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Genetic population structure of an autumn-emerging caddisfly with inherently low dispersal capacity and insights into its phylogeography

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Abstract. We used ecological information and current and historic species distribution models for the last glacial maximum (LGM) to develop hypotheses regarding the Pleistocene history of the montane, autumn-emerging caddisfly *Chaetopterygopsis maclachlani*. We used mitochondrial sequence data from the cytochrome oxidase I (mtCOI) gene of 282 specimens from populations across Europe to analyze the population structure and phylogeography of *C. maclachlani* and to test our hypotheses. We examined the population genetic structure with median-joining networks, analysis of molecular variance (AMOVA), exact tests of population differentiation, and Mantel tests of isolation by distance. Furthermore, we used tests for selective neutrality (Tajima's D , Fu's F_S) to infer potential population growth and expansion and Bayesian Skyline Plots to analyze past population dynamics. We found strong population structure with 47 different haplotypes that could be separated into a southeastern and a western clade. The western clade seems to have survived at least the LGM in an extra-Mediterranean refugium, independent of the southeastern clade. Within the 2 main clades, we found haplotype overlap between mountain ranges and a high proportion of private haplotypes (89%). As predicted for a montane species with very limited adult dispersal capabilities, many regions and populations are currently isolated and appear to be diversifying into independent lineages. We discuss speciation within the Chaetopterygini and the diversification of *C. maclachlani* in particular.

Key words: aquatic insect, *Chaetopterygopsis maclachlani*, genetic population structure, mtCOI, Trichoptera.

The occurrence of a species and the distribution of its genetic variation result from a species response to historic habitat contraction and expansion, e.g., from past climate change and the location, number, and size of refugia, and present environmental conditions, dispersal capability, and connectedness of populations (Hewitt 2004, Schmitt 2007, Hughes et al. 2009). Although the patterns of species responses to glacial cooling currently are understood only for individual

species, a growing consensus is that most terrestrial temperate species in central Europe must have survived glaciations in ≥ 1 refugia in more moderate climates in southern Europe (Hewitt 2004, Schmitt 2007). Some cold-tolerant or cold-hardy species were able to remain in place and survived glaciations in nunataks or cryptic northern refugia in the periglacial zone between northern and southern glacial expanses (Stehlik et al. 2002, Pauls et al. 2006, Mráz et al. 2007, Naciri and Gaudeul 2007). During interstadials, interglacials, or after glaciation, recolonization of central Europe started from these refugia (reviewed by Stewart et al. 2009). Stream insects with an aquatic larval stage and a terrestrial adult stage are interesting subjects for phylogeographic investigations because of

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their different dispersal pattern and the distinct linear structure of stream habitats.

The winged adult stage of aquatic insects often is considered the stage in which the main lateral dispersal among streams, catchments, or over long distances can occur (Bilton et al. 2001, Petersen et al. 2004, Hughes 2007). However, many aquatic insects inhabit large ranges but have brachypterous (short-winged) or apterous (wingless) adults, or emerge during cold periods of the year when dispersal generally is assumed to be poor. In caddisflies, brachyptery is rare (Giudicelli and Botoșaneanu 1999, Holzenthall et al. 2007, Graf et al. 2009). Moreover, most aquatic insect species of temperate regions emerge in summer when dispersal capacity can be considered highest for poikilothermic animals. Some exceptions are winter-emerging stoneflies (e.g., *Allocapnia* spp., Ross and Ricker 1971), caddisflies (e.g., *Melampophylax mucoreus*, Waringer and Graf 1997), and dipterans (e.g., *Bryophaenocladus thaleri*, Willassen 1996; *Diamesa mendotae*, Bouchard and Ferrington 2009). Thus, caddisflies of the Limnephilidae tribe Chaetopterygini present an interesting intermediate form: adults of these species emerge in autumn, are brachypterous with relatively short, rounded wings, and are poor fliers or are unable to fly (Botoșaneanu 1975, Wagner 1991, Malicky 1994). Like most autumn-emerging caddisflies, they generally are not seen flying, but rather are seen crawling through riparian vegetation and are rarely caught with light traps (Malicky 1987, Malicky and Krušnik 1991).

Many species of autumn-emerging Chaetopterygini caddisflies are endemic to single mountain ranges or small areas (Malicky 1994). However, other species are widespread across Europe. One such species is *Chaetopterygopsis maclachlani* Stein 1874 (Fig. 1A, 2). Disjunct insular highland or montane distributions can result either from past fragmentation of a previously broader, more coherent distribution range or from repeated long-distance dispersal events and founding of new populations in isolated patches of suitable habitat. Our first hypothesis is that short-winged, autumn-emerging caddisfly species should exhibit stronger genetic population structure than other montane caddisfly species that emerge in summer or are good fliers.

We followed the approach advocated by Richards et al. (2007) and used our genetic data to test 2 additional phylogeographic hypotheses derived from last glacial maximum (LGM) climate-niche models (Fig. 1B, C, Appendix 1). First, in line with the LGM climate niche distribution under the Community Climate System Model (CCSM) model (Fig. 1B) we hypothesized that *C. maclachlani* persisted in 2

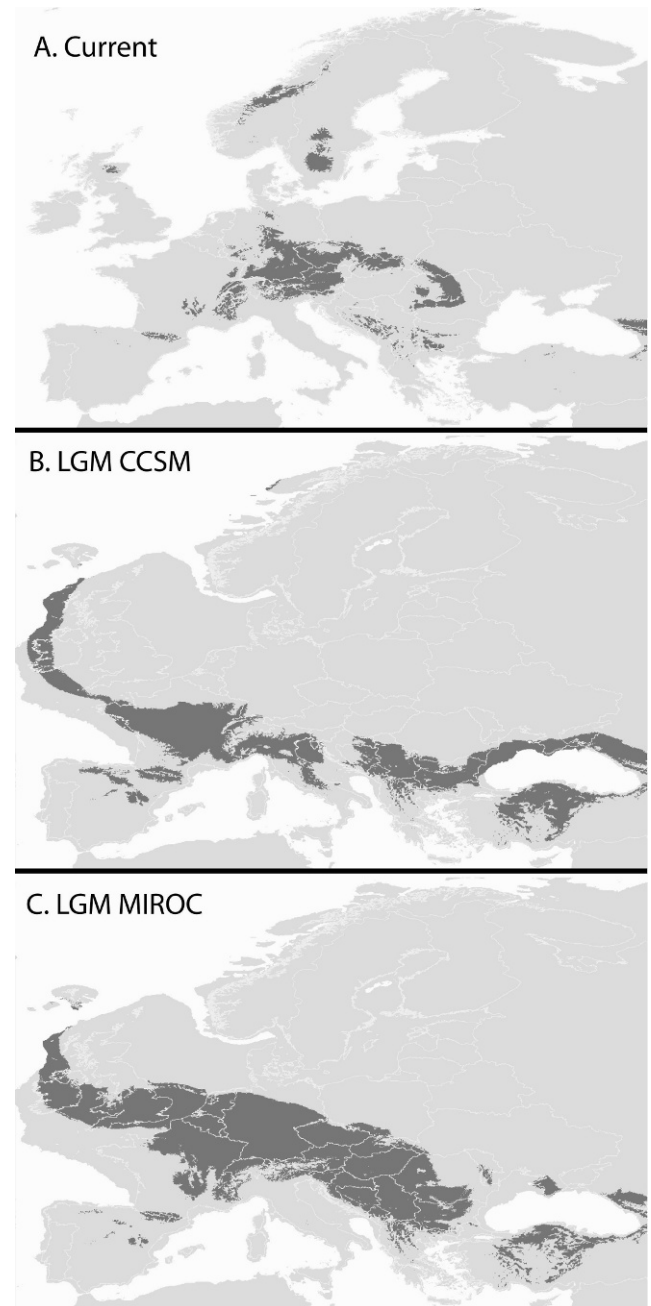


FIG. 1. Climatic niche model predictions for *Chaetopterygopsis maclachlani*. The range of the suitable climatic niche (black shading) is shown at present (A) and during the last glacial maximum (LGM) based on the Community Climate System Model (CCSM) (B) and the Model for Interdisciplinary Research on Climate (MIROC) (C). See Appendix 1 for details.

independent refugial areas, 1 in western and central Europe and 1 east of the present-day Adriatic Sea. Under this scenario, we would expect to see a deep split between eastern and western European populations. The Model for Interdisciplinary Research on

Climate (MIROC) offers an alternative scenario to this hypothesis. Under this model, *C. maclachlani* survived in a more-or-less contiguous metapopulation across central and eastern Europe (Fig. 1C). Under this scenario, we would predict shallow divergence and little genetic differentiation among central and eastern European populations of *C. maclachlani*. Second, we hypothesized that *C. maclachlani* was more widespread during the LGM and is currently a regressive species with a fragmented distribution, restricted to colder habitats, i.e., higher elevations. This scenario is consistent with both LGM niche models and should result in a pattern of demographic regression after the LGM.

