

# Molecular phylogeny and morphological analysis resolve a long-standing controversy over generic concepts in Ecdyonurinae mayflies (Ephemeroptera: Heptageniidae)

ZOHAR YANAI<sup>1</sup>, MICHEL SARTORI<sup>2,3</sup>, ROI DOR<sup>1</sup> and NETTA DORCHIN<sup>1</sup>

<sup>1</sup>Department of Zoology, Tel Aviv University, Tel Aviv, Israel, <sup>2</sup>Musée Cantonal de Zoologie, Palais de Rumine, Lausanne, Switzerland and <sup>3</sup>Département d'Ecologie et Evolution, Université de Lausanne, Switzerland

**Abstract.** Heptageniidae is a species-rich mayfly family (Ephemeroptera), whose taxonomy and phylogeny have been based almost exclusively on traditional morphological studies. Inconsistent use of diagnostic characters and the general lack of molecular studies have led to vague generic concepts, and the phylogenetic relationships among taxa in the family remain unclear. *Afronurus* Lestage is an Old World heptageniid genus of 66 species. The generic assignment of two species within this genus, *A. kugleri* Demoulin and *A. zebratus* (Hagen), has been the subject of much debate, because they share many apomorphic features that distinguish them from other congeners. We combined a thorough morphological study of all life stages of 28 representative heptageniid species with a molecular phylogenetic analysis of four mitochondrial and nuclear markers to resolve the generic position of *A. kugleri* and *A. zebratus* as well as the integrity of *Afronurus* and related genera. Our results confirm the monophyly of *Afronurus* and *Electrogena* and support the assignment of *A. kugleri* and *A. zebratus* to a newly described genus, *Anapos* Yanai & Sartori **gen.n.** We provide clear, diagnostic morphological characters for the genus and discuss the need for a thorough revision of generic concepts in the subfamily Ecdyonurinae.

This published work has been registered in ZooBank, <http://zoobank.org/urn:lsid:zoobank.org:pub:075E40C6-BAFE-4184-A2C2-E3BCFBC0BC15>.

## Introduction

Heptageniidae constitute one of the most species-rich families of mayflies (Ephemeroptera), currently with more than 600 described species in 36 genera (Barber-James *et al.*, 2008; Webb & McCafferty, 2008; Sartori, 2014a, 2014b, 2014c, 2014d). The family has a wide distribution in the Palaearctic Region, where the nymphs usually inhabit lotic habitats, and often comprise an important component of the mayfly community (Wang & McCafferty, 2004; Barber-James *et al.*, 2008). Taxonomic and phylogenetic studies of the family have relied almost exclusively on morphological characters of different life stages, including mouthpart structure, pronotum shape, leg setation, gill shape, tergal ornamentation and caudal-filament structure in nymphs,

and colouration, leg proportions and male genitalia structure in adults (e.g. Tshernova, 1974, 1976; Tomka & Zurwerra, 1985; Kluge, 1989, 1993, 2004; Wang & McCafferty, 2004; Webb & McCafferty, 2008). Egg chorionic structures also have been shown to be useful (e.g. Gaino *et al.*, 1987; Belfiore *et al.*, 2003). While these studies produced ample information on species morphology and distribution ranges, phylogenetic relationships in the family remain controversial. Molecular studies, which could help to resolve these relationships, have hardly been conducted in Heptageniidae, with the exception of Vuataz *et al.* (2011, 2013), who clarified the taxonomic status of Malagasy heptageniids and of European *Rhithrogena* Eaton species. The scarcity of phylogenetic information for Heptageniidae contrasts with the situation in other families, the phylogeny of which has been resolved recently at least to some extent (e.g. Gattolliat *et al.*, 2008; O'Donnell & Jockusch, 2008;

Correspondence: Netta Dorchin, Department of Zoology, Tel Aviv University, Tel Aviv 6997801, Israel. E-mail: ndorchin@tauex.tau.ac.il  
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Ogden *et al.*, 2009b; Rutschmann *et al.*, 2014), as well as the situation in Ephemeroptera as a whole (Ogden & Whiting, 2005; Ogden *et al.*, 2009a).

*Afronurus* Lestage and *Electrogena* Zurwerra & Tomka are two of the largest heptageniid genera, currently with 64 and 45 species, respectively (Barber-James *et al.*, 2013), both in the subfamily Ecdyonurinae as redefined by Webb & McCafferty (2008). *Afronurus* distribution spans the Afrotropical and Oriental Regions, whereas *Electrogena* has a narrower distribution in Europe and Central Asia (Webb & McCafferty, 2008; Fig. 1). Lestage (1924) erected *Afronurus* for the South African *Ecdyonurus peringueyi* Esben-Petersen, accompanied by a superficial description of adult morphological characters, including the shape of the wing and claw, and the tibia-tarsus proportion (reviewed by Schoonbee, 1968). Many species from Afrotropical and Oriental regions were later added to the genus or transferred to it from other genera (e.g. Corbet, 1960; Nguyen & Bae, 2003; Braasch, 2005; Sartori, 2014b). *Electrogena* was erected for species of the 'lateralis group' within *Ecdyonurus* Eaton, which formed a distinct clade among other European heptageniids based on the results of an enzyme-electrophoresis analysis (Zurwerra & Tomka, 1985). Unfortunately, the description of the new genus included very few morphological details of taxonomic use. The type species, *E. lateralis* (Curtis), was originally described under the genus *Baetis* Leach from an unspecified location in the U.K., with limited information about colour of adult body parts and without any illustrations. Subsequent publications included descriptions of additional characters and species (e.g. Bogoescu & Tabacaru, 1962; Braasch, 1980; Sowa, 1981; Belfiore, 1994) but none of them provided a clear and diagnostic description of the genus.

*Afronurus* and *Electrogena* belong to the 'Atopopus group' of Kluge (2004), or Atopopus/fg1, the autapomorphy of which is the division of the maxillary distal dentisetae into several branches, as opposed to entire or only apically divided dentisetae in other Ecdyonurinae (Sartori, 2014b). Kluge also included in this group *Atopopus* Eaton, *Thalerosphyrus* Eaton, *Notacanthurus* Tshernova and *Thamnodontus* Kluge. The Afrotropical genus *Notonurus* Crass recently was reinstated from its synonymy with *Compsoeuria* Eaton (Vuataz *et al.*, 2013) and should also be included in this group because it exhibits the abovementioned apomorphy (Sartori, 2014b, figs 55–56). The validity of the 'Atopopus group' has never been tested and the relationships among its genera are unclear. Similarly, the relationships of this group to other genera that do not possess this supposed apomorphy (e.g. *Compsoeuria*, *Compsoeurilla*, *Rhithrogeniella* and *Asionurus*) are unknown.

