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Amphinemura palmeni is a valid Holarctic stonefly species (Plecoptera: Nemouridae)

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Abstract

The taxonomic status of *Amphinemura palmeni*, a rare stonefly from northern Fennoscandia, was re-evaluated in a morphological study combined with analyses of mitochondrial COI and nuclear 28S sequences. Taxon sampling included *A. standfussi*, with which *A. palmeni* has been confused, the Nearctic *A. linda* and seven other *Amphinemura* species. *Amphinemura palmeni* is confirmed as a valid species, and *A. norvegica* and *A. linda* identified as junior synonyms. The species is illustrated with line drawings, photographs and SEM micrographs. Its COI haplotype diversity is compared with the intraspecific diversity in other stonefly species.

Key words: stoneflies, *Amphinemura*, *A. palmeni*, *A. norvegica*, *A. linda*, *A. standfussi*, Holarctic, Fennoscandia, Nearctic, COI, 28S, SEM, systematics, synonymy, validity

Introduction

Four species of the genus *Amphinemura* are known from Finland and northern Scandinavia. Three of these are widespread Palaearctic species: *A. standfussi* (Ris, 1902), *A. sulcicollis* (Stephens, 1835) and *A. borealis* (Morton, 1894). The fourth species, *A. palmeni* (Koponen, 1917) is listed as an endemic from northern Fennoscandia (Illies, 1978; Lillehammer, 1988), but there has been much uncertainty about its identity and its valid name.

Koponen (1917) described *Nemoura* (*Amphinemura*) *palmeni* on the basis of one male and one female specimen collected by Envald in Tuloma Lapland on the Kola Peninsula. The type locality, Lake Nuorti (Russian: Notozero), disappeared when the Verkhnetulomskoe Reservoir was constructed in the 1960s (Gusev *et al*., 2011).

According to Brinck (1949: 19) *A. palmeni* was a synonym of *A. standfussi*, "considering the description and the type material". Subsequently D. Tobias (1973) described *A. norvegica* from northern Norway. Meinander (1975) reported that Tobias and Baumann later checked the type of *A. palmeni* and thought that both *A. norvegica* and the Nearctic *A. linda* (Ricker, 1952: 22) were conspecific with *A. palmeni*. However, their work on this taxon was never published. Illies's catalogue (1966: 185) reproduced Brinck's opinion, but his chapter in Limnofauna Europaea (Illies, 1978) followed Meinander in considering *A. norvegica* a synonym of *A. palmeni*, without mentioning *A. linda*. Lillehammer (1988: 96) likewise considered *A. norwegica* (sic!) a synonym of *A. palmeni* and did not mention *A. linda*. However it is not clear whether he has studied specimens of *A. palmeni* himself, since his publications do not mention this and no specimens were found in the Plecoptera collection in Oslo (Boumans, 2011b).

We reconsider the taxonomic status of *A. palmeni* and *A. linda* on the basis of morphological study of the types and freshly collected specimens of these taxa as well as *A. standfussi*. In addition, we performed a molecular phylogenetic analysis of fragments of the mitochondrial gene cytochome oxidase I (COI) and the nuclear ribosomal gene 28S of the aforementioned species together with additional *Amphinemura* species.

Note: The type specimen label (see below) and some literature sources (Lillehammer, 1988; Fochetti & Tierno de Figueroa, 2004) state the publication year of *A. palmeni* as 1916. Volume 44 of the journal Acta Societatis pro fauna et flora Fennica was published in eight issues from 1916 to 1919. The description of *A. palmeni* appeared in issue 3 dated 1917.

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Material and methods

Amphinemura palmeni (Koponen, 1917), **species propria**
Nemoura (*Amphinemura*) *palmeni* Koponen, 1917: 13
Nemoura (*Amphinemura*) *linda* Ricker, 1952: 22, **syn. nov.**
Amphinemura norvegica D. Tobias, 1973

Type specimens. The type specimens of *A. palmeni* consist of two cleared genitalia preparations (Figs. 1–4): Microscope slide (Fig. 1) bearing the thorax and abdomen of 1 female (Fig. 2) and the abdomens of 2 males. One of the males is *A. palmeni* (Fig. 3), which we designate as the lectotype; the other is *A. standfussi* (Fig 4). Three handwritten labels: a) Mus. Zool. H:fors, Spec. typ. No 6651, Amphinemura palmeni Koponen b) Amphinemura palmeni Koponen (1916, p.13) c) A. palmeni, A. xx [undecipherable, possibly A. standfussi]. The types are located at the Zoological Museum of the University of Helsinki.

Paratopotype specimens of both *A. linda* and *A. norvegica*, identified by W. E. Ricker and D. Tobias at the time of their species description, were examined as part of this study:

Amphinemura linda: **USA**, Michigan: Montmorency Co. Hunt Creek, 7-IX-1940, E. Cooper, 1 ♂, 2 ♀. det. W. E. Ricker, col. Monte L. Bean Life Science Museum, Brigham Young University, Provo, Utah, USA.

Amphinemura norvegica: D. Tobias **Norway**, Finnmark: Sør-Varanger 'Elnelv' [= Ellenelva], 28-VII-1972, 1 ♂ 1 ♀; idem 23 VII 1973, 1 ♂ 1 ♀; all leg. & det. Tobias, col. Senckenberg Museum.

Other material. The following specimens were used for morphological investigation:

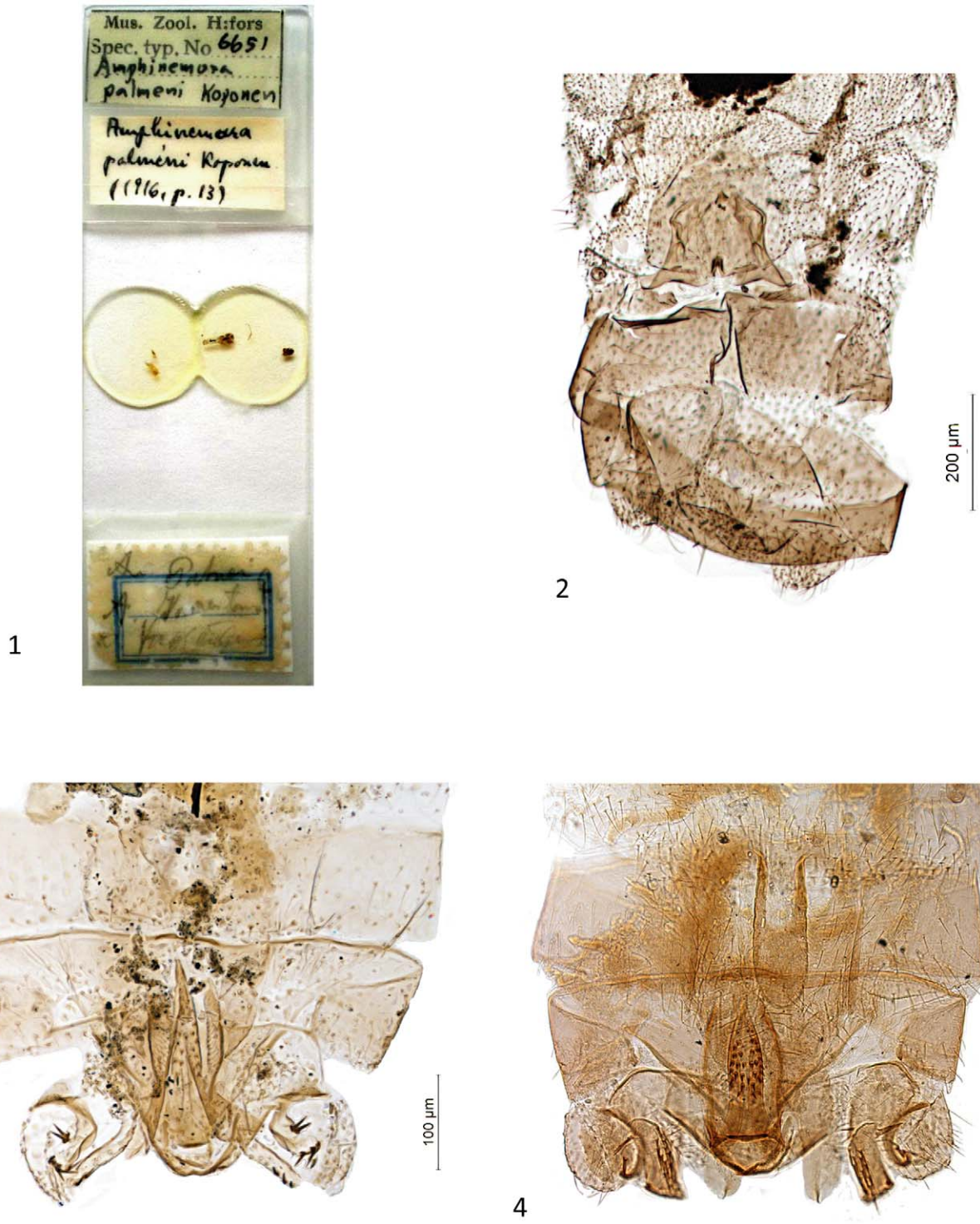
Amphinemura palmeni: **Norway**, Finnmark: Sør-Varanger, 30-VII-2010, Nordvest-bukta: Emanuelbekken N 69.3035° E 29.2632°, 62 m asl: 9♂ 6♀; idem, Ellenelva N 69.2132° E 29.1535°, 67 m asl : 1♂ 1♀. All leg. L. Boumans, S. Roth & T. Ekrem, det. L. Boumans, col. Natural History Museum, University of Oslo (ZMUN).

