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European phylogeography of *Rhyacophila tristis* Pictet (Trichoptera: Rhyacophilidae): preliminary results

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Abstract

We present preliminary results of a phylogeographic analysis of *Rhyacophila tristis*, a wide-spread European caddisfly. Mitochondrial sequence data (the second part of the mtCOI gene) of 52 of specimens were used to investigate large-scale population genetic patterns of Central European populations of the study species. The results show strong genetic differences between a western and an eastern lineage. The deep split most probably indicates that the identified lineages of *R. tristis* survived in independent Pleistocene refugia in the Alps and in the Carpathians, emphasizing the importance of these areas in the Pleistocene survival of aquatic mountain organisms.

Key words: glaciation, genetic divergence, Carpathians, Alps, Trichoptera

Introduction

The last decade revealed many phylogeographic aspects of the European terrestrial species. However, aquatic, and especially mountain aquatic organisms are still under-represented in the surveys. The genetic population structure among populations of European mountain species can be very different compared to more eastern, lowland species (Schmitt 2009). There are differences among mountain aquatic and terrestrial species (Pauls et al. 2006). Aquatic ecosystems, and especially fast flowing streams provide stable environments over long time periods, in contrast to the majority of terrestrial habitats, thus becoming refugia during glaciations (Malicky 1983).

The few existing studies on aquatic mountain species show that populations inhabiting distinct regions may be genetically very different (e.g., Pauls *et al*. 2006, 2009; Lehrian *et al*. 2009). The cryptic genetic diversity usually stands in contrast with the lack of strongly differentiating phenotypic characters. Nonetheless, careful analysis of phenotypic traits may reveal fine differences, enabling the distinction of cryptic entities at the species level (e.g., Bálint et al. 2009). The number of phylogeographic studies analysing widespread aquatic mountain species of Europe is very limited. The published studies focus on several Trichoptera (e.g., Pauls *et al*. 2006, 2009; Lehrian *et al*. 2009) and Ephemeroptera species (e.g., Williams *et al*. 2006). To help fill this gap we studied the wide-spread caddisfly *Rhyacophila tristis* Pictet.

Here we present preliminary results of a larger-scale investigation of *R. tristis* on its entire range of distribution. The present results are based on a limited number of specimens collected in Central

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Europe, which is only a fraction of the entire distribution range of the species. The analyses performed are also limited due to the sampling restricted to several mountains in Central Europe. Our hypothesis is that *R. tristis* populations inhabiting distinct Central European mountain ranges show stronger geographically associated genetic differences than terrestrial organisms occupying a similar range.

Materials and methods

Target species and collecting localities

Rhyacophila tristis is the most widely distributed species of the *Rhyacophila tristis* Group sensu Schmid (1970). The Group has an entirely euromediterranean (sensu Bănărescu 1991) distribution. The species inhabits fast-flowing streams at altitudes between ~300-2000 m a.s.l.

In this study nucleotide sequences of 52 specimens were analysed. These were obtained from the Alps, Black Forest, Carpathians, Rila and Pirin (Fig. 1) and are part of a larger collection that covers