We used mitochondrial cytochrome oxidase I (mtCOI) sequence data to examine the population structure and demographic history of the widespread autumn-emerging caddisfly *C. maclachlani* across its main distribution range in Europe. We explicitly tested the hypotheses outlined above. Our study allowed us to examine how species with low inherent dispersal capacity can occupy large ranges that were climatically dynamic in recent history. We predicted that because of the species' discontinuous distribution in cold headwater mountain streams and its low inherent dispersal capacity, which is a key ecological factor influencing the structure of a population (Hughes 2007), *C. maclachlani* would exhibit strong genetic population structure, indicative of long-term persistence in the vicinity of its current area of distribution.

Methods

Study species

We chose *Chaetopterygopsis maclachlani* Stein, 1874 (Trichoptera:Limnephilidae:Chaetopterygini) as a model species with which to examine how a species with intermediate dispersal capacity could occupy a large range in a historically dynamic landscape. The genus *Chaetopterygopsis* consists of 3 Eurasian species. *Chaetopterygopsis maclachlani* has 2 sister taxa in eastern Europe: *Chaetopterygopsis sisestii* Botoșaneanu, 1961 in the Carpathians and *Chaetopterygopsis siveci* Malicky, 1988 in Greece (Graf et al. 2008). *Chaetopterygopsis maclachlani* is distributed across western and central Europe and extends to the Ural and Baikal region in the east.

The larvae of *C. maclachlani* live in the springs, headwaters, and middle reaches of streams (eucrenal to metarhithral) (Graf et al. 2008). Thus, larvae occur over a large stretch of streams and have been observed 0.3 to 50 km from the spring (Pitsch 1993). Our observations showed that larvae often are found

in patches of moss of the genus *Fontinalis*, usually in areas of the stream with slower current or laminar flow. In the Vosges Mountains, larvae of *C. maclachlani* feed on bryophytes (64.5%) and to a lesser extent on coarse leaf detritus (35.5%), indicating a very specialized feeding behavior (Dangles 2002). *Chaetopterygopsis maclachlani* larvae almost always build their cases using leaves from *Fontinalis* mosses (Malicky 1994), and they seldom use other suitable organic material, such as pieces of plant leaves (Pitsch 1993, Waringer and Graf 1997). The case is almost always longer than the body and can be $>2\times$ the body length of the larvae in the later instars (SL, SUP, personal observation). Thus, the species also seems to be very specialized in its case-building. In addition, *C. maclachlani* is thought to have a preference for higher altitudes or regions that are close to higher mountain ranges (Pitsch 1993, Haase 1999, Graf et al. 2008), presumably because of a preference for colder water temperatures.

Population sampling

We sampled the central distribution range of *C. maclachlani* and examined a total of 282 specimens from 28 populations in 12 regions (Fig. 2, Appendix 2). *Chaetopterygopsis maclachlani* also has been recorded from the Pyrenees, the Jura, and southwestern Alps in the west, and in northern Poland, the Dinarids, the Ural, and the Baikal area in the east (Fig. 2) (Kumanski 1988, Mey 1991, Czachorowski et al. 1993, Medvedev 1998, Graf et al. 2008). Given the autumn emergence of the species, some regions probably are not as well studied as others, e.g., France and the former Soviet Union (Kumanski 1980, Malicky 2005). Presumably, the species also occurs in the western Alps, but we were unable to find any individuals there or in the Jura Mountains during several sampling trips. The main distribution range of *C. maclachlani* seems to be in the eastern parts of the European Highlands where we collected many specimens (Fig. 2).

Larval and adult specimens were sampled with hand nets and were stored in ethanol until deoxyribonucleic acid (DNA) extraction. Larvae and adults were identified with keys in Waringer and Graf (1997) and Malicky (2004), respectively. Vouchers are deposited at the Senckenberg Research Institute and Natural History Museum, Frankfurt, Germany.

DNA extraction, amplification, and sequencing

DNA extraction and polymerase chain reaction (PCR) amplification of the mtCOI fragment followed methods published in Lehrian et al. (2009). Primers

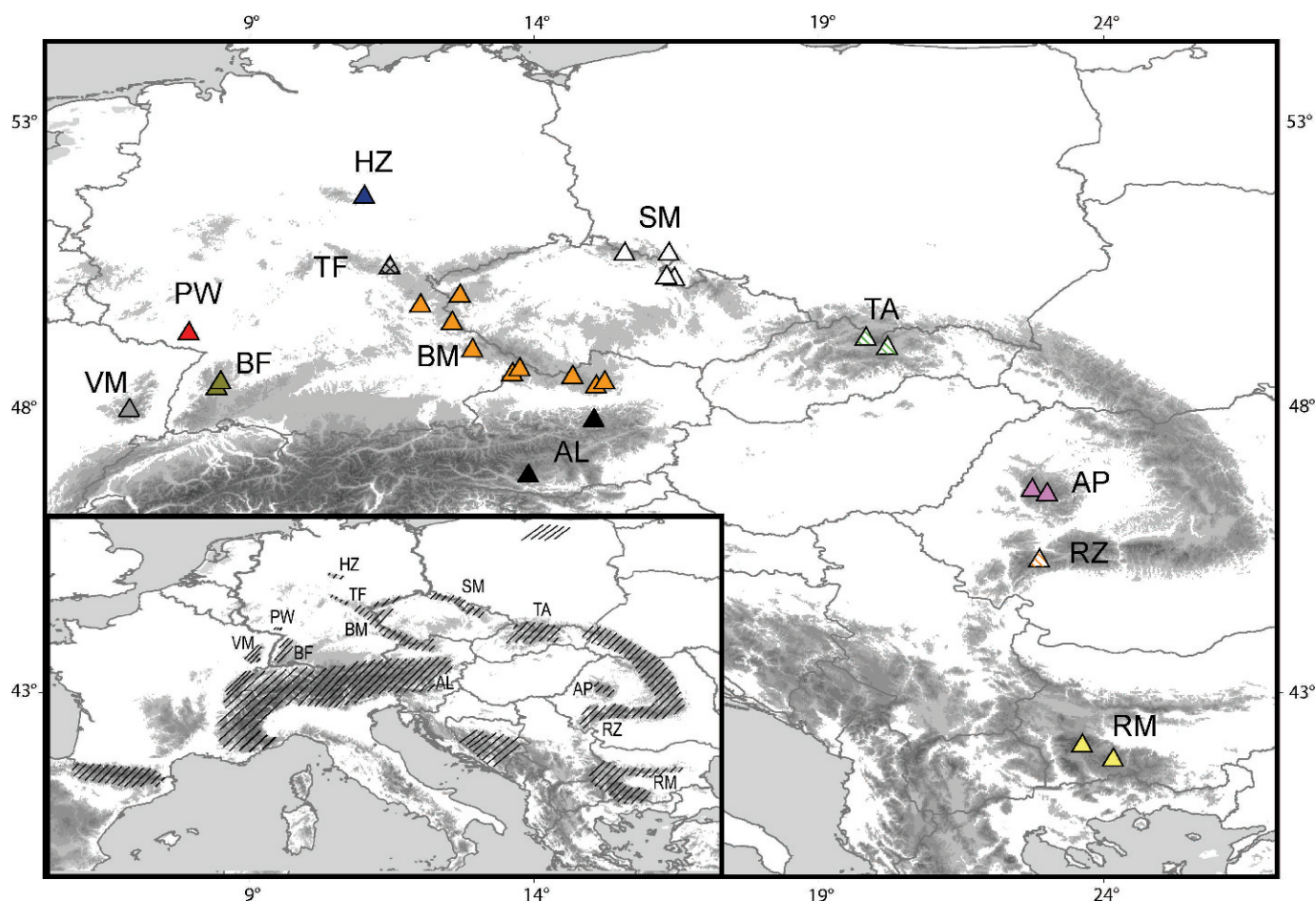


FIG. 2. Sampling sites (triangles, color-coded as in Fig. 3) and European distribution (hatched area on inset) of *Chaetopterygopsis maclachlani*. Details on sampling sites given in Appendix 2. The European distribution of *C. maclachlani* is based on literature, personal communications with specialists, and the authors' observations (Kumanski 1988, Mey 1991, Czachorowski et al. 1993, Pitsch 1993, Medvedev 1998, Haase 1999, Graf et al. 2008). HZ = Harz, SM = Sudety Mountains, TF = Thuringian Forest, BM = Bohemian Massif, PW = Pfaelzer Wald, TA = Tatra, AP = Apuseni, RZ = Retezat, BF = Black Forest, VM = Vosges Mountains, AL = Alps, RM = Rila-Rhodope Massif.