Among other unresolved systematic issues in Heptageniidae in general, and in the Ecdyonurinae in particular, are the relationship and generic assignment of two Mediterranean species, *Afronurus kugleri* Demoulin and *A. zebratus* (Hagen), which have been the subject for much debate over the past century. These species have been transferred repeatedly among several genera based on morphological characters and biochemical profiles (e.g. Eaton, 1885; Ulmer, 1920; Gaino & Belfiore, 1987; Hefti & Tomka, 1989; Kluge, 1989), but are currently placed in *Afronurus* based on characters of the male genitalia

and nymphal gills (Bauernfeind & Soldán, 2012). This is despite a suite of common morphological attributes that distinguish them from all other congeners (Hefti & Tomka, 1989; Sartori, 1992; Braasch, 2005).

*Afronurus kugleri* is endemic to the Northwestern Levant, where it is known from several locations in Iraq, Israel, Lebanon, Syria and Turkey (Demoulin, 1973; Al-Zubaidi *et al.*, 1987; Kazanci & Braasch, 1988; Koch, 1988; Fig. 1). Despite consistent doubts about its correct generic placement (Al-Zubaidi *et al.*, 1987; Gaino & Belfiore, 1987; Hefti & Tomka, 1989; Sartori, 1992), the taxonomic status of this species has remained almost unchanged since its description. *Afronurus zebratus* is known exclusively from the Mediterranean islands of Corsica and Sardinia (Fig. 1). Gaino & Belfiore (1987) redescribed the species and attributed it to *Electrogena*, mainly based on nymphal characters, although they recognized that characters of the male genitalia are atypical of that genus. Based on electrophoretic analyses, Hefti & Tomka (1989) showed that this species has low affinity to the other European genera included in the study (*Ecdyonurus*, *Electrogena*, *Epeorus*, *Heptagenia*, *Nixe* and *Rhithrogena*), and reassigned it to *Afronurus* based on morphological characters of the nymph and adult male. This combination was not adopted by subsequent authors (Belfiore, 1996, 1997; Kluge, 2004), and Braasch (2005) suggested that *A. kugleri* and *A. zebratus* actually deserve a separate genus. Lastly, *Electrogena zebrata* was again reassigned to *Afronurus* by Bauernfeind & Soldán (2012) based on several morphological characters.

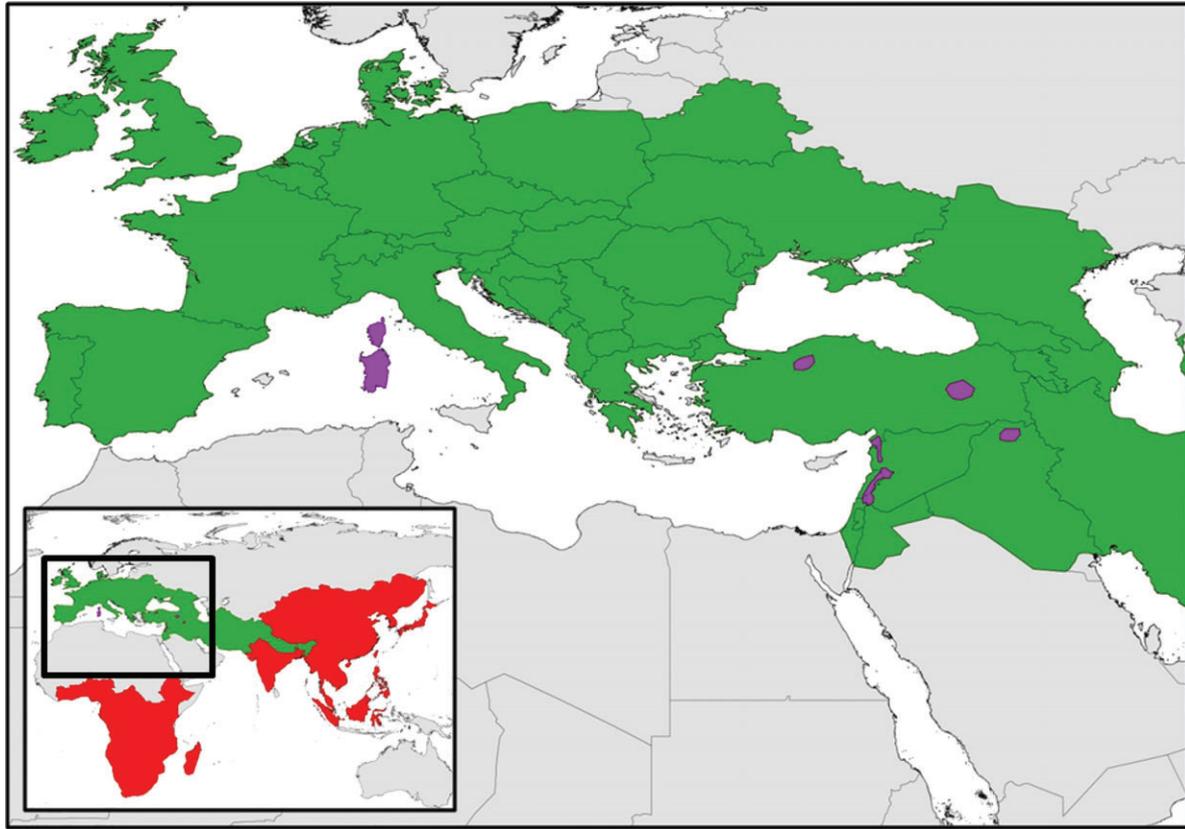
In the present study, we integrate a comprehensive morphological analysis of all life stages with a molecular analysis of four mitochondrial and nuclear genes to settle the taxonomic status of *A. kugleri* and *A. zebratus*. We assign the two species to a newly described genus, confirm the monophyly of this genus, as well as that of *Afronurus* and *Electrogena*, and discuss the need to revisit generic concepts in the family.

## Materials and methods

### Taxon sampling

Eggs, nymphs and adults of museum and newly collected specimens were examined. Nymphs were collected either with a hand net (500- $\mu$ m mesh size), or by manual search on pebbles and stones. Specimens were preserved in 70% ethanol and inspected in the lab under a Leica M125 stereomicroscope. *Afronurus kugleri* was collected in its type locality in 'En Jelabina, Israel, and in several additional sites in northern Israel. *Afronurus zebratus* specimens were collected in Corsica and Sardinia.

Additional *Afronurus* and *Electrogena* species, as well as other heptageniid genera, were selected for genetic analyses based on available material that best represents the distribution ranges of the relevant genera, including both Afrotropical and Oriental elements within *Afronurus*. *Afronurus peringueyi* and *Electrogena lateralis*, the type species of the genera, were sampled from South Africa and the UK, respectively. Additional samples of *E. lateralis* from continental Europe (France and



**Fig. 1.** Distribution map for *Afronurus* (red) and *Electrogena* (green) (based on Webb & McCafferty, 2008, figs 214, 226) and for *Anapos* **gen.n.** (purple): *Anapos kugleri* (Middle East) and *Anapos zebratus* (Corsica and Sardinia).

