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Phylogeography of the micro-endemic *Pedicia staryi* group (Insecta: Diptera): evidence of relict biodiversity in the Carpathians

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The Carpathian region is recognized as one of the most important hotspots for aquatic biodiversity in Europe. In the present study, 658-bp long nucleotide sequences from the mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene were used to study the phylogeographical patterns of the Carpathian endemic dipteran species belonging to the *Pedicia staryi* group. Molecular data support the taxon status of the allopatric sibling pairs of the morphologically highly similar *Pedicia apusenica*, *Pedicia staryi*, and *Pedicia lobifera*. This pattern is most likely the result of long-term isolation in so-called cumulative microrefugia in the Carpathians, caused by aridification and forest fragmentation in the Miocene-Pliocene period, in combination with the specific habitat requirements of these species (i.e. the wet and humid environments of forested headwater springs). Furthermore, molecular data reveal an important cryptic diversity in the case of the most wide-spread Carpathian *P. staryi*, as represented by highly divergent, allopatric populations from distant mountainous ranges, already recognized as important centres of endemism for aquatic insects. In addition, an unexpectedly high genetic diversity was identified in populations from the Rodnei Mountains, where the northern and southern slopes harbour highly divergent genetic lineages. This highlights the importance of this mountain range in the preservation of autochthonous diversity in the Carpathians. The present study provides important new evidence regarding the persistence of relic species in spring habitats in the Carpathians, with ancient divergence events that predate Quaternary glaciations and confirm their continuous presence during the Last Glacial Maximum in multiple isolated refugia, leading finally to a high genetic complexity in these particular aquatic ecosystems. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2015, **00**, 000–000.

ADDITIONAL KEYWORDS: cumulative microrefugia – Miocene – Pliocene – speciation – spring habitats.

INTRODUCTION

The history of European biota has been extensively explored with the aim of detecting glacial refugia and postglacial re-colonization routes, as revealed by a series of chorological, paleontological, morphological, and molecular data (Hewitt, 2004). During glacial periods, temperate taxa persisted in refugial areas, whereas, during interglacial periods, they expanded their ranges in newly available climatically suitable areas (Huntley & Webb, 1989; Bennett, Tzedakis & Willis, 1991). A number of re-colonization models explain post-glacial range expansion not only from well-known classical southern refugia (Iberian, Apennine, and/or the Balkan Peninsula), but also from some 'cryptic' refugia located at higher latitudes (Pauls, Lumbsch & Haase, 2006; Stewart *et al.*, 2010; Wielstra, Babik & Arntzen, 2015). Thus, the postglacial biodiversity of Europe is shaped primarily by species that generally have good dispersal capacities, with high potential to colonize deglaciated areas in relatively short periods of time (Schmitt & Varga, 2012; Tzedakis, Emerson & Hewitt, 2013).

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To understand the history of less mobile European organisms, it is necessary to focus on biota characterized by narrow ecological habitat niches that are relatively stable in time and space. These can be found, for example, among narrow specialists, inhabiting extreme habitats, such as thermal springs, karstic caves, and cold springs (Varga, 2010; Bálint *et al.*, 2011). The very high level of spatial patchiness of such habitats, in combination with their spatio-temporal stability, has driven the evolution of biota in a framework of ecological island syndrome (Pellissier *et al.*, 2012). In particular, the organisms inhabiting cold-stenotherm mountain streams show a high level of endemism across Europe. Because of the buffering of temperature conditions in such aquatic ecosystems, they have been able to survive glacial cycles in the vicinity of ice sheets. Thus, these species could reflect long evolutionary histories that often predate Pleistocene glaciations (Lehrian *et al.*, 2010; Pop, Pop & Csuzdi, 2010). In the present study, we investigate a group of Carpathian Diptera associated with such habitats, and characterize their diversity over several mountain massifs.

To date, less mobile organisms from the Carpathians have generally been neglected in phylogeographical studies. Previous research conducted on earthworms and cave insects revealed that the origin and evolution of Carpathian endemics is highly congruent with complex geomorphological or palaeoecological changes in this region (Pop *et al.*, 2010; Meleg *et al.*, 2013). The Paratethys repeated transgression phases in the Miocene and the volcanic activity in the Pliocene induced the formation of isolated enclaves, resulting in accelerated insular-like speciation in the case of some low dispersal capability groups (Pop, 1997; Pop *et al.*, 2010). The subtropical climate conditions characterizing that period (Svenning, 2003; Kvaček *et al.*, 2006) intensified the isolation of species adapted to cold that were restricted to the high mountain regions surrounding the Central Parathetys (the Alps, the western, and eastern Carpathians; Sedivá *et al.*, 2008). By the end of the Miocene, a cooling period started that lasted through the Pliocene and Pleistocene, with climate oscillations causing several alternating glacial and interglacial cycles. It ended with the rise of temperatures after the Last Glacial Maximum (Schmitt, 2007). In a series of range-restricted and cold-adapted endemics from the area, the Pleistocene climatic fluctuations only remodelled the distributions of pre-existing lineages and their level of differentiation (Hofman *et al.*, 2007).