Amphinemura linda: **Canada** Alberta: Minnewanka Creek, Baniff National Park, 22-VIII-1969, C. M. Yarmoloy, 3 ♂, 8 ♀; Northwest Territories: Stark River, Great Slave Lake, 6-IX-1988, G. F. Edmunds, Jr., 7 ♂, 16 ♀; Saskatchewan: Brokenhead River, 18-IX-1982, D. K. Burton, 2 ♂, 2 ♀; Stream Hwy 155, mile 98, near Île-à-la-Crosse, 10-VII-1974, L. Dosdall, 1 ♂, 1 ♀; Mistohay Creek, Hwy 226, 12-VIII-1975, D. Smith, 1 ♂, 1 ♀; Stream, 10 miles east of Squaw Rapids Dam, 12-VII-1974, L. Dosdall, 1 ♂, 1 ♀; **USA** Iowa: Winnesheik Co. Dunnings Spring, Decorah, 22-IX-2004, D. Heimdal, 2 ♂, 2 ♀; Same locality, 8-X-2011, M. W. Birmingham, 30 ♂, 42 ♀; Michigan: Mecosta Co. Paris,1-X-1973, A. Maki, 8 ♂, 37 ♀; Montmorency Co. Hunt Creek, 7-IX-1940, E. Cooper, 1 ♂, 2 ♀; South Dakota: Roberts Co. Sica Hollow State Park, 17-IX-1974, P. J. Johnson, 1 ♂; Wisconsin: Burnett Co. Stream, 11 miles southeast of Siren, 8-X-1966, D. Hansen, 2 ♂; Kewaunee Co. Little Scarboro Creek, north of Luxemburg, 9-IX-1989, J. Cahow, 1 ♂, 1 ♀; Lincoln Co. Ripley Creek, below Grandfather Falls, 1-VIII-1992, C. R. Nelson, 1 ♀; Same county, North Branch Prairie River, 20-VIII-1972, R. W. Baumann, 1 ♂. All housed at the Monte L. Bean Life Science Museum, Brigham Young University, Provo, Utah, USA.

Amphinemura standfussi: **Norway**, Troms, Skibotn, Brennfjellet, N 69.3260° E 20.3650°, 2-VIII-2010, 1♂ leg. B. Fromm; Finnmark, Sør-Varanger, Steinbekken N 69.23104° E 29.16092°, 63 m asl, 30-VII-2010, 1♂ 1♀, leg. L. Boumans, S. Roth & T. Ekrem; all det. L. Boumans.

All North American specimens used in the SEM photographs and line drawings are deposited at the Monte L. Bean Life Science Museum, Brigham Young University, Provo, Utah, USA. The Norwegian specimens used in SEM micrographs are housed at the Senckenberg Museum in Frankfurt am Main. The specimens used in colour photographs are housed at Natural History Museum, University of Oslo (ZMUN).

Appendix 1 lists all specimens used for DNA analyses with collecting data and GenBank accession numbers. The set of mitochondrial cytochrome oxidase subunit 1 (COI) sequences includes eight individuals of *A. palmeni*, four of *A. linda* and 33 of *A. standfussi*, as well as individuals from a number of other *Amphinemura* species: the two other species occurring in Scandinavia, *A. sulcicollis* and *A. borealis*, and the Nearctic species *A. nigritta* (Provancher, 1876), *A. delosa* (Ricker, 1952: 18), *A. banksi* Baumann & Gaufin, 1972, *A. appalachia* Baumann 1996 and *A. wui* (Claassen, 1936). This larger data set provides a wider sampling of genetic distances both within and among species in the genus. *Nemoura cinerea* (Retzius, 1783: 60) was designated as outgroup taxon. The data set of the nuclear marker 28S includes sequences of *A. palmeni*, *A. linda*, *A. standfussi*, *A. borealis*, *A. sulcicollis*, *A. nigritta* and the outgroup taxon *N. cinerea*.



FIGURES 1–4. *Amphinemura palmeni* types: 1: Appearance of the slide preparation. 2: Female abdomen, ventral view. 3: Male abdomen featuring the paraprocts in ventral view, slide photographed upside-down. 4: Male abdomen of *A. standfussi* included in the same preparation, ventral view, slide photographed upside-down. Stacked photographs by Louis Boumans.

Most 28S sequences and the majority of the COI sequences for European specimens were produced in Oslo (see below); part of the COI data was produced at the sequencing facility of the Canadian Centre for DNA Barcoding in Guelph and retrieved from the Barcode of Life Data System (BOLD) (cf. Ratnasingham & Hebert, 2007) in the framework of the barcoding project 'NorBol - Freshwater Insects'. These specimens are all deposited at the Natural History Museum, University of Oslo (ZMUN). Sequence and collecting data for the other North

American species, three Finnish *A. palmeni* specimens and the 28S sequences of *N. cinerea* and *A. sulcicollis* were retrieved from the online databases of BOLD and NCBI GenBank (details in Annex 1).

180 COI sequences of *A. linda* were publicly available from BOLD and NCBI in July 2012. These all are from Churchill, Manitoba in Canada, and represent two haploclades with little internal variation (cf. Zhou *et al.*, 2010). In order to facilitate calculations and visualisation, a single representative of each haploclade was included in our study. Likewise, for presentation purposes, only eight representative COI sequences of *A. delosa* from Maryland were included in the data matrix (as well as one from Pennsylvania).

DNA extraction, amplification and sequencing. For the sequence data produced in Oslo, we extracted DNA from the head or head plus prothorax using either the GeneMole DNA Tissue Kit and DNA extraction robot, or Qiagen DNeasy Blood and Tissue Kit, following the manufacturers' protocols. Skeleton parts were not crushed but retrieved after DNA extraction and stored with the remainder of the specimen.

COI was amplified with the primers LCO1490-L and HCO2198-L (Nelson *et al.*, 2007). 28S nuclear ribosomal DNA was amplified with the primers Road1a and Road4b (Crandal *et al.*, 2000; Whiting, 2002). PCR amplifications were set up in a 10 µl reaction volume containing 0.5 µl template, 0.3 µl of each primer 10 µM, 0.8 µl dNTP mix (2.5 µM of each nucleotide), 0.05 µl TaKaRa Ex Taq polymerase, 1 µl TaKaRa PCR buffer and 7.05 µl H2O. Temperature regimes were as follows. COI: initial denaturation at 94°C for 1', followed by 32 cycles of denaturation at 94°C for 45", annealing at 45°C for 45" and extension at 72°C for 45", and a final extension step at 72°C for 5'. For 28S: initial denaturation at 95°C for 2', followed by a step-down regime of 1 cycle of denaturation at 95°C for 40", annealing at 55°C for 40" and extension at 72°C for 1'30", 1 cycle with annealing at 52°C and 32 cycles with annealing at 48°C, and a final extension step at 72°C for 5'. PCR products were purified using ExoSAP-IT (Stratagene).

Part of the purified PCR products was sequenced using the ABI BigDye Terminator Cycle Sequencing Kits v3.1 (Applied Biosystems) following basically the manufacturer's instructions. Cycle sequencing products were cleaned using Sephadex (GE Healthcare) and subsequently analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) at the Natural History Museum in Oslo. Another part of the amplicons was sequenced externally at the sequencing facility ABI-lab of the University of Oslo.

