | OK412733 | OK412823 | AMGS |
| ***Aprionyx sp. 1*** | LEP046 | ISF84A | 10.XI.2018 | South Africa | Breede | Du Toits trib. | Jan Joubertsgat bridge | -33,9390 | 19,1604 | OK413033 | OK412762 | OK412852 | AMGS |
| ***Aprionyx sp. 1*** | LEP076 | ISF97C | 12.XI.2018 | South Africa | Eerste | Jonkershoek trib. | Eerste waterval | -33,9987 | 18,9832 | OK413016 | OK412786 | OK412874 | AMGS |
| ***Aprionyx sp. 2*** | LEP078 | ISF45A | 24.III.2018 | South Africa | Berg | Klein Berg trib. | Secret Falls - Site 2 | -33,1819 | 19,1271 | | OK412788 | OK412876 | AMGS |
| ***Aprionyx sp. 3*** | LEP077 | ISF41A | 23.III.2018 | South Africa | Breede | Watervalskloof | Eensgevonden farm | -33,7151 | 19,3712 | | OK412787 | OK412875 | AMGS |
| ***Aprionyx sp. 4*** | LEP017 | ISF151A | 29.V.2019 | South Africa | Breede | Molenaars | Molenaars old bridge | -33,7308 | 19,1181 | OK413059 | OK412736 | OK412826 | AMGS |
| ***Aprionyx sp. 5*** | LEP079 | ISF49C | 27.III.2018 | South Africa | Homtini | Millwood Creek | Jubilee Creek picnic spot | -33,8884 | 22,9662 | | OK412789 | OK412877 | AMGS |
| ***Aprionyx sp. 6*** | SMN778 | SMNS_EPH_005778_E-4 | 17.IX.2011 | South Africa | Breede | Steenboks | Bains Kloof Steenbok Park | -33,5563 | 19,1496 | - | | | SMNS |

| Taxa | DNA nr. | Catalogue nr. | Collection date | Country | River system | River name | Site name | Latitude | Longitude | COI | 16S rRNA | 28S rRNA | Institute/Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Aprionyx sp. 7*** | SMN230 | SMNS_EPH_007230_E | 23.XI.2011 | South Africa | Storms | Storms | Storms River Pass | -33,9886 | 23,9192 | - | | | SMNS |
| ***Aprionyx sp. 7*** | SMN231 | SMNS_EPH_007231_E-1 | 29.XI.2011 | South Africa | Sout | Wit | Kurland | -33,9267 | 23,4894 | - | | | SMNS |
| ***Aprionyx sp. 7*** | SMN235 | SMNS_EPH_007235_E-5 | 25.XI.2011 | South Africa | Lottering | Lottering | Keurbos hut | -33,9109 | 23,7421 | - | | | SMNS |
| ***Aprionyx sp. 7*** | SMN249 | SMNS_EPH_004249_E | 27.III.2009 | South Africa | Bloukrans | Bloukrans trib. | Bloukrans hut | -33,9174 | 23,6373 | - | | | SMNS |
| ***Aprionyx sp. 7*** | SMN567 | SMNS_EPH_004567_E | 01.IV.2009 | South Africa | Storms | Kortklofie | Sleepkloof hut | -33,9487 | 23,9155 | - | | | SMNS |
| ***Aprionyx sp. 8*** | SMN227 | SMNS_EPH_007227_E-3 | 17.IX.2011 | South Africa | Breede | Wit trib. | Bains Kloof | -33,6019 | 19,1105 | - | | | SMNS |
| ***Aprionyx sp. 9*** | SMN254 | SMNS_EPH_007254_E | 18.XI.2014 | South Africa | Eerste | Jonkershoek | Jonkershoek bridge | -33.9939 | 18.9750 | - | | | SMNS |
| ***Aprionyx sp. 10*** | SMN238 | SMNS_EPH_007238_E-1 | 04.XII.2011 | South Africa | Keiskamma | Tyume | Upper falls | -32,6054 | 26,9656 | - | | | SMNS |
| ***Aprionyx sp. 10*** | SMN242 | SMNS_EPH_007242_E-2 | 05.XII.2011 | South Africa | Keiskamma | Tyume | 3rd stream | -32,5877 | 26,9486 | - | | | SMNS |
| ***Aprionyx sp. 10*** | SMN243 | SMNS_EPH_007243_E-1 | 05.XII.2011 | South Africa | Keiskamma | Tyume | 4th stream | -32,5873 | 26,9455 | - | | | SMNS |
| ***Aprionyx sp. 10*** | SMN245 | SMNS_EPH_007245_E-2 | 05.XII.2011 | South Africa | Keiskamma | Tyume | 5th stream | -32,5866 | 26,9442 | - | | | SMNS |
| ***Aprionyx sp. 10*** | SMN246 | SMNS_EPH_007246_E-1 | 05.XII.2011 | South Africa | Keiskamma | Tyume | 1st stream | -32,5882 | 26,9550 | - | | | SMNS |
| ***Aprionyx sp. 10*** | SMN248 | SMNS_EPH_007248_E-2 | 06.XII.2011 | South Africa | Keiskamma | Tyume | Tor Doone | -32,5793 | 26,9321 | - | | | SMNS |
| ***Aprionyx sp. 11*** | SMN240 | SMNS_EPH_007240_E | 04.XII.2011 | South Africa | Keiskamma | Tyume | Madonna and child falls | -32,6071 | 26,9629 | - | | | SMNS |
| ***Aprionyx sp. 11*** | SMN247 | SMNS_EPH_007247_E-1 | 05.XII.2011 | South Africa | Keiskamma | Tyume | Arboretum | -32,5900 | 26,9355 | - | | | SMNS |
| **Outgroups** | | | | | | | | | | | | | |
| ***Choroterpes ndebele*** | LEP052 | GEN2589A | 19.IX.2019 | South Africa | Gourits | Buffelsklip trib. | Buffelsklip trib. at damaged bridge | -33,5426 | 22,9283 | OK413027 | OK412768 | OK412858 | AMGS |
| ***Choroterpes ndebele*** | LEP053 | GEN2589A | 19.IX.2019 | South Africa | Gourits | Buffelsklip trib. | Buffelsklip trib. at damaged bridge | -33,5426 | 22,9283 | OK413026 | OK412769 | OK412859 | AMGS |
| ***Choroterpes nigrescens*** | LEP056 | ISF28E | 16.III.2018 | South Africa | Breede | Baviaans | Genadendal school | -34,0311 | 19,5578 | OK413024 | OK412770 | OK412860 | AMGS |
| ***Choroterpes nigrescens*** | LEP057 | ISF40B | 22.III.2018 | South Africa | Breede | Molenaars | Molenaars River | -33,7036 | 19,2320 | OK413025 | OK412771 | OK412861 | AMGS |
| ***Choroterpes nigrescens*** | LEP059 | ISF43C | 23.III.2018 | South Africa | Breede | Holsloot | Dwarsberg | -33,7994 | 19,3139 | OK413023 | OK412772 | OK412862 | AMGS |
| ***Choroterpes nigrescens*** | LEP060 | ISF47B | 26.III.2018 | South Africa | Kaaimans | Kaaimans | Kaaimans (Seven Passes) | -33,9714 | 22,5473 | OK413022 | OK412773 | OK412863 | AMGS |
| ***Choroterpes nigrescens*** | LEP065 | ISF66C | 9.XI.2018 | South Africa | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 | OK413019 | OK412776 | OK412866 | AMGS |
| ***Choroterpes nigrescens*** | LEP066 | ISF105D | 17.XI.2018 | South Africa | Berg | Klein Kliphuis | Groot Winterhoek | -33,0468 | 19,1006 | OK413018 | OK412777 | OK412867 | AMGS |
| ***Choroterpes nigrescens*** | LEP092 | ISF12B | 23.XI.2017 | South Africa | Breede | Huis | Huis River at canal | -33,9199 | 20,7527 | | OK412794 | OK412880 | AMGS |
| ***Choroterpes nigrescens*** | LEP093 | ISF19B | 6.XII.2017 | South Africa | Elandsbos | Elandsbos | Lower site (T1) | -33,9642 | 23,7745 | | OK412795 | OK412881 | AMGS |
| ***Choroterpes nigrescens*** | LEP094 | ISF19B | 6.XII.2017 | South Africa | Elandsbos | Elandsbos | Lower site (T1) | -33,9642 | 23,7745 | | OK412796 | OK412882 | AMGS |
| ***Choroterpes nigrescens*** | LEP095 | ISF40H | 22.III.2018 | South Africa | Breede | Molenaars | Molenaars River | -33,7036 | 19,2320 | OK413014 | OK412797 | OK412883 | AMGS |
| ***Choroterpes nigrescens*** | LEP096 | ISF47B | 26.III.2018 | South Africa | Kaaimans | Kaaimans | Kaaimans (Seven Passes) | -33,9618 | 19,0690 | | OK412798 | OK412884 | AMGS |
| ***Choroterpes nigrescens*** | LEP097 | ISF49A | 27.III.2018 | South Africa | Homtini | Millwood Creek | Jubilee Creek picnic spot | -33,8884 | 22,9662 | | OK412799 | OK412885 | AMGS |
| ***Choroterpes nigrescens*** | LEP098 | ISF50B | 28.III.2018 | South Africa | Groot | Groot | Nature's Valley (Long bridge) | -33,9684 | 23,5595 | | OK412800 | OK412886 | AMGS |
| ***Choroterpes nigrescens*** | LEP099 | ISF117A | 20.XI.2018 | South Africa | Breede | Wit trib. | Tweede Tol (Wolwekloof) | -33,5680 | 19,1366 | | OK412801 | OK412887 | AMGS |