The *Pedicia staryi* group represents a well-suited case for investigating the evolutionary history of range restricted endemics in the Carpathian area and examining whether the divergence of less mobile European endemics occurred during or before the Pleistocene. This group inhabits wet and humid environments of headwaters, in the forested region of the Carpathians and the Apuseni Mountains, at altitudes between 1000 and 1500 m a.s.l. and is characterized by a high level of endemism. The *P. staryi* group is represented by five species of which three are endemic for the Carpathians: *Pedicia apusenica* Ujvárosi & Starý, 2003 for the Apuseni Mountains, *Pedicia lobifera* Savchenko, 1986 for the eastern Carpathians, and *Pedicia staryi* Savchenko, 1978, which shows disjunctive distribution, with populations in the northern part of the eastern Carpathians (Chornohora-Maramureș and Rodnei Mountains) and in the eastern part of the southern Carpathians (Bucegi Mountains). In the Bulgarian mountains, they are replaced by *Pedicia spinifera*. Finally, the last member of this species group, *Pedicia straminea*, is widely distributed in various headwater habitats at different altitudes in Europe (Oosterbroek, 2014).

In the present study, we aim to: (1) infer the phylogenetic relationship among the five species of the *P. staryi* group; (2) estimate the molecular divergence of the three Carpathian species; (3) build up their evolution history based on the genetic data; and (4) identify potential refugial areas.

The present analyses will therefore focus on three hypotheses:

1. Hypothesis 1: The present taxonomic definition within the *P. staryi* group is correct and reflects evolutionary relationships of taxa
   - i. Prediction 1: The group is monophyletic
   - ii. Prediction 2: Species level definition stands firm
2. Hypothesis 2: The *Pedicia staryi* group comprises additional cryptic taxa
   - i. Prediction 1: There are several allopatric lineages that are genetically well differentiated within *P. staryi*
3. Hypothesis 3: Wet and humid headwaters ecosystems in the Carpathians can accumulate genetic lineages over a long temporal range.
   - i. Prediction 1: Genetic distances between endemic taxa belonging to the *P. staryi* group reveal an ancient divergence that predates Pleistocene glaciations.

MATERIAL AND METHODS

SAMPLING

One hundred and twenty-six specimens representing the five species of the *P. staryi* group were collected between 2003 and 2013 (Fig. 1; see also Supporting

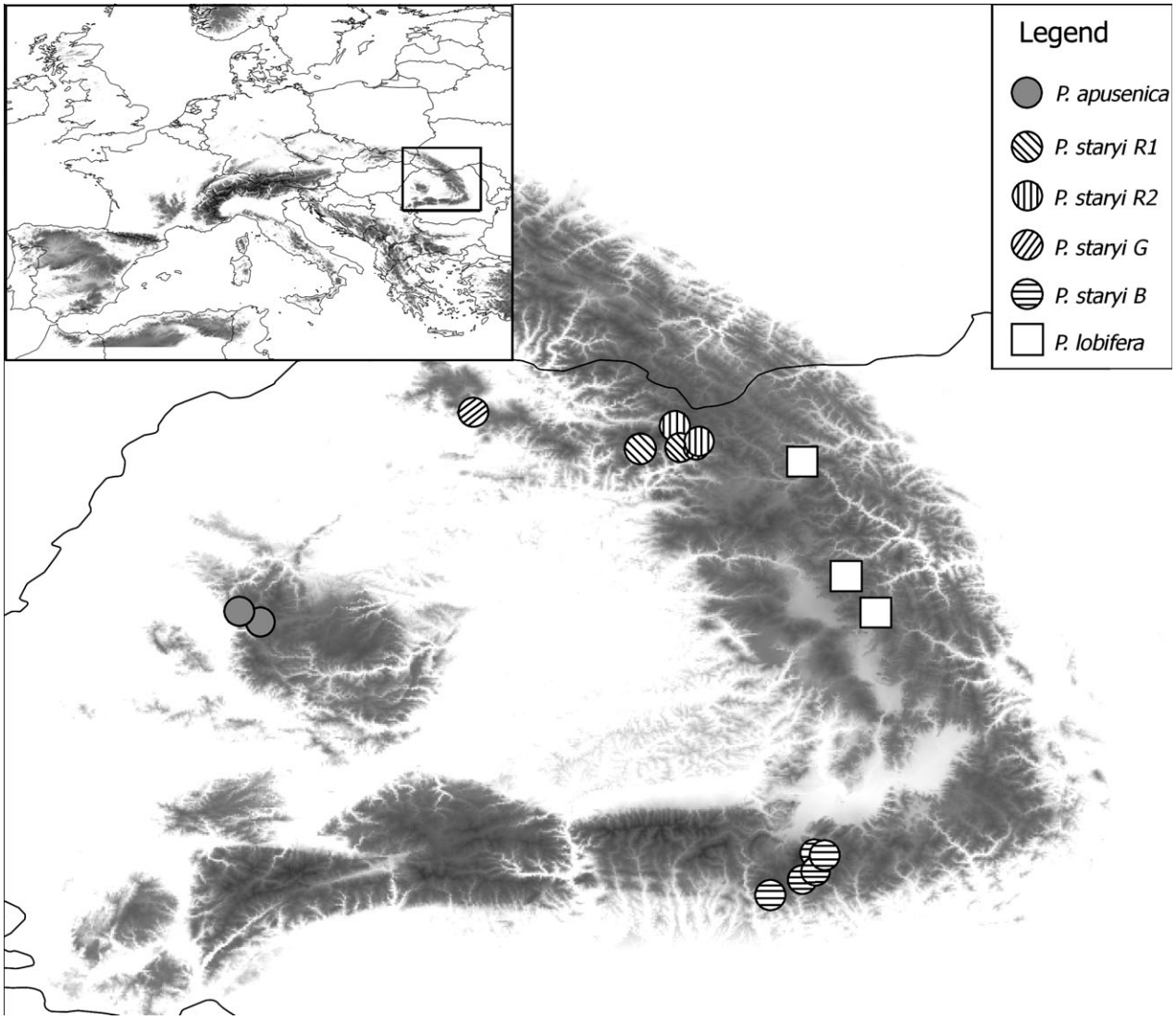

Figure 1. Map showing the Carpathian collection sites of the five lineages belonging to the *Pedicia staryi* group. *P. staryi* R1 corresponds to *staryiR1*, *P. staryi* R2 to *staryiR2*, *P. staryi* G to *staryiG*, and *P. staryi* B to *staryiB*.

