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Molecular phylogeny of the Raphidiidae (Raphidioptera)*

ELISABETH HARING[1,4], HORST ASPÖCK[2], DANIELA BARTEL[1,4] and ULRIKE ASPÖCK[3,4]

[1]Laboratory of Molecular Systematics, Museum of Natural History, Vienna, Austria, [2]Department of Medical Parasitology, Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna (MUW), Vienna, Austria, [3]Department of Entomology, Museum of Natural History, Vienna, Austria and [4]Department of Evolutionary Biology, University of Vienna, Vienna, Austria

Abstract. We present a molecular phylogeny of the family Raphidiidae including representatives of 21 of the 26 genera. Sequences from the nuclear gene for the large subunit ribosomal RNA (*28S* rRNA) and the mitochondrial *cytochrome c oxidase subunit 3* gene (*cox3*) were used. For the phylogenetic reconstructions we applied automated and manual approaches for sequence alignment and different evolutionary models and tree building algorithms. The trees based on the two alignment approaches were rather similar in their overall topology. A combination of both marker sequences increased the resolution of the trees. The six clades within the raphidiid family that emerged represent either single genera or groups of genera, namely: (i) the Nearctic genus *Agulla* Navás, (ii) the Nearctic/Central American genus *Alena* Navás, (iii) the Central Asiatic and Eastern Palaearctic genus *Mongoloraphidia* H. Aspöck & U. Aspöck, (iv) the Palaearctic *Puncha* clade, (v) the western Mediterranean *Ohmella* clade, and (vi) the Palaearctic *Phaeostigma* clade. The New World taxa *Agulla* and *Alena* are placed as successive out-groups to a monophyletic Palaearctic clade. The *Mongoloraphidia* clade is distributed in the eastern Palearctic while the remaining three clades are exclusively (*Ohmella* clade) or mainly distributed in the western Palaearctic. The early radiation of extant Raphidiidae is interpreted based on the phylogenetic tree obtained in the present study, and the geological and palaeobiological processes around the K–T boundary.

Introduction

Raphidioptera (snakeflies), together with Megaloptera and Neuroptera, constitute the Neuropterida, which is usually regarded as the sister group of the Coleoptera (with or without Strepsiptera) (Aspöck *et al*., 1991; Grimaldi & Engel, 2005; Cranston & Gullan, 2009; Cameron *et al*., 2009; Wiegmann *et al*., 2009). The position of the Raphidioptera within the Neuropterida is controversial. We have found convincing arguments for Raphidioptera being the sister group of Megaloptera + Neuroptera (Aspöck *et al*., 2001, 2003; Aspöck, 2002b; Haring & Aspöck, 2004), which was recently corroborated by Beutel *et al*. (2010) and Cameron *et al*. (2009). However, other authors have concluded that Raphidioptera is the sister group of Megaloptera (e.g. Beutel & Gorb, 2001; Wiegmann *et al*., 2009). Raphidioptera species are characterized by their long prothorax, their hyaline wings with a distinct pterostigma and the long ovipositor of the females (Aspöck *et al*., 1991; Aspöck & Aspöck, 2009). They are a relic group with only about 240 described extant species in two families: Inocelliidae (seven genera with about 30 species) and Raphidiidae (26 genera with about 210 species). To the uninitiated observer they all look rather similar, as 'living

Correspondence: Ulrike Aspöck, Department of Entomology, Museum of Natural History Vienna, Burgring 7, A-1010 Vienna, Austria. E-mail: ulrike.aspoeck@nhm-wien.ac.at; ulrike.aspoeck@univie.ac.at

*Raphidioptera have been infiltrating our lives for more than 40 years: we know their biology, their glacial refuges, we have pursued them back to the K–T impact and have interpreted them biogeographically, but have nevertheless been unable to crack the code of their global relationships. Jumping now into the molecules implies that we will end up in the deep phylogeny of a relic group – and still Raphidioptera remain a challenge... (Horst and Ulrike Aspöck)

fossils' they have not changed their appearance for almost an eternity: a 140 million year old female from the Jurassic of China and an extant female from Austria look very alike (Aspöck & Aspöck, 2007b).

Both extant families of Raphidioptera are distributed in the arboreal parts of the Northern hemisphere (Fig. 1), but are absent from the eastern and northern parts of North America. Fossil Raphidioptera, however, are known from South America and the eastern parts of North America (besides many places lying within the present distribution of the order). The western Palaearctic harbours the highest number of species. However, most of these are restricted to small areas in the mountain ranges of the peninsulas of Southern Europe, Northwest Africa, Anatolia and a few other parts of the Near East, respectively. No snakefly species are distributed across all three (southern) European peninsulas (i.e. the Iberian, Apennine and Balkan peninsulas), and very few species occur in more than one of them. Snakeflies in general are characterized by an extremely low dispersal capacity. The majority inhabit small areas. Several species exist that have been recorded from a single mountain only (stationary species). Rarely do species occupy large distribution areas. Examples are, on one hand, a few Euro-Sibirian elements with distributions covering large parts of Europe and northern Asia (they are particularly associated with the belt of coniferous forests), and, on the other hand, several Nearctic species occurring along the Rocky Mountains

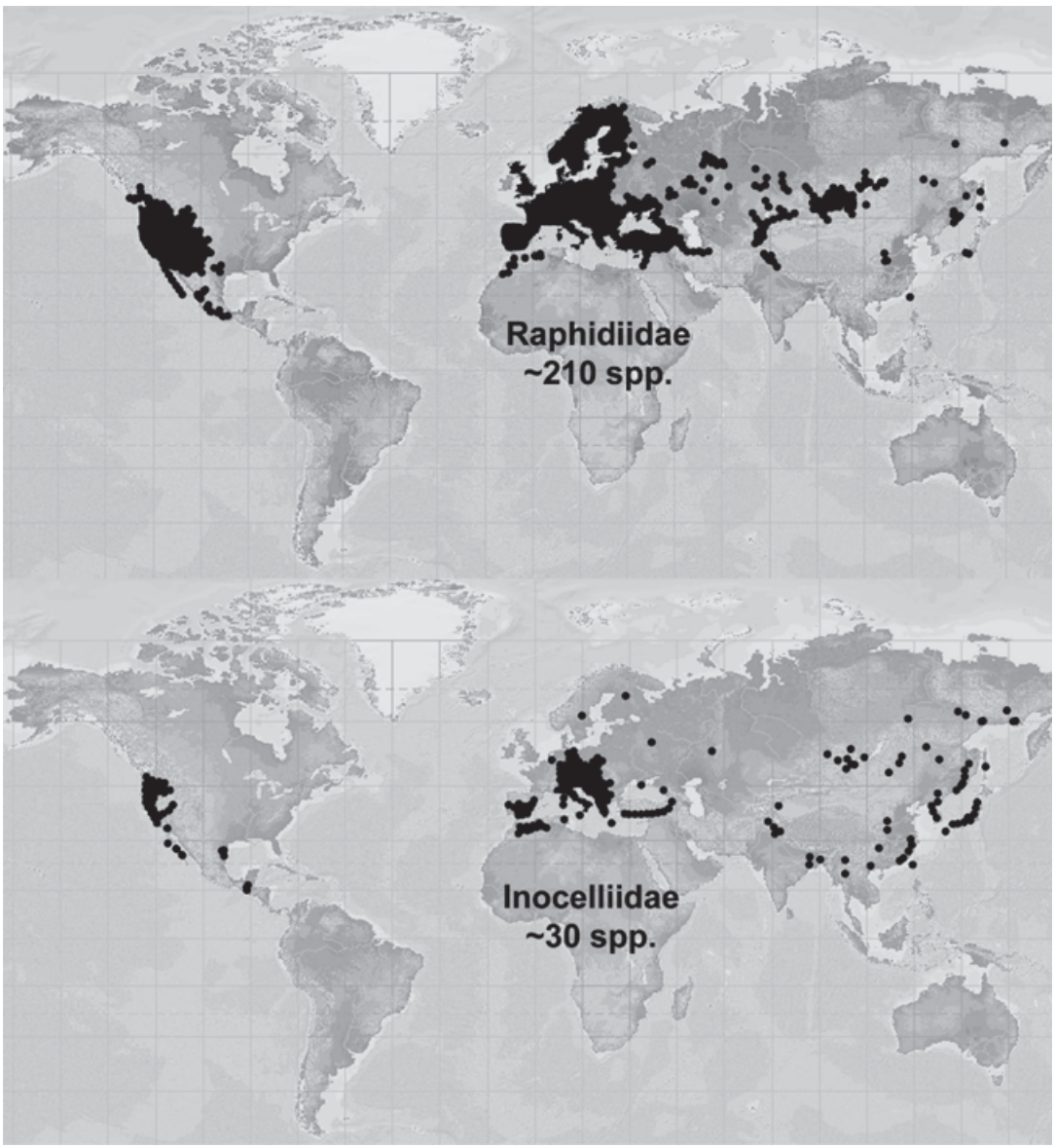

Fig. 1. The world distribution of the order Raphidioptera is characterized by the almost identical distribution areas of the two families, the Raphidiidae and the Inocelliidae.