Sequence analysis. Sequences were aligned with BioEdit version 7.1.3.0 (Hall, 1999) and Clustal X version 2.0.10 (Larkin *et al.*, 2007). COI and 28S were first analysed separately in order to evaluate the convergence of both data sets, and because of the narrower taxon sampling in the 28S matrix. COI haplotype differentiation within and between clades was calculated in Paup* 4.0b10 as both uncorrected p distance and Kimura two-parameter (K2P) distance in order to facilitate comparison with published distances in other taxa.

Distance and parsimony analyses were performed in Paup*; Bayesian analyses in MrBayes (Ronquist & Huelsenbeck, 2003) version 3.2. Models of sequence evolution were selected according to the Akaike information criterion implemented in MrModelTest 2.2 (Nylander, 2004) commands for Paup*.

Distance measures for the neighbour joining method in Paup* were based on the models selected with MrModeltest for the COI and 28S data (Table 1). We carried out heuristic searches under both optimality criteria (distance and parsimony) with tree bisection-reconnection branch swapping and 100 random addition sequence replicates. Bootstrapping (2000 replicates) was performed to obtain support values for branches.

TABLE 1. Evolution models used in Bayesian and distance-based phylogeny estimation.

data set	model	gamma shape value	p invariable	analysis
COI non-partitioned	GTR+I+G	1.8029	0.6308	distance
28S	GTR+I	NA	0.7804	distance
COI 1st and 2nd position	GTR+I	NA	NA	Bayesian
COI 3rd position	GTR+G	NA	NA	Bayesian
28S	GTR+I	NA	NA	Bayesian

For Bayesian analysis, the COI data were divided into two partitions, viz. a) 1st and 2nd codon position, and b) 3rd codon position, for which separate models were selected. 28S data were not portioned into stem and loop segments in view of the small number of substitutions in this data set. The selected models are listed in Table 1.

We ran two independent analyses consisting of four Markov chains that ran for 40×10^6 generations, sampled every 1000 generations, default priors, and the option "prset ratepr" set as "variable". After discarding the first 10 million generations, remaining trees from both analyses were combined and a 50% majority rule consensus tree was calculated. MrBayes and Tracer v1.5.0 (Drummond & Rambaut, 2007) were used to inspect trace plots and convergence diagnostics (standard deviation of split frequencies < 0.01 , effective sample size > 200) in order to ensure that the Markov chains had reached stationarity and converged on the parameter estimates and tree topology after the burn-in phase that was set at 25%. Calculations in MrModeltest, Paup* and MrBayes were performed at the Bioportal computer facility (http://www.bioportal.uio.no) at the University of Oslo, Norway.

In addition to separate analyses, COI and 28S sequences were concatenated for the taxa included in both sets. The concatenation was done with help of FASconCAT software (Kück & Meusemann, 2010). The two concatenated sequences stemmed from the same individual, except for *A. sulcicollis*, *A. nigritta* and *N. cinerea*. The combined matrix was analysed in MrBayes with three data partitions (28S, COI 1st and 2nd, and COI 3rd position), evolution models as in Table 1 and the MrBayes settings as described above for the separate data sets. After exclusion of the faster evolving 3rd codon position, the concatenated matrix was also analysed in Paup* under the parsimony criterion.

Results

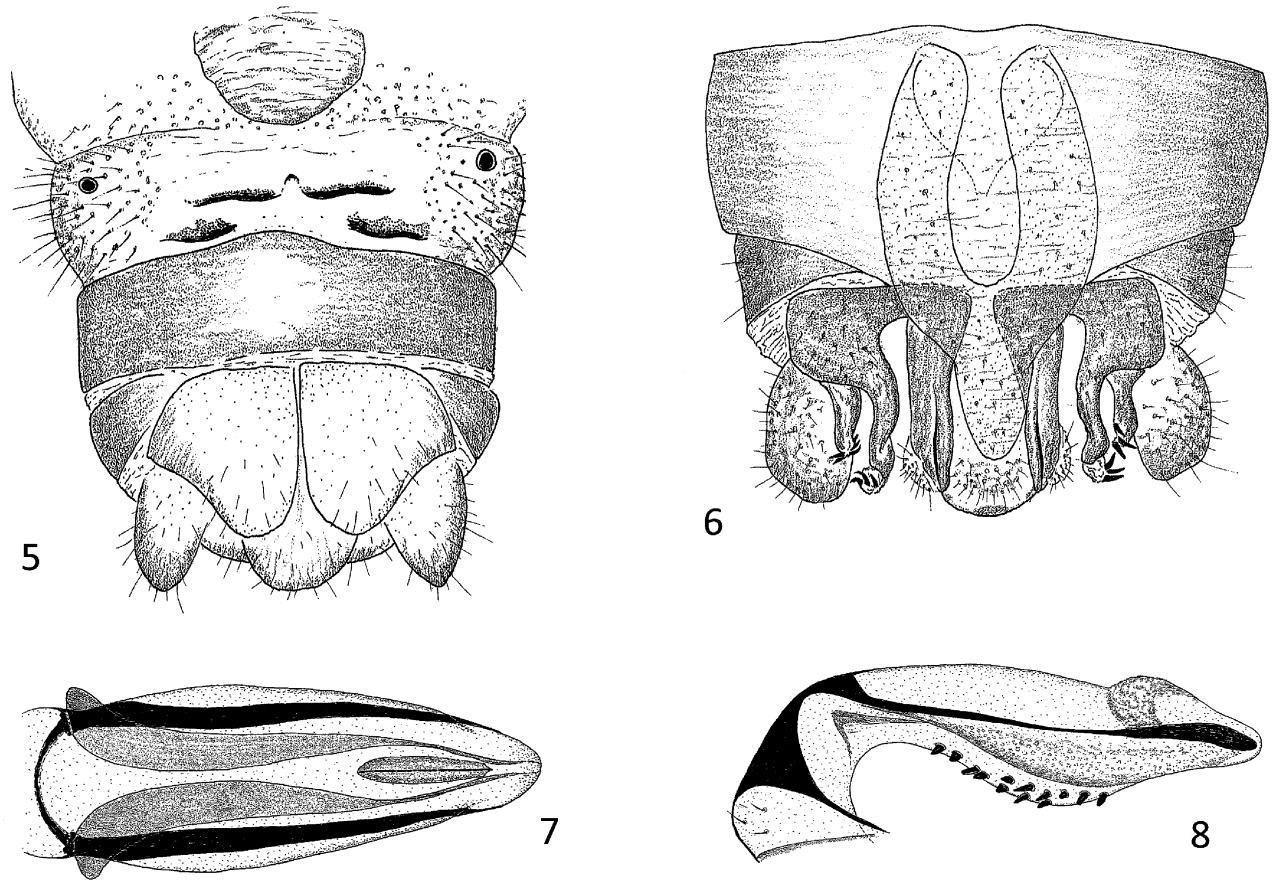
Morphology

The type specimens of Koponen (1917) belong to the same species as described by Tobias (1973). Both the male and the female specimen can be clearly distinguished from *A. standfussi*. In the male, the placement and number of spines on the median lobe of the paraproct provide good diagnostic characters: *A. standfussi* has a field of 8–14 smaller ventrally pointing spines on the central, posterior part of the lobe, and a second group of 3–4 outward pointing spines on the apex (i.e. dorsal). See Figs. 4 and 21; cf. also fig. 6 in Tobias (1973), figs. 1–4 in Lillehammer (1974) and fig. 74D in Tierno de Figueroa *et al.* (2003: 175). The median lobe of *A. palmeni* bears on the central part 2–6 ventrally pointing spines that are larger than in *A. standfussi*, in addition to 3–4 outward pointing spines at the apex. See Figs. 3, 6, 12, 13 and 20, and fig. 5 in Tobias (1973). The outer lobe of the paraproct bears 3–7 spines at the apex in both species.

