

Anthropogenic dispersal of a snakefly (Insecta, Neuropterida) – a singular phenomenon or a model case in Raphidioptera?

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Abstract

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The Mediterranean snakefly *Raphidia mediterranea* H. Aspöck, U. Aspöck & Rausch, 1977 – known from many parts of the Balkan Peninsula, several Aegean islands, southern parts of Italy, northwest of Anatolia and a few localities in Eastern Europe, yet not recorded in Central Europe – was surprisingly found with an astoundingly high population density on bushes in the yard of an old farmhouse at a comparatively high altitude (800 m) in Upper Austria, north of the Danube River, in 2013. This spectacular phenomenon was again observed in the following years (2014, 2015, 2016, 2017), and in 2016 the suspicion that the larvae develop in the straw of the thatched roof of the farmhouse could be confirmed by findings of larvae, pupae, and exuviae. It is most likely, that the occurrence of this *Raphidia* species in Austria is to be traced back to a human-caused introduction at some point in time. It remains, however, unknown when and specifically how this event might have occurred.

Morphologically no substantial differences were found between specimens from Greece, Italy and Upper Austria. In addition, the genetic uniformity (using 3 genes: *cox1*, *cox3*, and *28S*) of the populations was verified. This supports an earlier hypothesis that the occurrence of the species, as well as that of *R. mediterranea* in Italy, Anatolia and perhaps elsewhere, may be related to importation of goods involving wood or soil.

A molecular genetic analysis of several *Raphidia* species confirmed the present morphology-based concept of their systematic position.

The means of dispersal of Raphidioptera are largely unknown. We do not know of any other similar cases of anthropogenic dispersal of a snakefly, but it cannot be excluded that human activities may have played a greater role in the dispersal of Raphidioptera than previously assumed. Phylogenomic studies would therefore be promising to solve some of these questions.

Introduction

By the 1960s, it was revealed that the Balkan Peninsula was a distribution (and evolution) centre of the order Raphidioptera with an incredibly high number of spe-

cies (H. Aspöck and U. Aspöck 1965). In the course of the following years, extensive field studies were carried out in various parts of the Balkan Peninsula (H. Aspöck 1987, H. Aspöck et al. 1989, H. Rausch and R. Rausch 2004). These investigations led to the discovery of nu-

merous new species and among them a snakefly species, which – despite slight differences – was morphologically so similar to *Raphidia ophiopsis* Linnaeus, 1758, that we hesitated to separate it from *R. ophiopsis*. However, after discovery of unusually large populations of this taxon in various parts of Greece and in biotopes (e.g. in coastal areas with maquis vegetation) ecologically entirely different from those of *R. ophiopsis*, which is associated with coniferous trees, we decided to describe it as a subspecies of *Raphidia ophiopsis*: *R. ophiopsis mediterranea* (H. Aspöck et al. 1977). In the meantime, the taxon was surprisingly found in Italy (Apulia) and later in northwest Anatolia. In our monograph (H. Aspöck et al. 1991) we argued that the disjunct distribution could hardly be explained by natural dispersal and we therefore considered that human activities might have been a significant cause of the amplification of the distribution of *R. o. mediterranea*. One of our arguments was that *R. o. mediterranea* occurs on the eastern coast of the Apennine Peninsula around Brindisi, a region known for its intensive ship traffic with Greece, which dates back to antiquity. Subsequently, entomologists from Italy found *R. o. mediterranea* in western parts of the Apennine Peninsula, and from this they concluded that *R. o. mediterranea* had not been introduced from Greece to Italy by human activities, but that its occurrence in Italy was due to natural dispersal (Letardi 2002, Letardi and Pantaleoni 1996, Pantaleoni 2005). Meanwhile, the taxon was unexpectedly found in Romania (Kis 1984) and Hungary (Sziráki 1993a, b, 2010). Both latter authors studied the taxon carefully and arrived at the conclusion that *R. mediterranea* is a good