from the north of Mexico to Southwestern Canada. So far it is unknown why so many Raphidioptera species have such limited distributions, although it seems that identical or at least suitable ecological conditions exist in adjacent regions (Aspöck & Aspöck, 2007a).

All extant Raphidioptera need a cool period for proper development, although the rich Mesozoic snakefly fauna is also known from tropical habitats. This fauna died out at the end of the Cretaceous, probably because of the fundamental climate change after the K–T extinction event (Aspöck, 1998, 1999, 2004; Aspöck & Aspöck, 2007b). However, a part of the snakefly fauna, apparently a branch that was adapted to temperate and cold climates, survived the successive global cooling in the Tertiary. The larvae of Raphidioptera live under bark or in soil, preferably in the detritus around the roots of trees and shrubs. The larval development lasts two or three years in most species, and the larvae can survive extended periods without food. Thanks to these traits they appear to be well adapted for survival under extremely harsh conditions.

In the present paper we focus on the larger family Raphidiidae. In a monographic treatment of the order (Aspöck *et al*., 1991), eight putatively monophyletic groups of Raphidiidae were identified (Figure S1). The genus *Alena* (group VIII) from Mexico and southern U.S.A. was interpreted as the sister group of the rest. The large Asian genus *Mongoloraphidia* (VI) together with *Usbekoraphidia* (V) emerged as the sister group of the remaining genera comprising groups I, II, III, IV and VII. Within this almost exclusively Palaearctic group, the Nearctic *Agulla* (VII) was placed as the presumed sister group of group II from Western Europe. Group II has ever since been a problematic entity, as one of its eight genera, *Ohmella*, shows an amazing similarity with the Nearctic genus *Agulla.*

Recently a new species of *Alena* (subgenus *Aztekoraphidia*) was discovered in Mexico. This new species induced a new interpretation of the genital sclerite known as hypovalva (gonapophyses 9) as an amalgam of hypovalva + parameres (gonocoxites + gonapophyses complex 10) in *Alena*, as well as in the Palaearctic genera *Hispanoraphidia* (Iberian Peninsula) and *Harraphidia* (Iberian Peninsula and Northern Africa) (Aspöck & Contreras Ramos, 2004). This homology hypothesis contradicts the placement of *Alena* as a sister to all other Raphidiidae (Aspöck & Aspöck, 2007c).

In summary, because of the lack of sufficient informative morphological traits, the phylogenetic relationships among the genera of Raphidiidae remain controversial, especially with respect to the systematic positions of *Alena* and *Agulla*, as well as their relationships with the western Palaearctic genera *Ohmella*, *Hispanoraphidia* and *Harraphidia*.

In the present study we aim to establish a molecular phylogeny of the Raphidiidae, including 21 of the 26 genera. The study is based on the nuclear *28S rRNA* gene (*28S*) and the mitochondrial *cytochrome c oxidase subunit 3* (*cox3*) gene. Furthermore, using *28S* as a marker sequence, which is not protein coding, and thus contains several sections that are highly length variable, we tested different alignment procedures to assess the consequences with respect to the resolution of the ensuing phylogenetic trees.

Materials and methods

Sampling

The specimens analysed are listed in Table 1. Our set of samples comprises most genera of the family Raphidiidae with the exception of *Mauroraphidia* H. Aspöck, U. Aspöck & Rausch, 1983, *Africoraphidia* U. Aspöck & H. Aspöck, 1969, *Iranoraphidia* H. Aspöck & U. Aspöck, 1975, *Tauroraphidia* H. Aspöck, U. Aspöck & Rausch, 1982, and *Tadshikoraphidia* H. Aspöck & U. Aspöck, 1968, from which no material for sequencing was available from the authors. We tried to include two or more taxa of each genus (for at least one of the two marker genes), or, if this was not possible, to analyse two individuals of the same taxon to confirm authenticity and to make sure that no contamination had occurred. Most tissue samples were taken from ethanol-preserved adult specimens (64 individuals), but also one pupa and six larvae were sampled. Tissue samples were taken from the thorax (wing muscles) with sterile forceps. Vouchers are stored at the Entomological Department of the Museum of Natural History Vienna (NHMW). Remaining DNA extractions are stored at the laboratory of Molecular Systematics at the NHMW.

DNA extraction, PCR, cloning and sequencing

DNA extraction was performed using the DNeasy Blood & Tissue Kit (Quiagen) according to the manufacturer's instructions. The final volume of elution buffer was 50 μL. DNA solutions were stored in aliquots to avoid too frequent thawing. Control extractions with pure extraction buffer (without tissue) were prepared. PCR was carried out with an Eppendorf Thermocycler in a volume of 25 μL, containing 1 unit Dynazyme DNA polymerase (Finnzymes Oy), 1 μM of each primer and 0.2 mM of each deoxyribonucleotide triphosphate. The solutions were heated to 95°C (2 min) and then put through 30 reaction cycles: 95°C (10 s), annealing temperature (10 s), 72°C (1 min/1 kb expected length), followed by a final extension at 72°C (5 min). Control reactions to detect contaminations were carried out with: (i) control 'extractions' (without sample) instead of the template, and (ii) with distilled water instead of the template. PCR products were extracted from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen), and were either sequenced directly or cloned (TOPO TA Cloning Kit; Invitrogen). Sequencing of both cloned PCR products and gel-purified PCR products (both strands) was performed by AGOWA (Berlin, Germany).

Amplification of marker sequences

One nuclear and one mitochondrial marker sequence were used: (i) a partial sequence of the *cox3* gene that has also been

Table 1. Specimens investigated in the course of the present study and marker sequences with their GenBank accession numbers.