Secondly the outer lobe of the paraproct is roundish in posterior view in *A. standfussi* and L-shaped in *A. palmeni* (Figs. 20–21; not visible in the slide preparation of the type specimen; cf. figs. 5 and 6 in Tobias 1973 and fig. 3 in Lillehammer 1974). This character is useful under lower magnification, but can be misinterpreted if not viewed at the right angle. A third character is the shape of the epiproct in lateral view: the epiproct of *A. standfussi* is knife-shaped (Fig. 18), whereas it has a pre-distal dorsal hump in *A. palmeni* (Figs. 8, 10 and 17). See also figs. 4–5 in Tobias (1973) and figs. 144–145 in Lillehammer (1988: 93). However, this is a variable character because the hump is partly caused by a patch of hairs that is sometimes bulged upward and sometimes not. Moreover, some *A. standfussi* individuals also have a (less pronounced) dorsal bulge (Fig. 19), so that this character, if used on its own, can be misleading. In the examined collection (Annex 1), *A. standfussi* males with a slightly bulged epiproct were found only in northern Norway.

The females of *A. standfussi* and *A. palmeni* are distinguished by the different shapes of the subgenital plate. *Amphinemura standfussi* has a pair of lobe-shaped vaginal lobes, which are unpigmented and unsclerotised. To both sides of this pair is a smaller, usually sclerotised lobe. In *A. palmeni*, the vaginal lobes are fused with the neighbouring lobes, forming a single pair of broad, square pigmented and sclerotised lobes. In addition, the posterior edge of the 8th sternite bears a dark sclerotised, medially interrupted ridge. See Figs. 2, 5, 14, 22 and fig. 7 in Tobias (1973). In *A. standfussi* this ridge is not sclerotised and therefore not clearly distinguishable (Fig. 23).

We have examined all available specimens from Scandinavia and throughout North America and do not find any consistent morphological differences between *A. palmeni* and *A. linda*.



FIGURES 5–8. *Amphinemura palmeni* from Alberta, Canada: 5: Female abdomen, ventral view. 6: Male abdomen in ventral view. 7: Epiproct, dorsal view. 8: Epiproct, lateral view. Line drawings by Dagmar Tobias.

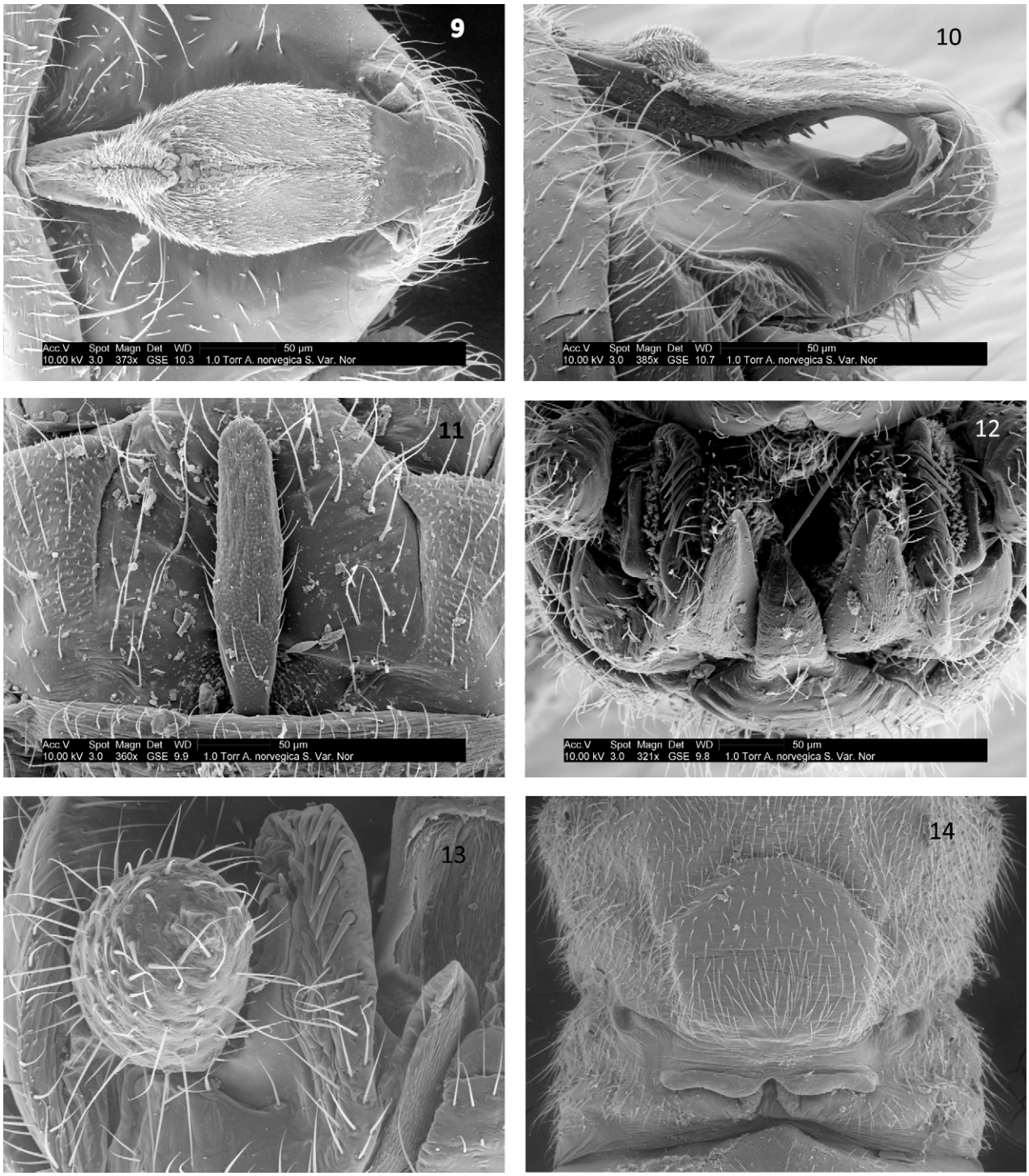
Molecular data

The COI alignment contains a total of 653 characters, of which 190 are parsimony-informative and 440 constant. The 28S alignment contains 796 characters, including 45 parsimony-informative and 715 constant bases. Figs. 24 and 25 show phylogenetic trees of both data sets with bootstrap support values and Bayesian posterior probabilities shown on the branches. Note that, as both the number of markers and our taxon sampling within the large genus *Amphinemura* were limited, these trees are only meant to illustrate the relative positions of *A. linda*, *A. palmeni* and *A. standfussi*.

For the twelve taxa included in the combined 28S + COI data matrix, the inferred tree for the combined data set has exactly the same topology as for 28S alone and full statistical support for all nodes above the species level. The support values are displayed in Fig. 25.

While neither the taxon sampling nor the number of characters is sufficient for phylogeny reconstruction, the COI data set supports the monophyly of the species included, with one exception: *Amphinemura delosa* and *A. nigritta* are not distinguished, and the sequences retrieved from GenBank appear to belong to a single species.