species and not a subspecies of *R. ophiopsis*. Aside from the known and corroborated morphological differences, an important argument for the status of a separate species was the sympatry of both taxa in Romania and Hungary. The arguments of Kis (1984) and Sziráki (1993a) were convincing and accepted by us (H. Aspöck and U. Aspöck 2007, 2013, 2014). Finally, in 2013 *R. mediterranea* was found in the yard and on the outer walls of an old farmhouse, now representing an open-air museum, at a considerably high altitude (800 m) in Upper Austria (Rausch et al. 2016). It was an absolute surprise to find this Mediterranean snakefly in a comparatively cold region of Austria (Figs 1, 2). Moreover, *R. mediterranea* occurs there in an extremely high population density. It was suspected that the larvae develop within the straw covering the roof (Rausch et al. 2016), and this could recently be confirmed (Gruppe et al. 2017) (Figs 3, 4). Thus, the question arose: How has *R. mediterranea* achieved the establishment of a stable population in a locality in Central Europe, which offers unfavourable climatic conditions compared to many other parts of Austria (Fig. 5)? To better evaluate the phylogeographic scenarios of this species, i.e. natural expansion of the distribution range vs. human mediated dispersal, we performed molecular genetic analyses of specimens from Austria, Greece and Italy. The specimens analysed genetically were compared morphologically with specimens from many localities covering the currently known distribution. Moreover, specimens of *R. ophiopsis* from Upper Austria and other parts of Central Europe were included to corroborate the morphological differences between the two taxa.