Taxon	Lab-code	Material	Geographic origin	Sequences	Accession numbers
Raphidiidae Latreille, 1810					
***Alena* Navás, 1916**					
Alena distincta (Banks, 1911)	Aledis-1	ad., EtOH	U.S.A., California, San Bernadino Co., Long Pine Canyon, 2005	cox, 28S-sf	HM543277, HM543380
	Aledis-2	ad., EtOH	U.S.A., California, San Bernadino Co., Long Pine Canyon, 2005	cox, 28S	HM543278, HM543341
Alena horstaspoecki U. Aspöck & Contreras-Ramos, 2004	Alehor-1	ad., EtOH	Mexico, Hidalgo, Huasca, 2003	cox, 28S	HM543279, HM543342
Alena americana (Carpenter, 1958)	Aleame-1	P, EtOH	Mexico, Morelos, Sierra de Tepoztlan, 1991	28S	HM543343
Alena infundibulata U. Aspöck, H. Aspöck & Rausch, 1994	Aleinf-1	L, EtOH	Mexico, Oaxaca, Sierra de Miahuatlan, 1991	cox	HM543280,
***Agulla* Navás, 1914**					
Agulla adnixa (Hagen, 1861)	Aguadn-1	ad., EtOH	U.S.A., Idaho, Latah Co., Moscow, 2008	cox	HM543272
	Aguadn-2	ad., EtOH	U.S.A., Idaho, Latah Co., Moscow, 2008	cox	HM543273
	Aguadn-3	ad., EtOH	U.S.A., Idaho, Latah Co., Moscow, 2008	cox	HM543274
	Aguadn-5	ad., EtOH	U.S.A., Idaho, Latah Co., Moscow, 2005	cox, 28S	HM543275, HM543340
	Aguadn-6	ad., EtOH	U.S.A., Idaho, Latah Co., Moscow, 2005	cox	HM543276
***Atlantoraphidia* H. Aspöck & U. Aspöck, 1968**					
Atlantoraphidia maculicollis (Stephens, 1836)	Atlmac-3	ad., EtOH	U.K., Surrey, Kew, Royal Botanic Gardens, 2007	cox, 28S	HM543281, HM543354
	Atlmac-4	ad., EtOH	Portugal, Viseu, Serra de Montemuro, 2007	cox, 28S	HM543282, HM543355
***Calabroraphidia* Rausch, H. Aspöck & U. Aspöck, 2004**					
Calabroraphidia renate Rausch, H. Aspöck & U. Aspöck, 2004	Calren-1	ad., EtOH	Italy, Calabria, Sila Grande, Fossiata, 2005	cox	HM543283
	Calren-2	ad., EtOH	Italy, Calabria, Sila Grande, above Fossiata, 2006	cox, 28S	HM543284, HM543357
***Dichrostigma* Navás, 1909**					
Dichrostigma flavipes (Stein, 1863)	Dicfla-1	ad., EtOH	Austria, Lower Austria, Dürnstein, 1999	cox	AY620050[a]
	Dicfla-2	ad., EtOH	Austria, Lower Austria, Dürnstein, 2005	cox	HM543285
	Dicfla-3	ad., EtOH	Austria, Lower Austria, Dürnstein, 2005	cox	HM543286
	Dicfla-4	ad., EtOH	Austria, Lower Austria, Dürnstein, 2005	cox	HM543287
	Dicfla-5	ad., EtOH	Austria, Lower Austria, Dürnstein, 2005	cox, 28S	HM543288, HM543378
***Harraphidia* Steinmann, 1963**					
Harraphidia laufferi (Navás, 1915)	Harlau-2	ad., EtOH	Portugal, Guarda, E Pinzio, (=NE Guarda), 2007	cox, 28S	HM543290, HM543352
***Hispanoraphidia* H. Aspöck & U. Aspöck, 1968**					
Hispanoraphidia castellana (Navás, 1915)	Hiscas-2	ad., EtOH	Portugal, Serra da Gardunha, above Alcongosta, 2007	cox, 28S	HM543291, HM543353
***Italoraphidia* H. Aspöck & U. Aspöck, 1968**					
Italoraphidia solariana (Navás, 1928)	Itasol-1	ad., EtOH	Italy, Calabria, Sila Grande, Fossiata, 2005	cox, 28S	HM543293, HM543356
	Itasol-2	ad., EtOH	Italy, Calabria, Sila Grande, Fossiata, 2006	cox	HM543294
***Mongoloraphidia* H. Aspöck & U. Aspöck, 1968**					
Mongoloraphidia botanophila H. Aspöck, U. Aspöck & Rausch, 1997	Monbot-1	ad., EtOH	Kyrgyzstan, Dzhalal-Abadskaya, Chatkal'skyi Khrebet, ESE of Passes Chap-Chyma, 1998	cox, 28S	HM543295, HM543349

Table 1. Continued.

Taxon	Lab-code	Material	Geographic origin	Sequences	Accession numbers
Mongoloraphidia kaspariani H. Aspöck, U. Aspöck & Rausch, 1983	Monkas-1	ad., EtOH	Kyrgyzstan, Dzhalal-Abadskaya, Chatkal'skyi Khrebet, Sary-Chelek, 1996	cox	HM543296
Mongolaraphidia monstruosa (H. Aspöck, U. Aspöck & Martynova, 1968)	Monmon-1	ad., EtOH	Kyrgyzstan, Dzhalal-Abadskaya, Ferganskiy Khrebet, Kara-Suu, 1995	cox, 28S	HM543297, HM543344
	Monmon-2	ad., EtOH	Kyrgyzstan, Dzhalal-Abadskaya, Ferganskiy Khrebet, Kara-Suu, 1995	cox, 28S-sf	HM543298, HM543381
Mongoloraphidia nomadobia H. Aspöck, U. Aspöck & Rausch, 1996	Monnom-1	dry	Kyrgyzstan, Alaikuu, near Eshigart, 2004	28S	HM543347
	Monnom-2	dry	Kyrgyzstan, Alaikuu, near Eshigart, 2004	cox, 28S	HM543299, HM543348
Mongoloraphidia nurgiza H. Aspöck, U. Aspöck & Rausch, 1997	Monnur-1	L, EtOH	Kyrgyzstan, Talasskaya, Khrebet Talasskiy, Alatau, 1995	cox, 28S	HM543300, HM543350
Mongoloraphidia pusillogenitalis (H. Aspöck, U. Aspöck & Martynova, 1968)	Monpus-1	ad., EtOH	Kyrgyzstan, Oshskaya Oblast, 25 km N Uzgen, 1996	cox, 28S	HM543301, HM543346
Mongoloraphidia sejde H. Aspöck, U. Aspöck & Rausch, 1995	Monsej-1	ad., EtOH	Kyrgyzstan, Dzhalal-Abadskaya, Khrebet Talasskiy Alatau, 1995	cox	HM543302
Mongoloraphidia manasiana H. Aspöck, U. Aspöck & Rausch, 1997	Monman-1	ad., EtOH	Kyrgyzstan, Chatkal	cox	AY620048[a]
Mongoloraphidia tienshanica H. Aspöck, U. Aspöck & Rausch, 1997	Montie-1	ad., EtOH	Kyrgyzstan, Dzhalal-Abadskaya, Chatkal'skyi Khrebet, ESE Chap-Chyma, 1998	cox, 28S	HM543303, HM543345
***Ohmella* H. Aspöck & U. Aspöck, 1968**					
Ohmella baetica bolivari (Navás, 1915)	Ohmbae-1	ad., EtOH	Portugal, Serra da Gardunha, above Alcongosta, 2007	cox, 28S	HM543305, HM543351
***Ornatoraphidia* H. Aspöck & U. Aspöck, 1968**					
Ornatoraphidia flavilabris (Costa, 1855)	Ornfla-1	ad., EtOH	Italy, Emilia-Romagna, Passo della Colla, 2005	cox, 28S	HM543306, HM543373
	Ornfla-2	ad., EtOH	Italy, Emilia-Romagna, Passo della Colla, 2005	cox	HM543307
	Ornfla-3	ad., EtOH	Italy, Calabria, Sila Grande, above Fossiata, 2006	cox	HM543308
***Parvoraphidia* H. Aspöck & U. Aspöck, 1968**					
Parvoraphidia microstigma (Stein, 1863)	Prvmic-1	L, EtOH	Greece, Phokis, NE Krokilio, 2006	cox, 28S	HM543319, HM543366
***Phaeostigma* Navás, 1909**					
Phaeostigma cyprica (Hagen, 1867)	Phacyp-1	ad., EtOH	Cyprus, SW Dhierona, Limassol-Forest, 2001	cox	HM543310
Phaeostigma grandii (Principi, 1960)	Phagra-1	ad., EtOH	Italy, Abruzzo, Montenerodomo, 2005	cox	HM543311
	Phagra-2	ad., EtOH	Italy, Abruzzo, Montenerodomo, 2005	cox, 28S	HM543312, HM543369
	Phagra-3	ad., EtOH	Italy, Abruzzo, Montenerodomo, 2005	cox, 28S	HM543313, HM543370
	Phagra-4	L, EtOH	Italy, Abruzzo, Montenerodomo, 2007	cox, 28S	HM543314, HM543371
Phaeostigma italogallica (H. Aspöck & U. Aspöck, 1976)	Phaita-1	ad., EtOH	Italy, Calabria, Sila Grande, Fossiata, 2006	cox	HM543315
	Phaita-2	ad., EtOH	Italy, Calabria, Sila Grande, above Fossiata, 2006	cox, 28S	HM543316, HM543367
	Phaita-3	L, EtOH	Italy, Abruzzo, Passo del Diavolo, 2007	cox	HM543317
Phaeostigma notata (Fabricius, 1781)	Phanot-1	ad., EtOH	Austria, Lower Austria, Dürnstein, 2005	cox, 28S	HM543318, HM543368

Table 1. Continued.

