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ORIGINAL ARTICLE

Glacial survival and post-glacial recolonization of an arctic–alpine freshwater insect (*Arcynopteryx dichroa*, Plecoptera, Perlodidae) in Europe

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This study is dedicated to the memory of Professor Dr Alfred Seitz.

ABSTRACT

Aim General models for understanding the climate-driven processes of post-glacial colonization in European arctic–alpine species are mainly derived from studies on temperate terrestrial taxa. However, cold-adapted freshwater species may tolerate or even thrive under colder climatic conditions as flowing water habitats are thermally buffered against freezing and extremely cold temperatures. Here, we investigate the European Pleistocene and Holocene history of the arctic–alpine stonefly *Arcynopteryx dichroa.*

Location Europe.

Methods We used two genetic data sets (mitochondrial sequence data and nuclear microsatellite data) to investigate the glacial survival and post-glacial recolonization routes of *A. dichroa*. We used species distribution models to critically evaluate our genetic data and phylogeographical interpretations.

Results Among 344 sequenced individuals from eight European mountain ranges, 80 unique haplotypes were detected. Of these, 77 haplotypes were endemic to a single mountain range, indicating almost complete lineage sorting. Both sequence and microsatellite data suggested strong population differentiation between mountain ranges. The genetic hotspots were found in the Carpathians, the Balkans and the eastern Alps. The Black Forest and Fennoscandian populations exhibited shared and closely related haplotypes, indicating ancestral polymorphism in two populations that became disjunct due to vicariance or resulting from rare long-distance dispersal among disjunct northern and southern periglacial populations.

Main conclusions *Arcynopteryx dichroa* is a glacial relict that survived glacial cycles through elevation shifts in isolated periglacial populations in the Pyrenees, the central European highlands, the Carpathians, the Balkans and the eastern Alps. The species probably recolonized the formerly glaciated Fennoscandian range from a refugium in the central European highlands, following the retreat of the ice sheet. This study suggests that aquatic organisms may have reacted differently to Pleistocene climate change compared with terrestrial species.

Keywords

Aquatic insects, COI sequences, Europe, Fennoscandia, microsatellites, phylogeography, Pleistocene, species distribution modelling.

INTRODUCTION

General models for understanding climate-driven processes of post-glacial colonization in central Europe are mainly derived from studies on temperate terrestrial taxa (Hewitt, 2004; Schmitt *et al.*, 2010). However, cold-adapted species, such as those found in arctic and alpine environments, may tolerate or even thrive under colder climatic conditions.

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Freshwater species that inhabit permanently flowing turbulent waters are also likely to tolerate a colder climate, as these waters are thermally buffered against freezing and extreme cold (Malicky, 1983; Pauls *et al.*, 2006). Thus, the larvae of cold-tolerant aquatic organisms would have been subject to much less severe temperature extremes during cold phases than terrestrial species (Malicky, 1983).

Following the general consensus on European biogeography and phylogeography (e.g. de Lattin, 1967; Hewitt, 2004), cold-adapted species may have wider distributions during glacial periods and experience range contractions during interglacials – the opposite pattern to temperate species. This should result in molecular signatures of population expansion during the last ice age (12,000–110,000 years ago), followed by a population decline during the Holocene as the climate warmed (Hewitt, 2000; Schmitt, 2007). However, recent work on terrestrial arctic and alpine invertebrate species has revealed that this pattern is not generally true (Schmitt *et al.*, 2010), and it is questionable if it is valid for cold-adapted insects with low dispersal capabilities. Moreover, some terrestrial and aquatic cold-tolerant invertebrates did not necessarily respond to climate change with large-scale latitudinal shifts (i.e. long-distance dispersal) but rather with intra-regional elevational shifts (Galbreath *et al.*, 2009), which often resulted in mountain system-specific genetic lineages (Pauls *et al.*, 2006; Schmitt, 2007; Lehrian *et al.*, 2010). It is most likely that responses to climate change are species-specific and primarily the result of the vulnerability, exposure and adaptability of a species to changing climatic conditions.