| Taxa | DNA nr. | Catalogue nr. | Collection date | Country | River system | River name | Site name | Latitude | Longitude | COI | 16S rRNA | 28S rRNA | Institute/Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Choroterpes nigrescens*** | LEP100 | ISF117A | 20.XI.2018 | South Africa | Breede | Wit trib. | Tweede Tol (Wolwekloof) | -33,5680 | 19,1366 | | OK412802 | OK412888 | AMGS |
| ***Choroterpes nigrescens*** | LEP101 | ISF122C | 20.XI.2018 | South Africa | Breede | Wit | Wit River (Tweede Tol) | -33,5702 | 19,1394 | | OK412803 | OK412889 | AMGS |
| ***Choroterpes nigrescens*** | LEP102 | ISF126C | 22.XI.2018 | South Africa | Breede | Krom | Krom River (Du Toit's Kloof) | -33,7262 | 19,1109 | OK413012 | OK412804 | OK412890 | AMGS |
| ***Choroterpes sp.*** | LEP063 | ISF51B | 7.XI.2018 | South Africa | Breede | Riviersonderend | Riviersonderendkloof | -34,0419 | 19,0363 | OK413021 | OK412774 | OK412864 | AMGS |
| ***Choroterpes sp.*** | LEP064 | ISF59B | 8.XI.2018 | South Africa | Palmiet | Palmiet | Palmiet | -34,0548 | 19,0363 | OK413020 | OK412775 | OK412865 | AMGS |
| ***Euthraulus elegans*** | LEP047 | ISF67B | 9.XI.2018 | South Africa | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 | OK413032 | OK412763 | OK412853 | AMGS |
| ***Euthraulus elegans*** | LEP048 | ISF93B | 11.XI.2018 | South Africa | Eerste | Jonkershoek | Jonkershoek 2 | -33,9958 | 18,9815 | OK413031 | OK412764 | OK412854 | AMGS |
| ***Euthraulus elegans*** | LEP049 | ISF105A | 17.XI.2018 | South Africa | Berg | Klein Kliphuis | Groot Winterhoek | -33,0468 | 19,1006 | OK413030 | OK412765 | OK412855 | AMGS |
| ***Euthraulus elegans*** | LEP050 | ISF126B | 22.XI.2018 | South Africa | Breede | Krom | Krom River (Du Toit's Kloof) | -33,7262 | 19,1109 | OK413029 | OK412766 | OK412856 | AMGS |
| ***Euthraulus sp.*** | LEP051 | ISF143B | 26.III.2019 | South Africa | Tugela | Twee Dassies | Twee Dassies at 2nd bridge | -29,2828 | 29,5158 | OK413028 | OK412767 | OK412857 | AMGS |
| ***Euthraulus sp.*** | LEP088 | CAW965W | 30.XI.2019 | Angola | Zambezi | Luio | Luio River at bridge | -13,1939 | 20,2235 | OK413015 | OK412791 | | AMGS |
| ***Euthraulus sp.*** | LEP089 | CAW968U | 2.XII.2019 | Angola | Zambezi | Luanginga | Luanginga below 2nd waterfall | -13,7118 | 21,2502 | OK413013 | OK412792 | | AMGS |
| ***Euthraulus sp.*** | LEP090 | CAW969V | 2.XII.2019 | Angola | Zambezi | Luanginga | Luanginga upstream of main waterfall | -13,7092 | 21,2482 | | OK412793 | | AMGS |
| ***Euthraulus sp.*** | LEP104 | ISF143B | 26.III.2019 | South Africa | Tugela | Twee Dassies | Twee Dassies at 2nd bridge | -29,2828 | 29,5158 | | OK412805 | OK412891 | AMGS |
| ***Euthraulus sp.*** | LEP108 | LIM968F | 12.IX.2017 | South Africa | Limpopo | Letaba | Klipkoppies | -23,9434 | 31,7314 | | OK412806 | OK412892 | AMGS |
| ***Euthraulus sp.*** | LEP110 | LIM974K | 13.IX.2017 | South Africa | Limpopo | Letaba | Mahlangeni, | -23,6505 | 31,1483 | | OK412807 | OK412893 | AMGS |
| ***Euthraulus sp.*** | LEP111 | LIM982E | 14.IX.2017 | South Africa | Limpopo | Luvuvhu | Dongadziva | -22,7098 | 30,8843 | | OK412808 | OK412894 | AMGS |
| ***Euthraulus sp.*** | LEP112 | LIM690T | 08.IX.2016 | South Africa | Komati | Sabie | Lisbon | -24,9700 | 31,4039 | | OK412809 | OK412895 | AMGS |
| ***Euthraulus sp.*** | LEP113 | LIM690T | 08.IX.2016 | South Africa | Komati | Sabie | Lisbon | -24,9700 | 31,4039 | | OK412810 | OK412896 | AMGS |
| ***Euthraulus sp.*** | LEP114 | LIM667V | 07.IX.2016 | South Africa | Komati | Sabie | Lower Sabie Bridge | -25,1214 | 31,9252 | | OK412811 | OK412897 | AMGS |
| ***Atalophlebia sp.*** | | Outgroup | | Australia | | | | | | KP697596.1 | | | Shackleton and Rees 2016 |
| ***Austrophlebioides sp.*** | | Outgroup | | Australia | | | | | | KP697456.1 | MG764392.1 | AY749929.1 | Shackleton and Rees 2016, Miller et al. 2018, Ogden and Whiting 2005 |
| ***Penaphlebia sp.*** | | Outgroup | | Chile/Argentina | | | | | | | AY749782.1 | | Ogden and Whiting 2005 |
| ***Thraulodes sp.*** | | Outgroup | | USA | | | | | | HM900404.1 | | AY749924.1 | Webb et al. 2012, Ogden and Whiting 2005 |