information, Table S1). All individuals of the three microendemic Carpathian species were collected in Romania: *P. apusenica* in the Apuseni Mountains ($N = 17$); *P. lobifera* in the eastern Carpathians ($N = 9$); and *P. staryi* in the northern part of the eastern Carpathians and the Bucegi Mountains ($N = 57$). The Bulgarian *P. spinifera* was represented by six specimens from the Rhodope Mountains. The 37 individuals of *P. straminea* were collected in the Italian and French Alps and in several locations in the Romanian Carpathians. Four additional species (*Pedicia littoralis*, Meigen, 1804, *Pedicia riedeli* Lackschewitz, 1940, *Pedicia nielseni* Slipka, 1955, and *Pedicia zernyi* Lackschewitz, 1940) representing the *P. littoralis* species complex were used as outgroups. The individuals were collected with entomological nets and stored separately in collection tubes containing 96% ethanol in the Zoological Museum of the Babeș Bolyai University, Cluj Napoca, Romania.

DNA SEQUENCING

Thorax-tissue samples were collected from all individuals and deposited in 96-well plates containing 30 μL of 96% ethanol. All steps of this procedure were executed under sterile conditions to avoid contamination. DNA extraction, polymerase chain reaction (PCR) amplification, gel electrophoresis for PCR product checking, sequencing PCR cycle, and sequencing all followed standard protocols employed

at the Canadian Centre for DNA Barcoding (Ivanova, Dewaard & Hebert, 2006; Ivanova & Grainger, 2007, 2012; Ivanova, De Waard & Hebert, 2012). The mitochondrial cytochrome *c* oxidase subunit I (mtCOI) sequences were amplified with the LepF1/LepR1 primers. When the amplification failed, the LCO1490_t1/HCO2198_t1, LepF1/C_ANTMR1D, MLepF1/HCO2198_t1, MLepF1/LepR1, and LepF1/MLepR1 primers were used as alternatives. Specimen collection data, photographs, sequences, PCR and sequencing primers, and trace files are available through the Barcode of Life Data Systems (BOLD; Sujeevan & Hebert, 2007) under project name: Tipuloidea of Europe (EUTIP).

Phylogenetic analysis and the estimation of divergence time

The phylogenetic analysis consisted of two steps. First, the phylogeny and the time of divergence were estimated for the five species of the group. Second, genetic structure and diversity were assessed for *P. apusenica and P. staryi*, two of the Carpathian microendemic species.

The sequences were aligned using CLUSTALW in MEGA, version 6 (Tamura *et al.*, 2013). The number of polymorphic sites and haplotypes, as well as haplotype (h) and nucleotide (p) diversities, were obtained with DNASP, version 5 (Librado & Rozas, 2009). The Hasegawa, Kishino and Yano (HKY) was selected as the most suitable substitution model from 88 distinct models, on the basis of the minimum value of the corrected Akaike information criteria using JMODELTEST (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). The potential saturation of the nucleotide substitution was checked for the whole sequences and also for two separate partitions (with codon positions 1+2 and codon position 3). Number of transitions and transversions for each pairwise comparison were plotted against the genetic distance calculated with the F84 model (closely related to HKY) of nucleotide substitution using DAMBE, version 5.1.2 (Xia & Xie, 2001; Xia, 2013).

The relationships between all five species of the *P. staryi* complex were inferred on the basis of the haplotype data set using a maximum likelihood (ML) and a Bayesian inference (BI) algorithm for the two separate partitions and for the whole sequences. Phylogenetic reconstructions were performed assuming a HKY model with a gamma-distributed variation rate across sites (G) and a proportion of invariable sites (I). The trees were rooted using sequences of the *P. littoralis* species group, described as the sister group of the *P. staryi* complex (Savchenko, 1986). The ML analysis was conducted in SEAVIEW, version 4 (Gouy, Guindon & Gascuel, 2010) using thhe PHYML option with the starting tree determined by a BIONJ analysis (http://www.atgc-montpellier.fr/bionj) and with bootstrap resampling of 1000 pseudoreplicates for the node support assessment. BI was performed using BEAST, version 1.7.4 (Drummond & Rambaut, 2007) using a Markov chain Monte Carlo (MCMC) method, with the Yule speciation process, to infer the phylogeny and the divergence time of the nodes. As a result of the absence of fossil records, a value of 0.0177 ± 0.00119 was employed as a lineage substitution rate, corresponding to a mean substitution rate of 3.54% per Myr characteristic to the cox gene of insects based on the Mid-Aegean trench calibration (Papadopoulou, Anastasiou & Vogler, 2010). Five independent runs were conducted with 10 000 000 iterations each, sampling every 1000th step and using two types of molecular clocks (strict and uncorrelated log-normal relaxed). The first 10% of the sampled trees from each run was discarded as burn-in, resulting in 45 000 trees for both clock models. Resulting parameters were checked with TRACER, version 1.5 (http://tree.bio.ed.ac.uk/software/tracer) and showed a sufficient number of MCMC steps with effective sample size larger than 200 for every parameter. LOGCOMBINER, version 1.7.4 was used to combine independent runs. The best fitting clock model for the dataset was selected by comparing the Bayes factor (BF) values. $2 \times \ln BF$ results (Kaas & Raftery, 1995; Brandley, Schmitz & Reeder, 2005) were used to determine the significance of the BF. Tree topologies and node ages were assessed as a means of the posterior estimates and 95% highest posterior density intervals (HPD) using TREEANNOTATOR, version 1.7.4 and were visualized with FIGTREE, version 1.4 (http://tree.bio.ed.ac.uk/software/figtree).