were Dave (5'-AGTTTATGACAGGAGCAATTACTAT-3', position 2059 at 3' end relative to *Drosophila yakuba*) and Inger (5'-AAAAATGTTGAGGGAAAAATGTTA-3', position 2735 at 3' end relative to *D. yakuba*) (Zhang and Hewitt 1996). Sequences were generated with the PCR primers by 2 commercial sequence services: Nano+Bio Center Kaiserslautern, Germany, and AGOWA GmbH Berlin, Germany.

Electropherogram traces were aligned, checked, and manually edited using Sequencher software (version 4.8; Gene Codes Corporation, Ann Arbor, Michigan). We used Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1997) to verify the identity of sequences. Sequences were aligned using CLUSTAL W with default parameters as implemented in BioEdit 7.0.9.0 (Hall 1999).

Data analysis

Haplotype distribution.—We used phylogenetic tree- and network-building techniques to visualize the relationships among haplotypes and their distribution. The sequence alignment was imported into DnaSP 4.50.2 (Rozas et al. 2003) to generate a haplotype matrix for calculating a median-joining (MJ) haplotype network (Bandelt et al. 1999) in Network 4.5.0.1 (Fluxus Technology, Suffolk, UK) using default settings. In addition, we constructed a minimum spanning network of haplotypes with 95% plausible parsimony links using TCS 1.21 (Clement et al. 2000).

We used Modeltest (Posada and Crandall 1998) to select the best-fitting model for our data. The

Hasegawa-Kishino-Yano + gamma + invariant sites (HKY + Γ + I) model was chosen based on the likelihood ratio test criterion in Modeltest. We calculated a Bayesian Metropolis-Coupled Markov-Chain Monte-Carlo (B/MCMCMC) phylogenetic inference of the 47 haplotypes based on the selected model using MrBayes 3.1 (Ronquist and Huelsenbeck 2003). Four chains were run for 5×10^6 generations. Trees were sampled every 1000th generation; the first 2.5×10^6 generations were discarded as burn-in.

Genetic population structure and differentiation.—Analysis of molecular variance (AMOVA) was calculated in Arlequin 3.11 (Excoffier et al. 2005) with 16,000 permutations. The distance applied was Kimura-2-Parameter distance. Our a priori grouping allowed us to test variation within vs among regions. We calculated exact tests of population differentiation (ETPD) (Raymond and Rousset 1995) and pairwise F_{ST} values as implemented in Arlequin 3.11 (Excoffier et al. 2005) to analyze differentiation between regions. We ran exact tests with 10,000 dememorization steps and a Markov Chain with a length of 100,000. We ran F_{ST} with 16,000 permutations. Significance of both was Benjamini-Yekutieli (B-Y) adjusted (Narum 2006). We applied a Mantel test to the matrices of pairwise F_{ST} values and geographical distances between all analyzed populations to assess isolation-by-distance in Arlequin 3.11 (Excoffier et al. 2005). We ran 10,000 permutations. We calculated geographic distances using ArcView® (ESRI ArcView 3.2, Redlands, California). We used Barrier 2.2 (Manni et al. 2004) to detect barriers to gene flow based on Monmonier's (1973) maximum difference algorithm. Barriers are computed associating a pairwise F_{ST} -distance matrix with a geographic data matrix.

Genetic population diversity.—Gene diversity, nucleotide diversity, and pairwise differences were calculated in Arlequin 3.11 using the default settings (Excoffier et al. 2005). More recently colonized regions are expected to have lower genetic diversity than refugial areas or older areas of occurrence because an expanding population contains only a subset of the haplotypes found in the ancestral population (Ibrahim et al. 1996).

Demographic history and population expansion.—To test for recent demographic expansion in each region and in the whole data set, we calculated Tajima's D and Fu's F_S in Arlequin 3.11 (Excoffier et al. 2005) with 16,000 permutations. These metrics are expected to have significantly negative values if the populations are not in equilibrium between mutation and drift, e.g., because of demographic expansion. These negative values can arise under selective effects, but they can also be indicative of population expansion or

bottlenecks (Tajima 1993a, 1996). Fu's F_S is especially sensitive to population expansions (Fu 1997).

Past population dynamics were analyzed using Bayesian Skyline Plots (BSP; Drummond and Rambaut 2007) implemented in BEAST 1.5.3 (Drummond et al. 2005). BSPs were calculated separately for the eastern and western clades of *C. maculachlani* and for the total data set containing all individual sequences. The relative rate test (Tajima 1993b) in MEGA 4.0 did not allow us to reject the hypothesis that our sequences were evolving in a clock-like manner. We used a divergence rate of 2.3%/my (10^6 y; Brower 1994) under the relaxed uncorrelated log-normal model. We chose a piecewise-constant skyline model for the analyses. We constructed an Unweighted Pair Group Method with Arithmetic mean (UPGMA) starting tree before each run. We ran tests for 100 million generations for each clade and the whole data set to ensure high effective sample sizes (ESS). We sampled chains every 10,000 states and removed 10% of the samples as burn-in. We did 2 independent runs for the eastern clade and 4 runs for the western clade and the complete data set. We combined the results of the independent runs in the program LogCombiner (version 1.4.8; <http://beast.bio.ed.ac.uk/LogCombiner>) to check the convergence and mixing of independent chains. We created BSPs in Tracer (version 1.4.1; <http://tree.bio.ed.ac.uk/software/tracer/>) based on the combined results.

Results

The final alignment was 497 base pairs (bp) long and contained 46 variable positions, which resulted in 47 unique haplotypes (GenBank accession numbers: HM005165–HM005211). The nucleotide composition showed the typical adenine thymine (AT)-rich signature of insect mitochondrial DNA (cytosine [C]: 18.13%, T: 41.02%, A: 24.88% and guanine [G]: 15.97%).

Haplotype distribution

We visualized geographic distribution of the 47 identified haplotypes by color coding in a MJ network (Fig. 3). The MJ and the TCS network were similar. The TCS network connected all haplotypes in 1 network with 95% confidence, but it built 1 loop more than the MJ network between H18 and H32 (red dotted line in Fig. 3). Maximum divergence between all haplotypes was 3.82% and the mean pairwise difference was 4.591 ± 2.262 bp. Two main clades were identified. Clade 1 (top) consisted of 33 haplotypes from the northern Carpathians and central Europe, whereas clade 2 (bottom) represented a