**Appendix B. Additional records: published records that were not located in the three museum collections, and also dubious samples found during the cataloguing process, considered only for distribution records.**

***Aprionyx tabularis* (Eaton, 1884) n. syn.**

SOUTH AFRICA: Western Cape. Material unknown, Great Winterhoek Mountains, Tulbagh, ix.1932, KH Barnard [Barnard 1940]; 1 ♀ imago, Franschhoek, Bosreserve, Upper Berg River, station 21, 1.xi.1950, Lund Univ. Exp. [Demoulin 1970].

***Aprionyx peterseni* (Lestage, 1924)**

SOUTH AFRICA: Western Cape. Material unknown, Rawsonville, Du Toits Kloof, 25.iii.1932, KH Barnard [Barnard 1940]; Material unknown, Kleinmond, Palmiet River, iii.1932, HG Wood [Barnard 1940]; Material unknown, Citrusdal, Elands Kloof, 5.iii.1933, HG Wood [Barnard 1940]; Material unknown, Great Winterhoek Mountains, Tulbagh valley, ii.1934, KH Barnard [Barnard 1940]; Material unknown, Swartberg Mountains, Meirings Poort, i.1935, KH Barnard and HG Wood [Barnard 1940]; 32 nymphs, Franschhoek Bosreserve, Upper Berg River, station 21, 1.xi.1950, Lund Univ. Exp. [Demoulin 1970]; 1 ♂ imago, 4 ♀ imagos, 1 ♂ subimago, 2 ♀ subimagos, 18 nymphs, Hermanus, Maanskynkop, station 93, 1.xii.1950, Lund Univ. Exp. [Demoulin 1970]; 1 nymph, Hermanus, station 94, 22.xii.1950, Lund Univ. Exp. [Demoulin 1970]; 1 ♀ subimago, 1 nymph, Langeberg, Tradouws Pass, station 111, 4.i.1951, Lund Univ. Exp. [Demoulin 1970]; 1 ♀ subimago, Natures Valley, Groot River, station 132, 11.i.1951, Lund Univ. Exp. [Demoulin 1970]; 4 nymphs, Stellenbosch, Jonkershoek, station 170, 4.ii.1951, Lund Univ. Exp. [Demoulin 1970]; 11 nymphs, Grabouw, Viljoens Pass, 11.ii.1951, Lund Univ. Exp. [Demoulin 1970].

***Aprionyx phoeocera* (Lestage, 1924) stat. rev.**

SOUTH AFRICA: Western Cape. Material unknown, Franschhoek, no date, KH Barnard [Barnard 1932]; material unknown, Stellenbosch, Jonkershoek, no date, AC Harrison & KH Barnard [Barnard 1932]; material unknown, Rawsonville, Du Toits Kloof, no date, AC Harrison [Barnard 1932]; material unknown, Rawsonville, Louws Hoek, no date, AC Harrison [Barnard 1932]; 1 ♀ subimago, Cape Peninsula, Hout Bay, Orange Kloof, station 26, 5.xi.1951, Lund Univ. Exp. [Demoulin 1970].

***Aprionyx rubicundus* Barnard, 1932**

SOUTH AFRICA: Western Cape. 1 ♂? imago (with the following two localities labelled for the same material), Oudebosch, Riviersonderend Mountains, xii.1920, and, Banhoek, Stellenbosch, 7.x.1929, KH Barnard [ethanol, AMGS; dubious]; material unknown, Worcester, Keeromberg, Nonna Kloof, ix.1930, KH Barnard [Barnard 1932]; material unknown, Wellington Mountains, Witte River, 1.x.1933, HG Wood [Barnard 1940]; material unknown, Franschhoek Pass, 1.x.1932, KH Barnard and HG Wood [Barnard 1940]; material unknown, Oudebosch, Riviersonderend Mountains, 1500 ft (ca. 457 m), i.1934, KH Barnard

[Barnard 1940]; material unknown, upper Olifants River, north of Ceres, x.1937, KH Barnard and CW Thorne [Barnard 1940].

***Aprionyx argus* Barnard, 1940**

SOUTH AFRICA: KwaZulu-Natal. 2 nymphs, Drakensberg, Cathkin Peak, 7500 ft (ca. 2286 m), 18.ii.1945, RS Crass [Crass 1947].

***Aprionyx natalicus* (Lestage, 1924)**

SOUTH AFRICA: Eastern Cape. Material unknown, Katberg [AMGS; Barnard 1932]; material unknown, Hogsback [AMGS; Barnard 1932]; material unknown, Amatolas, Fenfield, 13.ii.1942, RS Crass [Crass 1947]; material unknown, Hogsback, near Alice, ii.1942, J Omer-Cooper [Crass 1947b]; material unknown, Pirie Forest, ii.1944, J Omer-Cooper [Crass 1947b]. KwaZulu-Natal. 2 ♂ imagos, 1 ♂ subimago, 1 ♀ imago, Kranskop, xi.1917, KH Barnard (missing syntypes) [Lestage 1924]; 2 ♂ imagos, 2 nymphs, Royal Natal National Park, Dooley Mountains, the Cascades, station 257, 5500 ft (ca. 1677 m), 1.iv.1951, Lund Univ. Exp. [Demoulin 1970]; 1 ♀ imago, Royal Natal National Park, Tugela Valley, station 258, 3.iv.1951, Lund Univ. Exp. [Demoulin 1970]; material unknown, Curry's Post, xi-xii.1944, RS Crass [Crass 1947]; material unknown, Dargle, Furth stream, 5.xii.1944, RS Crass [Crass 1947]; material unknown, Maylands, Inzinga, 25.i.1945, RS Crass [Crass 1947].