Population structure and genetic diversity

The genealogy of the two morphologically very similar *P. apusenica* and *P. staryi* was further analyzed with the construction of a median joining network (MJN) using NETWORK, version 4.6.1.0 (http://www.fluxus-engineering.com/netwinfo.htm) (Bandelt, Forster & Röhl, 1999) and with the hierarchical analysis of the molecular variance (AMOVA) using ARLEQUIN, version 3.5 (Excoffier & Lischer, 2010). Populations were sorted into two groups corresponding to the current taxonomic status and into five groups suggested by the population structure analysis. The analysis was performed using Kimura-two-parameter (K2P) distance with 20,000 permutations.

The mean genetic distance between the *P. staryi* lineages (including *P. apusenica*) and the other species from the group was estimated using K2P distance implemented in MEGA, version 6 (Tamura *et al.*, 2013).

RESULTS

The COI alignment of the five species belonging to the *P. staryi* species group was 658 bp long. Of the 130 polymorphic positions, 129 were parsimony informative, resulting in 36 haplotypes with 0.938 haplotype diversity and 0.08633 nucleotide diversity. When *P. spinifera* and *P. straminea* were excluded, the number of variable positions for the sequences of the three Carpathian species changed to 94, with 92 being parsimony informative. Twenty-three haplotypes represented the three Carpathian species with 0.923 haplotype diversity and 0.06827 nucleotide diversity. In both cases, the nucleotide composition was biased towards a typical adenine thymine (AT)-rich composition specific to the mitochondrial DNA (mtDNA) of the insects (five species: %GC = 34.6; three species: %GC = 34.1).

No substitution saturation was observed in the case of the whole sequences (Fig. 2A) and in the case of the codon positions 1+2 (Fig. 2B). The plot for codon position 3 showed saturation at high genetic distances (Fig. 2C).

PHYLOGENETIC ANALYSIS

The phylogenetic trees constructed on the basis of the two separate partitions showed unresolved topologies (see Supporting information, Figs S1, S2); thus, we based any further analysis on the whole gene. The ML and BI analysis resulted in congruent tree topologies showing a monophyletic *P. staryi* species group but without a strong support of the bootstrap (BP) and posterior probability (PP) values (Fig. 3). *Pedicia spinifera* and *P. straminea* form two well resolved monophyletic groups (respectively, BP = 100% and PP = 1; BP = 93% and PP = 0.99). *Pedicia spinifera* represents the basal clade for *P. straminea* and the three Carpathian species that further fall into two groups with *P. straminea* as the basal clade for the five well supported lineages representing *P. lobifera*, *P. staryi*, and *P. apusenica* (Fig. 3).

Pedicia lobifera is monophyletic (BP = 100% and PP = 1.00). The topology of the trees shows four well supported operational taxonomic units. *Pedicia staryi* is represented by four separate lineages, hereafter referred to as *staryiR1*, *staryiG*, *staryiR2*, and *staryiB* (Fig. 3). The *staryiR1* + *staryiG* lineage consists of haplotypes from Rodnei and Gutâi Mountains and forms two distinct groups corresponding to the two mountain ranges. One haplotype from the Rodnei Mountains (*staryiR2*) is differentiated and is basal to the group formed by the third *P. staryi* lineage (*staryiB*) and *P. apusenica*. *StaryiB* is a genetically coherent and geographically well separated group consisting of haplotypes from the Bucegi Mountains. *Pedicia apusenica* is also well supported (BP = 99% and PP = 1.00) and monophyletic (Fig. 3).