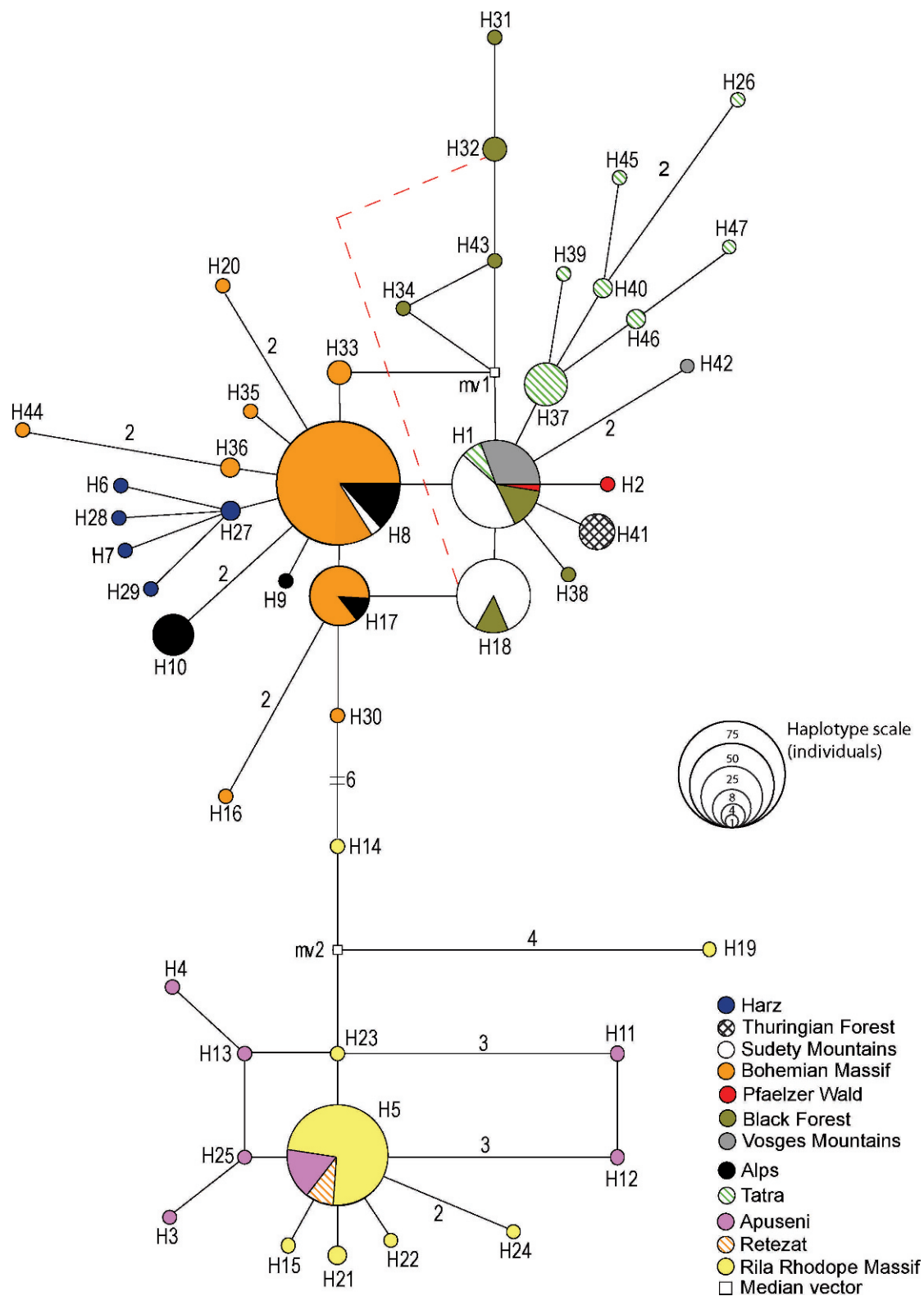


FIG. 3. Median-joining network of *Chaetopterygopsis maclachlani* haplotypes. Color-coding corresponds to origin of the specimens carrying a given haplotype. Circle diameter is relative to number of specimens carrying a haplotype. Hatch marks or numbers on connecting lines indicate number of base pair (bp) changes between haplotypes. If no number is shown, the number of bp changes is 1. The red dashed line shows the additional loop found in the network generated with TCS (Clement et al. 2000).

TABLE 1. Pairwise F_{ST} -values (bold indicates statistical significance). Significant results of exact tests for differentiation between regions are indicated with $^+$. Significance level after Benjamini–Yekutieli adjustment for both tests is $\alpha = 0.01037$.

Region	VM	PW	BF	HZ	TF	BM	AL	SM	TA	AP	RZ
Vosges Mountains (VM)											
Pfaelzer Wald (PW)	0.304										
Black Forest (BF)	0.256⁺	0.117									
Harz (HZ)	0.771⁺	0.602	0.588⁺								
Thuringian Forest (TF)	0.819⁺	0.878	0.492⁺	0.818⁺							
Bohemian Massif (BM)	0.628⁺	0.632⁺	0.603⁺	0.612⁺	0.765⁺						
Alps (AL)	0.902⁺	0.499⁺	0.517⁺	0.501⁺	0.685⁺	0.177⁺					
Sudety Mountains (SM)	0.209⁺	0.235	0.175⁺	0.690⁺	0.618⁺	0.512⁺	0.500⁺				
Tatra (TA)	0.441⁺	0.337	0.432⁺	0.671⁺	0.610⁺	0.699⁺	0.610⁺	0.499⁺			
Apuseni (AP)	0.902⁺	0.853	0.844⁺	0.858⁺	0.894⁺	0.915⁺	0.869⁺	0.898⁺	0.853⁺		
Retezat (RZ)	0.977⁺	0.981	0.868⁺	0.931⁺	1.000⁺	0.933⁺	0.899⁺	0.926⁺	0.885⁺	−0.008	
Rila Rhodope Massif (RM)	0.943⁺	0.936	0.907⁺	0.930⁺	0.947⁺	0.928⁺	0.912⁺	0.924⁺	0.908⁺	0.113	−0.101

southeastern lineage with 14 haplotypes. The clades were divergent by 6 mutational steps ($p = 1.21\%$). Clade 1 was characterized by 2 central haplotypes (H1, H8), that differed by 1 bp, each of which was connected to a number of haplotypes by 1 to 3 bp changes. Central haplotype H8 ($N = 75$) and haplotypes connected to it were associated with the Bohemian Massif, Alps, Sudety Mountains, or Harz. Central haplotype H1 ($N = 39$) and its connected haplotypes were associated with the Black Forest, Pfaelzer Wald, and Vosges Mountains in the west and the Thuringian Forest, Sudety Mountains, and Tatra in the east. Most haplotypes were private, i.e., specific to a single region (Fig. 3).

In clade 2 (southeastern lineage), 1 central haplotype (H5; $N = 53$) was present in all of the sampled mountain ranges in the region (Apuseni, Rila/Rhodope Massif, and Retezat Mountains). About $\frac{1}{2}$ of the other haplotypes in haplogroup 2 were singletons that diverged by 1 or 2 bp from H5. The remaining singleton haplotypes were connected to H5 by ≤ 6 bp. H21 was the only nonsingleton haplotype, was connected to H5 by 1 bp and was endemic to the Rila/Rhodope Massif.

Several regions exhibited relatively high levels of haplotype endemism. The Tatra Mountains had 7 private haplotypes (H26, H37, H39, H40, H46, H45, H47), 3 of which were not singletons. The Harz had 5 private haplotypes (H6, H7, H27, H28, H29), but only 1 of these was not a singleton. The Thuringian Forest had only a single private haplotype (H41). In total, 42 of 47 haplotypes (89%) were private to a single region.

The unrooted Bayesian 50% majority-rule consensus tree also showed that the 47 haplotypes clustered into 2 strongly supported clades (a southeastern clade and a western clade) with posterior probability = 1.00. The topology showed the same relationships

among haplogroups and haplotypes as the 2 haplotype networks and, therefore, is not shown.

Genetic population structure and differentiation

To avoid analytical issues with low sample sizes for some populations (SM2, BM1, BM2, BM8, PW, AP1, BF1), we decided not to analyze the data at the population level, but rather at the regional level. All specimens from distinct mountain ranges that were separated by valleys of lower altitude (<400 m asl) or other clear geological/orographic breaks (e.g., between Thuringian Forest and Bohemian Massif) were grouped into region/mountain range groups. This grouping is nonrandom but is based on the species preference for higher altitudes and its naturally insular distribution pattern.

AMOVA partitioned variation in our specimens among regions, among populations within regions, and within populations based on our a priori groupings. Most of the molecular variation observed in our data set could be attributed to variation among regions (85.79%, $\Phi_{CT} = 0.858$, $p < 0.001$), whereas very little variation occurred among populations within regions (1.37%, $\Phi_{SC} = 0.096$, $p = 0.005$). Variation within populations accounted for 12.85% ($\Phi_{ST} = 0.872$, $p < 0.001$) of the total variation.