***Aprionyx tricuspidatus* Crass, 1947**

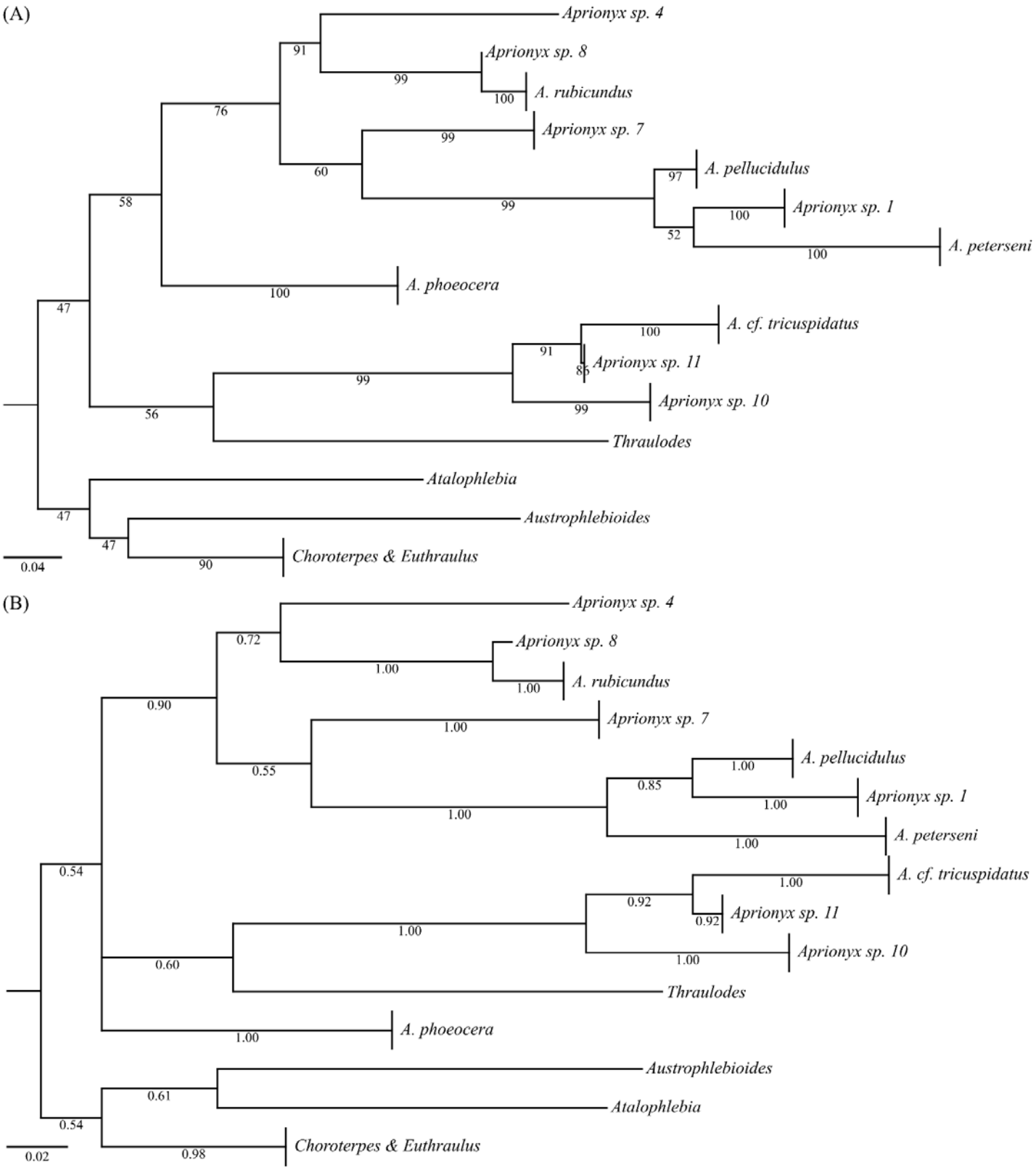
SOUTH AFRICA: Eastern Cape. 1 subimago (gender unknown), Amatola Mountains, Hogsback, 2.xii.40, RS Crass [Crass 1947]. KwaZulu-Natal. 1 nymph, Kamberg district, Kilmore, 9.iv.43, RS Crass [Crass 1947]; abundant material from various forest streams - data omitted, Curry's Post and Karkloof district, dates omitted, RS Crass [Crass 1947].