Switzerland) also were included. Other related genera from the 'Atopopus group' were represented by *Atopopus tarsalis* Eaton, *Thalerosphyrus lamuriensis* Sartori, and three unidentified *Notonurus* species from continental Africa and Madagascar. The 'Atopopus group' is therefore represented by all of the relevant genera except *Notacanthurus* and *Thamnodontus*, for which molecular data were unavailable. Other ecdyonurine taxa included in the analysis are *Asionurus* Braasch & Soldán, *Compsoneuria* Eaton, *Compsonhuriella* Ulmer, *Ecdyonurus* Eaton, *Parafronurus* Zhou & Braasch, and *Rhithrogeniella* Ulmer, together representing over 60% of the genera in the subfamily. The eight remaining ecdyonurine genera were not available for sequencing and their GenBank sequences did not meet the minimum requirement for two genes, and thus they were not included in the analysis. Collection sites of samples used in the molecular study are given in Table S1. *Heptagenia sulphurea* (Müller) was used as an outgroup, because it represents the sister subfamily Heptageniinae.

For the morphological study the following taxa were examined and compared to the focal species: *Electrogena lateralis* (material collected in Czech Republic, Scotland, Switzerland, adults and nymphs), *E. affinis* (Eaton) (Czech Republic, nymphs), *E. grandiae* (Belfiore) (Italy, nymphs), *E. ujhelyii* (Sowa) (Switzerland, adults and nymphs), *E. galileae* (Demoulin

(Israel, nymphs and eggs), *E. quadrilineata* (Landa) (Austria, Czech Republic, nymphs), *E. ujhelyii* (Czech Republic, Hungary, nymphs), *Afronurus peringueyi* (South Africa, nymphs), *A. barnardi* Schoonbee (Namibia, nymphs), *A. scotti* Schoonbee (South Africa, nymphs), *A. muehlenbergi* Puthz (Guinea, adults), *A. namnaoensis* Braasch & Boonsoong (Thailand, nymphs) and *Afronurus* spp. (Indonesia, Mozambique, South Africa, Tanzania, Thailand, adults, nymphs and eggs).

Voucher specimens are deposited in the Steinhardt Museum of Natural History in Tel Aviv University (SMNHTAU), in the Musée Cantonal de Zoologie in Lausanne (MZL) and in the Albany Museum in Grahamstown, South Africa (AMGS).

#### Morphological study

Specimens were studied under Leica M125 or M205 stereomicroscopes. Nymph and adult images were taken with a Visionary Digital LK system (Dun, Inc., VA, U.S.A.). Images of slide preparations were taken on an Olympus BX51 compound microscope. Scanning electron microscope images of eggs were taken with a JEOL 160 microscope at Lausanne University or a LEO 1525 microscope at Hamburg University. Terminology for egg structure follows Koss & Edmunds (1974).

### Molecular methods

DNA was extracted from 36 individuals using the DNAeasy blood and tissue kit (Qiagen Inc., Hilden, Germany) following the method outlined in Vuataz *et al.* (2011), summarized as follows. Whole individuals were soaked overnight in the extraction buffer with proteinase K at 56°C, after which they were taken out of the buffer and retained as vouchers for the DNA samples. The mitochondrial genes cytochrome c oxidase subunit I (*COI*) and 16S ribosomal RNA (*16S*), and the nuclear genes 28S ribosomal RNA (*28S*) and *histone 3 (H3)* were PCR-amplified with the primers specified in Folmer *et al.* (1994), Ogden & Whiting (2005), Pons *et al.* (2004) and Ogden & Whiting (2003), respectively (Table S2). PCR was conducted in a volume of 25 µL, consisting of 2 µL DNA template, 1 µL of 10 pmol/µL of each primer, 2.5 µL 10 mM dNTP solution, 3–3.5 20 mM MgSO<sub>4</sub>, 2.5 µL Taq buffer and 1 unit Taq purity (Hy Laboratories, Rehovot, Israel). Optimized PCR conditions included initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 47–54°C (Table S2) for 30–45 s and extension at 72°C for 1 min, with final extension at 72°C for 10 min.

PCR products were purified using either Qiagen MinElute PCR Purification kit (Qiagen) or Exonuclease and FastAP Thermo sensitive Alkaline Phosphatase (Thermo Scientific, Vilnius, Lithuania). Automated sequencing was carried out using the BigDye terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) on an ABI PRISM 3730xl DNA Analyzer and Sequencing Analysis Software v5.2 at Hy Laboratories.

### Phylogenetic analysis

We obtained sequences for 36 specimens, to which we added sequences of 21 specimens from GenBank (<http://www.ncbi.nlm.nih.gov/>). GenBank accession numbers of all sequences used in this study are given in Table S1. Altogether, the phylogenetic analyses were based on 52 samples representing 28 species, in an alignment in a total length of 1989 bp. Sequences were initially assembled and inspected using CodonCode Aligner v6.0.1, and subsequently aligned in Clustal Omega online (EMBL-EBI). We ran a separate analysis for each of the four genes as well as a joint analysis for the concatenated sequences.

The resulting data partitions were analysed by Bayesian analysis methods implemented in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The most appropriate model for each gene (or partition) was chosen by comparing their BIC scores in jModeltest 2.1.10 (v20160303; Guindon & Gascuel, 2003; Durrbin *et al.*, 2012) and the closest model available in MrBayes was implemented. The models chosen by jModelTest were HKY + I + G for *COI*, TrN + I + G for *16S* and *28S*, and TPM2uf + G for *H3* but the topological results were largely insensitive to the substitution model, as the resulting branching pattern did not vary depending on the models employed.

In each analysis, two independent runs with four chains were run for 10 million generations (sampling every 1000 generations). Convergence was assessed by examining stationarity in log-likelihood scores as the correlation of split frequencies between runs (AWTY; Nylander *et al.*, 2008) and by examining ESS (Effective Sample Size) in TRACER v1.6 (Drummond & Rambaut, 2007). The first 2500 trees (2 500 000 generations) were discarded as burn-in, and the remainder was used to estimate tree parameters and topology. We also conducted a maximum-likelihood analysis using the GTR + GAMMA model for each partition (gene) with 1000 bootstrap replicates using RAxML v8.1.21 (Stamatakis, 2014) on raxmlGUI 1.5 beta (Silvestro & Michalak, 2012).

Given the scarcity of informative morphological data in the studied group, the morphological characters were not combined with the molecular data into a total evidence analysis, but were subject to an ancestral state analysis in order to investigate the origin of three characters judged to be important in the phylogenetic history of the subfamily. These attributes are: (i) structure of the maxillary distal dentisetae in the nymph (0 = entire or apically divided, 1 = branched); (ii) arrangement of fine hair-like setae on the hind tibiae in the nymph (0 = no median row of hairs, 1 = median row of sparse hairs, 2 = median row of dense hairs); and (iii) arrangement of chorionic structures on the eggs (KCTs – see below) (0 = no/small structures, 1 = medium structures, 2 = large structures at equatorial area). An unordered, multistate ancestral state analysis for the three characters was performed based on the phylogenetic reconstruction using the maximum parsimony approach implemented in MESQUITE 3.04 (Maddison & Maddison, 2011).