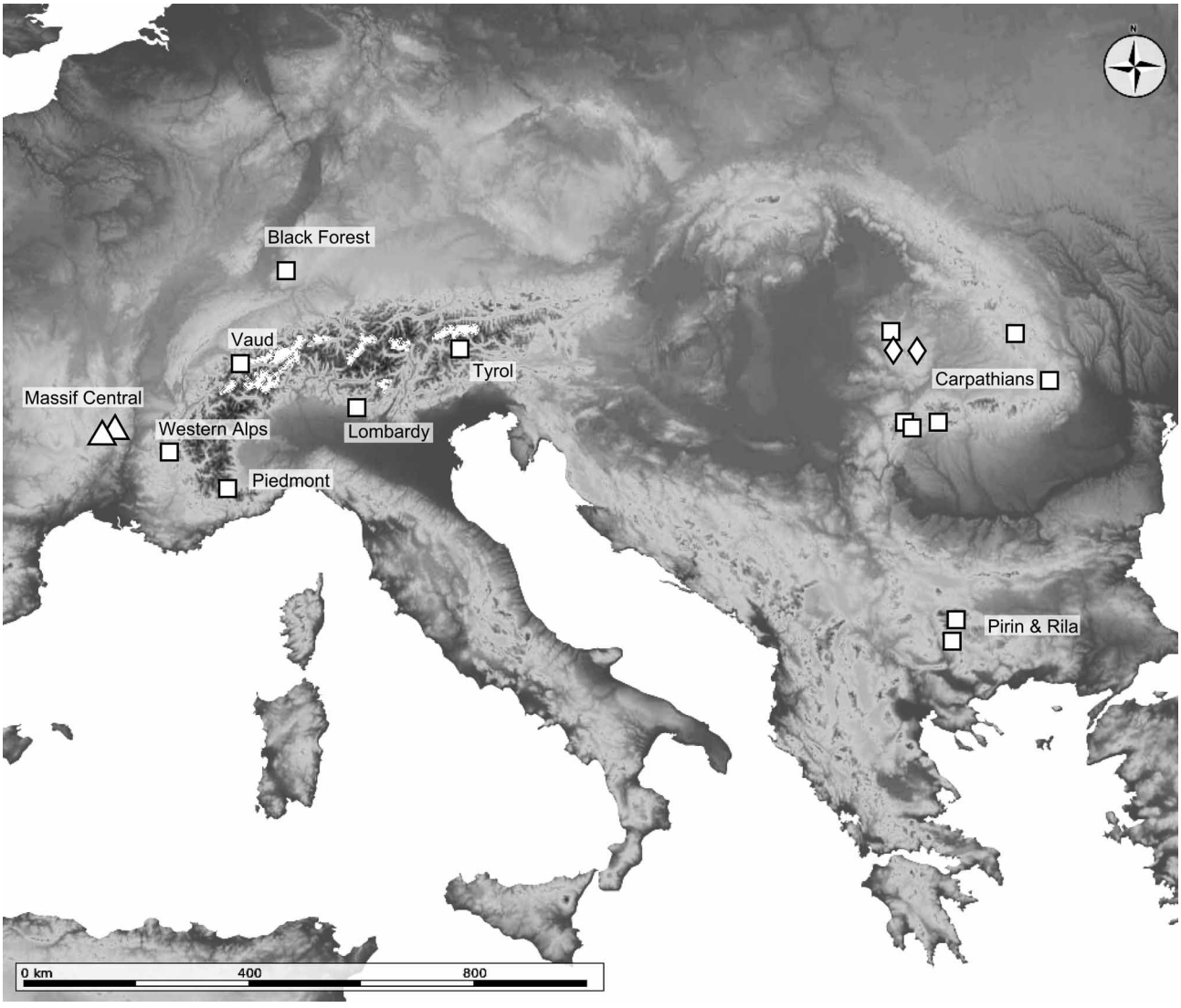

FIGURE 1. Collecting localities of *Rhyacophila tristis* specimens. Squares: *R. tristis*, triangles: *R. aquitanica*, diamonds: *R. orghidani*.

the entire distribution range of the species. Additionally, 3 specimens of each of the 2 closely related species *R. aquitanica* McLachlan and *R. orghidani* Botoşaneanu were used as outgroups. These were collected in the Massif Central, France, and in the Apuseni Mountains, Romania, respectively. Male specimens were identified based on genitalia structures (diagnostic characters that were emphasized by, e.g., Malicky 2005). The distinction of *R. tristis* females and larvae from those of *R. aquitanica* and *R. carpathica* is problematic. Their identity was confirmed by comparing the obtained nucleotide sequences with those from male *R. tristis*, *R. aquitanica* and *R. carpathica* (Botoşaneanu) using both phylogenetic and genetic distance criteria (Waringer *et al*. 2007, 2008).

Molecular methods and data analysis

DNA was obtained using phenol-chloroform extraction and ethanol precipitation (e.g., Reineke *et al*. 1998) and commercial DNA extraction kits (Qiagen DNeasy Blood & Tissue Kit). Whenever possible, DNA was extracted from fresh specimens. However, in a few cases DNA was successfully extracted from older specimens stored in 70% ethanol for 5 years.