Species with arctic–alpine distributions are generally considered cold-adapted, typically inhabiting arctic habitats as well as the alpine regions of more southern mountain ranges (de Lattin, 1967). Current distribution patterns of species adapted to high elevations and latitudes are often strongly disjunct (de Lattin, 1967), with a continuous distribution in the north and patchy distributions at high elevations further south. The stonefly *Arcynopteryx dichroa* (McLachlan, 1872) (Plecoptera, Perlodidae), until recently known as *Arcynopteryx compacta* (McLachlan, 1872) (Teslenko, 2012), is a good model with which to test the validity of the current phylogeographical paradigms regarding arctic–alpine invertebrate species. *Arcynopteryx dichroa* exhibits the typical arctic–alpine disjunction *sensu* de Lattin (1967), with a continuous distribution in the northern Holarctic and isolated distributions in the southern European mountains (Pyrenees, Carpathians, western Balkans, Bulgarian highlands), where it is restricted to the highest elevations. In the central European highlands the species is exclusively found in the Black Forest. Unlike other arctic–alpine species, *A. dichroa* is absent from the central Alps and only occupies the south-eastern Alpine piedmont (Illies, 1955; DeWalt *et al.*, 2012). All adult males, but also the females in some populations, are brachypterous, limiting the species' dispersal capability (Lillehammer, 1974). It is a relatively large predator and is adapted to springs and spring-fed brooks (Graf *et al.*, 1995). The species is restricted to cold waters and, as such, has adapted to conditions of extreme cold (Lillehammer, 1974).

We used mitochondrial sequence and nuclear microsatellite data to examine present-day population structure from a historical perspective. In addition, we employed species distribution models (SDMs; for methods and results see Appendix S1 in Supporting Information) to examine the geographical distribution of suitable climate regimes in the present and during the Last Glacial Maximum (LGM). We used these analyses to address the following research questions.

1. Is *A. dichroa* a glacial relict that survived glacial cycles through elevation shifts (*sensu* Galbreath *et al.*, 2009) in isolated periglacial populations? To address this first question we examined whether the present population structure is consistent with mountain system-specific lineages in isolated southern and eastern European populations that arose in independent glacial/interglacial refugia, and whether the SDM projects suitable climatic conditions in central European, extra-Mediterranean mountain ranges during the LGM (21,000 years ago).

2. Did *A. dichroa* recolonize the formally glaciated Fennoscandia in the post-glacial from the closest probable refugial areas in the central European highlands? Although other studies have shown that the geographically closest area is not always equivalent to the refugial region (Theissinger *et al.*, 2011), this recolonization scenario seems reasonable given the limited dispersal capability of the species. To address this second question we compared mitochondrial and microsatellite data for Fennoscandian samples with potential source populations.

MATERIALS AND METHODS

Sampling scheme

In any species, but particularly in those that inhabit large and complex ranges, it is best for phylogeographical research to take into account the entire range of the species (Bunje, 2005). Here, we present a dataset containing samples of all European mountain ranges inhabited by *A. dichroa*. In total, we collected 365 individuals from 46 European sampling sites (Fig. 1, Appendix S2). We also included some non-European samples.

Fieldwork

Larval and adult specimens of *A. dichroa* were collected manually from their preferred microhabitats with water nets. Collected specimens were identified in the field using a field microscope to ensure collection success. Specimens were stored in individual tubes in 96% ethanol and kept on ice until they were brought to the laboratory where they were maintained at 4 °C until DNA extraction was carried out.

Laboratory work

We used DNeasy tissue kits (Qiagen, Hilden, Germany) to extract whole genomic DNA following the manufacturer's

Figure 1 Sampling locations for *Arcynopteryx dichroa* (n = 45; Urals not shown) from seven mountain ranges within European ecoregions (dotted lines, modified from http://www.eea.europa.eu/data-and-maps/data/ecoregions-for-rivers-and-lakes). Mountain ranges: PY, Pyrenees; BF, Black Forest; EA, eastern Alps; CP, Carpathians; BU, Bulgarian highlands; WB, western Balkans; FS, Fennoscandia. Ecoregions: 1, Pyrenees; 2, Alps; 3, central European highlands; 4, Carpathians; 5, eastern Balkans; 6, Hellenic western Balkans; 7, boreal uplands. Note that *A. dichroa* is absent from the central and western Alps, as well as from all western and central European highlands except the Black Forest.

protocol. After removing the gut, DNA was extracted from the abdomen. Cleared abdomens were placed in individual tubes together with the torso and head as vouchers. All vouchers were labelled and stored at 8 °C in 96% ethanol in the Senckenberg Museum (voucher specimens SMF1967–SMF2104).