### Results

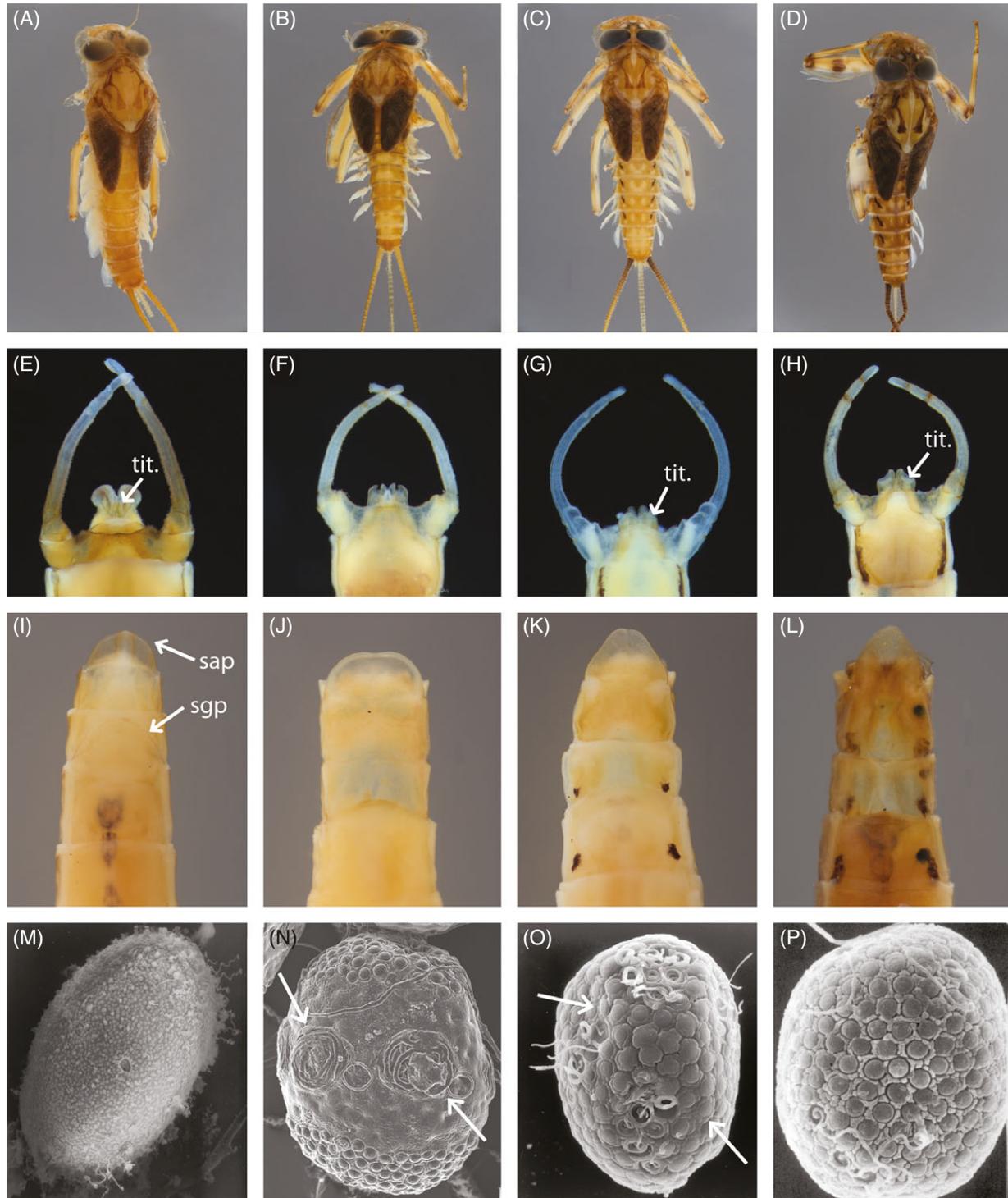
Our morphological and molecular analyses clearly support the assignment of *Afronurus kugleri* and *Afronurus zebratus* to a distinct genus, and we therefore allocate these species to a newly described genus as follows.

#### *Anapos Yanai & Sartori gen.n.*

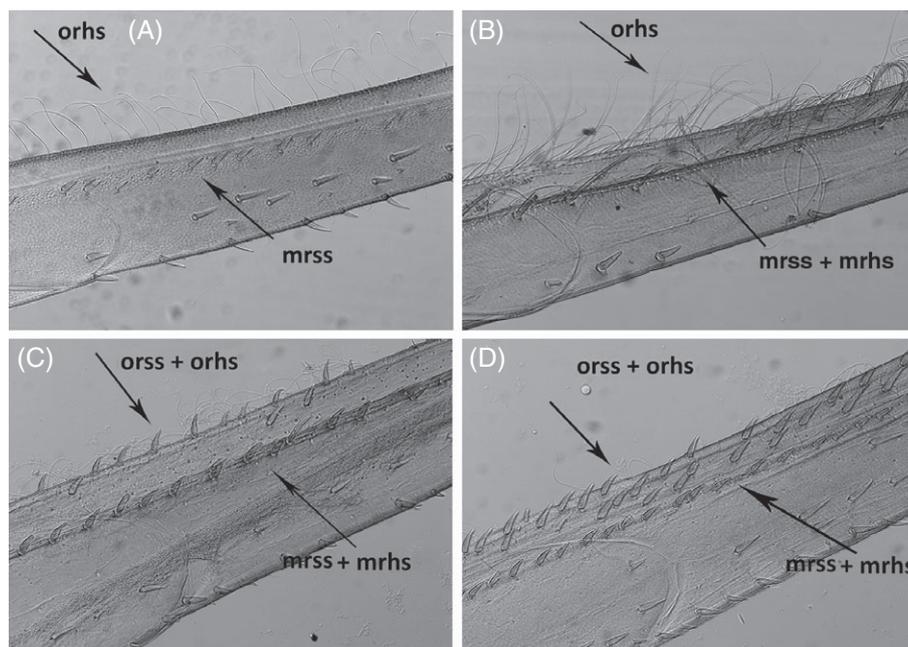
<http://zoobank.org/urn:lsid:zoobank.org:act:9B365908-7A4C-4D75-BED0-3CA3BF3C3DFC>

*Type species.* *Anapos zebratus* (Hagen, 1864) by present designation.

*Diagnosis. Nymph.* Body dorsoventrally flattened. Head capsule: broad (Fig. 2C, D), anterior margin slightly thickened. Labrum with distinct antero-median notch. Distal dentiseta of maxilla with five entire branches. Number of comb-shape setae on the crown of the galea-lacinia 16–19. Thorax: Pronotum broad, without postero-lateral expansions. Hind tibiae with three longitudinal rows of stout spines and two rows of fine setae (one of them sparse) (Fig. 3C, D). Claws with 3–4 denticles. Abdomen: Segments I–VII each with pair of flattened plate-like gills, all except gill VII with filamentous tufts; caudal filaments without whorls of setae.