High levels of differentiation between regions were observed in ETPD and pairwise F_{ST} values. ETPD revealed significant differentiation in 54 of 66 comparisons (82%) and pairwise F_{ST} values were significant for 57 of 66 comparisons (86%) after B–Y adjustment ($\alpha = 0.01037$) (Table 1). Populations in the Bohemian Massif and the Alps were significantly differentiated from populations in all other regions in both tests. In both tests, populations in the Vosges Mountains, the Black Forest, the Harz, the Thuringian Forest, the Sudety Mountains, and the Tatra also were

TABLE 2. Gene diversity, nucleotide diversity, and mean number of pairwise differences for each region. See Table 1 for region codes. n.a. = not available.

Region	N individuals	N haplotypes	Gene diversity	Nucleotide diversity	Pairwise differences
VM	13	2	0.154 ± 0.126	0.007 ± 0.008	0.308 ± 0.338
PW	2	2	1.000 ± 0.500	0.022 ± 0.031	1.000 ± 1.000
BF	17	7	0.824 ± 0.064	0.039 ± 0.026	1.772 ± 1.079
HZ	6	5	0.933 ± 0.122	0.029 ± 0.024	1.334 ± 0.955
TF	6	1	n.a.	n.a.	n.a.
BM	83	9	0.469 ± 0.061	0.014 ± 0.012	0.649 ± 0.509
AL	27	4	0.613 ± 0.071	0.025 ± 0.019	1.146 ± 0.765
SM	41	3	0.643 ± 0.033	0.017 ± 0.014	0.788 ± 0.584
TA	20	8	0.784 ± 0.084	0.030 ± 0.021	1.363 ± 0.878
AP	15	7	0.657 ± 0.138	0.039 ± 0.026	1.772 ± 1.086
RZ	5	1	n.a.	n.a.	n.a.
RM	47	8	0.314 ± 0.088	0.015 ± 0.013	0.672 ± 0.524
Western clade	215	33	0.821 ± 0.019	0.037 ± 0.024	1.698 ± 0.997
Eastern clade	67	14	0.376 ± 0.077	0.020 ± 0.015	0.902 ± 0.635
All	282	47	0.862 ± 0.012	0.100 ± 0.054	4.591 ± 2.262

significantly differentiated from populations in all other regions except from the Pfaelzer Wald, presumably because of small sample sizes at the region scale ($n = 2$). We detected 9 barriers to gene flow (Barrier 2.2). The 1st barrier was between the Pfaelzer Wald populations and all other populations, presumably because of the limited sample size. The 2nd barrier was between the Bohemian Massif and the Sudety Mountains, regions that split our samples into eastern and western groups. The 3rd barrier was detected between the Tatra and the Apuseni Mountains and coincided with the 2 main clades in the haplotype network. The probability associated with each remaining barrier decreased, and barriers 4 to 9 merely provided finer splitting of distinct geographic regions.

The Mantel test revealed significant isolation by distance (correlation coefficient $r = 0.318$, $p < 0.01$) in the whole data set. We used populations instead of regions to test for isolation by distance because use of geographic distance based on the geographic center of each region could falsify the correlation between geographic and genetic distance, especially in regions, such as the Bohemian Massif, where samples were widely separated. When clades were analyzed independently, an isolation-by-distance signal was observed in the western clade ($r = 0.286$, $p < 0.01$), but not in the southeastern clade ($r = 0.032$, $p > 0.05$). Therefore, we can infer that the western clade was comparatively closer to equilibrium between genetic drift and gene flow than the southeastern clade.

Genetic population diversity

Gene diversity was higher in the western lineage (0.821 ± 0.019) than in the southeastern lineage (0.376 ± 0.077) as a result of greater divergence between

haplotypes and a larger number of haplotypes. Gene diversity of all individuals was 0.862 ± 0.012 , but the nucleotide diversity was only 0.100 ± 0.054 , and the mean number of pairwise differences was 4.591 ± 2.262 (Table 2). Gene diversity was highest among individuals in the Pfaelzer Wald, the Harz, the Tatra, and the Black Forest. How genetically diverse the Pfaelzer Wald truly is remains in question because the total sample from the region contained only 2 specimens. We found 5 different haplotypes in 6 individuals in the Harz, whereas we found only 1 haplotype in 6 individuals in the Thuringian Forest (H41) and 1 haplotype in 5 individuals in Retezat (H5) populations. The difference among these 3 populations seems clear, but low sample sizes make it difficult to come to strong conclusions. We also found high numbers of haplotypes/individual in the Tatra Mountains and Black Forest (8 haplotypes in 20 individuals, and 7 haplotypes in 17 individuals, respectively). We found a high number of haplotypes/individual in the Apuseni Mountains (7 haplotypes in 15 individuals), but gene diversity was relatively low (0.657 ± 0.138).

Demographic history and population expansion

For the whole data set, Fu's F_S indicated a recent demographic change ($F_S = -21.529$, $p = 0.00025$). Tajima's D also was negative, a result indicating demographic change, but was not significant for the whole data set ($D = -1.088$, $p = 0.10956$; Table 3). At the regional scale, both Tajima's D and Fu's F_S were significantly negative for the Bohemian Massif ($D = -1.892$, $p < 0.05$; $F_S = -5.468$, $p < 0.01$) and the Rila Rhodope Massif ($D = -2.251$, $p < 0.01$; $F_S = -4.902$, $p < 0.01$), results indicating recent demographic expansion.

TABLE 3. Results of neutrality tests (Fu's F_S and Tajima's D) by region and the total data set. See Table 1 for region codes. Bold values are significant. Fu's F_S is significant at $p < 0.02$, Tajima's D is significant at $p < 0.05$. n.a. = not applicable.

	VM	PW	BF	HZ	TF	BM	AL	SM	TA	AP	RZ	RM	All regions
Fu's F_S	0.362	n.a.	-1.94	-3.047	n.a.	-5.468	0.640	1.277	-3.664	-2.264	n.a.	-4.902	-21.529
p	0.336	n.a.	0.081	0.003	n.a.	0.004	0.661	0.750	0.005	0.049	n.a.	0.001	>0.000
Tajima's D	-1.468	n.a.	0.639	-1.295	n.a.	-1.892	0.274	1.286	-1.016	-0.638	n.a.	-2.251	-1.088
p	0.063	n.a.	0.759	0.072	n.a.	0.007	0.648	0.897	0.173	0.287	n.a.	0.001	0.110

sion of *C. maclachlani* in these regions. The Harz populations had negative values in both tests, but only Fu's F_S was significant ($F_S = -3.047$, $p = 0.003$; $D = -1.295$, $p = 0.072$).

BSPs indicated demographic expansion of the eastern and western clades, and of both clades combined shortly after the end of the Riss glaciation (Fig. 4). The higher and lower 95% highest probability density (HPD) values showed broad variations in all 3 clades, presumably because of the limited resolution of the single locus data.

Discussion

Pleistocene persistence in 2 regions

The major split between western and southeastern populations of *C. maclachlani* is consistent with the 2-refugia hypothesis derived from the CCSM and is inconsistent with the alternative common refugia hypothesis based on the MIROC (Fig. 1B, C). Use of standard molecular clock rate estimates is much debated, and the standard rate of 2.3%/my can greatly underestimate true diversification rates (e.g., Gratton et al. 2008). We do not have paleontological or paleo-ecological benchmarks for calibrating our mutation rate, so we do not wish to overemphasize the dating of our results. However, based on both the conventional rate of 2.3%/my and a 10× higher rate suggested for recent diversification in the Clouded Apollo (*Parnassius mnemosyne*) (Gratton et al. 2008), the southeastern and western clade would have split well before the LGM. All haplotypes we observed in *C. maclachlani* differed by 1 to 19 mutational steps. Maximum divergence between all haplotypes was 3.82%, which would place the split between 165 ky (10^3 y) to 1.65 my ago with the above rates. The eastern clade would coalesce to ~200 ky or 2 my with the 10× rate. No haplotype overlap was found, so the species probably persisted in independent refugia for ≥1 glacial cycle, a result that provides further support for the 2-refugia scenario predicted under the CCSM LGM climate scenario. This persistence in independent refugia would have

given rise to the southeastern and western clades. Other European caddisfly species also show diversification events originating in the late Pliocene (*Drusus discolor*; Pauls et al. 2006), through the Pleistocene (*Drusus bosnicus*-group, *D. croaticus*, Previšić et al. 2009), and into the Holocene (*R. pubescens*, Engelhardt et al. 2008).