Taxon	Lab-code	Material	Geographic origin	Sequences	Accession numbers
***Puncha* Navás, 1915**					
Puncha ratzeburgi (Brauer, 1876)	Punrat-1	ad., EtOH	Austria, Lower Austria, Eichkogel, 2005	cox	HM543320
	Punrat-2	ad., EtOH	Austria, Lower Austria, Eichkogel, 2005	cox, 28S	HM543321, HM543358
***Raphidia* Linnaeus, 1758**	**Linnaeus, 1758**				
Raphidia ligurica Albarda, 1891	Raplig-1	ad., EtOH	Italy, Calabria, Sila Grande, Fossiata, 2006	cox, 28S	HM543322, HM543377
	Raplig-2	ad., EtOH	Italy, Calabria, Sila Grande, Fossiata, 2006	cox	HM543323
***Subilla* Navás, 1916**	**Navás, 1916**				
Subilla confinis (Stephens, 1836)	Subcon-1	L, living	Austria, Upper Austria, Maria Neustift, Hofberg, 2000	cox	AY620049[a]
	Subcon-2	ad., EtOH	Austria, Lower Austria, Klosterneuburg, 2005	cox, 28S	HM543324, HM543374
	Subcon-3	ad., EtOH	Austria, Lower Austria, Klosterneuburg, 2005	cox, 28S	HM543325, HM543375
***Tjederiraphidia* H. Aspöck, U. Aspöck & Rausch, 1985**					
Tjederiraphidia santuzza (H. Aspöck, U. Aspöck & Rausch, 1980)	Tjesan-1	ad., EtOH	Italy, Calabria, Aspromonte, W Montalto, 2006	cox	HM543326
	Tjesan-2	ad., EtOH	Italy, Calabria, Aspromonte, Gambarie –Montalto 2006	cox, 28S	HM543327, HM543365
***Turcoraphidia* H. Aspöck & U. Aspöck, 1968**					
Turcoraphidia amara (H. Aspöck & U. Aspöck, 1964)	Turama-1	ad., EtOH	Romania, Transylvania, above Rimetea, 2007	cox, 28S	HM543328, HM543372
***Ulrike* H. Aspöck, 1968**					
Ulrike syriaca (Steinmann, 1964)	Ulrsyr-1	ad., EtOH	Cyprus, SW Dhierona, Limassol-Forest, 2001	cox, 28S	HM543329, HM543376
	Ulrsyr-2	ad., EtOH	Cyprus, SW Dhierona, Limassol-Forest, 2001	cox	HM543330
***Venustoraphidia* H. Aspöck & U. Aspöck, 1968**					
Venustoraphidia nigricollis (Albarda, 1891)	Vennig-2	ad., EtOH	Italy, Calabria, Aspromonte, Scido, 2006	cox, 28S	HM543331, HM543363
	Vennig-3	ad., EtOH	Germany, Bavaria, Schweinfurt, Werneck, 2005	28S	HM543364
***Xanthostigma* Navás, 1909**					
Xanthostigma aloysiana (Costa, 1855)	Xanalo-1	ad., EtOH	Italy, Calabria, Aspromonte, Gambarie –Bagaladi, near M. Rosso, 2006	cox	HM543332
	Xanalo-2	ad., EtOH	Italy, Calabria, Sila, NW San Giovanni in Fiore, 2006	cox, 28S	HM543333, HM543359
Xanthostigma corsica (Hagen, 1867)	Xancor-1	ad., EtOH	Italy, Calabria, Aspromonte, E Montalto, 2006	cox, 28S	HM543334, HM543361
	Xancor-2	ad., EtOH	Italy, Calabria, Aspromonte, E Montalto, 2006	cox, 28S	HM543335, HM543362
Xanthostigma xanthostigma (Schummel, 1832)	Xanxan-1	ad., EtOH	Austria, Lower Austria, Klosterneuburg, 2005	cox, 28S	HM543336, HM543360
	Xanxan-2	ad., EtOH	Austria, Lower Austria, Klosterneuburg, 2005	cox	HM543337
***Inocelliidae* Navás**					
***Fibla* Navás, 1915**					
Fibla maclachlani (Albarda, 1891)	Fibmac-1	ad., EtOH	Italy, Sardinia, Gennargentu, Fonni, 2003	cox, 28S-sf	HM543289, HM543379

Table 1. Continued.

Taxon	Lab-code	Material	Geographic origin	Sequences	Accession numbers
***Inocellia* Schneider, 1843**					
Inocellia crassicornis (Schummel, 1832)	Inocra-1	ad., EtOH	Austria, Lower Austria, Scheibbs, Ginning, 1999	cox	AY620051[a]
	Inocra-2	ad., EtOH	Austria, Lower Austria, Eichkogel, 2005	cox, 28S	HM543292, HM543338
***Negha* Navás, 1916**					
Negha inflata (Hagen, 1861) "Northern Form" (Aspöck, 1974)	Neginf-1	ad., EtOH	U.S.A., Washington, Kittitas Co., 2005	cox, 28S	HM543304, HM543339
***Parainocellia* H. Aspöck & U. Aspöck, 1968**					
Parainocellia bicolor (Costa, 1855)	Parbic-1	ad., EtOH.	Italy, Abruzzo, Passo del Diavolo, 2005	cox, 28S-Y	HM543309, HM543271

[a] Sequence from Haring & Aspöck (2004).

Abbreviations of specimens are derived from genus/family (first three letters) and species (second three letters) names plus number; L, larva, P, pupa; sf, small fragment; cox, *cox3* gene; 28S, *28S rRNA* gene; 28S-Y, putative pseudogene.

used in a previous study on Neuropterida (Haring & Aspöck, 2004) and (ii) a partial sequence of *28S* spanning the 5′ part of D2 to the 3′ end D7a (notation according to Gillespie *et al*., 2006). The primers were designed in this study (Table 2). The fragment lengths of *cox3* sequences ranged from 703 to 712 bp (depending on the primers used). The annealing temperature for *cox3* primers was 54°C. The *28S* sequence was in general obtained by the amplification of two overlapping fragments produced with the primer pairs Raph28S-1+/Raph28S4− (5′-section) and Raph28S3+/Raph28S6− (3′-section). The overlap between the two fragments was ∼ 700 bp. The complete sequences ranged from ∼ 1.5 to 2 kb in length. Alternative primers used for the amplification of smaller fragments from samples with poor DNA quality are also listed in Table 2. The annealing temperature for *28S* primers was 56°C.

Table 2. Primer sequences (5′–3′).

cox3		
cox3-fw	TAGTTGATTATAGACCATGACC	Haring & Aspöck (2004)
cox3-rev	ACATCAACAAAATGTCAATATCA	Haring & Aspöck (2004)
Raph-cox3fw	TAGTCCATGACCHTTAACAGG	Haring & Aspöck (2004)
28S		
Raph28S-1+	CAGGGGTAAACCTGAGAAA	Present study
Raph28S-2−	ACATGCTAGACTCCTTGGT	Present study
Raph28S-3+	AGCTTTGGGTACTTTCAGGA	Present study
Raph28S-4−	AGCGCCAGTTCTGCTTACC	Present study
Raph28S-5+	ACGTGGAGAAGGGTTTCGT	Present study
Raph28S-6−	GGAATAGGAACCGGATTCCC	Present study
Raph28S-7−	AGGAACCGGATTCCCTTTCG	Present study
Raph28S-10−	TAGGATCGACTGACTCGTGTG	Present study
Raph28S-9−	CCATCCATTTTCAGGGCTA	Present study
Raph28S-8+	ATGGGTGAGATCTCCGGC	Present study