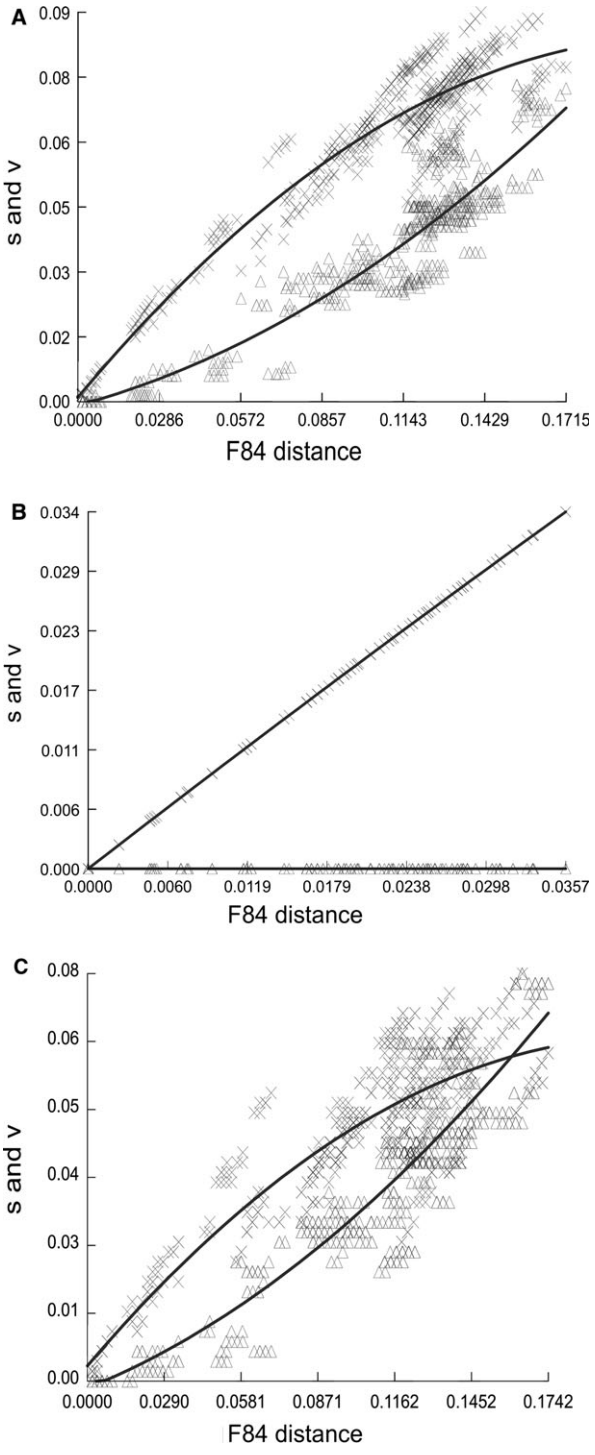

Figure 2. DAMBE substitution saturation plots for whole sequences (A), codon positions 1+2 (B), and codon position 3 (C). The number of transitions (s) and transversions (t) is plotted against the F84 distance.

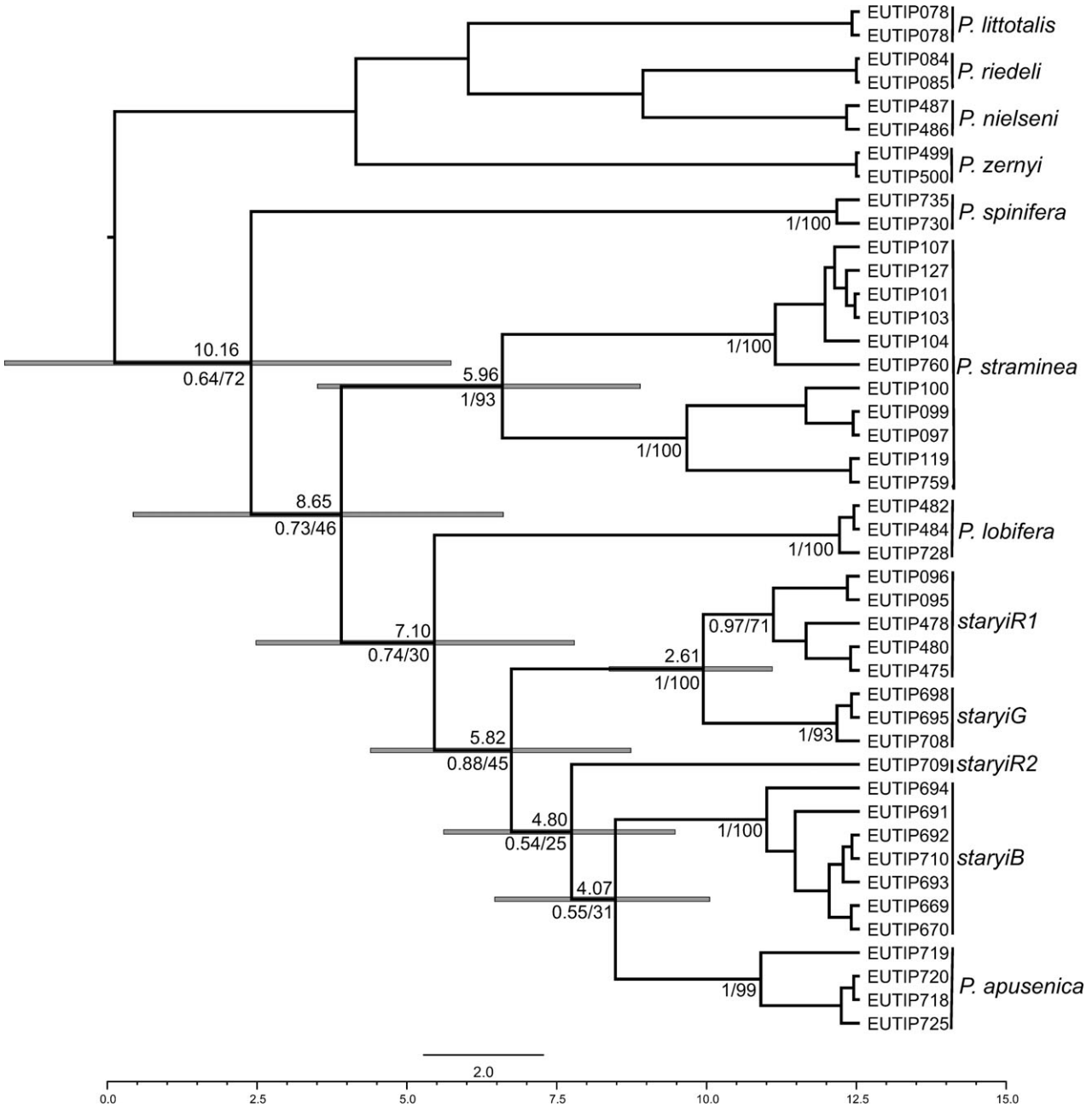

Figure 3. Bayesian inference (BI) tree showing the phylogenetic relationship among the five species of the *Pedicia staryi* group. Posterior probabilities (PP) and bootstrap values (BP, %) for the nodes are shown under the branches. The values above the branches show the divergence time (Mya). The bars indicate the 95% highest posterior density interval for the divergence time estimates.