DNA was extracted from the abdomen of the male specimens. As Trichoptera generally don't feed as adults, this is considered a contamination-safe method. In addition, the genitalia of males were macerated after proteinase K digestion, making conventional KOH maceration unnecessary. In the case of females the 2 hind legs were used for DNA extraction, as fertilized eggs in the abdomen may interfere with further molecular analyses of nuclear DNA. DNA was obtained from the abdomen of each larva after its digestive tract and parasites were carefully removed.

A part of the mitochondrial cytochrome oxydase I (COI) was amplified using universal primers Jerry (Simon *et al*. 1994) and S20 (Pauls *et al*. 2006). PCR was performed in 25 μL reaction volumes following Bálint (2008). PCR products were purified using commercial column-based gel extraction kits (Fermentas). Fragments were sequenced on an automatized ABI Prism 310 one-capillary and an automatized Beckman-Coulter CEQ 8800 eight-capillary sequencer at the Molecular Biology Center, Babeş-Bolyai University. The sequences were submitted into GenBank (http://www.ncbi.nlm.nih.gov/) with accession numbers HM204650-HM204704 (*R. orghidani* and *R. tristis*) and FJ514788, FJ514790, FJ514791 (*R. aquitanica*).

The sequences were aligned using the Clustal W algorithm (Thompson *et al*. 1994). The aligned sequences were manually checked for errors using the tracefiles in MEGA v. 4 (Tamura *et al*. 2007). We obtained an alignment containing 456 bp-long sequences for the 52 specimens. Sequence identities were checked using BLAST (Altschul et al. 1997). The nucleotide substitution model was selected using the Akaike Information Criterion in jModelTest (Posada 2008).

Bayesian inference of phylogenetic relationships was accomplished with MrBayes 3.2 (Huelsenbeck & Ronquist 2001), using the previously selected substitution model (GTR+Γ). Two simultaneous and independent analyses were run for 4,000,000 generations. Trees were sampled in every 100 generations. The first 25% of the resulting trees were discarded as burn-in. The MCMC sampling was run using 12 chains (1 cold and 11 heated). The consensus tree was visualized in FigTree 1.2.1 (Rambaut 2009).

Overall mean Jukes-Cantor distances were calculated in DnaSP 4 (Rozas *et al*. 2003). Population genetic structures were analysed by AMOVA (Analysis of MOlecular VAriance) as implemented in Arlequin 3.11 (Excoffier et al. 2005) with 10,100 random permutations for statistical significance. Two major regions were designated for AMOVA. The western region contained sampling sites from the Alps and the Black Forest. Carpathian and Bulgarian sites were assigned to the eastern region.

Results and Conclusions

The overall mean Jukes-Cantor distance among the sequences was *d*=0.02138. The specimens comprised 19 nucleotide haplotypes, and clustered into 2 major clades (Fig. 2). These clades entirely corresponded to western (Alps, Black Forest) and eastern (Carpathians, Pirin, Rila) populations, respectively. The Jukes-Cantor nucleotide diversity was *d*=0.0097 and *d*=0.0092 within the western and eastern groups, respectively, and *d*=0.0235 between the 2 clades.

No geographically associated genetic structure was found among the Carpathian specimens. Specimens of the western sampling sites formed 2 well-supported groups. One group contains specimens from the Western Alps, Lombardy, Piedmont and the Swiss canton of Vaud. The second group is formed from specimens collected in Tyrol, in the Black Forest and in Vaud.

Almost all genetic variation was attributed to differences between the 2 major regions (Table 1). Gene flow between the eastern and western populations seems completely stopped (F_{ST}=0.983 at p<0.001). There were no shared haplotypes between these regions.

TABLE 1. Analysis of molecular variance of *Rhyacophila tristis* populations.