## Appendix C. List of localities where *Aprionyx* specimens were collected. Trib. = tributary.

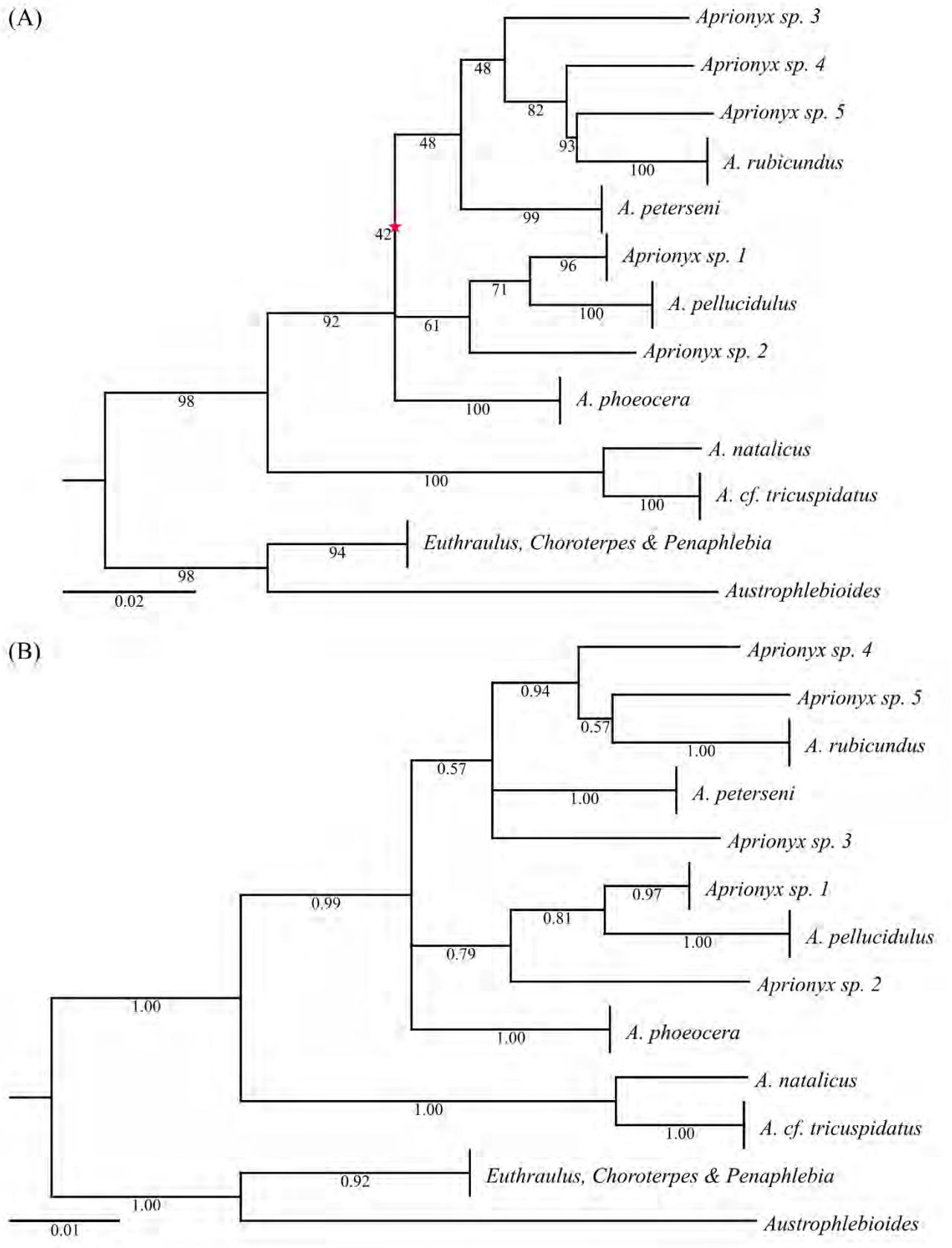
| Province | River System | River name | Site name | Latitude | Longitude |
|---|---|---|---|---|---|
| Western Cape | Berg | Wemmershoek | La Ferme | -33,8490 | 19,0498 |
| Western Cape | Berg | Bakkerskloof | Bakkerskloof weir | -33,8222 | 19,0462 |
| Western Cape | Berg | Kasteelskloof | Kasteelskloof weir | -33,8222 | 19,0584 |
| Western Cape | Berg | Klein Berg trib. | Secret Falls - Site 1 (stream) | -33,1846 | 19,1361 |
| Western Cape | Berg | Klein Berg trib. | Secret Falls - Site 2 (waterfall) | -33,1819 | 19,1271 |
| Western Cape | Berg | Berg | Berg (upstream of dam) | -33,9618 | 19,0690 |
| Western Cape | Berg | Berg trib. | Wolwekloof (upstream of weir) | -33,9441 | 19,0244 |
| Western Cape | Berg | Hugos | Element Adventures | -33,7407 | 19,0561 |
| Western Cape | Berg | Klein Kliphuis | Groot Winterhoek | -33,0468 | 19,1006 |
| Western Cape | Breede | Tradouw trib. | Piekniekbos | -33,9651 | 20,7046 |
| Western Cape | Breede | Huis | Hiking trail waterfall | -33,9310 | 20,7769 |
| Western Cape | Breede | Huis | Huis River at canal | -33,9199 | 20,7527 |
| Western Cape | Breede | Molenaars | Molenaars River | -33,7036 | 19,2320 |
| Western Cape | Breede | Watervalskloof | Eensgevonden - Doug's Farm | -33,7151 | 19,3712 |
| Western Cape | Breede | Holsloot | Dwarsberg (camp 4) | -33,7994 | 19,3139 |
| Western Cape | Breede | Riviersonderend | Riviersonderendkloof | -34,0419 | 19,0363 |
| Western Cape | Breede | Du Toits trib. | Jan Joubertsgat bridge | -33,9390 | 19,1604 |
| Western Cape | Breede | Breede trib. | Tolhuis (Ceres - Michell's Pass) | -33,3911 | 19,2852 |
| Western Cape | Breede | Wit | Wit River (Tweede Tol) | -33,5702 | 19,1394 |
| Western Cape | Breede | Wit trib. | Tweede Tol (Wolwekloof) | -33,5680 | 19,1366 |
| Western Cape | Breede | Krom | Krom River (Du Toit's Kloof) | -33,7262 | 19,1109 |
| Western Cape | Breede | Klip | Klip River (Du Toit's Kloof) | -33,7241 | 19,1830 |
| Western Cape | Breede | Keisie trib. | Silver Sream Waterfall | -33,7712 | 20,0907 |
| Western Cape | Breede | Molenaars | Molenaars at old bridge | -33,7308 | 19,1181 |
| Western Cape | Eerste | Jonkershoek | Jonkershoek 1 | -33,9950 | 18,9793 |
| Western Cape | Eerste | Jonkershoek | Jonkershoek 2 | -33,9958 | 18,9815 |
| Western Cape | Eerste | Jonkershoek trib. (Eerstewaterval) | Jonkershoek trib. (crossing) | -33,9971 | 18,9840 |
| Western Cape | Eerste | Jonkershoek trib. (Eerstewaterval) | Eerste waterval | -33,9987 | 18,9832 |
| Western Cape | Eerste | Jonkershoek trib. (Tweedewaterval) | Tweede waterval | -34,0042 | 18,9938 |
| Western Cape | Eerste | Jonkershoek trib. (Swartboskloof) | Swartboskloof | -33,9968 | 18,9580 |
| Western Cape | Groot | Groot | Nature's Valley (Long bridge) | -33,9684 | 23,5595 |
| Western Cape | Homtini | Millwood Creek | Jubilee Creek picnic spot | -33,8884 | 22,9662 |
| Western Cape | Palmiet | Palmiet | Palmiet (at ‘no swimming’ sign) | -34,0548 | 19,0363 |
| Eastern Cape | Keiskamma | 2nd stream | Lower site (H1) | -32,5944 | 26,9562 |
| Eastern Cape | Keiskamma | 2nd stream | Middle site (H2) | -32,5931 | 26,9581 |
| KwaZulu-Natal | Tugela | Mandleni tributary stream | Kranskop stream | -28,9567 | 30,8774 |