Divergence time estimation

Time of divergence was inferred with the uncorrelated lognormal relaxed clock based on the likelihood differences that suggested the model as being significantly more adapted to our data ($2 \times \ln BF > 10$). Molecular dating estimates that the common ancestor of the three Carpathian species diverged from *P. straminea* 8.65 Mya (95% HPD, 5.95–12.12 Mya). It further shows that *P. staryi* and *P. apusenica* share a common ancestor that splits from *P. lobifera* 7.10 Mya (95% HPD, 4.76–10.07 Mya). The split between the *staryiR1* + *staryiG* and the other three lineages took place 5.81 Mya (95% HPD, 3.82–8.16 Mya). *Pedicia apusenica* and *staryiB* diverged

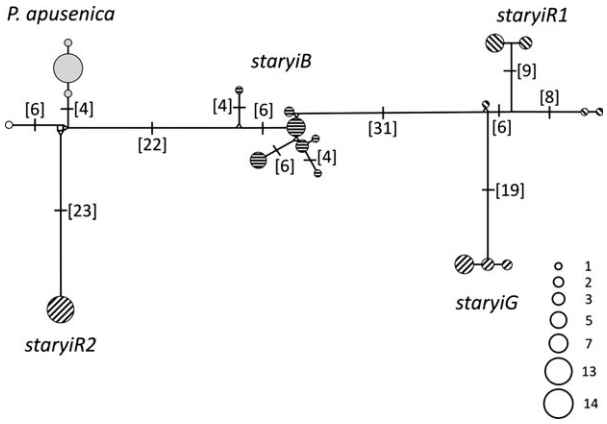

Figure 4. Mitochondrial DNA median-joining network. Circles represent the different haplotypes, with the area being proportional to the frequency of the haplotypes as shown to the lower right. Numbers on the branches show the mutational steps between the haplotypes.

from the *staryiR2* 4.80 Mya (95% HPD, 3.08–6.09) and they split 4.07 Mya (95% HPD, 2.50–6.09) (Fig. 3).

Population structure and genetic diversity

The MJN defined five well separated haplogroups with the shortest tree having 153 estimated numbers of substitutions. The lowest number of nucleotide changes between haplogroups was observed between *staryiR1* and *staryiG* (Fig. 4). The *P. apusenica* haplotypes form one divergent group and *P. staryi* sequences are grouped into four separate genetic lineages corresponding to three geographically distant mountain ranges and with two haplogroups from the Rodnei Mountains (Fig. 4). The genealogy of the network is similar to the phylogenetic tree topologies, with the difference being that the *staryiR2* haplotype is linked to the *P. apusenica* and *staryiB* haplogroups.

AMOVA showed the highest amount of variation when the five clades (*P. apusenica*, *staryiB*, *staryiG*, *staryiR1*, and *staryiR2*) were treated separately. In this case, the highest diversity was explained by the variation among groups (79.55%). By contrast, when the a priori grouping was based on the current taxonomic status (two species: *P. staryi* and *P. apusenica*), the variation among groups explained only 6.59% of the overall variation and, among populations within groups, the variation was 77.80% (Table 1).

The high level of differentiation of *P. lobifera*, *P. apusenica* and the four cryptic groups of *P. staryi* is also confirmed by the high pairwise K2P distances (Table 2).

DISCUSSION

Phylogenetic relationship within the *P. staryi* species group

The present study is the first assessment of the phylogenetic relationship of the five species belonging to the *P. staryi* species complex based on mtDNA sequence data. Our results are in agreement with the findings of Savchenko (1986), and indicate the

Table 1. Molecular variance analysis inferred with two groups (*Pedicia apusenica* and *P. staryi*) and five lineages (*P. apusenica, staryiG, staryiR1, staryiR2 and staryiB*)

Group composition	Among groups	Among population	Within population	F_{sc}	F_{st}	F_{ct}
Five lineages	79.55	5.4	15.05	0.26	0.84	0.79
Two species	6.59	77.8	15.61	0.83	0.84	0.06

Table 2. Pairwise K2P distances between species and cryptic lineages within *Pedicia staryi* species group

	Pedicia apusenica	*Pedicia lobifera*	*Pedicia straminea*	*Pedicia spinifera*	*staryiB*	*staryiG*	*staryiR1*
Pedicia apusenica							
Pedicia lobifera	9.57						
Pedicia straminea	11.43	11.92					
Pedicia spinifera	12.36	12.58	15.27				
staryiB	6.3	9.65	10.59	12.86			
staryiG	9.5	11.2	11.72	13.86	8.22		
staryiR1	9.97	12.52	13.18	13.97	9.27	6.02	
staryiR2	6.24	11.14	12.14	13.23	7.64	8.99	9.79