Geographically, the split lies between the Tatra Mountains in the Western Carpathians and the Apuseni and Retezat Mountains in the Southern Carpathians. The break we observed in *C. maclachlani* is common in plants that inhabit the Tatra Mountains and the rest of the Carpathians. This phylogeographical boundary between the Eastern Carpathians and Tatra Mountains was first recognized by Woloszczak in 1896 (Ronikier et al. 2008). Large genetic divergence between Tatra populations and populations from the rest of the Carpathians is reported for some plants (Mráz et al. 2007, Ronikier et al. 2008, Tollefsrud et al. 2008, Liepelt et al. 2009) and, to some extent, the bank vole (Kotlík et al. 2006). For aquatic insects, the only genetic studies known to us that sampled across this region were the *D. discolor* studies by Pauls et al. (2006, 2009). In both cases, haplotype overlap was found between the regions and divergence was limited to 2 bp among the Carpathian and Tatra Mountain clades. However, Botoșaneanu (1975) found a morphology-based speciation gradient that was related to features of the female and male genitalia for *Annitella* species (Chaetopterygini) along the main axis of the Carpathians. He assumed that *Annitella* originated from the Balkans and colonized the Carpathians and the central European Highlands gradually after the Würm glaciations. Another Chaetopterygini, *Psilopteryx psorosa*, can be separated into regional subspecies along a geographic gradient in the Carpathians and the adjoining mountain ranges (Mey and Botoșaneanu 1985). Unfortunately, we cannot fully resolve the genetic break between the Eastern Carpathians and the Tatra Mountains because we could not find any individuals during our sampling campaign in the Eastern Carpathians.

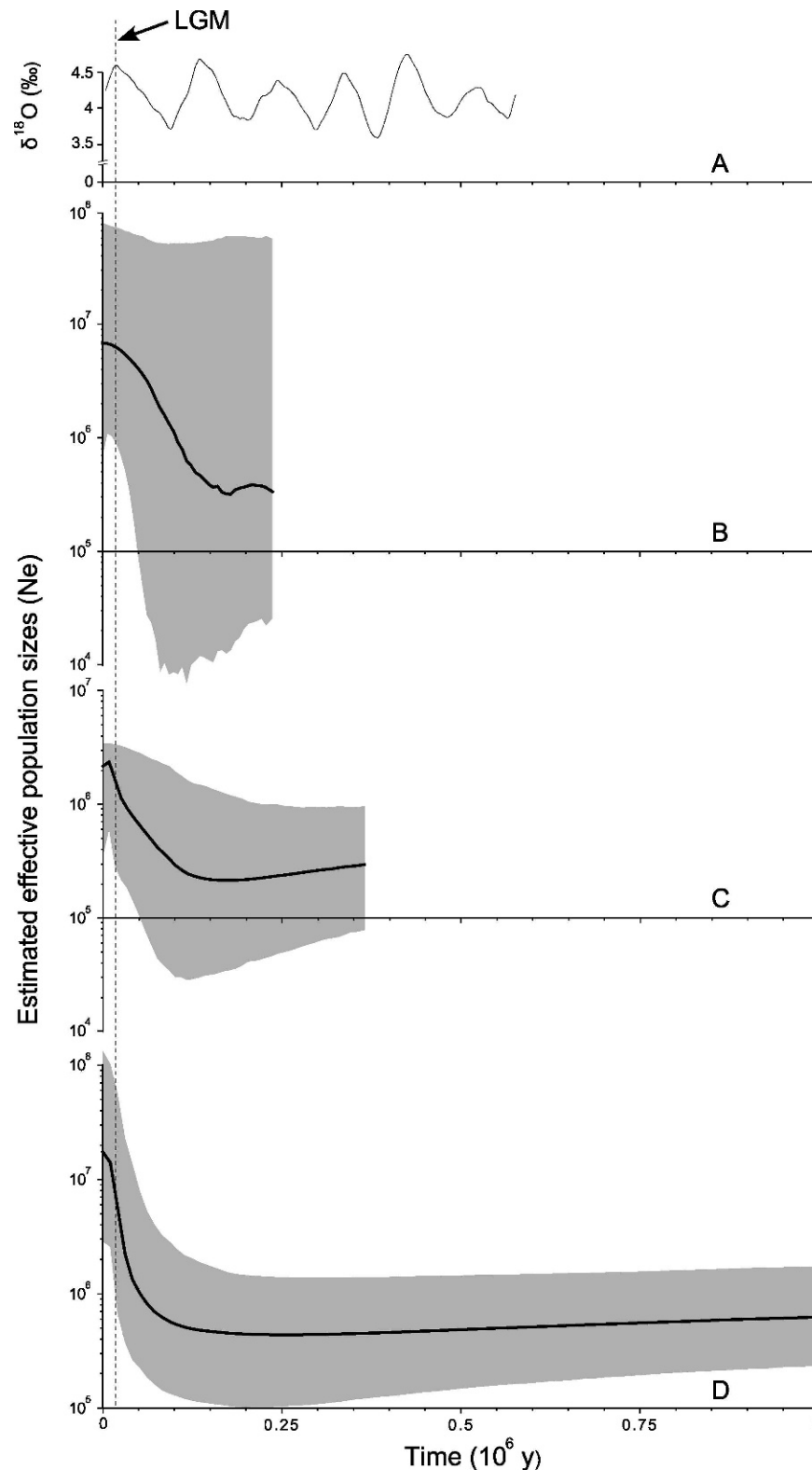


FIG. 4. Demographic changes of *C. macLachlani* populations estimated by Bayesian Skyline Plots. A.—Climatic oscillations presented as temporal change in benthic $\delta^{18}\text{O}$ ‰ isotope records of Lisiecki and Raymo (2005) plotted as the moving average of the data through time. B–D.—Mean and 95% confidence intervals (gray shading) for effective sample size (N_e) for the eastern clade (B), western clade (C), and total data set (D) plotted through time. The demographic changes reflected by the graphs are estimated back in time to the mean timing of the coalescent event. LGM = last glacial maximum.

Centers of high diversity often are regarded as refugial regions because they harbor much of the genetic diversity from which newly colonized regions take their source. Newly founded populations frequently have signals of reduced genetic diversity through founder effects or bottlenecks until they reach equilibrium (e.g., Ibrahim et al. 1996). The Carpathian basin, including the Apuseni Mountains, often has been considered a glacial refugium and a genetic center (Botoșaneanu 1975, Deffontaine et al. 2005, Sommer and Benecke 2005, Malicky 2006, Pauls et al. 2006, 2009, Ursenbacher et al. 2006, Bálint et al. 2008). The refugial sources for the southeastern lineage in the more southern Retezat and the Balkans could have been in the Apuseni. As expected for refugia, gene diversity is higher in the Apuseni than in the other southeastern lineage regions. This hypothesis also is consistent with H5 as the central and presumably ancestral haplotype that can be found in all of the southeastern ranges. As is typical for recent haplotypes, only haplotypes that have few base pair changes and are at the tips of the network are derived from the putative ancestral haplotype H5. An alternative hypothesis is that genetic diversity is high in the Apuseni Mountains because they support multiple local refugial populations. Under this scenario, the Southern Carpathians and the Balkan Mountains could have been recolonized from a common refugial source south of the Carpathians. This scenario is consistent with the CCSM LGM distribution, which indicates that suitable source areas existed in the vicinity of the western Balkan Mountains in present-day Bulgaria and the Banat Mountains in southwestern Romania. Recent disjunction between the Carpathians and the Balkan Mountains and signs of recolonization from a Pleistocene refugia south of the Carpathians have been identified in the case of *Erebia euryale* (Schmitt and Haubrich 2008). This hypothesis also is consistent with the current distribution of haplotype H5.