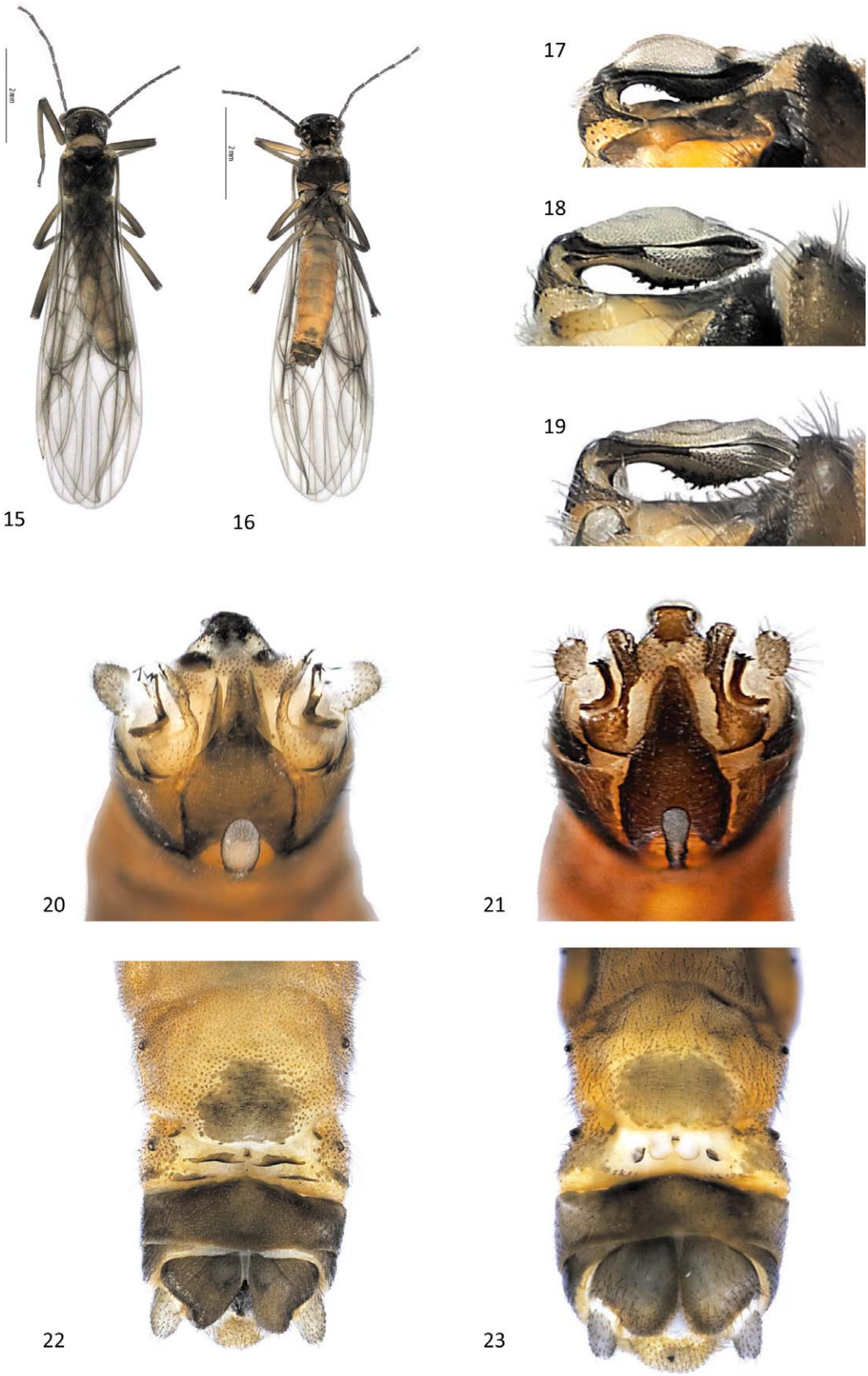
The monophyly of the nine sequences of *A. wui* has 81% bootstrap support in parsimony, and 92% in distance analysis, which both identify *A. appalachia* as the sister clade of *A. wui* (not shown in Fig. 24). This monophyly is not retrieved in the Bayesian analysis. Bayesian analysis also supports a monophyletic clade (*A. appalachia* + *A. wui*) but does not resolve the position of *A. appalachia* within this clade. The tree diagram output by MrBayes (Fig. 24) suggests that *A. appalachia* is embedded in the *A. wui* clade, but note that this interpretation is both lacking statistical support in Bayesian analysis and contradicted by MP and distance analysis (not shown).



FIGURES 9–14. *Amphinemura palmeni* 9: Epiproct, dorsal. 10. Epiproct, lateral. 11. Vesicle. 12. Male terminalia, posterior view. 13. Left cercus and paraproct. 14. Female pregenital and subgenital plate. 9–12 specimen from Sør-Varanger, Norway, 13–14 specimens from Dunnings Spring, Iowa. SEM micrographs by Michael Standing.

The 28S data (Fig. 25) and the 1st and 2nd codon position in COI indicate deep genetic diversification within *Amphinemura*: The distances between some species of this genus are almost as large as between *N. cinerea* and the *Amphinemura* species. (In COI, saturated substitution makes the 3rd codon position unsuitable for evaluating the deeper relationships in our data matrix.)

Table 2 shows pairwise differences between the mitochondrial haplotypes of *A. linda*, *A. palmeni* and *A. standfussi*. The larger distances in *A. linda* (K2P 1.7) are those between Iowa and one of the two lineages found in Manitoba. Sequence divergence between *A. linda* and *A. palmeni* ranges from K2P 3.9 to 5.2, with one of the clades in Manitoba being closest *A. palmeni*.



FIGURES 15–23. 15–16. *Amphinemura palmeni* female, dorsal and ventral habitus Sør-Varanger, Norway. 17. Epiproct, lateral *A. palmeni* from Dunnings Spring, Iowa. 18. Idem, *A. standfussi* from Sør-Varanger. 19. Idem, *A. standfussi* from Skibotn, Norway. 20. Male terminalia, posterior view *A. palmeni* from Dunnings Spring. 21. Idem, *A. standfussi* from Sør-Varanger. 22. Female abdomen, ventral *A. palmeni* from Sør-Varanger. 23. Idem, *A. standfussi* from Sør-Varanger. Photographs by Karsten Sund.

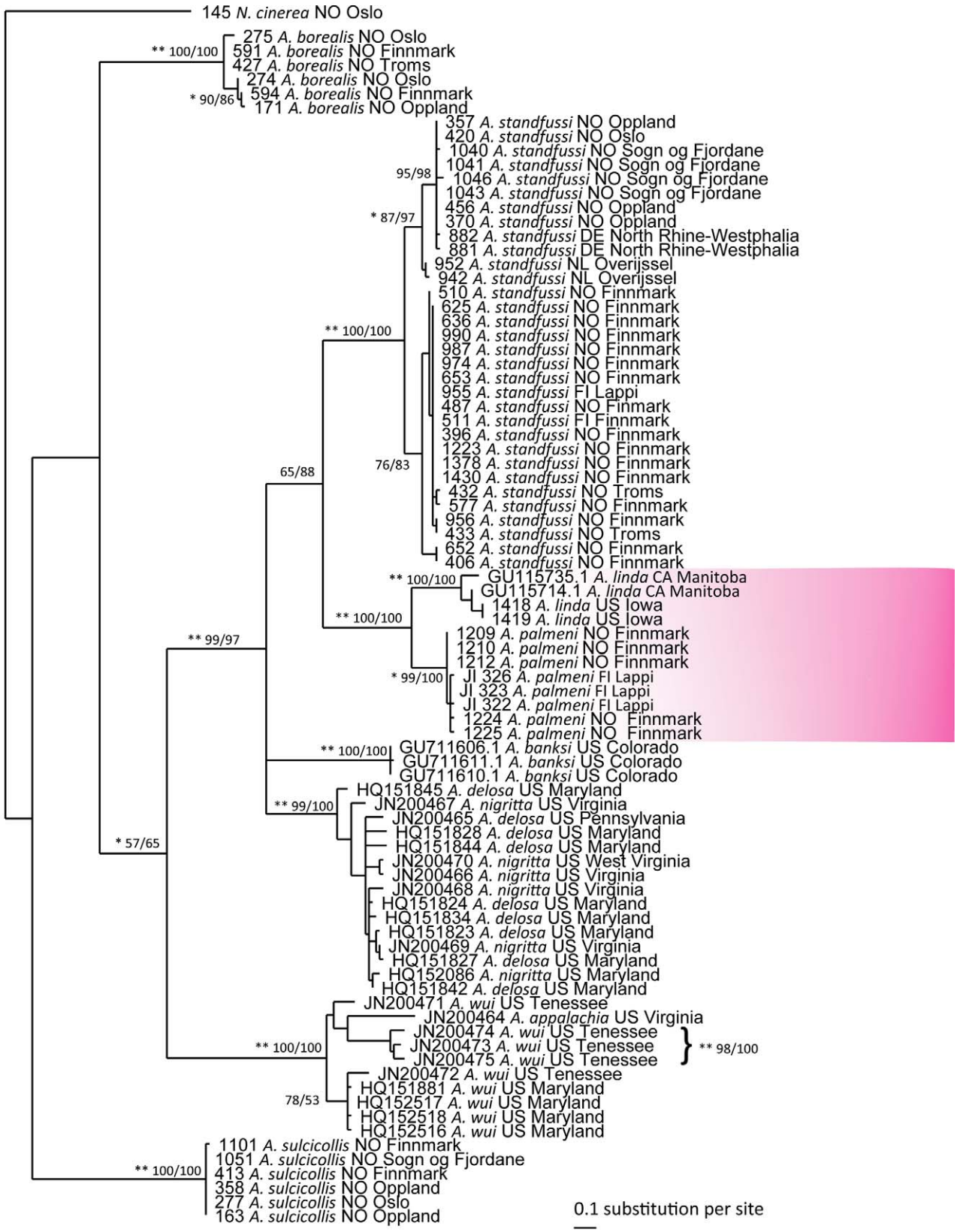


FIGURE 24. Bayesian tree of 84 *Amphinemura* specimens with *Nemoura cinerea* as outgroup, based on a 653 bp fragment of COI. Support values: *, ** indicate Bayesian posterior probability >0.95 and >0.99 respectively; MP and NJ bootstrap percentages are shown in this order separated by a slash. Bootstrap values for minor intraspecific nodes not shown.