Phylogenetic analyses

Alignment and further processing for the phylogenetic reconstruction were executed for each gene separately. For the *cox3* sequences the alignment was straightforward as there were no insertions or deletions. Reading frames of all sequences proved to be intact, resulting in the correct translation into amino acid sequences. For *28S* sequences, with their extensive length variation, optimal alignment and character choice (exclusion of positions with suspicious homology statements) procedures are needed. There are two main approaches to alignment and character exclusion procedures: manual and computer based (Kjer *et al*., 2007). To test for differences between these strategies, two methods were tested with respect to resolution and node support in the subsequent phylogenetic reconstruction of the phylogeny of Raphidioptera. For the manual approach, a prealignment was performed with ClustalX (Thompson *et al*., 1997) and edited manually in BioEdit v5.0.9 (Hall, 1999). The final alignment was screened for ambiguously aligned sections, which were excluded in the subsequent phylogenetic analyses. For computer-based alignment, we used RNAsalsa beta version 0.8.1 (Stocsits *et al*., 2009) with subsequent automated character exclusion according to the output of aliscore (Misof & Misof, 2009). RNAsalsa is a new structural alignment tool that includes already known rRNA secondary structure information into the alignment process of sequences from taxa with unknown structure. Conserved structural features are checked via primary sequence variation and the detection of consistent and compensatory substitutions. Subsequently, highly variable regions within these genes are folded by minimizing free-energy algorithms. The result is an individual secondary structure for each sequence. In the last step a final alignment is done by taking both structure and sequence information of each position into account. For a more detailed discussion of RNAsalsa we refer to Stocsits *et al.* (2009).

Sequence and secondary structure predictions for *Apis mellifera* (Gillespie *et al*., 2006) was incorporated as constraints into the alignment process. All sequences were prealigned with MAFFT v6 using default settings (Katoh & Toh, 2008; MAFFT v6, http://align.bmr.kyushu-u.ac.jp/mafft/online/server). The prealignment, primary sequence and secondary structure information of *Apis mellifera* were used as the input file in RNASALSA (beta version 0.8.1). The alignment was performed using default parameter values for substitution costs, gap penalties and base pairing occurrence stringency.

Character exclusion

ALISCORE v02 (Misof & Misof, 2009) was chosen for the identification of randomness in the alignment generated with RNASALSA. ALISCORE identifies randomly similar sections in multiple sequence alignments based on pairwise comparison within a sliding window and a Monte Carlo resampling approach. A sliding window size of $w = 4$ was used, gaps were treated as ambiguities ($-N$ option) and the maximum number of possible random pairwise comparisons ($-r$ option) was analysed. Both RNASALSA and ALISCORE can be freely downloaded from the homepage of the Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany (http://www.zfmk.de).

Nucleotide frequency estimation

Non-stationarity of nucleotide frequencies among taxa is observed frequently in *28S* data sets. After the exclusion of randomly similar sections and MP uninformative sites, the alignment was checked for non-stationarity of nucleotide frequencies using the χ^2 test, implemented in PAUP (*4.0 beta10; Swofford, 2002). The final alignments used for tree calculations can be downloaded from the internet site http://www.nhm-wien.ac.at/pub/Raphidioptera_Alignments_for_download.zip.

Tree reconstruction

To infer the phylogenetic relationships, Bayesian analyses were performed on the data sets using MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001), applying the GTR + γ model of sequence evolution for nucleotide sequences according to the Akaike information criterion, as implemented in the program MRMODELTEST v2.2 (Nylander *et al*., 2004). For the *cox3* nucleotide data set the model parameter was set to nucmodel = codon (code metmt). For the analyses based on deduced amino acid sequences of *cox3*, the model parameter was set to mixed (aamodel), in order to determine the most probable model of protein evolution during the analyses, and a γ correction was used with four categories. The extent of invariant sites was not estimated during the Bayesian analyses, as it was shown that this parameter strongly correlates with the estimation of the shape parameter of the gamma distribution (Yang, 1996; Kelchner & Thomas, 2007). In combined data sets all parameters were unlinked and the rates were set to vary freely under a flat Dirichlet prior. For all analyses two independent runs starting with random trees were performed for at least 2 million generations (sometimes up to up to 6 million, depending on time constrains), each with four Markov chains, and with a sampling frequency of every 100th generation. Model parameter values were treated as unknown and estimated separately in each run. The trees generated prior to stationarity were discarded as burn-in and were not included in the calculation of the consensus trees.

In addition, other tree-building algorithms were employed, the results of which can be found in the Supporting Information. The distance-based neighbor-joining algorithm (Saitou & Nei, 1987), and equally weighted maximum parsimony (MP) analyses for amino acid, nucleotide and combined datasets were conducted with PAUP (Swofford, 2002). For the MP analyses based on *28S*, gaps were treated as missing characters. MP trees were calculated using heuristic search with the tree bisection reconnection branch-swapping algorithm, with a random taxon addition sequence (1000 replicates). Nodal support was evaluated with nonparametric bootstrapping, based on 1000 replicates with ten random-addition sequences and a tree bisection reconnection branch-swapping algorithm. Concerning support values, we would like to emphasize that posterior probability values of Bayesian analyses cannot be directly compared with bootstrap values, and that the two types of values must be interpreted differently. Moreover, as Simmons *et al.* (2004) outlined, Bayesian support values should not be interpreted as probabilities that clades are correctly resolved. However, support values are the only a priori criterion to evaluate a tree representing an unknown phylogeny.

The sequences are deposited at GenBank under the accession numbers listed in Table 1. Out-group sequences from GenBank: *28S*, *Sialis hamata* (AY521793); *cox3*, *Crioceris duodecimpunctata* (CriduoGB; Coleoptera; AF467886), *Sialis lutaria* (Sialut-1; Megaloptera; AY620054), *Corydalus* sp. (Corspe-1; Megaloptera; AY620052) and *Archichauliodes guttiferus* (Arcgut-1; Megaloptera; AY620199).

Results

Marker sequences, samples and out-group selection

The *cox3* sequence was obtained from 70 individuals (Table 1). A section of the *28S* sequence was determined for 41 individuals representing Inocelliidae (four genera) and Raphidiidae (21 genera) (Table 1). The *28S* sequence from one species (*Parainocellia bicolor*, Parbic-1) was highly aberrant, with several smaller and larger deletions. We did not succeed in isolating another sequence from this individual. The same sequence was obtained in four independent approaches, either from an amplification product using the external primers or from two overlapping fragments. As this sequence can be considered as a putative pseudogene (28S-Y in Table 1), we excluded it from further analyses. For three individuals only the 5′ part of the *28S* sequence could be

amplified: *Alena distincta* (Aledis-1), *Mongolaraphidia monstruosa* (Monmon-2) and *Fibla maclachlani* (Fibmac-1). The first two proved to be almost identical to sequences of other individuals of the same species (Monmon-1 and Aledis-2, respectively; data not shown). The *28S* sequence of *Fibla maclachlani* (Fibmac-1) appeared most closely related to *Negha inflata* (Neginf-1), a finding also confirmed by the *cox3* tree. The *28S* sequence of three individuals of *Alena americana*, *Mongoloraphidia nomadobia* and *Venustoraphidia nigricollis* (Aleame-1, Monnom-1 and Vennig-3, respectively) was not included in the final data set of the comparative analysis, because the *cox3* sequences could not be obtained from them. The *28S* sequences (Monnom-1 and Vennig-3) proved to be very similar to that of each conspecific specimen (Monnum-2 and Vennig-2), with some substitutions occurring mainly in the highly variable sections. Among the *Alena 28S* sequences, *Alena americana* (Aleame-1) is most similar to *Alena horstaspoecki* (Alehor-1) (data not shown).

In the first analysis of the *28S* data set a species of the related order Megaloptera (*Sialis hamata*) was used as the out-group. However, because of the high divergence of *Sialis hamata* no unambiguous alignment could be obtained (data not shown). Nevertheless, in this tree the family Inocelliidae branched off from the basal node with high support values (the overall topology of the remaining tree being roughly the same as in the following analyses). The same result was obtained with the *cox3* data set using one coleopteran sequence (*Crioceris duodecimpunctata*) as out-group, and also including three sequences from megalopteran species (families Corydalidae and Sialidae). Despite the fact that there is low resolution within the family Raphidiidae, the branching pattern is clear concerning the first split within the Raphidioptera: the family Inocelliidae appears as the sister group to all remaining Raphidioptera, with high support (Figure S2). Therefore, for all further tree calculations, the family Inocelliidae was used as the out-group.