In the western clade, high levels of diversity and the presence of 2 central ancestral haplotypes (H1 and H8) indicate recent migration and colonization events from very recently split lineages. Haplotype H1 was observed in the Sudety Mountains and Tatra Mountains in the east and the Vosges Mountains, Pfälzer Wald, and Black Forest in the west. The Thuringian Forest population presumably also was descendent from the H1-associated refugial source. Thus, these regions and, presumably, the regions in between are remnants of a once more widespread lineage of *C. maclachlani*. Based on the current distribution, it appears that H1 became extinct in the intermittent region between the Thuringian Forest and the Black

Forest. Whether the extinction was a consequence of competition or a climate change event cannot be resolved. The intermittent region is now occupied by a different, recently split lineage derived from H8. This lineage might have been from a 2nd independent refugial source or might have been a postglacially fragmented lineage associated with the Eastern Alps, Bohemian Massif, and the Harz. The lack of intermediate populations, e.g., in the Thuringian Forest, that carry haplotypes probably derived from H8 is inconsistent with a migratory movement from north to south or south to north between the Harz and the Bohemian Massif. Therefore, we currently favor the scenario of a relatively recent fragmentation with incomplete lineage sorting among the 2 central European subclades (see below). However, we were able to sample only a single population in the Thuringian Forest. Therefore, this potential secondary contact zone might not be sufficiently sampled to draw conclusions because alternative migratory pathways might have existed along the Thuringian Massif.

Postglacial population dynamics

Given the poor dispersal capacity of autumn-emerging caddisflies in general and of *C. maclachlani* in particular, fragmentation of a more cohesive distribution range seems to be a likely explanation for the high level of population differentiation within the western clade. *Chaetopterygopsis maclachlani* is a cold-tolerant species and might have occupied a wide range at lower elevations during cooler periods in the past as predicted by both climate niche models (Fig. 1B, C). This period of broad distribution could have been followed by extinction of several populations between mountain ranges during warmer phases. At the same time, some individuals could have survived by moving to higher altitudes. This scenario of shifts to lower altitudes at cooler stages in the past is inferred for many cold-adapted and alpine species (Volckaert et al. 2002, Muster and Berendonk 2006, Schmitt et al. 2006).

Our results suggest that *C. maclachlani* experienced demographic expansion in all parts of its range. The standard substitution rate (2.3%/my) used to estimate the timing of population events indicates that these expansions coincided with the end of the Würm glaciation, but the general scenario is independent of the method for calculating dates (standard or otherwise-calibrated molecular clock). The mean estimated effective population size of the western lineage also suggests that this long phase of population expansion was followed by a very recent decline shortly after the end of the Würm glaciation. It is plausible that *C.*

maclachlani had a widespread, cohesive distribution throughout some cold periods of the Pleistocene before being split into 2 main refugial areas. In response to a warming postglacial climate, the species retreat to higher elevations caused fragmentation and forced the species into headwater systems. In a few regions, this process might have gone hand in hand with a demographic expansion if less cold-tolerant competitors remained downstream. In most regions, the isolated headwater populations would be experiencing a range regression. The main split into a southeastern and a western clade, and a very recent demographic decline in western clade is consistent with such a scenario.

Diversification of autumn-emerging caddisflies

Adult dispersal is the main source for gene flow among populations of aquatic insects (e.g. Bilton et al. 2001, Hughes 2007, Hughes et al. 2009). Our study revealed strong population structure and genetic differentiation within and among mountain ranges and populations of *C. maclachlani*. Clear patterns of genetic population structure caused by limited gene flow are common in insects that inhabit high montane regions and sky islands or in species with other types of fragmented distributions (e.g., Mardulyn 2001, Engelhardt et al. 2008). Our results agreed with our prediction that we would find strong genetic differentiation among populations and regions of *C. maclachlani*, presumably because of its poor flying capabilities, and thus, limited lateral dispersal capacity. Nevertheless, *C. maclachlani* was able to colonize a very wide range in central Europe, presumably during the glacial stages of the Pleistocene, and is now retreating to higher altitude, isolated populations.

The degree of differentiation of *C. maclachlani* among regions is similar to that of other European caddisflies that are considered better fliers based on their wing shape, emergence period, and adult flight behavior (Pauls et al. 2006, 2009, Bálint 2008, Engelhardt et al. 2008). *Chaetopterygopsis maclachlani* populations in most regions are significantly separated from those in other regions (ETPD: 82% of pairwise tests were significant, pairwise F_{ST} : 86%). Very similar patterns were observed at the regional scale for *D. discolor* (ETPD: 83%, F_{ST} : 87%) and *R. pubescens* (ETPD: 72%) across Europe (Pauls et al. 2006, Engelhardt et al. 2008), *D. romanicus* (ETPD: 100%) in the Carpathians and Southern Balkan regions (Pauls et al. 2009), and the *Rhyacophila aquitanica*-complex (ETPD: 93%, pairwise F_{ST} : 98%) among the Massif Central, Black Forest, Vosges Mountains,

Southern Alps, and the Carpathians (Bálint 2008). Eighty-nine percent of *C. maclachlani* haplotypes are private to a single region, compared with 83% in *D. discolor*, 92% in *D. romanicus*, and 83% in *R. aquitanica* (Pauls et al. 2006, 2009, Bálint 2008). Eighty-six percent of *R. pubescens* haplotypes are private across its range north of the Alps (Engelhardt et al. 2008). Thus, many species of caddisflies with different inherent levels of dispersal capability show high degrees of differentiation in Europe, results suggesting limited gene flow among central European mountain ranges. However, differentiation was much lower and fewer haplotypes were private in the putatively best flier, *Hydropsyche tenuis* (Lehrian et al. 2009). These results follow the predictions of the “Widespread gene flow with isolation by distance model” (*H. tenuis*) and the “Headwater model” (all other species discussed above) for genetic structure of aquatic organisms (Finn et al. 2007, Hughes et al. 2009).

Regardless how the current genetic population structure arose, many regions and populations currently appear to be isolated and diversifying into independent lineages that could potentially evolve further into subspecies or ultimately species. Given enough time, genetic and morphological differences are likely to accumulate because of *C. maclachlani*'s poor dispersal capacity. *Chaetopterygopsis maclachlani* adults, like most insects, are incapable of flying great distances at very low temperatures, which are common in autumn when they emerge; females of *C. maclachlani* are unable to fly (Wagner 1991). Caddisfly larvae generally disperse only short distances by drifting downstream or moving upstream (Malicky 1994, Petersen et al. 2004). Such limited dispersal capacity should lead to isolation among disjunct populations, genetic drift, and ultimately to lineage diversification and speciation. Other autumn-emerging caddisflies also show evidence of recent or ongoing diversification. The species complex *Chaetopteryx rugulosa* Kolenati is an example of ongoing speciation. The subspecies are readily distinguishable at the edge of their distribution ranges (Malicky et al. 1986), but the different subspecies can mate and produce fertile offspring. In the center of the overlapping ranges of several subspecies, the morphologies introgress and distinction of lineages becomes very difficult. This pattern suggests a progressively evolving species complex, not established by allopatric fragmentation of populations (Malicky 2005, Malicky et al. 1986). In another closely related species complex, the *Chaetopteryx villosa* group, allopatric speciation seems to be the main factor driving the morphological differentiation

(Malicky et al. 1986, Malicky 2005). These species also hybridize, resulting in intermediate populations in areas of contact between lineages (Malicky 2005). The patchy distribution of *C. maclachlani* populations and the high level of private haplotypes observed might indicate a very early stage of speciation. The genetic divergence between the 2 lineages of *C. maclachlani* is only 1.21%, but the largest interspecific distance between 2 other Limnephilidae, *Drusus nigrescens* and *Drusus monticola*, also was 1.21%, probably indicating recent speciation (Waringer et al. 2007). The interspecific distance between species of the *Chaetopteryx villosa* group can be as low as 0.44%, but generally ranges from about 5 to 8% (H. Malicky, Lunz, Austria, and SUP, unpublished data).