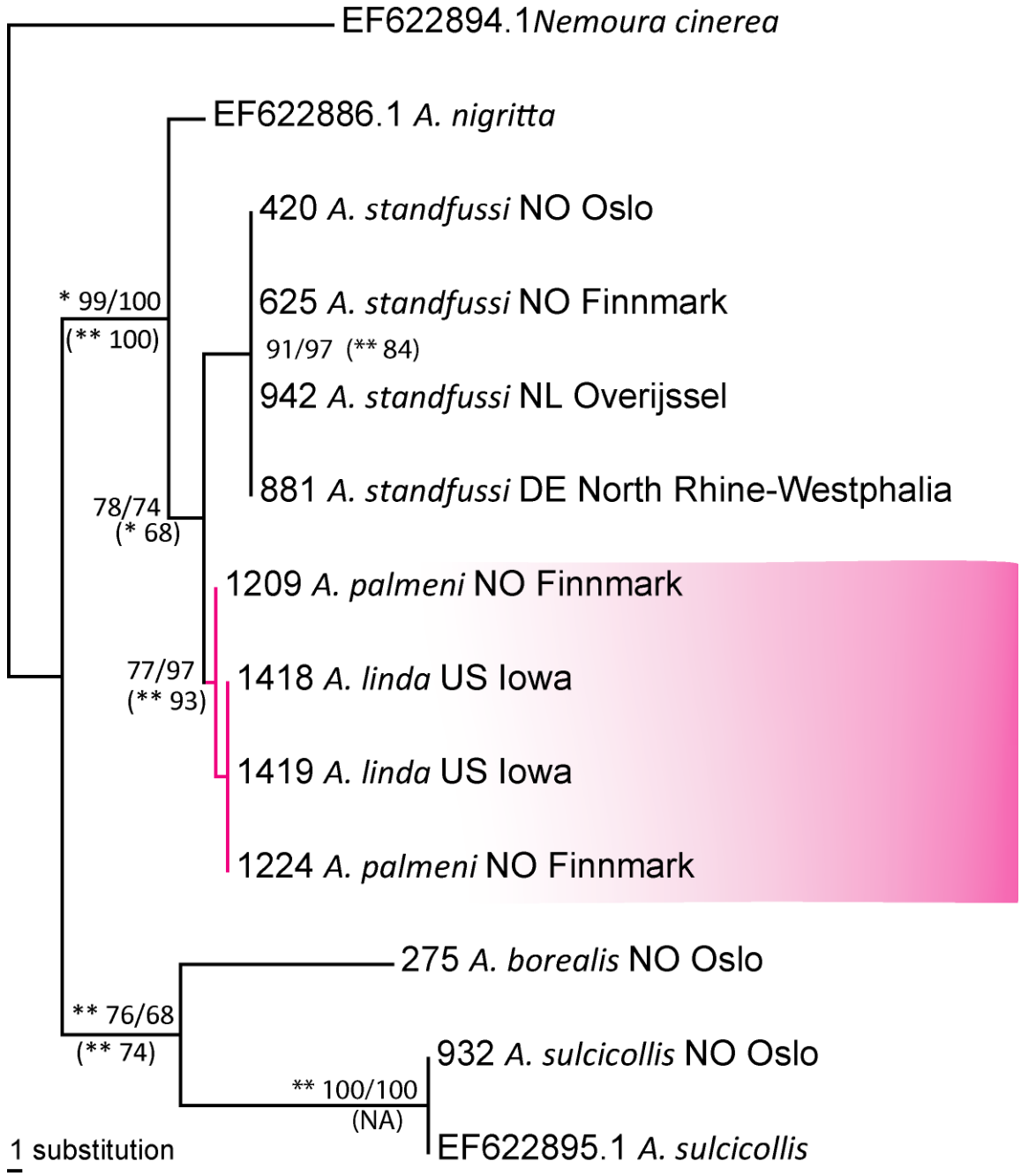


FIGURE 25. Phylogenetic tree of 12 *Amphinemura* specimens with *Nemoura cinerea* as outgroup, based on 796 bp fragment of 28S. Single most parsimonious tree (length 102 steps, CI 0.89, RI 0.88). Support values: above branches: *, ** indicate Bayesian posterior probability >0.95 and >0.99 respectively; MP and NJ bootstrap percentages are shown in this order separated by a slash; below branches and between brackets: Bayesian and MP support values of the tree with the same topology based on concatenated 28S and COI, with exclusion of the 3rd codon position in parsimony analysis. Support for clustering of identical 28S sequences not shown.

The 28S fragment does not distinguish between *A. palmeni* and *A. linda*, but shows a single base difference between the two Norwegian individuals tested. *Amphinemura standfussi* is the closest outgroup species in our data set. The mitochondrial sequences of *A. standfussi* from north-western Europe form two major haploclades, one found in western Europe and southern Scandinavia, the other in northern Scandinavia. Specimens with typical epiprocts (Fig. 18) were found in both clades, but specimens with bulged epiprocts (Fig. 19) all belong to the northern clade. The maximum K2P distance between these two clades is 2.8.

In order to evaluate the genetic distance between *A. linda* and *A. palmeni*, we compared it with intraspecific variation in COI in other *Amphinemura* species. A limitation is that none of the species was sampled over its entire or even a large geographic range. Nonetheless, pairwise K2P distances up to 6.0 are found among *A. wui* individuals from a single locality in the Great Smoky Mountains National Park, in Tennessee.

TABLE 2. Pairwise differences between the haplotypes of *A. linda*, *A. palmeni* and *A. standfussi*. The upper rows are uncorrected p distances, the lower K2P distances (italicized).

	A. linda	*A. palmeni*	*A. standfussi*
A. linda	0.0–1.7%		
	0–1.7		
A. palmeni	3.7–5.0%	0.0–0.3%	
	3.9–5.2	*0.0–0.3*	
A. standfussi	8.8–10.8%	7.5–9.3%	0.0–2.8%
	9.4–11.6	*7.9–9.7*	*0.0–2.8*

Discussion

The description and drawings of Koponen (1917) appear to belong to *A. standfussi* rather than *A. palmeni.* Such a mistake seems unlikely, however, because Koponen was very familiar with *A. standfussi*, an ubiquitous species in Finland, and he erroneously referred to the same specimens as *A. triangularis* (Ris, 1902) in a previous publication (Koponen, 1914). Inspection of the type specimens has identified *A. palmeni* as a valid species and *A. norvegica* as its junior synonym as stated by Meinander (1975) and Lillehammer (1988).

The minimal genetic distance in COI of 3.7% (3.9 K2P) between *A. palmeni* and *A. linda* requires some discussion. Since the early DNA barcoding studies of Hebert *et al.* (2003; 2004) a threshold of 3% is often cited as distinguishing intraspecific and interspecific haplotype variation. However, maximal values for intraspecific distance vary widely, and there are no universal distance-based thresholds (Galtier *et al.*, 2009). Previous studies on stoneflies illustrate this point: On the one hand, Fochetti *et al.* (2009) found very low sequence divergence (max. 1.3% K2P distance in COI) in the stonefly genus *Tyrrhenoleuctra* Consiglio, 1957. This genus encompasses five hypothesised species, some of which are difficult to distinguish on morphological grounds. Commenting on the remarkably low rates of molecular evolution in their study, the authors suggest a peculiar low evolutionary pace in stoneflies compared with other insects. On the other hand, Graf *et al.* (2008) found up to 2.7% divergence for *Siphonoperla montana* (Pictet, 1841) sampled in the eastern Alps. Mynott *et al.* (2011) report minimum interspecific sequence divergences ranging from 7.2% to 19.5% and maximum intraspecific sequence divergences ranging from 0.6% to 5.8% for thirteen species of *Riekoperla* McLellan, 1971 from the alpine areas of New South Wales and Victoria, Australia. Our study shows up to 5.6% distance in *A. wui* from a single collecting site (assuming all specimens were correctly identified), and 2.8% in *A. standfussi* from Scandinavia. High intraspecific variation may result from the low agility of many stonefly species, leading to populations being isolated on relatively small geographic scales.