Phylogenetic relationships among Raphidioptera

The alignment of *cox3* had a length of 658 nucleotides. The final *28S* alignments, after removing the variable regions, had lengths of 1676 (manual) and 1667 nucleotides (automated approach). The *cox3* trees are based on a somewhat larger sample (taxa and individuals): two more taxa of the family Inocelliidae are included (*Parainocellia bicolor* and *Fibla maclachlani*), *Agulla* is represented by five individuals, and several other genera are represented by additional species (*Alena infundibulata*, *Phaeostigma cyprica*, *Mongoloraphidia kaspariani*, *Mongoloraphidia sejde* and *Mongoloraphidia tienshanica*).

The *cox3* data set was intended to provide phylogenetic information at lower taxonomic levels (within genera or between closely related genera), whereas the *28S* sequences should resolve deeper nodes. However, the *cox3* distances were surprisingly high, even within genera (Table S1). For example, intrageneric p-distances between species of *Mongoloraphidia* range from 6.2 to 13.5%, within *Phaeostigma* the range is 7.4–16.1% and within *Xanthostigma* it is 13.5–14.9%. Comparing these distances with those between genera (10.4–19.0%) it becomes clear that the poor resolution in the trees based on *cox3* is the result of sequence saturation. These intergeneric distances overlap even with the range of distances between the Raphidiopteran families Inocelliidae and Raphidiidae (18.1–23.7%). This is also illustrated in the *cox3* tree (Figure S2). Moreover, the paraphyly of the genera *Phaeostigma* and *Xanthostigma*, which was found in several of the trees, might also result from saturation in the *cox3* sequences.

On the basis of a sample of sequences from individuals of which both sequences were available, we calculated trees with the two marker sequences (*cox3* and *28S*). Besides the model-based Bayesian analysis, we also calculated trees using other tree-building methods (MP and neighbour-joining analyses). Comparisons of results from the various algorithms, alignment procedures and gene combinations are given in Table S2. Concerning the protein coding *cox3* gene, we also compared the performance between nucleotide and deduced amino acid sequences. Although a high proportion of the trees obtained quite low support values for several nodes (bootstrap percentages and Bayesian posterior probability values), all trees are in general very similar concerning the division in main lineages: the first split (node 1) separates the genus *Agulla* from the remaining taxa, followed by the genus *Alena* (node 2). The remaining four lineages represent: the genus *Mongoloraphidia* (node 3); a clade combining *Puncha*, *Italoraphidia*, *Calabroraphidia*, *Venustoraphidia* and *Xanthostigma* (‘*Puncha* clade’, node 4); a clade comprising *Ohmella, Harraphidia*, *Hispanoraphidia*, *Atlantoraphidia* (‘*Ohmella* clade’, node 5); and a clade comprising the remaining genera *Phaeostigma*, *Dichrostigma*, *Ornatoraphidia*, *Parvoraphidia*, *Raphidia*, *Subilla*, *Tjederiraphidia*, *Turcoraphidia* and *Ulrike* (‘*Phaeostigma* clade’, node 6). The relationships between the latter four clades must be considered as an unresolved tetratomy, as in most trees support of the respective nodes was low. Table S2 summarizes the results indicating the support values of those nodes in the various analyses. Support values of main nodes representing the *Agulla* clade and the *Alena* clade (i.e. the monophyly of these genera) are not included in Table S2, as they were always maximal (100%, 1.00).

The tree with the best support values (posterior probability values for all six nodes = 1), was the Bayesian inference tree based on combined *cox3* (DNA) and *28S* (manual alignment) sequences (Fig. 2). It has the same topology as the corresponding tree based on automated alignment, but node support in the latter tree is lower for nodes 2 and 5, whereas nodes 1, 3, 4 and 6 obtained the highest support in both trees. With respect to the internal branching of clades, both trees have the same topology within the *Mongoloraphidia* clade and the *Ohmella* clade. Differences are found within the *Phaeostigma* and *Puncha* clades; however, within these clades support values are rather low in the tree based on automated alignment.

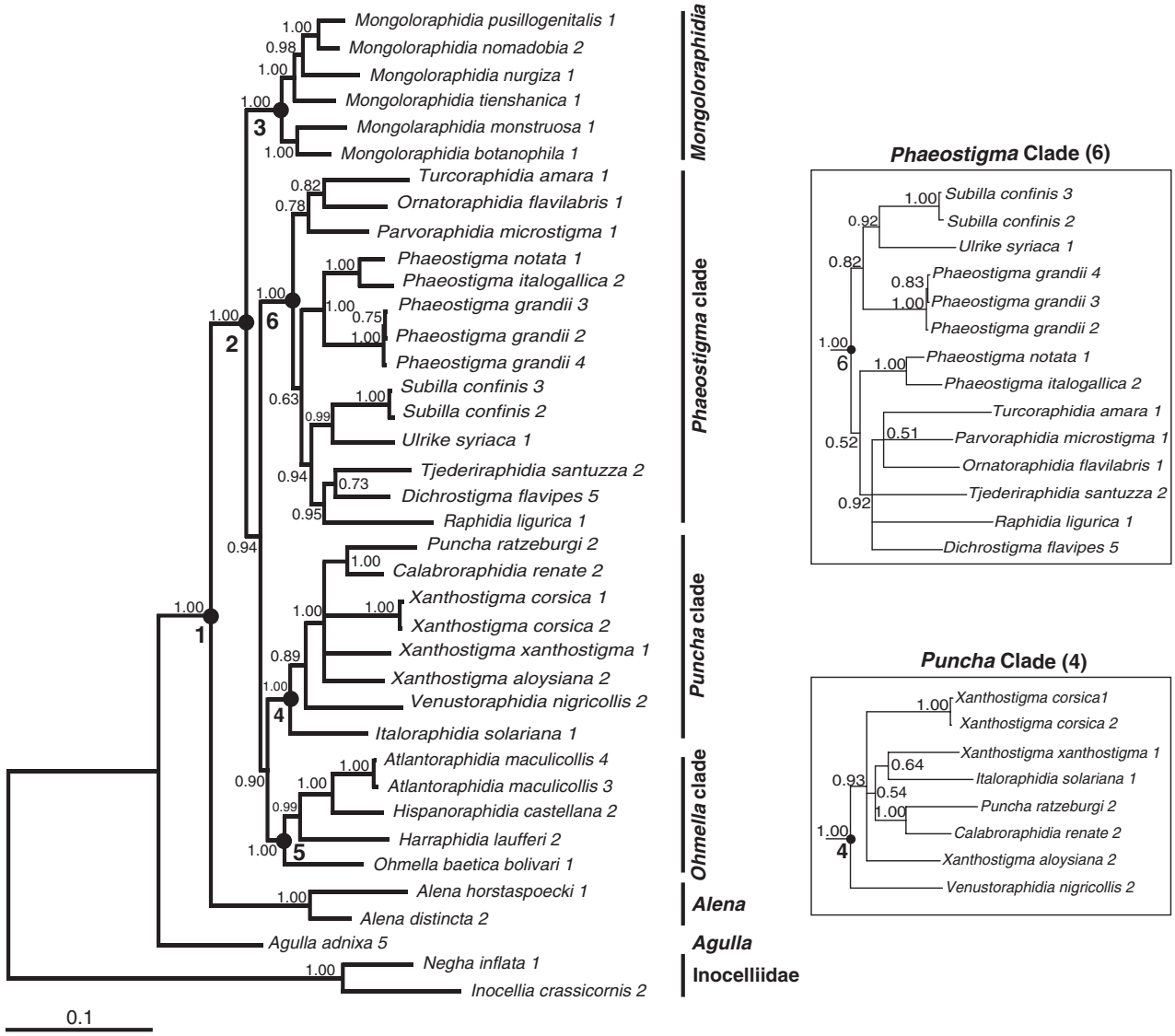

Fig. 2. Bayesian inference tree based on combined *cox3* (DNA) plus *28S* sequences. Nodes mentioned in the text and in Table S2 are indicated. The partial trees on the right show the topological changes in the trees obtained from automated alignment.