	Variance components		*Percentage of variation*
Among major regions	160.6039	(Va)	93.85
Among populations within mountain ranges	7.56918	(Vb)	4.42
Within populations	2.94717	(Vc)	1.72

Our hypothesis on the strong genetic differentiation of eastern and western populations is supported by the results. The absence of a link between Alpine and Carpathian populations is quite common in the case of (semi)aquatic species. Similar differentiation patterns were observed in the case of the caddisflies *Drusus discolor* (Rambur) (Pauls *et al.* 2006), *R. aquitanica* and *R. carpathica* (Bálint *et al.* 2008), and *Chaetopterygopsis maclachlani* Stein (Lehrian *et al.* 2010). This stands in contrast with the majority of terrestrial species, where the connection between the Alps and the Carpathians is common (e.g., Varga & Schmitt 2008; Schmitt *et al.* 2009). The results present evidence for Pleistocene extramediterranean refugia for *R. tristis* at least during the Würm glaciation in the Carpathians. There are 7 mutation steps between the Carpathian and Alpine clades. There are also 7 mutation steps between the Carpathian clade and the sequenced 3 Bulgarian specimens. These relatively deep splits suggest an early divergence of the Carpathian clade from Alpine and Bulgarian populations. However, we have to emphasize that the divergence of the Carpathian and Balkan populations should be treated with care due to the very limited number of sequenced Bulgarian specimens.

The possibility that *R. tristis* recolonized the Carpathians post-glacially from a Balkan refuge cannot be excluded, as the number of analysed individuals is very low for Bulgaria. However, no shared haplotypes were found between the Carpathian and Bulgarian populations. Based on the preliminary results it seems plausible that glacial refugia existed for *R. tristis* both in the Carpathians and on the Balkan Peninsula. The importance of the Carpathians in the Pleistocene survival and differentiation of (semi)aquatic mountain species was already underlined by a number of studies (e.g., Bálint *et al.* 2009; Pauls *et al.* 2009; Ujvárosi *et al.* 2010).

The genetic structure observed among populations in the western group can be explained by the existence of multiple extramediterranean glacial refugia around the Alps. Areas north of the Alps were certainly not repopulated from the Carpathians. Recolonization of these areas from the Italian peninsula seems also unlikely, but this possibility cannot be entirely excluded due to the small number of analysed Southern Alps specimens. *Rhyacophila tristis* is also present on the Iberian Peninsula. However, recolonization from this direction is unlikely as the species is completely missing from the Massif Central. There are no known links between the Pyrenees and the Western Alps, as the Massif Central is inhabited exclusively by the closely related *R. aquitanica* according to our present knowledge.