Considering that the genetic distance between *A. palmeni* and *A. linda* is within the range of intraspecific variation reported for various other stonefly species, while we found no difference in either morphology or 28S, we confirm the position stated in Meinander (1975) and consider also *A. linda* to be a junior synonym. This implies that *A. palmeni* belongs to the group of stoneflies with a Holarctic distribution.

In the Nearctic, *A. palmeni* (as *A. linda*) is widely distributed in Canada and the northern USA (Stark *et al.*, 1986; DeWalt *et al.*, 2012). In the Palaearctic it has only been reported from Fennoscandia, more particularly northernmost Norway and Finland (Meinander, 1975; Lillehammer, 1988; Kuusela, 1996) and the Russian Murmansk oblast (Koponen, 1917). Only few observations have ever been recorded (Boumans, 2011b, 2011a), and *A. palmeni* is listed as vulnerable on the Norwegian Red List (Kjærstad *et al.*, 2010). It has not yet been reported from Sweden, but can probably be found in Swedish Lappland as it has been collected in Finland only two kilometres north of the Swedish border (Jari Ilmonen's collection, see Annex 1). *Amphinemura palmeni* does not figure on the checklists of European Russia (L. A. Zhiltzova, 1966; V. Teslenko & Zhiltzova, 2009), the Russian

Far East (Levanidova & Zhiltzova, 1979; L. Zhiltzova & Zapekina-Dulkeit, 1986; V. A. Teslenko, 2009) or Mongolia (Surenkhorloo, 2009). The record from Latvia (Fochetti & Tierno de Figueroa, 2004) is probably an error. A reliable Plecoptera checklist has not been published for this country (pers. comm. Mārtiņš Kalniņš, February 2012).

The small number of records from northern Europe (and perhaps Asia) may be partly due to the taxonomic confusion that surrounded this species. In the identification key for Fennoscandia (Lillehammer, 1988: 91–94), females of *A. palmeni* are hard to recognise and males can be confused with northern *A. standfussi* specimens with a humped epiproct. The illustrations we present here may be helpful in finding *A. palmeni*, possibly also in the Ural Mountains or some mountain ranges in northern Asia. This would produce a more complete picture of its current distribution in the Palaearctic and its phylogeographic history.

Nymphs of *A. palmeni* have not been described. We note that distinguishing the nymphs of even the two distantly related species *A. standfussi* and *A. sulcicollis* is difficult due to intraspecific variation in the characters currently used in identification keys (Koese, 2008: 78, 112). The latter species are often found together with *A. palmeni* in Scandinavia (Tobias, 1973). Collecting adult specimens is therefore currently the best way to find new localities for *A. palmeni* in the Palaearctic.

Other findings: Our analysis of COI haplotypes shows that *A. standfussi* is one of the stonefly species that colonised the Scandinavian Peninsula from the south as well as the northeast, as suggested by Lillehammer (1988: 27). The failure to distinguish the Nearctic species *A. delosa* and *A. nigritta* with the published COI data calls for further investigation. Since these species are very different morphologically, misidentification of some of the sequenced specimens is a likely explanation.

Acknowledgements

Michael W. Burmingham made a special collecting trip to an isolated spring in Iowa to procure fresh specimens of *A. linda* for DNA studies. The late William E. Ricker loaned paratopotype specimens used in his original description of *A. linda* from Michigan. Klaus Enting, Bram Koese and Pieter Jan Nellestijn helped in various ways to collect samples of *A. standfussi* from Germany and the Netherlands. Bastian Fromm contributed samples from Troms, Norway. Steffen Roth and Torbjørn Ekrem are thanked for their invaluable help during fieldwork in Finnmark in 2010. In addition they provided large numbers of stoneflies from malaise traps set up in that province. Karsten Sund took excellent photographs. Larry Huldén from the Finnish Museum of Natural History in Helsinki was very helpful in finding and sending the type specimens of *A. palmeni*. Mārtiņš Kalniņš provided information on the Latvian stonefly checklist. Juho Paukkunen provided useful information of the type locality of *A. palmeni*. Valentina Teslenko helped with information on the Russian checklists; Judith Osswald advised on the preparation of illustrations with Adobe Illustrator and Hallvard Elven on the use of Tracer. John Brittain and Arild Johnsen gave feedback on the manuscript. Dagmar Tobias allowed us to borrow her paratopotype specimens of *A. norvegica* and also provided her line drawings of *A. linda* from Alberta, Canada. Scanning electron micrographs were taken by Michael Standing at the Brigham Young University, Electron Microscope Laboratory, Provo, Utah. Boris Kondratieff and Jari Ilmonen allowed us to include their unpublished COI barcode sequences of *A. banksi* and *A. palmeni* in our analyses. Jeff Webb, former manager of the EPT section in the barcoding facility was very helpful in facilitating the communication between contributors of the BOLD database. The work in Norway was funded by the Natural History Museum of the University of Oslo.

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APPENDIX 1. Specimens used in molecular analyses. Sex: M = male, F = female; Stage A = adult, N = nymph. Collections: ZMUN = Natural History Museum Oslo (ZMUN), CSU = Colorado State University, Dept. of Bioagri. Sci. and Pest Management, US EPA = United States , Environmental Protection Agency, USNM = National Museum of Natural History, Smithsonian Institution, BIO = Biodiversity Institute of Ontario, Ilmonen = Research Collection of Jari Ilmonen.

taxon	country	state/province	latitude N	longitude E	sex	stage	det	coll.	cat.nr.	COI	28S
A. appalachia	USA	Virginia	36.393	-81.351		A	Boris Kondratieff	USNM		JN200464	
A. banksi	USA	Colorado				A	B. Kondratieff	CSU		GU711610	
A. banksi	USA	Colorado				A	B. Kondratieff	CSU		GU711611	
A. banksi	USA	Colorado				A	B. Kondratieff	CSU		GU711606	
A. borealis	Norway	Oppland	61.57245	9.86139	M	A	L. Boumans	ZMUN	171	JX495647	
A. borealis	Norway	Oslo	59.96982	10.72854	M	A	L. Boumans	ZMUN	274	JX495644	
A. borealis	Norway	Oslo	59.96982	10.72854	F	A	L. Boumans	ZMUN	275	JX495655	JX460909
A. borealis	Norway	Troms	69.2570	20.3850	F	A	L. Boumans	ZMUN	427	JX495646	
A. borealis	Norway	Finnmark	69.82281	23.47890	M	A	L. Boumans	ZMUN	591	JX495640	
A. borealis	Norway	Finnmark	69.2102	23.7620	M	A	L. Boumans	ZMUN	594	JX495660	
A. borealis	Norway	Oslo	59.84615	10.81364	Ex	A	L. Boumans	ZMUN	932	not seq	JX460913
A. delosa	USA	Maryland	39.384	-77.35		N	Istvan Turcsanyi	US EPA		HQ151827	
A. delosa	USA	Maryland	39.201	-77.401		N	Istvan Turcsanyi	US EPA		HQ151828	
A. delosa	USA	Maryland	39.251	-77.281		N	Istvan Turcsanyi	US EPA		HQ151823	
A. delosa	USA	Maryland	39.384	-77.35		N	Istvan Turcsanyi	US EPA		HQ151824	
A. delosa	USA	Maryland	39.111	-77.29		N	Istvan Turcsanyi	US EPA		HQ151834	
A. delosa	USA	Maryland	39.111	-77.29		N	Istvan Turcsanyi	US EPA		HQ151842	
A. delosa	USA	Maryland	39.251	-77.2812		N	Istvan Turcsanyi	US EPA		HQ151844	
A. delosa	USA	Maryland	39.251	-77.2812		N	Istvan Turcsanyi	US EPA		HQ151845	
A. delosa	USA	Pennsylvania	42.717	-79.572		A	Boris Kondratieff	USNM		JN200465	
A. linda	USA	Iowa	43.31127	-91.79021	F	A	R. Baumann	ZMUN	1418	JX460956	JX460917
A. linda	USA	Iowa	43.31127	-91.79021	F	A	R. Baumann	ZMUN	1419	JX460957	JX460918
A. linda	Canada	Manitoba	58.754	-93.949		A	R. Edward Dewalt	BIO		GU115714	
A. linda	Canada	Manitoba	58.754	-93.949		A	R. Edward Dewalt	BIO		GU115735	
A. nigritta	USA	West Virginia			M	A	Boris Kondratieff	USNM		JN200470	
A. nigritta	USA	Virginia	38.062	-79.224		A	Oliver S. Flint, Jr.	USNM		JN200466	
A. nigritta	USA	Virginia	37.075	-81.214		A	Oliver S. Flint, Jr.	USNM		JN200467	
A. nigritta	USA	Virginia	38.594	-77.152		A	Oliver S. Flint, Jr.	USNM		JN200468	
A. nigritta	USA	Virginia	38.594	-77.152		A	Oliver S. Flint, Jr.	USNM		JN200469	
A. nigritta	USA	Maryland	38.511	-77.0291		N	Istvan Turcsanyi	US EPA		HQ152086	