**Fig. 2.** Ecdyonurinae nymphs, adults and eggs. Nymph, habitus: (A) *Electrogena lateralis*; (B) *Afronurus* sp.; (C) *Anapos kugleri*; (D) *Anapos zebratus*. Adult male terminalia (ventral view; tit. = titillators): (E) *Electrogena lateralis*; (F) *Afronurus muehlenbergi*; (G) *Anapos kugleri*; (H) *Anapos zebratus*. Adult female terminalia (ventral view; sgp = subgenital plate, sap = subanal plate): (I) *Electrogena lateralis*; (J) *Afronurus muehlenbergi*; (K) *Anapos kugleri*; (L) *Anapos zebratus*. Egg (arrows = KCTs): (M) *Electrogena galileae*; (N) *Afronurus* sp.; (O) *Anapos kugleri*; (P) *Anapos zebratus* (modified from Gaino *et al.*, 1987). [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)].



**Fig. 3.** Ecdyonurinae nymphs, hind tibiae: (A) *Electrogena lateralis*; (B) *Afronurus scotti*; (C) *Anapos kugleri*; (D) *Anapos zebratus*. Abbreviations: orhs: outer row of hair-like setae; orss: outer row of spine-like setae; mrhs: middle row of hair-like setae; mrss: middle row of spine-like setae.

**Adult male.** Penis lobes short, apex constricted, not expanded laterally, dorsal medio-apical sclerites well developed. Base of penis lobes with two median spindle-shaped sclerotized titillators (Fig. 2G, H).

**Adult female.** Subgenital plate weakly developed, not extending beyond mid-length of segment VIII. Subanal plate well developed, rounded at apex, entire or weakly indented (Fig. 2K, L).

**Eggs.** Chorionic surface entirely covered by large round knob-terminated coiled threads (KCTs) (Fig. 2O, P).

**Etymology.** *Anapos* is the river god of eastern Sicily in the Greek mythology, used here in reference to a region within the distribution range of the new genus. The gender is masculine.

***Anapos zebratus* (Hagen, 1864) comb.n.**

*Baetis zebrata*; Hagen (1864).

*Heptagenia zebrata*; Eaton (1871).

*Ecdyurus zebrata*; Eaton (1885).

*Ecdyonurus zebratus*; Ulmer (1920).

*Heptagenia fallax*; Grandi (1953).

*Electrogena zebrata*; Gaino & Belfiore (1987).

*Afronurus zebratus*; Hefti & Tomka (1989).

*Electrogena zebrata*; Belfiore (1996, 1997).

*Ecdyonurus (Electrogena) zebrata*; Kluge (2004).

*Afronurus zebratus*; Bauernfeind & Soldán (2012).

**Material examined.** FRANCE: 1 ♂, 2 ♀, Corse-du-Sud, Rizzanese River, 500 m (a.s.l.), 14.ix.1983 (*Giudicelli*) (MZL); 2 nymphs, Corse-du-Sud, Porto River, bridge before Ota, 210 m,

6.vii.1985 (*Sartori*) (MZL); 1 nymph, Corse-du-Sud, Taravo River, Taravo, 30 m, 8.vii.1985 (*Sartori*) (MZL); 1 nymph, 4 subimago ♂, 9 subimago ♀, 1 ♂, Haute-Corse, Fango River, Galeria, 16 m, 23.v.1986 (*Studemann & Landolt*) (MZL); 2 ♂, Haute-Corse, Fango River, Montestremo, 200 m, 24.v.1986 (*Studemann & Landolt*) (MZL); 5 nymphs, Corse, Regino, Lozari, 18 m, 8.v.2005 (*Carvalho & Vuataz*) (SMNHATAU); 23 nymphs, Haute-Corse, Fiume Secco, 5 km east of Calvi, 10 m, 11.iv.2006 (*Gattolliat & Gattolliat*) (MZL).

***Anapos kugleri* (Demoulin, 1973) comb.n.**

*Afronurus kugleri*; Samocha (1972; *nomen nudum*).

*Afronurus kugleri*; Demoulin (1973).

*Afronurus kugleri*; Moubayed (1986).

*Afronurus kugleri*; Koch (1988).

*Electrogena kugleri*; Kluge (1989).

*Afronurus kugleri*; Sartori (1992).

*Electrogena kugleri*; Belfiore (1994).

*Afronurus kugleri*; Bauernfeind & Soldán (2012).

**Material examined.** *Holotype*, ♂, ISRAEL: Golan Heights, 'En Jelabina, 103 m, 10.ii.1970 (*Samocha*) (SMNHATAU); ISRAEL: 11 nymphs, Golan Heights, Wadi Zavitan, 270–350 m, 5.v.1990 (*Sartori*) (MZL); 2 nymphs, Golan Heights, 'En Aleiqa, 530 m, 7.v.1990 (*Glassmann & Sartori*) (MZL); 2 nymphs, Kinnarot Valley, Nahal Yehudiyya, st.1, –180 m, 7.v.1990 (*Glassmann & Sartori*) (MZL); 5 nymphs, 3 subimago ♂, 2 ♂, 3 ♀, Kinnarot Valley, Upper Jordan River, st.5 Almagor Bridge, –160 m, 7.v.1990 (*Glassmann & Sartori*) (MZL); 2 nymphs, Hula Valley, Upper Jordan River, st.3