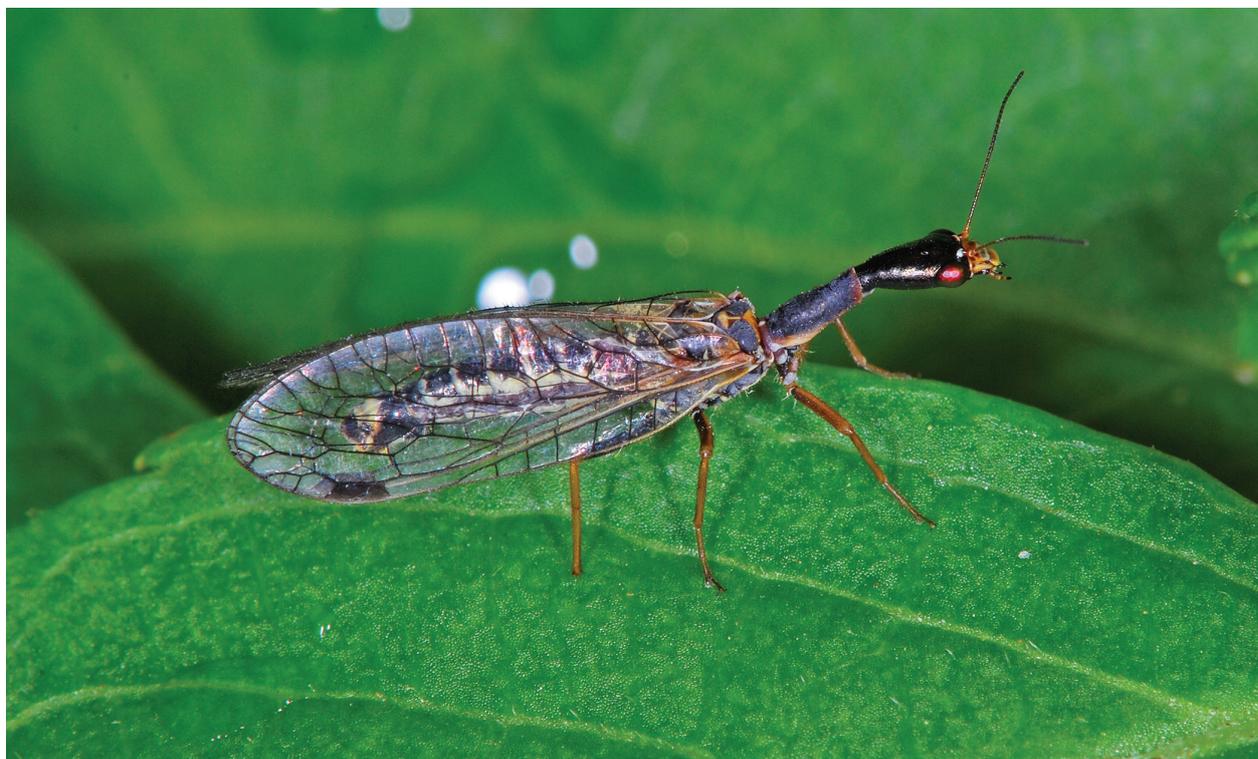


Figure 1. *Raphidia mediterranea*, male, from Pelmberg (Upper Austria). Photo H. Bruckner.



Figure 2. *Raphidia mediterranea*, female, from Pelmberg (Upper Austria). Photo H. Bruckner.



Figure 3. *Raphidia mediterranea*, full-grown larva, from Pelmberg (Upper Austria). Photo H. Bruckner.



Figure 4. *Raphidia mediterranea*, pupa, from Pelmborg (Upper Austria). Photo H. Bruckner.

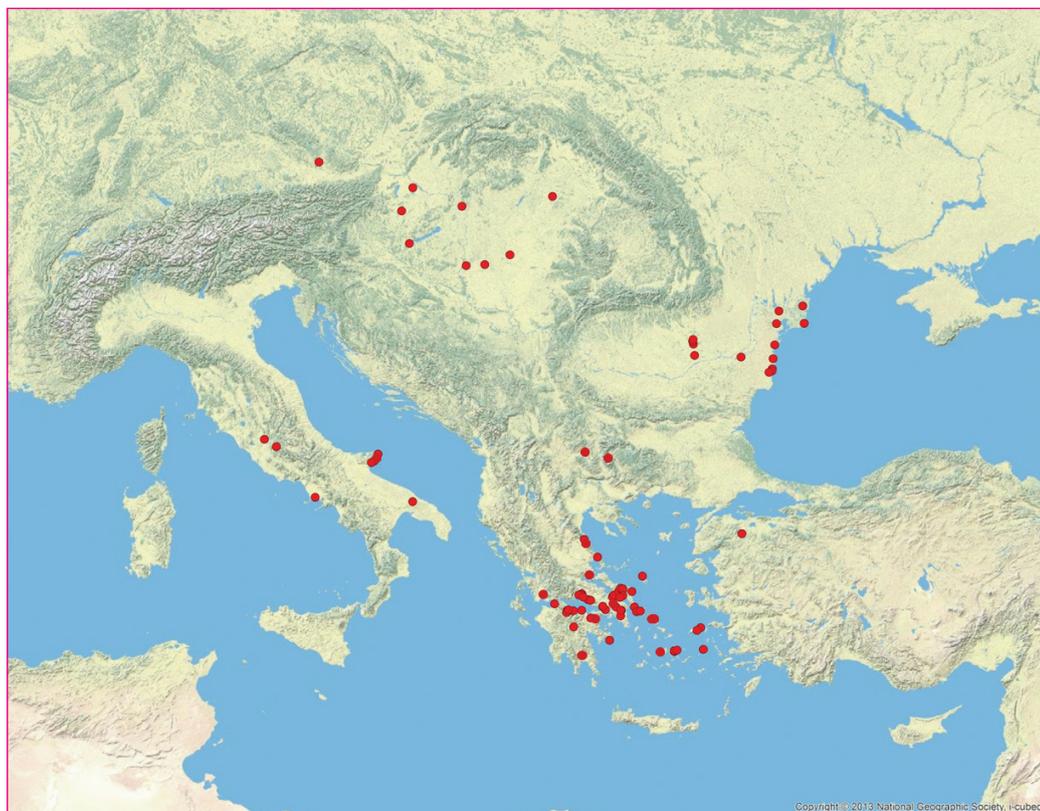


Figure 5. Known distribution of *Raphidia mediterranea* H.A. & U.A. & Rausch. Source of the map see under Material and methods.