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APPENDIX 1. (continued)

taxon	country	state/province	latitude N	longitude E	sex	stage	det	coll.	cat.nr.	COI	28S
A. nigritta											EF622886.1
A. palmeni	Norway	Finnmark	69.30357	29.26322	M	A	L. Boumans	ZMUN	1209	JX460949	JX460915
A. palmeni	Norway	Finnmark	69.30357	29.26322	F	A	L. Boumans	ZMUN	1210	JX460950	
A. palmeni	Norway	Finnmark	69.30357	29.26322	M	A	L. Boumans	ZMUN	1212	JX460951	
A. palmeni	Norway	Finnmark	69.21324	29.15357	F	A	L. Boumans	ZMUN	1224	JX460953	JX460916
A. palmeni	Norway	Finnmark	69.21324	29.15357	M	A	L. Boumans	ZMUN	1225	JX460954	
A. palmeni	Finland	Lappi	68,44799805	22,58200073	M	A	Aki Rinne	Ilmonen		JX495649	
A. palmeni	Finland	Lappi	68,48000336	22,10400009	M	A	Jari Ilmonen	Ilmonen		JX495656	
A. palmeni	Finland	Lappi	68,44799805	22,58200073	F	A	Aki Rinne	Ilmonen		JX495653	
A. standfussi	Norway	Oppland	61.4177	8.8940	F	A	L. Boumans	ZMUN	357	JX460920	
A. standfussi	Norway	Oppland	61.40147	8.89218	M	A	L. Boumans	ZMUN	370	JX495635	
A. standfussi	Norway	Finnmark	70.43792	30.88085	M	A	L. Boumans	ZMUN	396	JX460922	
A. standfussi	Norway	Finnmark	70.17757	28.61053	M	A	L. Boumans	ZMUN	406	JX460923	
A. standfussi	Norway	Oslo	60.01754	10.78501	F	A	L. Boumans	ZMUN	420	JX460924	JX460910
A. standfussi	Norway	Troms	69.3260	20.3650	M	A	L. Boumans	ZMUN	432	JX460925	
A. standfussi	Norway	Troms	69.3260	20.3650	M	A	L. Boumans	ZMUN	433	JX495648	
A. standfussi	Norway	Oppland	61.4025	8.8050	M	A	L. Boumans	ZMUN	456	JX495651	
A. standfussi	Norway	Finnmark	70.02787	23.39469	M	A	L. Boumans	ZMUN	487	JX495642	
A. standfussi	Norway	Finnmark	69.44852	23.68362	M	A	L. Boumans	ZMUN	510	JX495662	
A. standfussi	Norway	Finnmark	69.44852	23.68362	M	A	L. Boumans	ZMUN	511	JX460930	
A. standfussi	Norway	Finnmark	69.44852	23.68362	F	A	L. Boumans	ZMUN	577	JX460931	
A. standfussi	Norway	Finnmark	70.32584	25.40609	F	A	L. Boumans	ZMUN	625	JX460932	JX460911
A. standfussi	Norway	Finnmark	70.21086	24.88571	F	A	L. Boumans	ZMUN	636	JX460933	
A. standfussi	Norway	Finnmark	70.17757	28.61053	M	A	L. Boumans	ZMUN	652	JX460934	
A. standfussi	Norway	Finnmark	70.34693	25.43628	M	A	L. Boumans	ZMUN	653	JX495657	
A. standfussi	Germany	North Rhine-Westphalia	50.4281	6.5194	M	A	K. Enting	ZMUN	881	JX460936	JX460912
A. standfussi	Germany	North Rhine-Westphalia	50.4281	6.5194	M	A	K. Enting	ZMUN	882	JX460937	
A. standfussi	Netherlands	Overijssel	52.43182	6.89482	F	A	L. Boumans	ZMUN	942	JX460938	JX460914
A. standfussi	Netherlands	Overijssel	52.43182	6.89482	F	A	L. Boumans	ZMUN	952	JX495652	
A. standfussi	Finland	Lappi	69.45461	26.29408	M	A	B. Koese	ZMUN	955	JX495643	

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APPENDIX 1. (continued)

taxon	country	state/province	latitude N	longitude E	sex	stage	det	coll.	cat.nr.	COI	28S
A. standfussi	Finland	Lappi	69.45461	26.29408	F	A	B. Koese	ZMUN	956	JX495634	
A. standfussi	Norway	Finnmark	70.44121	26.80466	M	A	L. Boumans	ZMUN	974	JX495636	
A. standfussi	Norway	Finnmark	70.43792	30.88085	M	A	L. Boumans	ZMUN	987	JX495654	
A. standfussi	Norway	Finnmark	69.26932	29.11891	F	A	L. Boumans	ZMUN	990	JX495639	
A. standfussi	Norway	Sogn og Fjordane	61.4356	6.7800	M	A	Sivertsen, Mossestad, Stok	ZMUN	1040	JX460945	
A. standfussi	Norway	Sogn og Fjordane	61.4356	6.7800	F	A	Sivertsen, Mossestad, Stok	ZMUN	1041	JX460946	
A. standfussi	Norway	Sogn og Fjordane	61.4356	6.7800	M	A	Sivertsen, Mossestad, Stok	ZMUN	1043	JX495638	
A. standfussi	Norway	Sogn og Fjordane	61.2747	7.1557	F	A	Sivertsen, Mossestad, Stok	ZMUN	1046	JX495659	
A. standfussi	Norway	Finnmark	69.21324	29.15357	F	A	L. Boumans	ZMUN	1223	JX460952	
A. standfussi	Norway	Finnmark	69.44497	29.89904	F	A	L. Boumans	ZMUN	1378	JX460955	
A. standfussi	Norway	Finnmark	69.20992	23.75766	F	A	L. Boumans	ZMUN	1430	JX460958	
A. sulcicollis	Norway	Oppland	61.56366	9.90555	F	A	L. Boumans	ZMUN	163	JX495650	
A. sulcicollis	Norway	Oslo	59.96982	10.72854	M	A	L. Boumans	ZMUN	277	JX495655	
A. sulcicollis	Norway	Oppland	61.4177	8.8940	F	A	L. Boumans	ZMUN	358	JX495637	
A. sulcicollis	Norway	Finnmark	70.47263	25.07285	F	A	L. Boumans	ZMUN	413	JX495645	
A. sulcicollis	Norway	Sogn og Fjordane	61.2750	7.1557		N	Sivertsen, Mossestad, Stok	ZMUN	1051	JX495641	
A. sulcicollis	Norway	Finnmark	69.16986	29.00162	M	A	L. Boumans	ZMUN	1101	JX495658	
A. sulcicollis											EF622895.1
A. wui	USA	Maryland	39.6061	-76.5913		N	Istvan Turcsanyi	US EPA		HQ151881	
A. wui	USA	Maryland	39.688	-76.7509		N	Istvan Turcsanyi	US EPA		HQ152517	
A. wui	USA	Maryland	39.688	-76.7509		N	Istvan Turcsanyi	US EPA		HQ152518	
A. wui	USA	Maryland	39.688	-76.7509		N	Istvan Turcsanyi	US EPA		HQ152516	
A. wui	USA	Tennessee	35.452	-83.124		A	Boris Kondratieff	USNM		JN200471	
A. wui	USA	Tennessee	35.408	-83.277		A	Boris Kondratieff	USNM		JN200472	
A. wui	USA	Tennessee	35.452	-83.124		A	Boris Kondratieff	USNM		JN200473	
A. wui	USA	Tennessee	35.452	-83.124		A	Oliver S. Flint, Jr.	USNM		JN200474	
A. wui	USA	Tennessee	35.452	-83.124		A	Boris Kondratieff	USNM		JN200475	
N. cinerea	Norway	Oslo	59.88221	10.83824	M	A	L. Boumans	ZMUN	145	JX495661	
N. cinerea											EF622894.1