We generated mitochondrial (mt) DNA sequence data from the 'DNA barcode' region (Hebert *et al.*, 2003) of the cytochrome *c* oxidase subunit I (*mtCOI*) gene. We used the primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer *et al.*, 1994) to produce a 658 base pair (bp) fragment. Polymerase chain reaction (PCR) was performed using PuReTaq Ready-To-Go PCR Beads (GE Healthcare Lifesciences, Freiburg, Germany). Cycling conditions followed Hebert *et al.* (2003). Amplified fragments were purified using NucleoSpin Extract II kits (Macherey & Nagel, Düren, Germany) and were sequenced on a 16 capillary sequencer (3130 Genetic Analyzer, Applied Biosystems, Carlsbad, CA, USA). The sequences of each individual were aligned in the SeqMan software (DNASTAR, Lasergene, Madison, WI, USA). The starting alignment was generated using Clustal W (Thompson *et al.*, 1994) as implemented in BioEdit 9.0 (Hall, 1999). The final alignment was 620 unambiguous base pairs in length.

Microsatellite genotyping was performed using six species-specific primer pairs (Arco_8, Arco_79, Arco_123, Arco_126, Arco_102, Arco_157), following Theissinger *et al.* (2009). PCR product (1 μL) was added to 11.7 μL HiDi-formamide and 0.3 μL ROX 500 standard (Applied Biosystems) and genotyped on a 3130 Genetic Analyzer. Loci were scored using the software genemapper 4.0 (Applied Biosystems) and were analysed for possible null alleles using microchecker 2.2.3 (van Oosterhout *et al.*, 2004) for each sampling site. We additionally estimated the genotyping error rate by randomly re-amplifying 32 individuals (10%) across all loci (Bonin *et al.*, 2004).

The *mtCOI* sequence data

We sequenced 335 individuals of *A. dichroa* from seven ecoregions and 45 sampling sites across Europe (Fig. 1), including one population from the Urals (n = 5; Appendix S2). We also analysed sequences from two sites from the far east of Siberia (n = 8) and one Alaskan specimen. These sequences (n = 9) were only used in the analyses of haplotype relationships, i.e. the median joining network and beast analysis (see below).

We calculated the overall haplotype diversity with Arlequin 3.5 (Excoffier *et al.*, 2005). A median-joining (MJ) network (Bandelt *et al.*, 1999) was constructed with the default settings in Network 4.516 (Fluxus Technology, Suffolk, UK). We colour-coded the origin of each specimen carrying a given haplotype to illustrate haplotype distributions, identify the number of haplotypes, and tally the number of endemic haplotypes. Final illustrations were generated using Network Publisher (Fluxus Technology Ltd, Clare, Suffolk, UK) and Illustrator CS5 (Adobe, San Jose, CA, USA).

We conducted a spatial analysis of molecular variance (SAMOVA) to test whether geographical sample distributions corresponded to the genetic variance in our sampling. We used the software SAMOVA 1.0 (Dupanloup *et al.*, 2002) which, given an a priori number of groups (K), uses a simulated annealing procedure to define the group composition in which populations within a group are as genetically homogeneous as possible (θ_{SC} minimized) and groups are maximally differentiated from each other (θ_{CT} maximized). The analysis was run for $K = 2$ to $K = 20$ and the significance of fixation indices was tested with 1000 permutations. We then estimated the genetic differentiation among clusters with analysis of molecular variance (AMOVA) based on F_{ST} and pairwise F_{ST} values among mountain ranges using ARLEQUIN 3.5 (Excoffier *et al.*, 2005).