A morphological study of *C. maclachlani* adult genitalia across the range would be very interesting for determining whether the genetic divergences correlate with morphological features. Unfortunately our sampling of adults was too limited to accomplish this task. In general, the Chaetopteryginae present an interesting group of caddisflies for evolutionary studies. It would be particularly interesting to study the comparative phylogeography and population genetics of species across hybridization zones to examine species boundaries and demographic history of the group. Based on varied results observed in montane aquatic insect phylogeography to date, we can expect that these poorly studied species will have previously unknown and unexpected phylogeographic patterns, which could greatly improve our understanding of the aquatic biota's history in Europe and thus our management potential concerning cold-adapted species at risk through anthropogenic disturbances or global change.

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APPENDIX 1. Climate Niche Modeling

We estimated climate niche models with the maximum entropy method implemented in Maxent (Phillips et al. 2006). All climatic data (19 bioclimatic variables) with a resolution of 5 km² (2.5 arc min) were obtained from the WorldClim global climatic database (Hijmans et al. 2005; available from: <http://www.worldclim.org>). We generated climate niche models for the last glacial maximum (LGM) under 2 scenarios, a Community Climate System Model (CCSM) and a Model for Interdisciplinary Research on Climate (MIROC), using default settings in Maxent. We based models on 43 sites from our own sampling (Appendix 2) or from literature data (see Table A1 below). The models were cross-validated in 10 parallel runs. We created binary distribution maps with equal test sensitivity and specificity logistic thresholds. Both models predicted a contiguous potential LGM range across much of central and northwestern Europe, and isolated refugia in the Pyrenees. However, the CCSM predicted a break in the potential LGM range near the present-day Adriatic Sea and the Northern Dinaric Alps. The MIROC also predicted a break, but much further east associated with the present-day extent of the Black Sea.

TABLE A1. Additional observations used for species distribution modeling. Geographic coordinates are given in decimal degrees.

Country	Latitude (°N)	Longitude (°E)	Source
France	46.7051	6.20883	Global Biodiversity Information Facility (GBIF)
Austria	46.7667	13.95	http://www.zobodat.at
Austria	46.79	13.71	http://www.zobodat.at
Austria	47.87	15.89	http://www.zobodat.at
Austria	47.8333	15.6833	http://www.zobodat.at
Austria	48.4072	14.6839	http://www.zobodat.at
Austria	48.5219	14.6672	http://www.zobodat.at
Bulgaria	42.06	23.52	Inferred from Kumanski 1988
Bulgaria	42.59	23.25	Inferred from Kumanski 1988
Bulgaria	42.6	23.23	Inferred from Kumanski 1988
Romania	46.81	25.81	Inferred from Ciubuc 1993
Romania	47.35	25.63	Inferred from Ciubuc 1993
Romania	47.36	25.1	Inferred from Ciubuc 1993
Romania	46.69	22.62	Inferred from Ciubuc 1993
Romania	46.32	25.6	MB, personal observation

APPENDIX 2. *Chaetopterygopsis maclachlani* material used in our study. Country codes are based on International Organization for Standardization (ISO) 3166 codes. Geographic coordinates are given in decimal degrees. N = number of individuals in each collection; * denotes number of adults. See Fig. 2 for region abbreviations. Regions are arranged from west to east. Lat = latitude, Long = longitude, Alt = altitude.

Region	N	Population, country, mountain/ region, locality or stream	Lat (°N)	Long (°E)	Alt (m asl)	Collectors**	Number of different/private haplotypes (Haplotype[N])
Vosges Mountains (VM)	13	VM, F, VM, Moyen-Rupt	48.04	6.88	850	FG ^a	2/1 H1(12), H42(1)
Pfäelzer Wald (PW)	2	PW, DE, PW, Helmbach	49.35	7.94	~400	AS ^b	2/1 H1(1), H2(1)
Black Forest (BF)	17	BF1, DE, BF, Brotenaubach	48.72	8.44	900	SUP, SL, AS	7/5 H1(6), H18(4), H31(1), H32(3), H34(1), H38(1)
	16	BF1, DE, BF, Brotenaubach	48.72	8.44	900	SUP, SL, AS	H1(6), H18(4), H31(1), H32(3), H34(1), H38(1)
	1	BF2, DE, BF, tributary to Kaltenbach	48.63	8.41	776	KT ^c , JT ^d	H43(1)
Harz (HZ)	6	HZ, DE, Harz, Kalte Bode	51.75	11.00	–	MH ^e	5/5 H6 (1), H7 (1), H27 (2), H28 (1), H29(1)
Thuringian Forest (TF)	6	TF, DE, TF, nameless brook near Steinheid	50.47	11.11	621	KT, JT	1/1 H41(6)
Bohemian Massif (BM)	83	BM0, CZ, Slavskovsky les, Pramensky potok	50.04	12.72	758	SL, NG ^f	9/7 H8(7), H16(1), H17(9), H20(1), H30(1), H33(3)
	2	BM1, DE, Bavarian Forest, Groetschenreuth, nameless brook	49.86	12.03	~500	SL, DB ^g	H8(1), H17(1)
	1	BM2, DE, Oberpfälzer Wald, Lindau, nameless brook	49.55	12.55	600	SUP	H8(1)
	6	BM3, DE, Bavarian Forest, Trautmannsried, nameless brook	49.08	12.88	750	SL, DB	H8(5), H17(1)
	5	BM4, DE, Bavarian Forest, Gegenbach	48.75	13.75	~700	SL, DB	H8(5)
	23	BM5, DE, Bavarian Forest, Neustifter Bach	48.65	13.62	500	SL, DB	H8(19), H17(3), H35(1)
	11	BM6, AT, Muehlviertel, Maltisch	48.58	14.64	~900	SUP, WG ^h , KT	H8(9), H36(2)
	11	BM7, AT, Muehlviertel, Hoellbach	48.42	15.08	~900	SUP, WG, KT	H8(11)
	2	BM8, AT, Muehlviertel, Hoellbach 2	48.42	15.09	822	JT	H8(1), H44(1)
Alps (AL)	27	AL1, AT, AL, Rohrwiesteich	47.85	15.05	1000	HM ⁱ	4/2 H8(10), H9(1), H10(5)
	16*	AL2, AT, nameless brook near St, Lorenzen	46.86	13.92	~1300	SUP, WG, KT	H8(5), H10(3), H17(3)
	11						

APPENDIX 2. Continued.

Region	N	Population, country, mountain/ region, locality or stream	Lat (°N)	Long (°E)	Alt (m asl)	Collectors**	Number of different/private haplotypes (Haplotype[N])
Sudety Mountains (SM)	41						3/0
	6	SM1, CZ, Krkonosky, Labe	50.74	15.59	838	SL, NG	H18(6)
	28	SM2, CZ, Orlicky hory, Lusino Udoli, tributary to Bela	50.75	16.39	868	SL, NG	H1(13), H8(1), H18(14)
	2	SM3, CZ, Orlicky hory, Trckov, nameless brook	50.35	16.42	850	SL, NG	H18(1), H1(1)
	5	SM4, CZ, Orlicky hory, Serlissky Mlyn, tributary to Bela	50.33	16.37	875	SL, NG	H1(3), H18(2)
Tatra (TA)	20						8/7
	7*	TA1, PL, High Tatra, Chochołowska Dolina, nameless brook	49.27	19.83	1000	BS ^j	H1(2), H26(1), H37(2), H39(1), H40(1)
	13	TA2, SK, High Tatra, Batizovce- Valicky, nameless brook	49.08	20.19	~800	JT	H(1), H37(7), H40(1), H45(1), H46(2), H47(1)
Apuseni (AP)	15						7/6
	2	AP1, RO, AP, Gilău, nameless brook	46.50	23.00	1350	SUP, LU ^k , MB	H3(1), H4(1)
	13	AP2, RO, AP, Someșul Cald	46.64	22.74	1145	MB, KT	H5(9), H11(1), H12 (1), H13(1), H25(1)
Retezat (RZ)	5						1/0
	5	RZ, RO, RZ, nameless brook	45.37	22.87	2100	SUP, LU	H5(5)
Rila-Rhodope Massif (RM)	47						8 / 7
	30	RM, BG, Rila Mountains, Jakoruda, nameless brook	42.09	23.62	1950	MB, LU	H5(25), H15(1), H19(1), H21(1), H22(1), H23(1)
	17	RP, BG, Rhodope Mountains, Batak-Dospat, nameless brook	41.81	24.11	1560, 1700	MB, LU	H5(14), H14(1), H21(1), H24(1)

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