Asteret Fortress, 60 m, 7.v.1990 (*Glassmann & Sartori*) (MZL); 3 nymphs, same locality, 7.v.1991 (*Glassmann & Sartori*) (MZL); 1 nymph, same locality, 8.vii.2014 (*Yanai*) (SMNHTAU); 2 nymphs, same locality, 29.vii.2015 (*Yanai*) (SMNHTAU); 1 nymph, Hula Valley, Nahal Rosh Pinna, 60 m, 7.v.1990 (*Glassmann & Sartori*) (MZL); 4 nymphs, Hula Valley, Nahal Hermon, below waterfall, 260 m, 9.v.1990 (*Sartori*) (MZL); 2 nymphs, 1 subimago ♂, 2 subimago ♀, Hula Valley, Nahal Senir (Hasbani), st.3b, 110 m, 10.v.1990 (*Reuven & Sartori*) (MZL); 1 subimago ♂, 9 ♀, same locality, 10.v.1991 (*Reuven & Sartori*) (MZL); 1 nymph, same locality, 9.vi.2014 (*Yanai*) (SMNHTAU); 2 nymph, same locality, 29.vii.2015 (*Yanai*) (SMNHTAU); 3 nymphs, Hula Valley, Nahal Senir (Hasbani), st.4, 105 m, 10.v.1990 (*Reuven & Sartori*) (MZL); 4 nymphs, same locality, 10.v.1991 (*Reuven & Sartori*) (MZL); 1 nymph, Hula Valley, Nahal Dan, st.4, 170 m, 10.v.1990 (*Reuven & Sartori*) (MZL); 1 nymph, Hula Valley, Nahal Dan, st.6, 120 m, 10.v.1990 (*Reuven & Sartori*) (MZL); 3 nymphs, 3 ♀, same locality, 10.v.1991 (*Reuven & Sartori*) (MZL); 1 ♀, Hula Valley, Nahal Dan, st.1, 19.vi.1990 (*Reuven*) (MZL); 1 nymph, Golan Heights, 'En Divsha, 92 m, 26.iii.2014 (*Yanai*) (SMNHTAU); 2 nymphs, Hula Valley, Nahal Daliyot, downstream to road 92, -177 m, 26.iii.2014 (*Yanai*) (SMNHTAU); 1 nymph, Upper Galilee, Nahal 'Ammud, hidden pool, 475 m, 20.v.2014 (*Yanai*) (SMNHTAU); 2 nymphs, Upper Galilee, Nahal Meron, 'En Yaqim, 543 m, 20.v.2014 (*Yanai*) (SMNHTAU); 1 nymph, Kinnarot Valley, Park HaYarden, old gristmill, -200 m, 27.v.2014 (*Yanai*) (SMNHTAU); 1 nymph, Hula Valley, Upper Jordan River, near Park HaYarden, -203 m, 27.v.2014 (*Yanai*) (SMNHTAU); 1 nymph, Hula Valley, Nahal Senir (Hasbani), Beith Hillel, 82 m, 9.vi.2014 (*Yanai*) (SMNHTAU); 2 nymphs, Golan Heights, Nahal Jelabina, old gristmill, 76 m, 10.vi.2014 (*Yanai*) (SMNHTAU); 2 nymphs, Golan Heights, 'En Mimom, 290 m, 22.vi.2014 (*Yanai*) (SMNHTAU); 2 nymphs, Golan Heights, Nahal Jelabina, old gristmill, 76 m, 9.vi.2014 (*Yanai*) (SMNHTAU); 1 nymph, Golan Heights, 'En Tina, hiking trail, 71 m, 17.vii.2014 (*Yanai*) (SMNHTAU); 2 nymphs, Hula Valley, Upper Jordan Valley, HaDodot bridge, -161 m, 29.vii.2015 (*Yanai*) (SMNHTAU); LEBANON: 1 nymph, 1 ♂, South Lebanon, El Litani, 14.vii.1980 (*Dia*) (MZL).

#### Phylogenetic analysis

Our Bayesian phylogenetic reconstruction (Fig. 4) clearly supports the monophyly of the Ecdyonurinae but not the 'Atopopus group' (i.e. *Afronurus*, *Anapos*, *Atopopus*, *Electrogena*, *Notonurus* and *Thalerosphyrus*), the members of which are mixed with representatives of the supposedly unrelated *Asionurus*, *Compsoneria*, *Compsoneriella* and *Rhithrogeniella*. The position of *Parafronurus* with regard to the 'Atopopus group' was not resolved by the phylogenetic analysis. *Electrogena* and *Afronurus* form strongly supported monophyletic groups [Bayesian posterior probability (PP) = 1.0 and 0.99, respectively] and the taxonomic integrity of all sampled species within the two genera is also validated, although relationships within *Electrogena* are not fully resolved. The focal species, *Anapos kugleri* and

*A. zebratus*, clustered together to form a distinct and highly supported monophyletic clade (PP = 1.0) with similar strong support for each of the two species. The *A. zebratus* clade is further divided into individuals from Corsica and Sardinia. The maximum-likelihood analysis yielded similar topology but with lower support values (indicated on the Bayesian tree in Fig. 4).

#### Morphological analysis

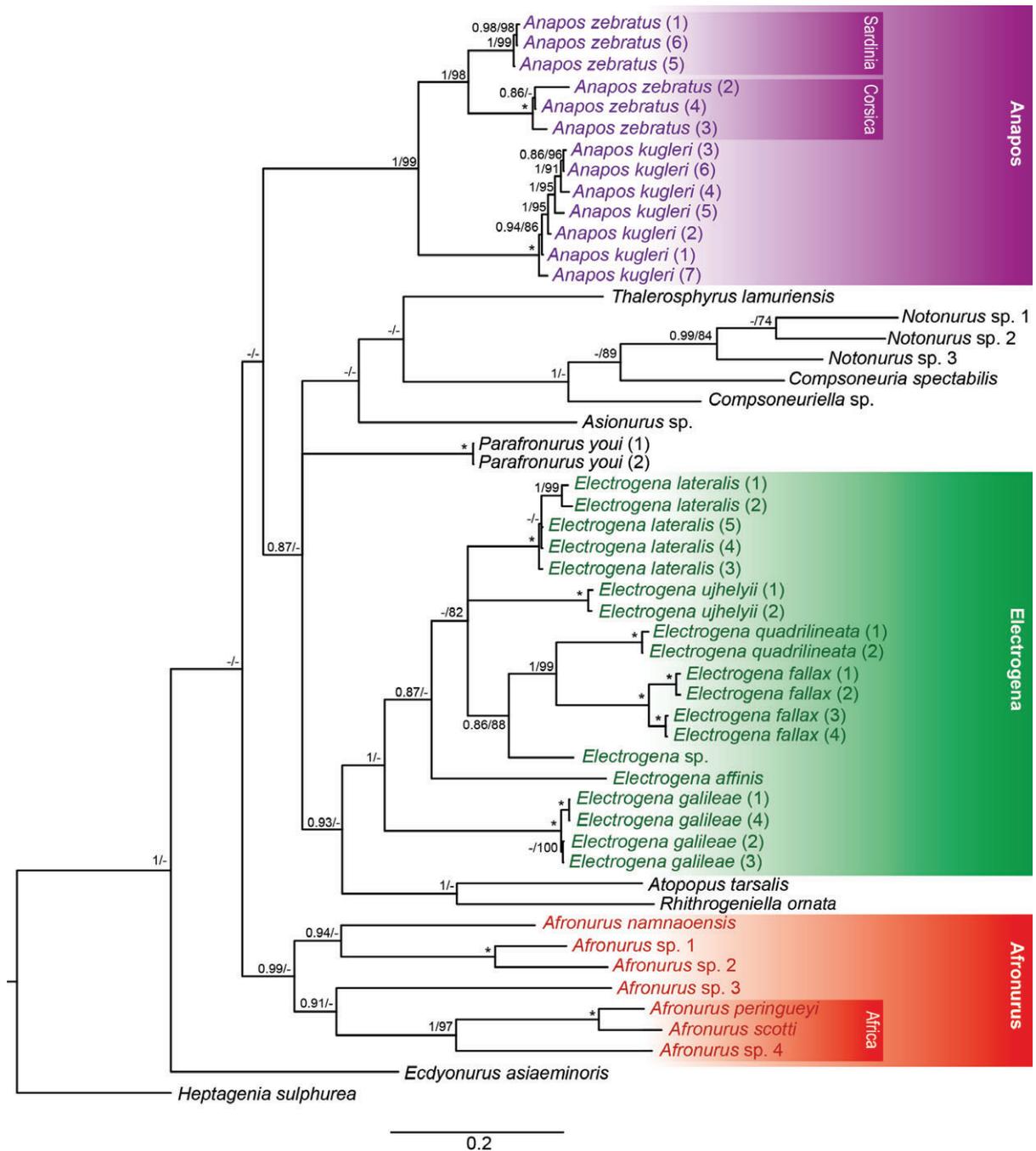
Our detailed morphological study revealed important similarities and dissimilarities between *Anapos kugleri*, *A. zebratus*, and *Afronurus* and *Electrogena* species. At the nymphal stage, *A. kugleri* and *A. zebratus* are unique in the arrangement of setae and spines on the hind tibia: both have three rows of thick spines and two rows of thin hair-like setae (Fig. 3C, D). By contrast, *Afronurus* and *Electrogena* have no outer row of spines (Fig. 3A, B), and *Electrogena* species have only one row of fine hair-like setae. Other differences include the number of teeth on tarsal claws (3–4 in *A. kugleri* and *A. zebratus*, 1–3 in *Electrogena*, 4–6 in *Afronurus*), number of distal dentisetal branches (5 in *A. kugleri* and *A. zebratus*, 3–5 in *Electrogena*, 6–7 in *Afronurus*), number of comb-shaped setae on the galea-lacinia crown (16–19 in *A. kugleri* and *A. zebratus*, 14–16 in *Electrogena*, 18–21 in *Afronurus*), and the anteromedian notch on the labrum, which is present in all *Afronurus* species and in *A. kugleri* and *A. zebratus*, but absent in *Electrogena*.