We inferred demographic history using selection tests that have a demographic bias, i.e. Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1997), and mismatch distributions (Rogers & Harpending, 1992) as implemented in ARLEQUIN 3.5. We estimated the timing of divergence of the 80 haplotypes of *A. dichroa* in BEAST 1.5.2 (Drummond *et al.*, 2005). We applied a molecular rate of evolution of 3.54% sequence divergence Myr^{-1} (Papadopoulou *et al.*, 2010) to date our ultrametric tree. The substitution model was set to GTR-I as suggested by the Akaike information criterion implemented in JMODELTEST (Posada, 2008). Three independent runs (20 million generations each) were performed on the dataset. Chains were sampled every 2000 states; the initial 10% of the samples were removed as burn-in. A UPGMA (unweighted pair-group method using arithmetic averages) starting tree was constructed before each run. The results of the independent runs were combined in the program LOGCOMBINER 1.5.2 (http://beast.bio.ed.ac.uk/LogCombiner) to check for convergence and mixing of independent chains. The 27,000 trees resulting from the three independent runs were summarized in a consensus tree using TREEANNOTATOR 1.5.2 (http://beast.bio.ed.ac.uk/TreeAnnotator). The ultrametric consensus tree was visualized in FIGTREE (http://beast.bio.ed.ac.uk/FigTree). The output plots were edited and combined in INKSCAPE 0.47 (http://www.inkscape.org). Past demographic growth was analysed with Bayesian skyline plots (BSP; Drummond & Rambaut, 2007).

Microsatellite data

We genotyped 360 individuals from 45 sites across seven European ecoregions, plus five individuals from the Urals (Appendix S2). Linkage disequilibrium between pairs of loci was tested in the web-based version of GENEPOP (Raymond & Rousset, 1995) using default parameters and applying a Bonferroni correction for multiple comparisons (Rice, 1989). Due to homozygote excess, five loci showed signs of null alleles in some – but not all – populations across the species' range. We therefore used the program FREENA (Chapuis & Estoup, 2007) to calculate null allele frequencies and to estimate global and pairwise F_{ST} values *sensu* Weir (1996) with the excluding null alleles (ENA) method as described by Chapuis & Estoup (2007). This method was found to correct efficiently for the positive bias induced by the presence of null alleles on F_{ST} estimation (Chapuis & Estoup, 2007). We conducted a Mantel test between pairwise F_{ST} uncorrected for null alleles and F_{ST} corrected for null alleles with 10,000 random permutations to test whether they were significantly correlated. We used expected heterozygosity (H_E) as a measure of genetic variability within populations as it is robust to the presence of null alleles (Chapuis *et al.*, 2009).

The number of alleles (N_A) and the number of private alleles (N_P) per population were calculated using the software CONVERT 1.31 (Glaubitz, 2004). Expected (H_E) and observed (H_O) heterozygosity as well as departure from Hardy–Weinberg equilibrium (HWE) were calculated for each locus, over all loci for each site with $n \geq 10$, and across mountain ranges using default parameters in GENEPOP (Raymond & Rousset, 1995). To visualize the genetic variation among 365 individuals across six microsatellite loci, a factorial correspondence analysis (FCA) of the microsatellite data was carried out using the program GENETIX 4.04 (http://kimura.univ-montp2.fr/genetix/), which graphically projects the individuals on the factor space defined by the similarity of their allelic states: there are as many factors as alleles across all loci. We plotted the first and second factors on a 2D graph.

We applied a Bayesian spatial modelling of the genetic population structure using the program BAPS 5 (Corander *et al.*, 2008) to define population partitioning based on microsatellite data. BAPS uses Bayesian mixture modelling to describe variation within subpopulations using a separate joint probability distribution over multiple loci. We used the group analysis mode, treating individuals of each site as separate groups ($n = 46$). We applied the spatial model that uses individual georeferenced multilocus genotypes to assign a biologically relevant non-uniform prior distribution over space of clustering solutions, thereby increasing the power to correctly detect the underlying population structure (Corander *et al.*, 2008). The program was initially run in fixed K modus with $K = 46$. Thereafter, we repeated the analysis with the five best partitions ($K = 26$ to $K = 30$), applying a vector for repeating the run five times for each K. For each K value, BAPS determines the optimal partitions, stores these internally, and, after all K values have been processed, it merges the stored results according to the log-likelihood values. We calculated the admixture of individuals, excluding populations with fewer than three specimens, to estimate gene flow rates (r) between given clusters, i.e. the relative average amounts of ancestry in the source cluster among the individuals assigned to the target cluster (Corander & Marttinen, 2006). The setting for estimation of admixture coefficients for individuals was 100, and the number of reference individuals from each population was set to 200 with 100 iterations.