Material and methods

Morphological studies

Numerous adults of both sexes of *Raphidia mediterranea* from many localities in Greece, Italy, Anatolia, as well as specimens of *R. ophiopsis* from Upper Austria and other parts of Central Europe were compared with imagines from Pelmberg (Upper Austria) based on the well-known morphological characters of male and female genitalia (H. Aspöck et al. 1991). Genital segments were cleared in KOH and processed in the usual manner described elsewhere.

The distribution map was provided with ArcGis/ArcMap ver. 10.3.1.4959 based on the distribution records provided in the Suppl. material 2. Source of the map: National Geographic-Weltkarte - Content may not reflect National Geographic's current map policy. Sources: National Geographic, Esri, DeLorme, HERE, UNEP-WCMC, USGS, NASA, ESA, METI, NRCAN, GEBCO, NOAA, increment P Corp.

Molecular genetic analysis

For DNA analysis samples of four individuals of *Raphidia mediterranea* were selected, which had been collected in Pelmberg (Upper Austria), Gargano (Italy) and Zachlorou (Peloponnesus, Greece). Moreover, five representatives of the genus were included: *Raphidia ophiopsis* Linnaeus, 1758, *Raphidia alcoholica* H. Aspöck & U. Aspöck, 1969, *Raphidia ulrikae* H. Aspöck, 1964, *Raphidia ariadne* H. Aspöck & U. Aspöck, 1964, and *Raphidia ligurica* Albar-da, 1891. A list of specimens analysed with exact localities is given in Table 1. Tissue samples were taken from one leg of alcohol-preserved specimens with sterile forceps. Vouchers are stored at the Entomological Department of the Museum of Natural History Vienna (NHMW). Remaining DNA is stored in the DNA and Tissue Collection of the Central Research Laboratories at the NHMW.

Marker sequences and laboratory procedures

Two mitochondrial marker sequences were amplified using primers listed in Table 2: (1) A partial sequence of the *cytochrome c oxidase subunit 3* gene (*cox3*) which

has been also used in a previous study on Neuropteri-da, as well as Raphidioptera (Haring and Aspöck 2004; Haring et al. 2011) and (2) the complete sequence of the *cytochrome c oxidase subunit 1* gene (*cox1*) plus partial sequences of the adjacent tRNA genes. In addition, a partial sequence of the *28S rRNA* gene (*28S*) was used as a nuclear marker sequence. The fragment lengths of *cox1* sequences ranged from 1604–1610 bp (due to indels in the flanking tRNA genes). The amplicon length of the *cox3* sequence was 712 bp. The amplicon length of the *28S* sequence was 1155–1161 bp.

DNA extraction was performed using the DNeasy-Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The final volume of elution buffer was 40 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 in an Eppendorf Thermocycler in a volume of 25 µl, containing Taq Polymerase (1.25 units/reaction; QIAGEN, Hilden, Germany) 1 µM of each primer, and 0.2 mM of each dNTP, 1.5 mM MgCl₂, 5 µl Q-Solution, 2.5 µl 10x PCR buffer and 1 µl of template DNA. The PCR protocols were as follows: *cox3*: initial denaturation 94° C (3 min); 35 cycles: 94° C (60 s) / 50° C (30 s) / 72° C (60 sec); final extension at 72° C (10 min). *cox1*: initial denaturation 94° C (3 min); 35 cycles: 94° C (60 s) / 50° C (30 s) / 72° C (60 sec); final extension at 72° C (10 min). *28S*: initial denaturation 94° C (3 min); 35 cycles: 94° C (60 s) / 55° C (30 s) / 72° C (60 sec); final extension at 72° C (10 min). Negative PCR controls were carried out to screen for contaminated reagents: (1) control extractions without tissue and subsequent PCR reactions (i.e. instead of template DNA); (2) PCR reactions with distilled water instead of template. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) prior to sequencing. Sequencing (both directions) was performed at Microsynth (Vienna, Austria) using the PCR primes as well as various internal primers (Table 2). Sequences obtained in the present study are deposited in GenBank under the accession numbers listed in Table 1.