Adult males of *A. kugleri* and *A. zebratus* differ from those of *Electrogena* in the shape of the penis lobes, shape of titillators, which are blade-like in *Electrogena* but spindle-shaped in *Anapos*, and the arrangement of apical sclerites; they can be separated from those of *Afronurus* by the presence of well-developed titillators (Fig. 2E–H).

Adult females of the two *Anapos* species are similar to those of *Afronurus* species in having a subgenital plate that reaches the middle of sternite VIII at most, whereas in *Electrogena* the subgenital plate is well developed and almost reaches the posterior margin of sternite VIII. The subanal plate is elongated, with rounded apex in *A. kugleri*, *A. zebratus* and *Electrogena* spp., but wide, straight or even concave in *Afronurus* (Fig. 2I–L).

Finally, eggs of *A. kugleri* and *A. zebratus* are entirely covered by large knob-terminated coiled threads (KCTs) that are surrounded by small tubercles (Fig. 2O, P), whereas those of *Electrogena* are either entirely smooth or covered by minute tubercles and small KCTs (Gaino *et al.*, 1987; Fig. 2M) and those of *Afronurus* are covered by minute tubercles and two types of KCTs: medium-sized on each pole, and large, oval ones in the equatorial area (Belfiore *et al.*, 2003; Fig. 2N).

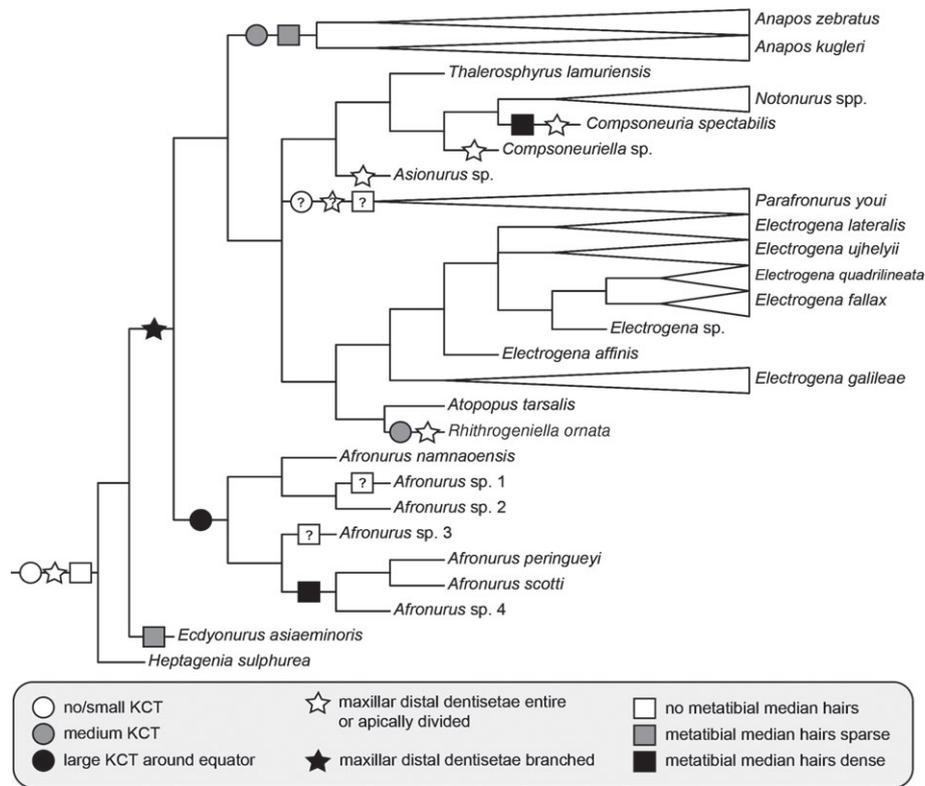
*Anapos* is closely related to *Afronurus* and *Electrogena*, from which it can be distinguished by the setation of hind legs in the nymphal stage, and the shape of penis lobes and presence and shape of titillators in the adult male. This latter character distinguishes *Anapos* from all other genera of the 'Atopopus group', except *Parafronurus*. At the nymphal stage, *Anapos* differs from other genera as follows: *Atopopus* is an Oriental genus exclusively found in Borneo and the Philippines, with peculiar morphological features, such as thickened



**Fig. 4.** Consensus tree for Ecdyonurinae based on Bayesian analysis, showing *Afronurus* spp. (red), *Electrogena* spp. (green), *Anapos* spp. (purple) and related genera based on concatenated sequences (1989 bp) of four genes (*COI*, *16S*, *28S* and *H3*). Posterior probabilities above 0.85 and maximum-likelihood bootstrap values above 70 are indicated next to the nodes.

head margin, shortened legs, and gill I reduced to a bunch of fibrillae (Wang & McCafferty, 1995; Sartori *et al.*, 2007). *Thalerosphyrus* (Oriental) and *Notonurus* (Afrotropical) have acute supracoxal spurs (Sartori, 2014b, 2014d). *Notacanthurus* is an East Palaearctic genus whose nymph has a median longitudinal abdominal ridge (Tshernova, 1974). *Thamnodontus* was considered by Kluge (2004) to occupy a plesiomorphic

position within the group because it shares no apomorphies with any of the abovementioned genera, but has very different genitalia, which are well developed and laterally expanded. Finally *Parafronurus* differs from *Anapos* by the presence of swimming setae on the caudal filaments and the shape of the gills in the nymph, the size and position of titillators in males, and the subgenital plate in females (Zhou & Braasch, 2003).



**Fig. 5.** Ancestral state reconstruction of three morphological traits under maximum parsimony. Characters are presented on an ultrametric topology of the Bayesian consensus tree with character states indicated on the relevant nodes. Question marks indicate unknown character states.