To estimate genetic differentiation among sampled mountain ranges we conducted an AMOVA using both the infinite allele model (Kimura & Crow, 1964) implemented in F_{ST}

and the stepwise mutation model (Kimura & Ohta, 1978) implemented in R_{ST}, with the program ARLEQUIN 3.5 (Excoffier *et al.*, 2005). Significance for fixation indices was established with 1000 permutations. To test for local population differentiation and to determine if gene flow is restricted among populations within mountain ranges we calculated pairwise F_{ST} (Weir, 1996) and R_{ST} (Weir & Cockerham, 1984) among all populations ($n = 45$) using ARLEQUIN with default parameters.

RESULTS

The *mtCOI* sequence alignment file and the microsatellite raw data table are both available at http://metacat.senckenberg.de/knb/metacat/bikf.152.4/bikf.

The *mtCOI* sequence data

We detected 80 unique haplotypes (GenBank accession numbers JF312785–JF312864), of which 77 were endemic to a single mountain range, indicating almost complete lineage sorting among regions. The number of variable sites was 72 out of 620 bp. The maximum number of base pair changes between haplotypes was 24. Overall haplotype diversity (H_d) was 0.9507.

The *mtCOI* MJ network (Fig. 2) was characterized by eight haplogroups, mainly corresponding to mountain ranges.

1. The southern and western Carpathian (CP) range formed a diverse group, with a H_d of 0.312.

2. The Bulgarian (BU) site from the Pirin Mountains (BU_2; Appendix S2) differed by 4 bp from the southern and western Carpathian haplogroup. Two Carpathian sites (CP_1, CP_14; Appendix S2) shared a haplotype (H19) with one Bulgarian site (BU_1; Appendix S2).

3. The northern and eastern Carpathians (CP_15–CP_17; Appendix S2) were separated from the southern and western Carpathians by 6 bp.

4. The Black Forest (BF; $H_d = 0.214$) grouped together with the Fennoscandia (FS) sites ($H_d = 0.072$), exhibiting two shared haplotypes, H06 and H08, and were also close to the Ural (UR) haplotypes ($H_d = 0.4$).

5. The eastern Alps (EA) were separated from the nearest haplogroup by 5 bp and exhibited an H_d of 0.4.

6. The Pyrenees (PY) samples ($H_d = 0.125$) were connected to the remainder of the network by the same median vectors as haplogroup 7.

7. Western Balkans (WB) haplotypes ($H_d = 0.250$). The Pyrenees (6) and western Balkans (7) haplotypes were separated by 6–10 bp.

8. The Far East Siberian (FES) haplogroup ($H_d = 0.444$), including one Alaskan (AL) specimen, was separated from other clades by 6–9 bp.

The SAMOVA grouped the 46 sites into six clusters, which mainly represented the sampled mountain ranges (Fig. 3a), with two exceptions: (1) BU_1 was included in the Carpathian group, while BU_2 formed a separate group; (2) the Black Forest, Fennoscandia and the Urals were grouped in the same cluster. Due to the distance between these mountain ranges, we proposed eight geographical groups, corresponding to the eight sampled European mountain ranges: PY, BF, EA, CP, WB, BU, FS and UR. This hierarchical structure was used for all subsequent population differentiation analyses.

The AMOVA for the eight geographical groups suggested strong differentiation between mountain ranges ($F_{ST} = 0.207$, $P < 0.0001$) as well as among sites within mountain ranges ($F_{SC} = 0.202$, $P < 0.0001$). Pairwise F_{ST} values among the eight mountain ranges ranged from 0.436 to 0.924, with a median of 0.783 (lower quartile 0.673, upper quartile 0.828; Table 1). All pairwise combinations of mountain ranges had significant F_{ST} values.

Mismatch distributions in the eight mountain ranges showed a multimodal pattern in the Pyrenees, the Black Forest, the eastern Alps, the Carpathians and the Bulgarian highlands. Unimodal patterns were observed in the western Balkans, Fennoscandia and the Urals (Table 2), indicating recent demographic expansions in these regions (Rogers & Harpending, 1992). Neutrality tests showed significant negative values for both Tajima's D and Fu's F_S in the Carpathian populations. The Fennoscandian range exhibited a significant negative F_S value (Table 2). The BSP (Fig. 4b) indicated population growth in central Europe starting at the end of the Riss (*c.* 150 ka) and stagnating at the end of the Würm (10 ka). The haplotype phylogeny (Fig. 4c) showed that all regional haplogroups were monophyletic, with the exception of the Carpathians (paraphyletic with Bulgarian highlands) and the Black Forest and Fennoscandia (both polyphyletic with respect to one another). However, only the eastern Alps, the Urals, and the western Balkans represented highly supported clades (posterior probability, PP > 0.95). Moreover, the connection of the Black Forest, Fennoscandia and the Urals, as well as the deepest split, separating the Eastern Alps, far east Siberia and Alaska from the rest of Europe, were significantly supported.