Table 1. Specimens analysed genetically in the present study and GenBank accession numbers for *Cox1*, *Cox3* and *28S* sequences.

Species	Sampling locality	Labcode	GenBank		
			Cox1	Cox3	28S
<i>Raphidia mediterranea</i>	Austria, Upper Austria, Pelmberg near Hellmonsödt	Ramed-1	MF975675	MF975666	MF975657
<i>Raphidia mediterranea</i>	Austria, Upper Austria, Pelmberg near Hellmonsödt	Ramed-2	MF975676	MF97567	MF975658
<i>Raphidia mediterranea</i>	Greece, Peloponnesus, Kato Zachlorou	Ramed-3	MF975677	MF975668	MF975659
<i>Raphidia mediterranea</i>	Italy, Puglia, Gargano, Punta Lunga	Ramed-4	MF975678	MF975669	MF975660
<i>Raphidia ariadne</i>	Greece, Crete, W Omalos	Raari-1	MF975679	MF975672	MF975661
<i>Raphidia alcoholica</i>	Greece, Phokis, S Pendency	Raalc-1	MF975680	MF975671	MF975662
<i>Raphidia ophiopsis</i>	Germany, Bayerischer Wald, Ruckwiesberg	Raoph-1	MF975681	MF975670	MF975663
<i>Raphidia ulrikae</i>	Austria, Styria, Gulsen near Kraubath	Raulr-1	MF975682	MF975673	MF975664
<i>Raphidia ligurica</i>	Italy, Sila Grande, near Viváio	Ralig-1	MF975683	MF975674	MF975665

Table 2. Primers used.

Gene	Primer	Sequence (5'-3')	Reference
Cox3 external primers			
	Arth-cox3-fwd	5'-TAGTTGATTATAGACCATGACC-3'	a
	Arth-cox3-rev	5'-ACATCAACAAAATGTCAATATCA-3'	a
Cox3 internal primers			
	Raph-cox3-1+	5'-ACAAATTCCTTTATTAAATAC-3'	present study
	Raph-cox3-2-	5'-CAWGTAAATTGTTAATCCTGA-3'	present study
Cox1 external primers			
	Tyr-myr-1+	5'-CCCATAAATAAATTTACAGTTTA-3'	present study
	Leu-Myr-1-	5'-GCACTATTCTGCCATATTAG-3'	present study
Cox1 internal primers			
	Raph-cox1-int1+	5'-TAGCAGGAGCTACTACTACT-3'	present study
	Raph-cox1-int2-	5'-ATATAAATTCTGGATGTCC-3'	present study
	Raph-cox1-int5+	5'-CGAATACCTTTATTTGTATGATC-3'	present study
	Raph-cox1-int4-	5'-AGAATAGGGTCTCCTCCTCC-3'	present study
28S internal primers			
	Raph-28S1+	5'-CAGGGGTAAACCTGAGAAA-3'	b
	Raph-28S-4-	5'-AGCGCCAGTTCTGCTTACC-3'	b
28S external primers			
	Raph28S-3+	5'-AGCTTTGGGTACTTTCAGGA-3'	b
	Raph28S-2-	5'-ACATGCTAGACTCCTTGGT-3'	b

a: Haring and Aspöck (2004); b: Haring et al. (2011)