The ancestral state analysis (Fig. 5) indicates that branched maxillary distal dentisetae is the character state commonest for most ingroup taxa in our analysis, and that this state appeared for the first time at the split between *Ecdyonurus* and the rest of Ecdyonurinae. Apparently, four independent reversals occurred in *Rhithrogeniella* and in the related genera *Asionurus*, *Compsoeuria* and *Compsoeuriella*. Metatibial setation in the nymph appeared independently four times in the history of the subfamily (in *Ecdyonurus*, *Anapos*, African *Afronurus* spp. and *Compsoeuria*), and is particularly dense in the latter two. The ancestral state of structures on the egg chorion surface is that of small or no KCTs, whereas the *Afronurus* clade is unique for the large KCTs at the equatorial area of the egg. The rare situation of medium-sized KCTs covering the entire chorion surface is shared by two unrelated genera, *Anapos* and *Rhithrogeniella*. This analysis does not provide any insight into the position of *Parafronurus* because none of the three characters are currently known for this genus.

## Discussion

### Generic concepts within Ecdyonurinae

Heptageniid taxonomy traditionally has relied on morphological descriptions yet comprehensive descriptions of all life stages are unavailable for many species. Throughout the years, different taxonomists have used different types of morphological

characters to distinguish genera or species groups, while ignoring other characters. This situation, together with the general lack of molecular systematic studies of the family, have caused much confusion with regard to generic concepts and hindered proper inference of phylogenetic relationships among genera. In the present study we analysed all available morphological characters and used four mitochondrial and nuclear genes to reconstruct the phylogeny of the 'Atopopus group' (Kluge, 2004) in order to resolve long-standing generic concepts within it.

Our phylogenetic analysis did not support the 'Atopopus group' based on the available representatives, which are mixed together with other genera, suggesting no clear division within the subfamily (Fig. 4). Furthermore, the apomorphy of the 'Atopopus group' suggested by Kluge (2004) (i.e. branched maxillary distal dentisetae) evidently constitutes a homoplasious character that is shared by most genera in the subfamily and therefore cannot serve as a reliable character for generic grouping. *Ecdyonurus* is a sister group to the rest of the studied genera, and *Afronurus* and *Anapos* also occupy basal positions relative to other genera that were traditionally considered to belong in the 'Atopopus group'.

The newly described genus *Anapos* constitutes a monophyletic clade that is clearly distinct from the close genera *Electrogena* and *Afronurus*, thus confirming previous morphological and biochemical observations (Hefti & Tomka, 1989; Sartori, 1992) as well as our own findings, which suggested that *A. kugleri*

and *A. zebratus* justify a separate genus. An apparently closely related species, *Afronurus madli* Kazanci, was described from Turkey based only on adults, and the author claimed that it was related to *A. kugleri* and *A. zebratus* (Kazanci, 1992), a position that was also expressed by Bauernfeind & Soldán (2012). Indeed, the presence of titillators in the male excludes this species from Eurasian *Afronurus*, but their position and the shape of the subgenital plate in the female resemble the situation in *Parafronurus* rather than that in *Anapos*. As nymphs are yet unavailable for study, it is currently impossible to draw any conclusions about the position and affinities of *A. madli* and it should be considered as *incertae sedis* until they are found and studied.

The historical confusion regarding the correct generic placement of *A. kugleri* and *A. zebratus* resulted from the poorly defined concepts of the genera to which they were originally assigned. Our morphological analysis offers a useful suite of characters which could be referred to in the future to correctly assign species to genera in this group. *Afronurus* is a good example of how poorly defined concepts may hamper our understanding of evolutionary processes. Titillators are present in adult males of all *Electrogena*, *Parafronurus* and *Anapos* species but only in a few Asian *Afronurus* species (Belfiore *et al.*, 2003; Wang & McCafferty, 2004). Based on the findings of the present study, it is likely that *Afronurus* species with and without titillators actually belong to different genera. Our molecular reconstruction suggests that the Oriental species within *Afronurus* constitute a paraphyletic group with regard to the Afrotropical species (represented by *Afronurus* sp. 4, *A. peringueyi* and *A. scotti*). *Afronurus namnaoensis* (Thailand), *Afronurus* sp. 1 (Thailand) and *Afronurus* sp. 2 (Sumatra) form a sister group to *Afronurus* sp. 3 (Borneo) + Afrotropical *Afronurus* species (Fig. 4). As two of the Oriental species do possess titillators (Braasch & Boonsoong, 2010, Figs 2 and 3), and no information is available regarding the adults of *Afronurus* sp. 1 and *Afronurus* sp. 3, further morphological and genetic studies of Oriental *Afronurus* species are needed to clarify the relationships within this genus.

#### Ecology and distribution of *Anapos* *gen.n.*

*Anapos* has a fragmented circum-Mediterranean distribution but the distribution ranges of the two included species are similar in climatic and landscape features and are much closer to each other geographically than to the distribution ranges of *Afronurus* species (Fig. 1). This implies that *Anapos* is more closely related to European or circum-Mediterranean genera than to Afrotropical or Oriental ones. It is possible that additional cryptic or undescribed species that belong to *Anapos* will be found to occupy habitats along the southern Mediterranean coast or on Mediterranean islands, which are largely understudied.

*Anapos zebratus* is clearly divided into genetically distinct Corsican and Sardinian populations. Such clear separation between populations from the two islands has already been demonstrated for *A. zebratus* (under the name *Electrogena zebrata*) as well as for other Ephemeroptera species by Gattolliat *et al.* (2015). These authors studied the Corsican mayfly

fauna and found that it was strongly influenced by the island's geological history. Nevertheless, our thorough study of specimens from the two populations failed to find any morphological differences between them, suggesting a relatively recent split. Indeed, Gattolliat *et al.* (2015) dated the appearance of *A. zebratus* to approximately 30 Ma, while the populations of Corsica and Sardinia were separated only 6.2 Ma, shortly before the Messinian salinity crisis.

The typical habitats of *A. kugleri* and *A. zebratus* are small- and mid-sized streams in regions of Mediterranean climate, with cool, turbulent water, and pebble and stone substrate.

An interesting observation is that, unlike most mayfly species, the last instar nymph of *A. kugleri* moults into the subimago underwater (Sartori, 1992), a rare phenomenon that has been reported for certain species from the families Leptophlebiidae, Leptohephidae, Ephemeridae, Baetidae, Caenidae, Oligoneuridae and Heptageniidae (Kennedy, 1926; Ide, 1930; Hall *et al.*, 1975; McClure & Stewart, 1976; Edmunds & McCafferty, 1988; Bauernfeind & Humpesch, 2001; Reisinger *et al.*, 2011). It is noteworthy that underwater moulting to subimago has also been reported for *Electrogena* (Kimmins, 1941) but never for any of the dozens of *Afronurus* species. At present, no information is available on the subimago moulting habits of *A. zebratus* and it would be interesting to find whether it resembles *A. kugleri* in this regard.

#### Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/syen.12203

**Table S1.** Samples used in the molecular analyses (names as in Fig. 4) and GenBank accession numbers.

**Table S2.** Primers used in the molecular analyses, with PCR annealing temperatures.

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