Microsatellite data

All microsatellite loci were highly variable across all populations ($n = 46$), with a total of 165 alleles over six loci (minimum 10 alleles for Arco_79; maximum 47 alleles for Arco_157). No linkage was observed between any pairs of loci. Our genotyping error rate was 0.5% and should not bias our results (Bonin *et al.*, 2004).

All samples were collected from discrete stream sites that were initially treated as individual populations. Within streams, 15 sites showed significant deviations from HWE (Appendix S2). When sites from the same mountain range were pooled into regional groups strong deviations from HWE were noticeable for all mountain ranges (Appendix S2), probably due to a Wahlund effect. Nevertheless, MICROCHECKER indicated the possible presence of null alleles at all loci except one (Arco_157), but only one locus (Arco_126)

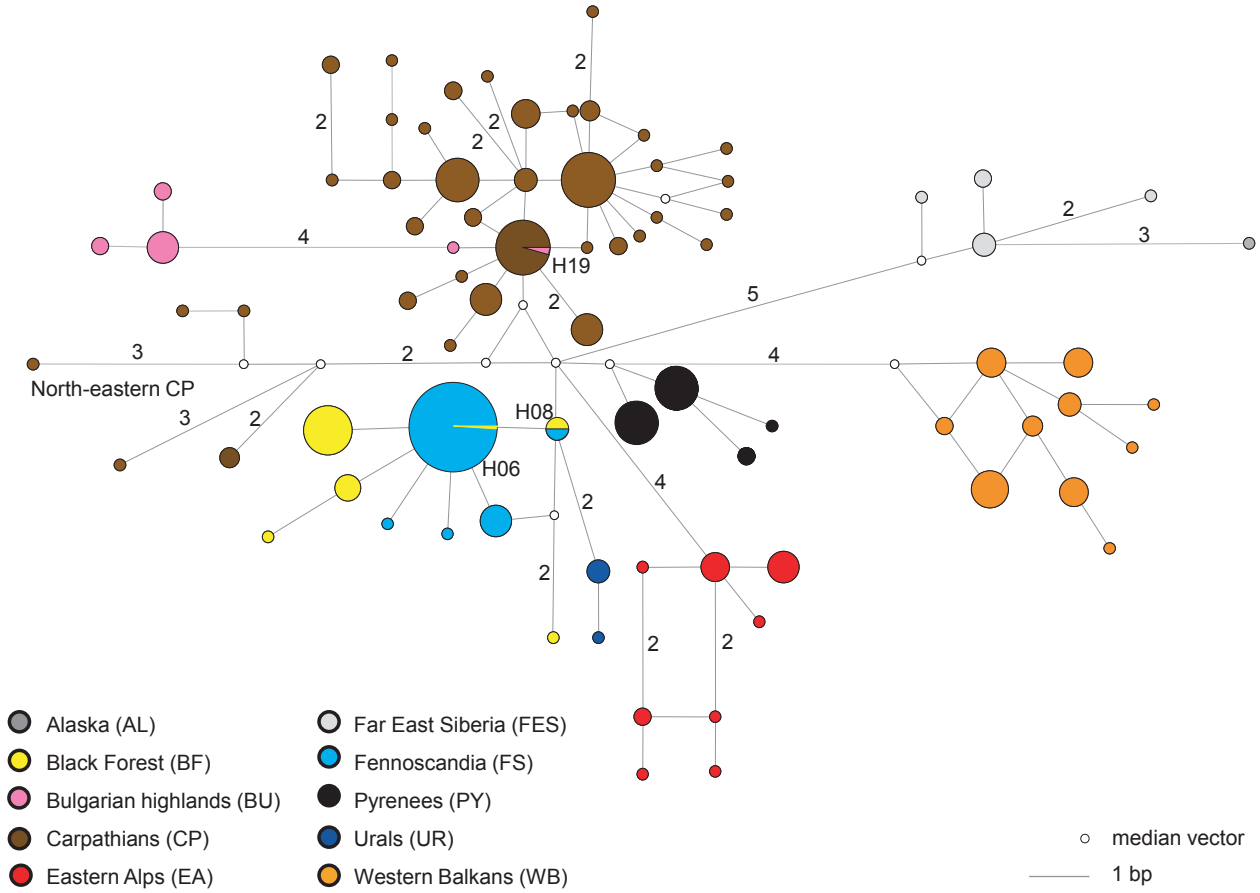

Figure 2 Median joining haplotype network constructed using NETWORK based on 620 bp cytochrome *c* oxidase subunit I sequence data for *Arcynopteryx dichroa* across eight mountain ranges and 46 sampling localities in Europe (n = 335). Also included are specimens from the far east of Siberia (n = 8) and one single specimen from Alaska for comparison of genetic variance. Haplotype circle size denotes the number of sampled individuals. Colours correspond to different mountain ranges. The number of base pair changes (no number = 1 bp) and the number of shared haplotypes among mountain ranges are given.