**Appendix D. Data matrix of the characters used in the morphological phylogeny. Missing data = ?; gap = -.**

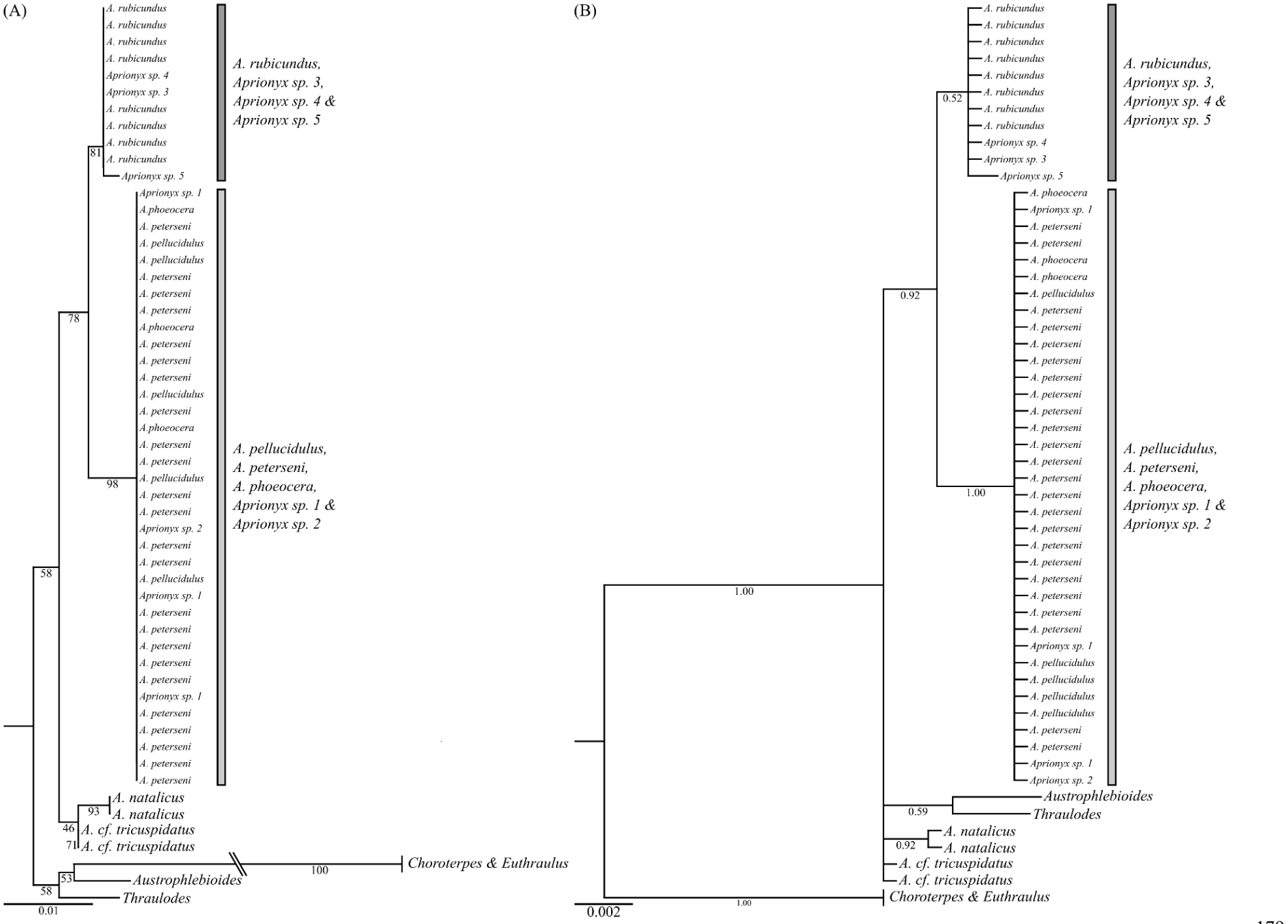
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ingroup** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| ***Aprionyx tabularis*** | 0 | 0 | 0 | 0 | - | 1 | ? | 1 | 1 | 0 | 0 | 2 | 0 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 1 | 1 | 1 | 1 | - | 0 | - |
| ***Aprionyx pellucidulus*** | 0 | 0 | 1 | 0 | - | 1 | 0 | 1 | 1 | 0 | 0 | 2 | 0 | 1 | 1 | 1 | 2 | 0 | 1 | 0 | 2 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | - | 1 | 0 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | - | 1 | 0 |
| ***Aprionyx peterseni*** | 0 | 0 | 1 | 2 | - | 1 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 1 | 2 | 0 | 1 | 0 | 2 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 2 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | - | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - | 1 | 0 |
| ***Aprionyx phoeocera*** | 0 | 1 | 1 | 0 | - | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 0 | 0 | 1 | 0 | 0 | 2 | 2 | 1 | 0 | 0 | 1 | 2 | 1 | 0 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | - | 1 | 1 |
| ***Aprionyx rubicundus*** | 0 | 0 | 1 | 1 | - | 1 | 0 | 1 | 2 | 0 | 1 | 3 | 0 | 1 | 1 | 1 | 2 | 0 | 2 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | - | 0 | - |
| ***Aprionyx argus*** | 0 | 1 | 2 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 3 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | - | 0 | 0 | 0 | ? | 0 | ? | ? | ? | 0 | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | 1 | ? | ? | ? | ? | 1 | 1 | 1 | 0 | 1 | ? | ? |
| ***Aprionyx natalicus*** | 0 | 1 | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 3 | 0 | 1 | 0 | 1 | 2 | 0 | 0 | - | 0 | 0 | 0 | 1 | 0 | ? | ? | ? | 0 | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | 1 | ? | ? | ? | ? | 1 | 1 | 1 | 0 | 0 | ? | ? |
| ***Aprionyx tricuspidatus*** | 0 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | - | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 3 | 0 | 0 | 1 | 2 | 2 | 3 | 2 | ? | ? | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 4 | 0 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | - |
| **Outgroup** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| ***Acanthophlebia*** | 0 | 0 | 1 | 1 | - | 1 | 1 | 1 | 2 | 0 | 0 | 3 | 0 | 1 | ? | 1 | 1 | 0 | 0 | - | 0 | 0 | ? | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 2 | 1 | ? | 1 | ? | ? | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | ? | 1 | 0 | 0 | - | - | 0 | - |
| ***Austrophlebioides*** | 0 | 0 | 1 | 0 | - | 1 | 0 | 0 | 2 | 1 | 0 | 3 | 1 | 0 | ? | 1 | 1 | 0 | 1 | - | 0 | 1 | ? | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | ? | ? | 0 | ? | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ? | 1 | 0 | 0 | - | - | 0 | - |
| ***Choroterpes*** | 1 | 0 | 0 | 0 | - | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - | - | - | - | - | 0 | 1 | 0 | 1 | 0 | 1 | - | - | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | - | - | 0 | - |