monophyletic character nature of the *P. staryi* species group, although this has to be tested further and confirmed with a phylogenetic study including all species of the subgenus *Crunobia*. The Balkan endemic *P. spinifera* is the earliest diverging lineage of the group. *Pedicia straminea* is the sister species of the three Carpathian taxa. It also shows a high level of divergence in relation to the other species. The support for the *P. lobifera*, *P. staryi*, and *P. apusenica* lineages shows high PP and BP values (Fig. 3) and *P. lobifera* is shown to be the sister species of the paraphyletic group containing four well supported *P. staryi* clades and *P. apusenica.* The present study provides important molecular evidence supporting the taxon status of the sibling species *P. apusenica–P. staryi*. It also confirms a close relationship between *P. apusenica* and *P. staryi*, instead of *P. spinifera*, as had been suggested previously (Ujvárosi & Starý, 2003). However, the low support of nodes on the phylogenetic trees shows that the relationship between the species is not clearly resolved by the mtCOI data at hand. A similar pattern of low clade support was observed in several studies (Bell *et al.*, 2004; Trontelj, Machino & Sket, 2005; Pollard *et al.*, 2006). This phenomenon can be explained by processes such as substitution saturation, lineage sorting or long branch attraction (Rokas & Carroll, 2006; Struck *et al.*, 2007; Whitfield & Kjer, 2008; Patel, Kimball & Braun, 2013). In our case, the low support of the deep nodes is most likely a result of the saturation shown by codon position 3 at high genetic distance. The unresolved relationship can be solved by inferring phylogenies using a multilocus approach (Anderson, Stur & Ekrem, 2013) or the complete sequence of the mitochondrial genome (Chen *et al.*, 2014).

Genetic population structure and divergence in the Carpathians

The lack of fossil data for a reliable calibration of the molecular clock led to the use of several standard mutation rates (Brower, 1994; Papadopoulou *et al.*, 2010) estimated in case studies regarding insects (Ho & Lo, 2013). However, these methods should be used with caution because many studies show unequal evolutionary rates for the same gene sequences using calibrations based on the time of different geological events (Andújar, Serrano & Gómez-Zurita, 2012). In the present study, we used a mean mutation rate on the basis of the Mid-Aegean Trench calibration based on Papadopoulou *et al.* (2010). This is only speculative because of the lack of fossil evidence, although our results are in concordance with other studies that show divergence predating Pleistocene glaciations in the Carpathian area (Kotlík & Berrebi, 2002; Pabijan, Wandycz & Hofman, 2013) and are highly congruent with some major palaeoecological changes during the Miocene and Pliocene periods.

Based on our calibration, the molecular clock estimates that the divergence within the Carpathian species and their main lineages started in the Upper Miocene and the Middle Tortonian and ended in the Pleistocene. The ancestor of the three Carpathian endemic species (*P. lobifera*, *P. staryi*, and *P. apusenica*) diverged approximately 8.65 Mya (5.95–12.12 Mya) from *P. straminea*. A similar divergence period in this region was shown for amphibians *Bombina bombina* and *Bombina variegata* (approximately 8.96 Mya, 4.93–12.74 Mya) (Pabijan *et al.*, 2013). At that time, approximately 8–9 Mya, the climatic conditions in Central Eastern Europe were characterized by a major aridification that led to forest fragmentation and an open vegetation with predominant grasslands (van Dam, 2006; Böhme, Bruch & Selmeier, 2007; Bruch, Uhl & Mosbrugger, 2007; Böhme, Winklhofer & Ilg, 2011; Bruch, Utescher & Mosbrugger, 2011). As a result of the subtropical conditions, species adapted to cold were probably restricted to forest patches in the mountain regions surrounding the Central Parathetys (Kvaček *et al.*, 2006). *Pedicia lobifera* appears to be the earliest diverging lineage within the Carpathian group (7.1 Mya, 4.76–10.07 Mya). This evolutionary event can coincide with another aridification after a short cooler and humid climate between 7 and 8 Mya (van Dam, 2006). It may also have been the result of the isolation in the insular enclaves separated by branches and transgressions of the Paratethys, Sarmatian, Pannonic, and Transylvanian Lakes (Rögl, 1999; Harzhauser & Mandic, 2008; Pop *et al.*, 2010; Stoica *et al.*, 2013). The diversification of the five lineages representing *P. staryi* and *P. apusenica* started at the beginning of the Messinian salinity crisis, approximately 5.81 Mya (3.82–8.16 Mya), when the lineage of *staryiR1* and *staryiG* split from the lineage of *P. apusenica*, *staryiR2*, and *staryiB*. Similar lineage diversification was observed for that period in the case of the alpine newt *Mesotriton alpestris* Laurenti, 1668 (Sotiropoulos *et al.*, 2007) and the freshwater flatworm *Crenobia alpina* Dana, 1766 (Brändle *et al.*, 2007), which share identical habitats with members of the *P. staryi* complex. The split between *staryiR2* and the clade formed by *staryiB* and *P. apusenica* took place approximately 4.80 Mya (3.08–6.94 Mya), and *StaryiB* and *P. apusenica* diverged approximately 4.07 Mya (2.50–6.09 Mya). Kotlík & Berrebi (2002) found a divergence between two lineages of the Danubian rheophilic barb (*Barbus petenyi* Heckel, 1852) from the same period, when the climatic conditions of the Carpathian region where dry and seasonally homogenous (van Dam,

2006). At the end of the Pliocene, the global climate started cooling, resulting in the beginning of the glaciations; thus, the split between *staryiR1* and *staryiG* lineages (2.88 Mya, 1.46–4.17 Mya) is probably the result of the Late Pliocene glaciations. In that time, a number of other cold-adapted aquatic insects, such as caddis flies, had undergone important genetic structuring in a series of isolated glacial refugia identified already in the Carpathians (Pauls *et al.*, 2008, 2009; Bálint *et al.*, 2009).

The MJN shows that *staryiR1*, *staryiG*, *staryiR2*, *staryiB*, and *P. apusenica* are genetically distant cryptic units with an island-like distribution and without any sign of gene flow. The deep divergence is also supported by the high proportion of the variance (79.55%) shown by AMOVA, and the observed pairwise distances (ranging between 4.91% and 9.55%) that are over the 2% sequence divergence threshold generally used for delimiting cryptic entities of aquatic insects (Zhou *et al.*, 2009). All these results suggest the need for a taxonomic revision of the *P. staryi* group.