exhibited null allele frequencies > 0.2 (FS, N_A = 0.236; PY, N_A = 0.235). Simulations show that the bias induced by null alleles for population structure estimates is negligible at frequencies below 0.2 (Dakin & Avise, 2004). To ensure that potential null alleles had no effect on our data, we also compared global F_{ST} values with and without using the ENA correction method as calculated in FREENA. The two runs showed similar results (F_{ST} = 0.30 and 0.31, respectively).

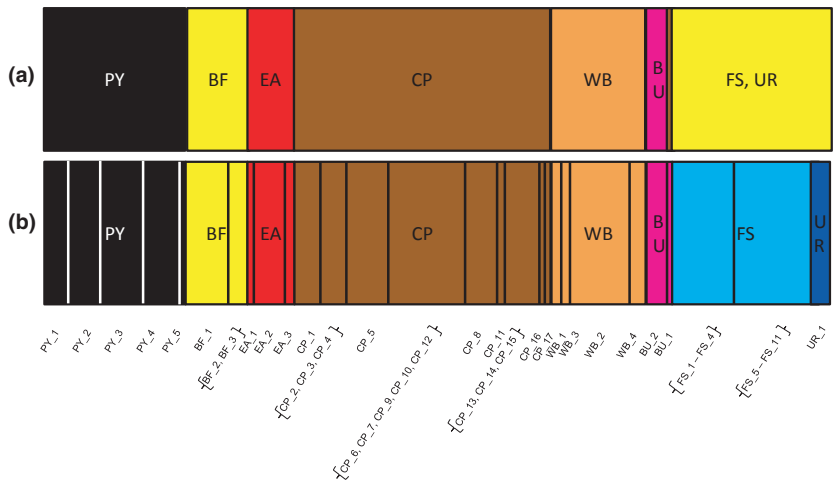

Figure 3 Clustering of individuals into groups of populations for *Arcynopteryx dichroa*. The partitioning is based on (a) sequence data (using SAMOVA) and (b) microsatellite data (using BAPS); abbreviations refer to sampling sites in Appendix S2. Sites in brackets belong to the same cluster. The colours correspond to sampled mountain ranges. PY, Pyrenees; BF, Black Forest; EA, eastern Alps; CP, Carpathians; WB, western Balkans; BU, Bulgarian highlands; FS, Fennoscandia; UR, Urals.

Table 1 Pairwise F_{ST} values for the eight European mountain ranges of *Arcynopteryx dichroa* based on cytochrome *c* oxidase subunit I sequence data as implemented in Arlequin 3.1.1.

	PY	BF	EA	CP	WB	BU	FS	UR
PY	0							
BF	0.673*	0						
EA	0.782*	0.775*	0					
CP	0.562*	0.600*	0.684*	0				
WB	0.723*	0.803*	0.827*	0.712*	0			
BU	0.783*	0.795*	0.840*	0.578*	0.828*	0		
FS	0.851*	0.436*	0.903*	0.671*	0.891*	0.924*	0	
UR	0.773*	0.679*	0.813*	0.621*	0.818*	0.860*	0.917*	0

PY, Pyrenees; BF, Black Forest; EA, eastern Alps; CP, Carpathians; WB, western Balkans; BU, Bulgarian highlands; FS, Fennoscandia; UR, Urals.
*$P < 0.05$.