Discussion

The phylogeny presented here represents the first DNA-based hypothesis of the phylogenetic relationships of Raphidiidae. Altogether, Bayesian inference analysis of the combined data set (*cox3* plus *28S*) yielded the best resolution, suggesting that the saturation effects of *cox3* are to some degree compensated for by the more conserved (and also longer) *28S* sequences. However, sequence saturation, as especially apparent in the analyses of the *cox3* data set, might not be the only reason for the poorly supported relationships among clades 3–6. Another possibility is rapid cladogenesis in the early evolution of the Palearctic lineage. This is suggested by the longer branches leading to the deeper nodes (1 and 2), compared with the structure of the remaining nodes (3–6).

The manual approach for the *28S* alignment resulted in better node support than the automated approach. One might ask whether preconceived notions of the relationships between taxa might have influenced the manual alignment, thus leading to better supported nodes. However, previous views of raphidiopteran relationships were somewhat vague, and in some respect conflicting, and the resulting topology disagrees in several aspects with previous assumptions. Moreover, a potentially biased alignment does not necessarily result in better node support. The alignment generated by the automated approach was used for tree reconstruction without further adjustment; the same is the case concerning the exclusion of positions, which was performed automatically without any manual corrections.

Morphological synapomorphies supporting nodes in the molecular tree of Raphidiidae

On the basis of morphological characters, the monophyly of the Raphidioptera is well established. In addition, without doubt, both Inocelliidae and Raphidiidae are monophyletic, each characterized by a number of striking morphological, anatomical and biological autapomorphies (Aspöck *et al*., 1991, 2001; Aspöck, 2002a; Aspöck & Aspöck, 2004). The corroboration of this monophyly in the present molecular approach is unsurprising.

The six clades within Raphidiidae that were retrieved by the present molecular analysis represent either single genera or groups of genera (Fig. 2): (i) the Nearctic genus *Agulla*, (ii) the Nearctic/Central American genus *Alena*, (iii) the Central Asiatic and Eastern Palaearctic genus *Mongoloraphidia*, (iv) the Palaearctic *Puncha* clade, (v) the western Mediterranean *Ohmella* clade, and (vi) the Palaearctic *Phaeostigma* clade. The geographic distributions of these clades are depicted in Fig. 3 and in Figures S3–5. Concerning the monophyly of genera in the DNA-based tree (Fig. 2), only *Xanthostigma* does not form a monophylum (which is not plausible at all, as the monophyly of the genus is well established morphologically). The three *Xanthostigma* species are placed in an unresolved tetratomy within a clade also including a lineage combining *Calabroraphidia* and *Puncha. Alena*, *Mongoloraphidia* and *Phaeostigma* are monophyletic in this tree, which is also the case (with the exception of *Phaeostigma*) in all other analyses.

The monophyly of each of the four aforementioned groups and their sister-group relationships seem plausible, although they do not entirely conform with previous, mainly morphology-based groupings (I–VIII; see Introduction, Figure S1 and Aspöck *et al*., 1991; Aspöck, 1998). In the following text we discuss autapomorphies/synapomorphies, mainly based on genital sclerites (Aspöck, 2002c; Aspöck & Aspöck, 2008), that might support the clades resulting from the present analysis (Fig. 2).

1. *Agulla* (Fig. 3): this genus is sister to all other extant Raphidiidae in the molecular trees. This is the real surprise of our analysis, because previously *Alena* was the most eccentric genus of the family Raphidiidae with respect to the appearance and shape of genital sclerites. Autapomorphies of *Agulla*: male genital sclerites – gonocoxites plus gonapophyses-complex 10 (parameres) represented as flat 'ribbed' sclerites, covered with little teeth (e.g. *Agulla bractea*; Aspöck *et al*., 1991: vol. 2, p. 213, figs 1399, 1400).
2. *Alena* is placed as the sister to a clade comprising all the Palaearctic Raphidiidae (Fig. 3). Autapomorphies of *Alena*: male genital sclerites – tergite and sternite of segment 9 separated, not forming a ring as in all other Raphidioptera (this ring is one of the autapomorphies of the order). Fusion (or amalgamation) of gonocoxites plus gonapophyses-complex 10 (parameres) with gonapophyses 9 (hypovalva), thus looking like apices of the hypovalva [e.g. *Alena* (*Aztekoraphidia*) *horstaspoecki*; Aspöck & Contreras Ramos, 2004, p. 131, figs 7 and 9; and *Alena* (*Aztekoraphidia*) *caudata*; Aspöck *et al*., 1991, vol. 2, p. 242, figs 1929, 1930]. Broad insertion of gonapophyses 9 on basal element of gonocoxites 9. Synapomorphies of *Alena* and the Palaearctic clade: male genital sclerites – presence of a basal sclerite of gonocoxites 9, which may be separated (e.g. in *Alena distincta*; Aspöck *et al.*, 1991, vol. 2, p. 240, figs 1907, 1908) or amalgamated (e.g. in *Phaeostigma pilicollis*; Aspöck *et al.* 1991, vol. 2, p. 91, fig. 511). However, lack of these basal sclerites in *Agulla* may be a reduction, meaning that possession of these basal

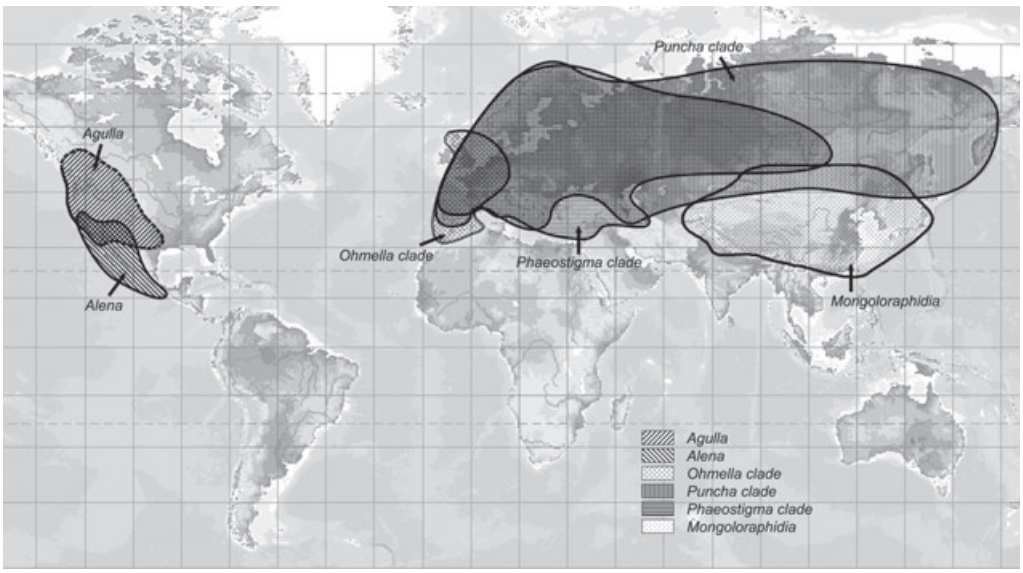

Fig. 3. Distribution patterns of the clades resulting from the present analysis. The contours of the single clades, however, comprise all known species constituting these monophyletic groups, not just the ones included in the analysis.

sclerites would be a plesiomorphic trait. Synapomorphies of the Palaearctic clade: male genital sclerites – amalgamation of gonocoxite 9 and tergite 9, at least along a small zone (e.g. *Phaeostigma pilicollis*; Aspöck *et al*., 1991, vol. 2, p. 91, fig. 511).