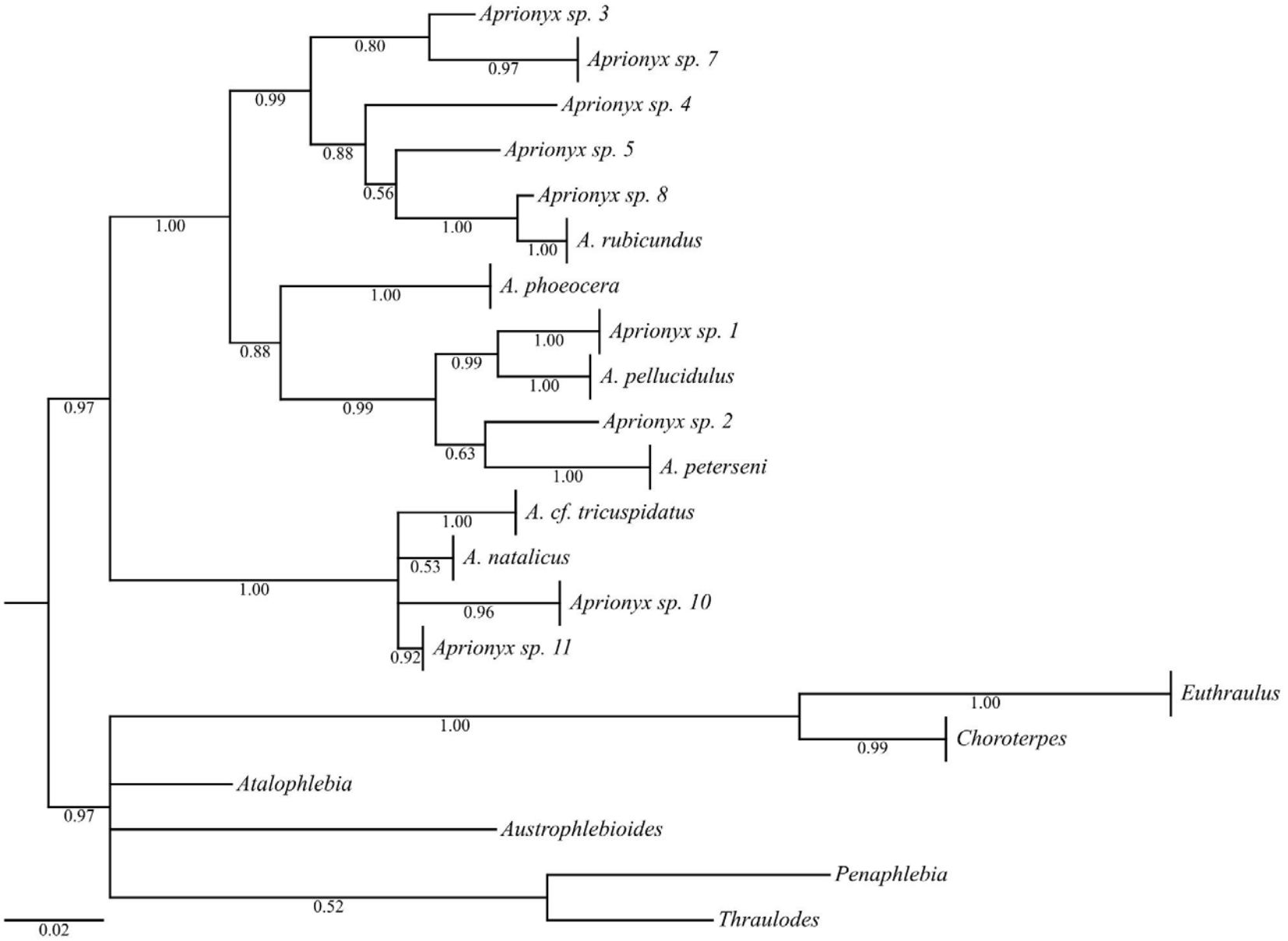

**Appendix E. COI analyses: (A) Maximum likelihood inferred tree with UFBoot (ultrafast bootstrap) values; (B) Bayesian inferred tree with PP (posterior probability) values, rounded to two decimals. Branches that terminate in a vertical bar indicates a collapsed monophyletic species clade.**



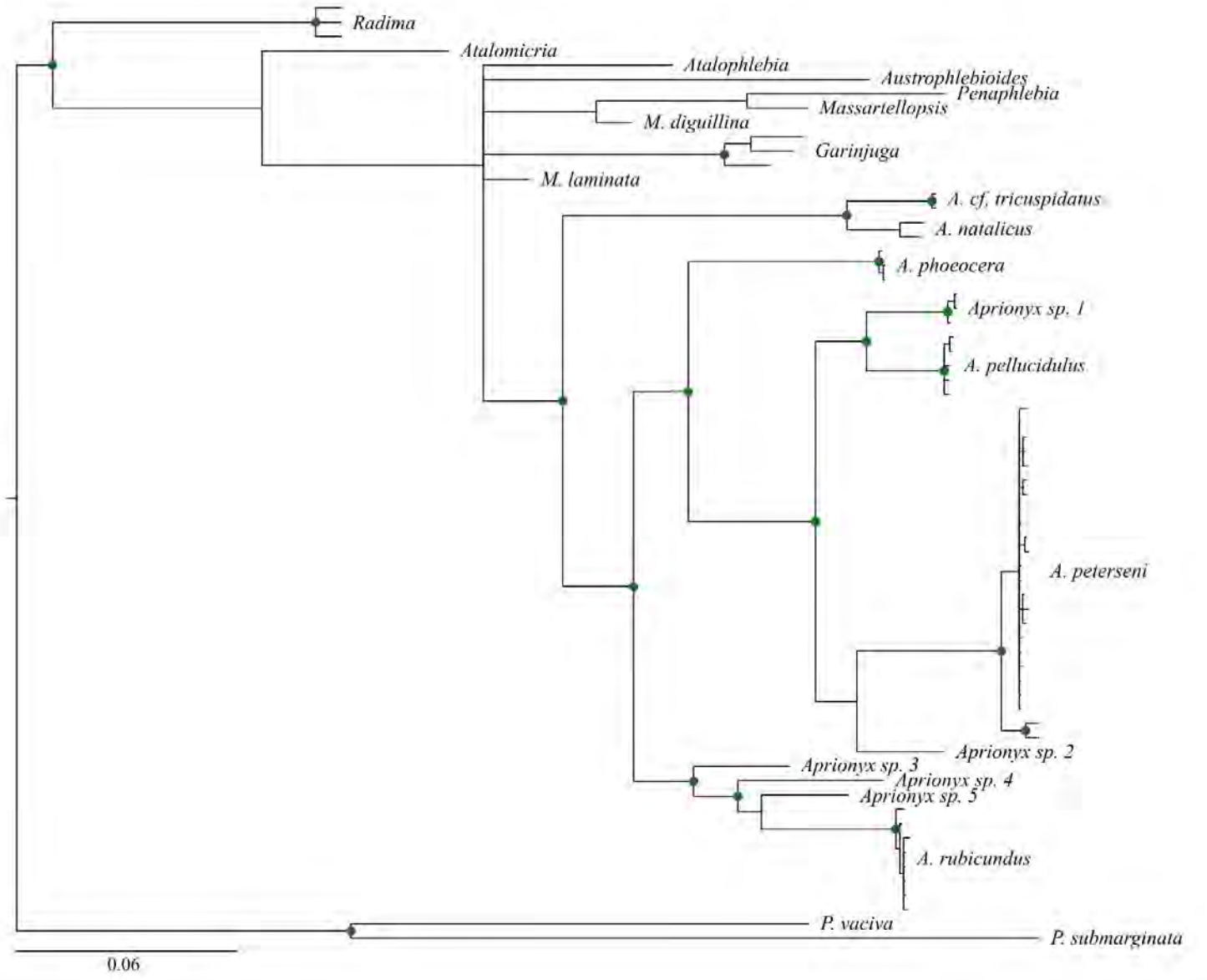

**Appendix F. 16S analyses: (A) Maximum likelihood inferred tree with UFBoot (ultrafast bootstrap) values (red star: a branch not visible because of its shortness); (B) Bayesian inferred tree with PP (posterior probability) values, rounded to two decimals. Branches that terminate in a vertical bar indicates a collapsed monophyletic species clade.**