Phylogenetic analyses

Raw sequences were manually aligned in BioEdit v.7.1.3 (Hall 1999) and checked for errors. The alignment was straightforward for the three marker sequences and was done in BioEdit v.7.1.3. In *cox1* and *cox3* sequences there were no insertions or deletions, and in the *28S* gene there were only a few indels allowing clear assessment of positional homology. The final alignment of the complete *cox1* gene had a length of 1534 positions. The *cox3* gene had 667 positions and the *28S* gene 1144 positions. As outgroup, *Agulla*, another genus of Raphidiidae was used. The *cox3* and *28S* sequences have been published in our previous paper (Haring et al. 2011; HM543275; HM543340; *Agulla adnixa*). The complete *cox1* sequence was derived from GenBank (FJ207460.1; *Agulla* sp.). By comparing this sequence with published partial *cox1* sequences of *Agulla adnixa* (e.g., KR141904.1), we deduced that the sequence FJ207460.1 is derived from *Agulla adnixa* (which has an identical sequence). As a result, in the concatenated data set, the outgroup sequence was derived from different individuals of the same species, which however appears to be unproblematic in this case.

Bayesian Inference (BI) was used for calculating phylogenetic trees. For BI the best fitting substitution model was determined for each of the three genes as well as codon positions of the protein coding genes by jModelTest v.2.1.5 (Darriba et al. 2012) with the corrected Akaike information criterion (AICc). The BI analyses were calculated using MrBayes v.3.2.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Phylogenetic trees were also calculated from a combined alignment in which all three marker sequences were concatenated (length of alignment: 3345 positions). BI analyses were

run for 7×10^6 generations (2 runs each with 4 chains, one of which was heated), sampling every hundredth tree. The first 25% of trees were discarded as burnin and from the remaining trees a 50% majority rule consensus tree was calculated. In addition, Neighbour Joining (NJ) trees (Saitou and Nei 1987) were calculated. Nodal support of NJ trees was evaluated with nonparametric bootstrapping based on 1000 replicates. These trees are shown to illustrate p distances among taxa in comparison of the three marker sequences.

Results

In former studies (H. Aspöck et al. 1991 and unpublished), based on male and female genitalia, populations of *Raphidia mediterranea* from various localities in Greece, Anatolia, and Italy could not be differentiated from each other. This was confirmed again on the basis of more material, particularly specimens from Pelmburg (Upper Austria) whose morphological characters coincide perfectly with those figured in H. Aspöck et al. (1991). Specimens of the genus *Raphidia* from other parts of Central Europe (except *Raphidia ulrikae*) proved to be conspecific with *Raphidia ophiopsis*.

The DNA sequence analysis revealed that the four specimens of *R. mediterranea* are identical in *cox1* and *28S*, while in *cox3* a single substitution differentiating Ramed-4 from the other (identical) sequences was found. In general, the variation within *28S* was extremely low. Except *R. ligurica*, which shows distances to the other ingroup taxa of 1.08 and 1.35%, respectively, sequences of all other ingroup species differ with p distances below

1% or are even identical. Between *Raphidia* and *Agulla* 28S distances ranged from 6.0 to 6.5%. Concerning the mitochondrial marker sequences, p distances between *R. mediterranea* and *R. ophiopsis* (the closest relative) were 5.08% in *cox1* and 5.62% in *cox3*, while the other species of *Raphidia* differed between 8.63–14.83% (*cox1*) and 7.46–16.94% (*cox3*) from *R. mediterranea*. Distances between species in the various gene sequences are illustrated by the NJ trees in Suppl. material 1.

To assess the systematic position of *R. mediterranea* not only on the basis of morphological characters, we performed a phylogenetic analysis based on three genes (*cox1*, *cox3*, 28S). The two mt sequences resulted in trees in which the sister group of *R. mediterranea* is *R. ophiopsis*. In most analyses, *R. alcoholica* is the sister group of those two lineages, followed by *R. ariadne*; only in the BI tree of *cox1* the relationships were unresolved (Suppl. material 1). With respect to the relationships of the other species there is a difference concerning *R. ligurica* and *R. ulrikae* depending on the marker sequence and the method applied. In some trees *R. ulrikae* splits from the most basal node, in others it is *R. ligurica*. Yet, in all trees this node is poorly supported. The tree based on 28S sequences (Suppl. material 1) is congruent with the mt based trees, yet, due to the low variation within this gene, the amount of phylogenetic information is limited. In a BI tree based on the combined marker genes (Fig. 6) all nodes are highly supported.