3. *Mongoloraphidia* (Fig. 3) is one of the monophyletic groups within the Palaearctic clade. *Mongoloraphidia* is an extremely heterogeneous genus (awaiting unknown species to be described), which has been hypothesized previously to be monophyletic (Aspöck *et al*., 1999). Autapomorphies of *Mongoloraphidia*: male genital sclerites – basal part of ectoproct withdrawn into tergite 9 and loss of gonocoxites plus gonapophyses-complex 10 (parameres). This loss remains, however, a matter of interpretation in the tracing of gonocoxites plus gonapophyses-complex 10 in the compound of the apices of the gonapophyses 9 [e.g. *Mongoloraphidia* (*Alatauoraphidia*) *zhilzovae*; Aspöck *et al*., 1991, vol. 2, p. 202, figs 1189, 1190, labelled as 'h'], analogous with *Alena* (see above). The sister-group relationships of the species within *Mongoloraphidia* resulting from this analysis partly lack plausibility, as *Mongoloraphidia monstruosa* and *Mongoloraphidia nurgiza* belong to the subgenus *Kirgisoraphidia*, which represents one of the best-established monophyletic groups within the genus.

Monophyly of the rest of the Palaearctic clade (i.e. *Ohmella* clade + *Puncha* clade + *Phaeostigma* clade) is supported only weakly. Up to now, no obvious synapomorphy of this Western Palaearctic clade has been recognized. Male genital sclerites: a sclerite, which is hypothesized as (a re-expression of) the gonocoxite-complex 11 ('sclerite H' in Principi, 1961, p. 103, fig. V, and p. 105, fig. VII; Principi, 1966, p. 370, fig. IV), is interpreted as being possibly synapomorphic. It is only observable in a single species of the Western Palaearctic clade, e.g. in *Atlantoraphidia maculicollis* (Aspöck *et al*., 1991, vol. 2, p. 173, fig. 1036, labelled 'p', fig. 1037, 'Parameren'), or in *Italoraphidia solariana* (Aspöck *et al*., 1991, vol. 2, p. 178, figs 1069, 1070, labelled 'p').

4. *Puncha* clade: the clade comprises taxa with a predominately European distribution – *Italoraphidia* + [*Venustoraphidia* + (*Xanthostigma*, *Calabroraphidia*, *Puncha*)] (Fig. 3; Figure S4). Only one species of the genus *Xanthostigma* (*Xanthostigma xanthostigma*) is distributed from Europe to the Far East, whereas all other species of this genus are limited to Europe. The genera *Venustoraphidia* and *Puncha* occur in Central Europe, and in parts of the Apennines Peninsula and the Balkan Peninsula, and *Italoraphidia* and *Calabroraphidia* are endemic to southern Italy, with a very restricted distribution area. A sister-group relationship of *Italoraphidia*, *Puncha* and *Calabroraphidia* has been hypothesized already by Rausch *et al.* (2004), but no synapomorphies have yet been found for the *Puncha* clade.
5. *Ohmella* clade: this clade includes *Ohmella* + (*Harraphidia* + (*Hispanoraphidia* + *Atlantoraphidia*)). This grouping also makes sense phylogeographically as all species have a western Mediterranean distribution (Fig. 3; Figure S3). Synapomorphies of the *Ohmella* clade: male genital sclerites – tergite 9 angled, sternites 8 and 9 overlappingly connected (because of a reduction of intersegmentale sternite 8/9), see e.g. *Atlantoraphidia maculicollis* and *Africoraphidia spilonota* (Aspöck *et al*., 1991, vol. 2, p. 173).
6. *Phaeostigma* clade: in this clade the genera *Phaeostigma*, *Raphidia*, *Subilla*, *Dichrostigma*, *Ornatoraphidia*, *Ulrike*, *Tjederiraphidia*, *Parvoraphidia* and *Turcoraphidi*a are united. Like the *Puncha* clade, it is a predominately European group (Fig. 3; Figure S5) – only one species (*Raphidia ophiopsis*) also occurs in Northeast Asia. Synapomorphies of the *Phaeostigma* clade: male genital sclerites – gonapophyses 9 amalgamated to unpaired sclerite (hypovalva) (e.g. *Phaeostigma pilicollis* and *Venustoraphidia nigricollis*; Aspöck *et al*., 1991, vol. 2, p. 91, fig. 511 and p. 180, Fig. 1076).

Phylogenetic considerations

Both extant families are represented as fossils from the early Tertiary as well as from Baltic amber (Engel, 1995, 2003; Aspöck & Aspöck, 2004; Grimaldi & Engel, 2005). The oldest fossils that can definitely be assigned to Raphidioptera are from Lower Jurassic deposits (Lias), which are 180–200 million years old. However, as fossils of their probable sister group, Megaloptera + Neuroptera, are known from the Permian, the Raphidioptera must have already existed in that geological period. In the Mesozoic, several raphidiopteran families were present (Engel, 2002; Aspöck & Aspöck, 2004) that exhibited amazing similarities to present-day raphidiopterans, and occurred in a significantly greater diversity than today, and inhabited tropical regions (Aspöck, 1998, 1999, 2004; Aspöck & Aspöck, 2004, 2005, 2009). This Mesozoic snakefly fauna largely died out at the end of the Cretaceous. Extant snakeflies depend on cold climates, larvae may starve for weeks and months, the number of larval instars is not fixed and larvae (as well as pupae) live in protected microhabitats. The proposed asteroid impact at the K–T boundary (Pope *et al*., 1997; Kring, 2000; but see also Keller *et al*., 2009) seems to be a likely explanation for the disappearance of all snakeflies living in tropical climates (Aspöck, 1998, 1999, 2004). If rapid climate cooling is assumed as the cause of extinction of many raphidiopteran lineages, only those representatives that were already adapted to a cold climate (with distinctive seasons and winter temperatures, which are essential for their development) could have survived. Considering the globally mild climate conditions at the end of the Cretaceous, it could be hypothesized that this should most likely have occurred in the northern regions of Laurasia.

Connecting these geological and paleobiological considerations with our phylogenetic tree, we can hypothesize a phylogeographic scenario for the radiation of the lineages leading to extant Raphidioptera. The first question is when the radiation of the crown group Raphidiidae started. Did the two New World

lineages of the raphidian radiation (*Agulla* and *Alena*) separate from the rest before or after the K–T event? This question cannot be decided on the basis of the sequence data; however, as the split of Laurasia into North America, Greenland and Eurasia, leading to the final opening of the northern Atlantic, occurred between 60 and 50 Mya (Harland, 1967; Pomerol, 1975), the New World lineages must have diverged prior to this time. However, as the opening of the North Atlantic started much earlier, and was a lengthy process, one cannot rule out that this branching started earlier.

Within the Palearctic clade there is a biogeographic pattern. The *Mongoloraphidia* clade is distributed in the eastern Palearctic, whereas the other three clades are exclusively (*Ohmella* clade) or mainly distributed in the western Palearctic. The *Puncha* and the *Phaeostigma* clades contain one species each that has an extensive distribution range reaching from Europe to the northeast of Asia, whereas the remaining taxa of these clades are restricted to Central or Western Europe. However, these two species are exceptions from an otherwise clear geographic pattern, and must be attributed to much later (Pleistocene) dispersal events. The remaining taxa can be divided into an eastern (*Mongoloraphidia*) and a western (*Ohmella, Puncha* and *Phaeostigma* clades) radiation. These radiations might have happened early within a rather short period of time, as the branches leading to clades 3 and 4 are rather short. Also, within the clades the basal branches are short, whereas the terminal branches are long. This scenario presumes a fast dispersal of early lineages, which stands in contrast to the present-day low dispersal capacity of most taxa. However, the ability to disperse may change over time, as exemplified by *Xanthostigma xanthostigma* and *Raphidia ophiopsis.* Moreover, dispersal might be possible only under very specific climatic conditions. Thus, the reason for the persistence of many lineages of snakeflies in rather restricted areas, as observed today, might be a consequence of climatic changes throughout the Cenozoic, as well as their ability to survive unfavourable periods.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2010.00542.x

Figure S1. Hypothetical phylogenetic tree of the family Raphidiidae based on holomorphological characters, modified after Aspöck *et al.* (1991).

Figure S2. Neighbour-joining tree based on *cox3* (nucleotide) sequences using p-distances to illustrate intergeneric distances, as well as distances between families.

Figure S3. *Ohmella* clade map.

Figure S4. *Puncha* clade map.

Figure S5. *Phaeostigma* clade map.

Table S1. Uncorrected (p) distance matrix of *cox3* sequences.

Table S2. Comparisons of the results from various tree-building algorithms, marker genes (gene combinations) and alignment approaches.

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