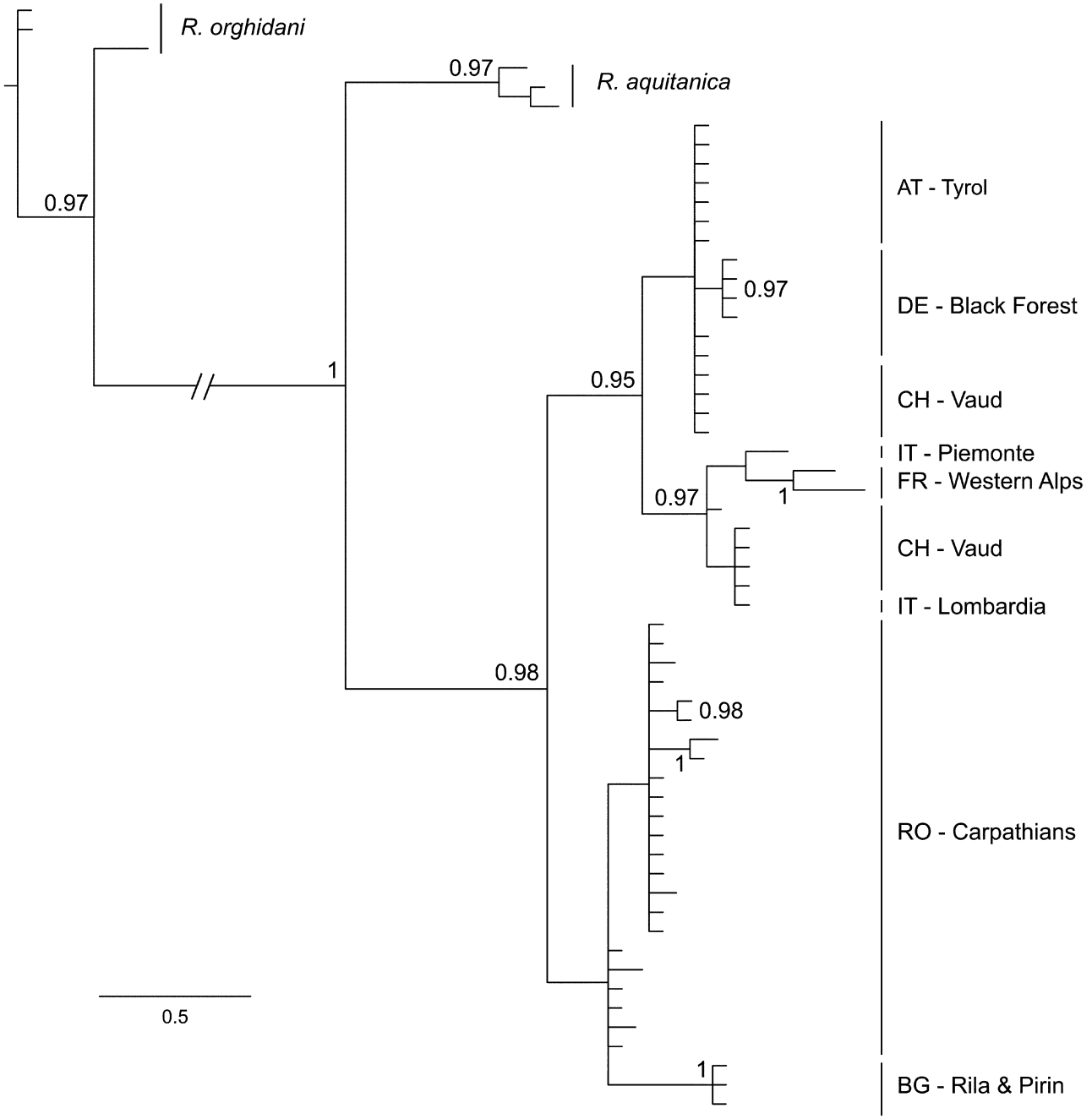

FIGURE 2. Phylogenetic relationships of *Rhyacophila tristis* specimens based on B/MCMC inference. Posterior probability values *pp*>0.95 are shown.

The 2 distinct western *R. tristis* lineages coexist at the sampling locality in Vaud, Switzerland (Figs. 1, 2). This area may be a secondary contact zone for the western 2 lineages. Secondary contact between eastern and western lineages in this region of the Alps is a common phenomenon, which has also been discussed for Limnephilidae (Graf *et al*. 2009) and *Rhyacophila simulatrix* (Graf & Waringer 2005). One of these probably extended its range from a refuge east of the Alps. The second lineage most probably came from the south-western parts of the Alps. Engelhardt (2009) showed that areas in the Western Alps served as refugia also for the closely related *R. pubescens* Pictet. This region is known as refugia for terrestrial species, too (e.g., the Marbled White butterfly, Habel *et al*. 2005). The geographic origin of the source populations can be determined only after the analysis of more specimens collected in areas around the Alps, Tatra and Dinaric Alps.

The phylogeographic analysis of *R. tristis* across its entire distribution range may bring new information about the diversity of the European mountain aquatic fauna. It is especially important to include more populations in the analyses, collected on the 3 Mediterranean peninsulas. This may help to understand the role of extramediterranean versus Mediterranean refugia in the recolonization of aquatic habitats after glaciations.

It is plausible that further cryptic taxonomic entities are present within what is currently recognized as *R. tristis*. Kumanski (1987) already described a very similar species from Bulgaria (*R. pseudotristis*). We hypothesize that molecular analyses of populations inhabiting distinct mountain ranges especially on the southern areas of Europe will reveal old cryptic taxonomic entities.

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