Discussion

The discovery of an isolated and unusually large population of *Raphidia mediterranea* – a Mediterranean species which has never been found elsewhere in Central Europe – in a farmhouse in a comparatively climatically unfavourable part of Upper Austria had raised the question concerning the origin of this population. It was assumed that morphological and/or genetic differences would be found, if the species had reached Upper Austria long ago by natural means of expansion of the distribution range. Therefore, specimens of the population from Upper Austria were compared with specimens from Greece and Italy. In the present study, the morphology-based results were clearly confirmed by molecular genetic analyses: The four specimens of *R. mediterranea* (two from Pelmburg (Austria), one from Greece, one from Italy) had almost identical sequences. It is legitimate to conclude that these populations were not separated long ago. The other species of *Raphidia* are clearly separated (see Fig. 6).

Substantial differences could not be found in morphological characters, particularly in male and female genitalia, or in the sequences of three genes (*cox1*, *cox3*, 28S). This implies that all presently known and examined populations of *R. mediterranea* originated from a single glacial refugium. This refugium can reasonably be assumed to be in the south of the Balkan Peninsula as a part of the large balkanopontomediterranean refugium (H. Aspöck

et al. 1991). From there the species reached other parts of Europe (and Anatolia) not long ago. Natural dispersal from the south of the Balkan Peninsula to isolated areas of the north of the Balkan Peninsula, to southern parts of Italy, to parts of Eastern Europe and particularly parts of Central Europe is highly unlikely. Consequently, an anthropogenic introduction into various regions is highly probable. *Raphidia mediterranea* is a euryoecious species, whose larvae live mainly in the detritus of roots of bushes, but sometimes also under bark.

In Greece, the species occurs in many regions, in various habitats at altitudes of 10 – 1200 m and often in high population densities. Thus, it might have been occasionally transported to new habitats by ships carrying wood or soil. This could have occurred already in antiquity and throughout the past centuries.

The discovery of the isolated population of *R. mediterranea* in Upper Austria and the failure to detect any morphological or genetic differences between these vastly distant populations supports our previous hypothesis (Aspöck et al. 1977, 1980, 1991, 2001) of unintentional introduction by human activities. A natural dispersal – per continuitatem or by wind – can convincingly be excluded. How did *R. mediterranea* come to Upper Austria? We now know definitely that the larvae develop within the straw of the roof (Gruppe et al. 2017). Thus, it is a reasonable assumption that this snakefly was introduced with straw from somewhere on the Balkan Peninsula. The straw presently on the roof is from Austria, but in the past it may have been imported. It is also possible that live adults (theoretically one female would be sufficient) were introduced (e.g. via car, truck or bus) from the Mediterranean region to Pelmburg and subsequently the female laid eggs in the straw. Until now, no studies have been published indicating that larvae develop in straw on roofs. In Central Europe thatched roofs have become rare, but in eastern and southeastern parts of Europe such roofs are still frequent in certain regions. It would be easy and exciting to examine these habitats for snakeflies. It would particularly be interesting to know whether other species of Raphidioptera can also develop in straw of thatched roofs where they would feed on mites, spring-tails, Psocoptera, larvae of beetles and other small arthropods living in the straw.

Concerning the systematic position of *R. mediterranea*, the phylogenetic analysis based on three genes confirmed our view of the systematics of *R. ophiopsis*, *R. mediterranea*, *R. alcoholica*, *R. ariadne*, *R. ulrikae* and *R. ligurica* (H. Aspöck et al. 1991, 2001). It is of particular interest that *R. alcoholica* is the sister species of *R. ophiopsis* + *R. mediterranea*, thus confirming the close relationship of the three taxa once regarded as subspecies of *R. ophiopsis*.