The cumulative nature of refugia in the Carpathian

The Carpathians are recognized as one of the largest hot spots for aquatic biodiversity in Europe, with important numbers of cold-adapted endemic species and range-restricted phylogenetic lineages that are often related to Pleistocene glaciations (Pauls *et al.*, 2006). However, the existence of several deeply divergent lineages suggests long evolutionary histories (Bálint *et al.*, 2011) and a continuous presence dating back to the Pliocene or, in certain cases, even to the Miocene. The strongly variable morphological and genetic structures identified within some fish species (Kotlík & Berrebi, 2002), aquatic insects (Bálint *et al.*, 2011) or terrestrial species from the region with limited dispersal ability (Varga, 2010; Ronikier, 2011) are important arguments in support of the hypothesis that these areas should not simply be viewed as 'glacial refugia' but rather as 'long term' or 'cumulative refugia' (Médail & Diadema, 2009; Tzedakis *et al.*, 2013). Thus, the Pleistocene climate change influenced population divergence and induced further diversification of the existing genetic structures (Hofman *et al.*, 2007).

The geographical projection of several range-restricted genetic lineages or endemic elements in the Carpathians shows a cumulative pattern in some well-defined mountain ranges. In a recent study, Bálint *et al.* (2011) confirmed the northern part of the Eastern Carpathians as one of the major biodiversity hotspots of the Carpathian endemic caddisflies. The importance of the Rodnei Mountains and the surrounding regions (Chornohora and Haghimaș Mts) with respect to maintaining an important number of range-restricted elements was also highlighted for some dipterans (Starý & Ujvárosi, 2005) and a series of endemic plant species (Ronikier, 2011). An important number of deeply divergent, relic-like genetic lineages in this area can be detected in some widely distributed species, such as in the case of a terrestrial animal (Schmitt & Varga, 2012) and plant species (Ronikier, Schneeweiss & Schönswetter, 2012). In concordance with these findings, the northern part of the eastern Carpathians was confimed to be genetically the most diverse area in the present study, where three well differentiated lineages of *P. staryi* and the range restricted endemic *P. lobifera* were detected in a limited geographical space covering the Gutâi, Rodnei, and Haghimaș Mountains. We observed two cryptic groups from the Rodnei Mountains that probably diverged during the Messinian salinity crisis. During the cold periods of Pleistocene, this region was partly glaciated (Urdea *et al.*, 2011), thus causing further isolation (probably with local extinctions) between the two lineages distributed in deep valleys on the northern (*staryiR2*) and the southern (*staryiR1*) slopes of this relatively high and steep mountain range (with altitudes of approximately 2000 m a.s.l.) (Fig. 3). The third lineage (*staryiG*) of the eastern Carpathians diverged from the western lineage of the Rodnei Mountains at the beginning of the Pleistocene glaciations approximately 2.88 Mya (1.46–4.17 Mya). This genetically differentiated group shows an insular-like distribution in the Gutâi Mountains without any shared haplotypes with the populations from the Rodnei Mountains, indicating long-lasting isolation. The deep divergence between *staryiG*, *staryiR1* and *staryiR2* shown in the present study indicates the possibility that survival in several microrefugia detected within this region might have resulted in a cumulative pattern during several palaeogeographical cycles dating well in advance of the Pleistocene glaciations.

The Bucegi Mountains are in a central position between the eastern and southern Carpathians. They are characterized by high altitudes, with peaks of approximately 2500 m, and diverse habitats. Although having a smaller number of range restricted endemics, the importance of the region as a refugium is supported by several studies (Pauls *et al.*, 2008; Ujvárosi *et al.*, 2010; Andújar *et al.*, 2012; Stachurska-Swakoń, Cieślak & Ronikier, 2012), suggesting high diversity as a result of allopatric speciation. The results of the present study, showing a well differentiated *staryiB* lineage, correspond to previous findings (Bálint *et al.*, 2011) that validate the region as an important diversification centre for cold-adapted aquatic insects.

The Apuseni Mountains are also recognized as an important centre of endemism in the Carpathians (Trontelj *et al.*, 2005; Pop *et al.*, 2010), being characterized by lower altitudes and a highly fragmented insular-like relief. The *P. staryi* group is represented here only by *P. apusenica*, which diverged from the other members of the group in the early Pliocene, confirming the role of the region as long lasting refugia, as indicated not only by other aquatic organisms (Kotlík & Berrebi, 2002; Sedivá *et al.*, 2008), but also by some terrestrial endemics, such as several endemic earthworm species (Pop *et al.*, 2010).

The cumulative pattern of diversity and distribution of such range-restricted endemics underlines the importance of some mountain areas in the preservation of the present autochthonous aquatic diversity. Particular centres for diversification hosting several endemic species in the Carpathians are the northern Carpathians (Chornohora-Maramureș-Rodnei), the southern Carpathians (Bucegi Mountains), and the Apuseni Mountains. The present study supports the high conservation value of cold-stenotherm aquatic habitats in the Carpathians. The future of these highly specialized range restricted endemics depends on the proper management of these unique ecosystems in Europe.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Bayesian inference (BI) tree based on codon positions 1+2, showing the phylogenetic relationship among the five species of the *Pedicia staryi* group.
Figure S2. Bayesian inference (BI) tree based on the codon position 3, showing the phylogenetic relationship among the five species of the *Pedicia staryi* group.
Table S1. List of BOLD sequence codes and collection data for individuals of *P. staryi* group used in the study.