(A)
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
Aprionyx sp. 4
Aprionyx sp. 3
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
Aprionyx sp. 5
A. rubicundus, Aprionyx sp. 3, Aprionyx sp. 4 & Aprionyx sp. 5
Aprionyx sp. 1
A.phoeocera
A. peterseni
A. pellucidulus
A. pellucidulus
A. peterseni
A. peterseni
A. peterseni
A.phoeocera
A. peterseni
A. peterseni
A. peterseni
A. pellucidulus
A. peterseni
A.phoeocera
A. peterseni
A. peterseni
A. pellucidulus
A. peterseni
A. peterseni
Aprionyx sp. 2
A. peterseni
A. peterseni
A. pellucidulus
Aprionyx sp. 1
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
Aprionyx sp. 1
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. pellucidulus, A. peterseni, A. phoeocera, Aprionyx sp. 1 & Aprionyx sp. 2
A. natalicus
A. natalicus
A. cf. tricuspidatus
A. cf. tricuspidatus
Austrophlebioides
Thraulodes
Choroterpes & Euthraulus
81
78
98
58
93
46
71
53
58
100
0.01
(B)
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
A. rubicundus
Aprionyx sp. 4
Aprionyx sp. 3
Aprionyx sp. 5
A. rubicundus, Aprionyx sp. 3, Aprionyx sp. 4 & Aprionyx sp. 5
A. phoeocera
Aprionyx sp. 1
A. peterseni
A. peterseni
A. phoeocera
A. phoeocera
A. pellucidulus
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
A. peterseni
Aprionyx sp. 1
A. pellucidulus
A. pellucidulus
A. pellucidulus
A. pellucidulus
A. peterseni
A. peterseni
Aprionyx sp. 1
Aprionyx sp. 2
A. pellucidulus, A. peterseni, A. phoeocera, Aprionyx sp. 1 & Aprionyx sp. 2
Austrophlebioides
Thraulodes
A. natalicus
A. natalicus
A. cf. tricuspidatus
A. cf. tricuspidatus
Choroterpes & Euthraulus
0.52
0.92
1.00
1.00
0.59
0.92
1.00
0.002

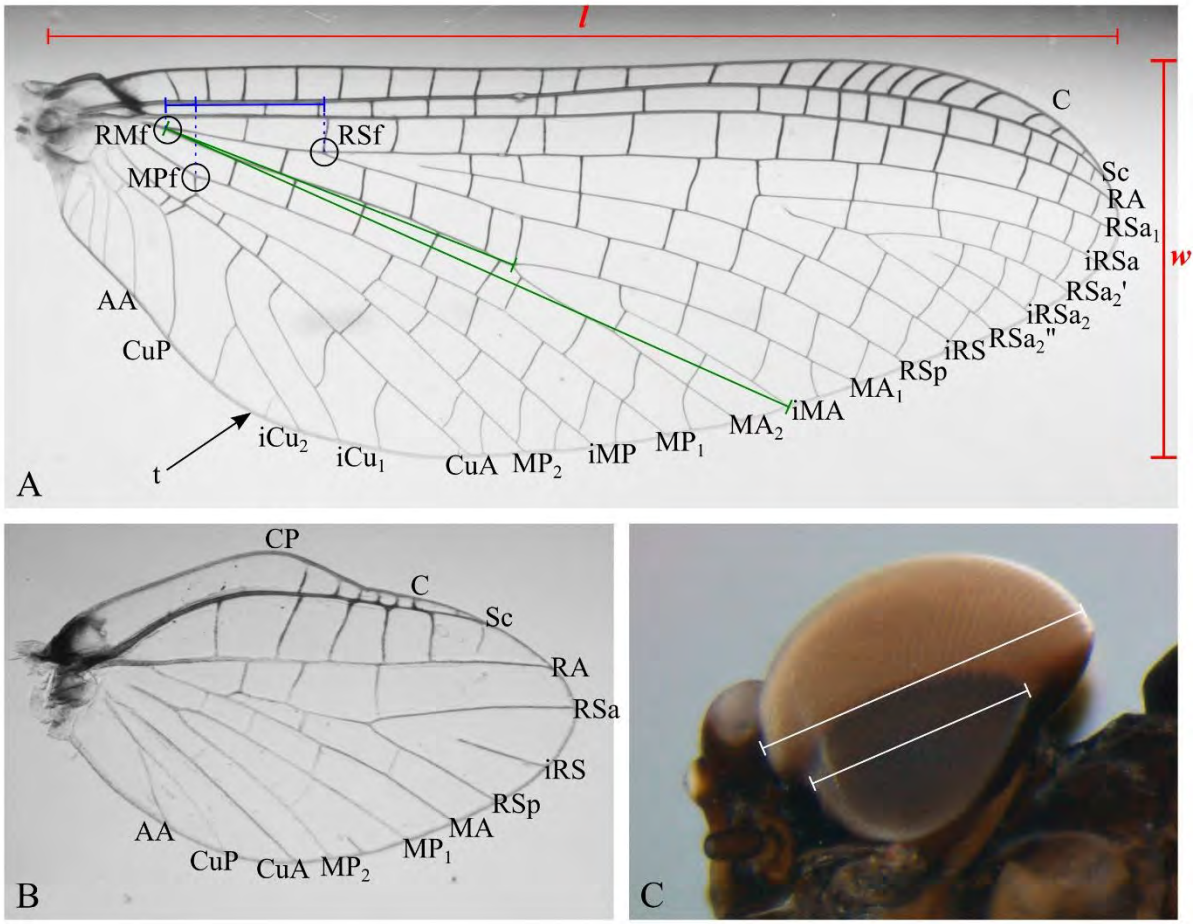
**Appendix G. 28S analyses: (A) Maximum likelihood inferred tree with UFBoot (ultrafast bootstrap) values; (B) Bayesian inferred tree with PP (posterior probability) values, rounded to two decimals. Recovered *Aprionyx* as polyphyletic and with six branches arising from a polytomic node, rooted on the longest of the six branches (i.e., *Choroterpes* and *Euthraulus* group).**




**Appendix H. Bayesian inferred tree of COI/16S/28S-combined, with PP (posterior probability) values, rounded to two decimals. Branches that terminate in a vertical bar indicates a collapsed monophyletic species clade.**




**Appendix I. The Bayesian inference tree of the data used for the molecular divergence time analysis. Green circles specify nodes with a posterior probability above 0.9.**




**Appendix J. Wing venation terminology and measurements. A) Forewing: green lines = measuring the distance of MA fork between RS-MA fork and the wing margin; blue lines = measuring where $MP_2$ is attached to $MP_1$ between the RS-MA fork and RS fork; red lines = the length and width of the wing. B) Hind wing. C) Taking the lengths of the dorsal and ventral portions of the males' eyes. CP = costal process; MPf = $MP_2$ attached to $MP_1$; RMf = RS-MA fork; RSf = RS fork; t = tornus; the rest follow Kluge (2004) with exception explained in section 3.2.3.**