We know little about the formation of distribution patterns of Raphidioptera. Snakeflies are generally characterised by low, in many cases extremely low, expansivity, and many species have hardly enlarged their distribution beyond their glacial refugial areas. In Central Europe, 16

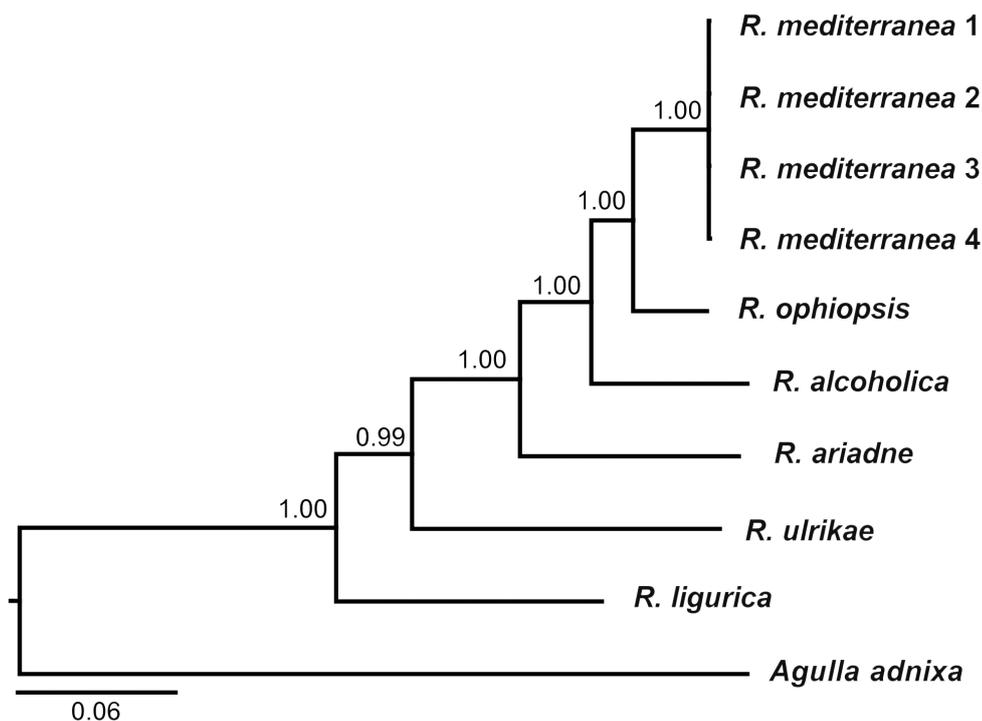


Figure 6. Phylogenetic tree based on BI analysis of six species of *Raphidia* (with *Agulla adnixa* as outgroup). Posterior probability values are indicated at the nodes.

species of Raphidioptera (13 Raphidiidae and 3 Inocelliidae) occur, 10 of these are of Mediterranean origin and presumably have reached Central Europe after the last glacial period, i.e. within the past 10,000 years (H. Aspöck 2008, 2010, H. Aspöck et al. 1991, 2001, H. Aspöck and U. Aspöck 2015). At least in Austria, *R. mediterranea* must be regarded as a human introduced neozoon.

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Supplementary material 1

BI and NJ trees

Authors: Horst Aspöck, Ulrike Aspöck, Axel Gruppe, Marcia Sittenthaler, Elisabeth Haring

Data type: molecular data

Explanation note: BI and NJ trees calculated with *cox1*, *cox3* and *28S* sequences, respectively. Posterior probability values (BI trees) and bootstrap values (in %, NJ trees) are indicated at the nodes.

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Supplementary material 2

Records of *Raphidia mediterranea*

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Data type: Microsoft Excel Worksheet (.xlsx)

Explanation note: Records of *Raphidia mediterranea* documented in the distribution map (Fig. 5).

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