Table 2 Results for demographic expansions for *Arcynopteryx dichroa* in the eight sampled European mountain ranges, calculated with Arlequin 3.1.1, showing patterns of mismatch distributions (MD), Harpending's raggedness index (HRI) and results from neutrality tests (Tajima's *D* and Fu's F_S) with levels of significance.

	PY	BF	EA	CP	WB	BU	FS	UR
MD	multi	multi	multi	multi	uni	multi	uni	uni
HRI	0.683***	0.212	0.060	0.043	0.070	0.124	0.300	0.200
D	−0.171	−0.596	−0.113	−1.748*	0.049	−0.798	−1.106	−0.817
F_S	1.361	−0.261	−2.371	−26.718***	−2.615	−0.289	−2.097*	0.090

MD: uni, unimodal distribution; multi, multimodal distribution.
*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$.
PY, Pyrenees; BF, Black Forest; EA, eastern Alps; CP, Carpathians; WB, western Balkans; BU, Bulgarian Highlands; FS, Fennoscandia; UR, Urals.

Furthermore, pairwise F_{ST} values corrected for null alleles by the ENA were strongly correlated with the uncorrected F_{ST} values in the Mantel test ($R^2 = 0.99$, $P < 0.0001$). This suggests that all populations were similarly affected and thus null alleles should not bias the results (Vandewoetijne & Dyck, 2010). Consequently, we used the original data set for subsequent analyses.

Per site H_E ranged from zero (FS_2, FS_4: homozygotic at every locus) to 0.917 (CP_4); for mountain ranges H_E ranged from 0.237 (Fennoscandia) to 0.826 (Carpathians) (Appendix S2). The number of private alleles within mountain ranges (N_P, Appendix S2) ranged from zero (Fennoscandia, Urals) to 38.2% (eastern Alps). Of the 14 alleles found in Fennoscandia (Appendix S2), 18.2% were shared with the Pyrenees, 10.8% with the Black Forest, 5.4% with the eastern Alps, 9.5% with the Carpathians, 15.2% with the western Balkans, 28.6% with Bulgarian highlands and 31.3% with the Urals.

The spatial structuring program baps 5.0 grouped the 45 sampling sites into 28 clusters ($P = 0.96$). Only six of these clusters contained multiple sites, albeit always from the same mountain ranges or regions (Fig. 3b): BF_2 and BF_3 (northern Black Forest); CP_2, CP_3 and CP_4 (western Carpathians); CP_6, CP_7, CP_9, CP_10 and CP_12 (south-western Carpathians); CP_13, CP_14 and CP_15 (southern and eastern Carpathians); FS_1 to FS_4 (southern Fennoscandia); and FS_5 to FS_11 (northern Fennoscandia). Running baps with the same settings but without spatial references for sampling localities produced similar clustering results ($P = 0.99$). In contrast to the *mtCOI* sequence data, the Black Forest and the Fennoscandian sites were genetically distinct based on microsatellite data. The baps analysis indicated very low gene flow rates (r) between all cluster pairs ($0.05 > r > 0.0002$), except between southern and northern Fennoscandia ($r = 0.1$).

The FCA (Fig. 5), based on 48.61% of the overall genetic variation, supported the separation of the Black Forest and the Fennoscandian individuals as indicated by the spatial structuring results (Fig. 3b). The most diverse populations were found in the eastern Alps, indicated by the widespread scatter plot.

The AMOVA for the eight geographical groups suggested strong differentiation between mountain ranges ($F_{ST} = 0.455$, $P < 0.0001$; $R_{ST} = 0.491$, $P < 0.0001$) and among sites within mountain ranges ($F_{SC} = 0.257$, $P < 0.0001$; $R_{SC} = 0.221$, $P < 0.0001$). Population pairwise F_{ST} and R_{ST} values within each mountain range also indicate strong divergence among sites within mountain ranges (F_{ST} and $R_{ST} > 0.15$; Conner & Hartl, 2004) for 53.9 and 59.3% of pairwise comparisons, respectively (Appendix S3).

DISCUSSION

Glacial survival in Europe

Our genetic data support the hypothesis that *A. dichroa* survived glacial cycles in isolated extra-Mediterranean mountain ranges. Based on *mtCOI* sequence data we observed

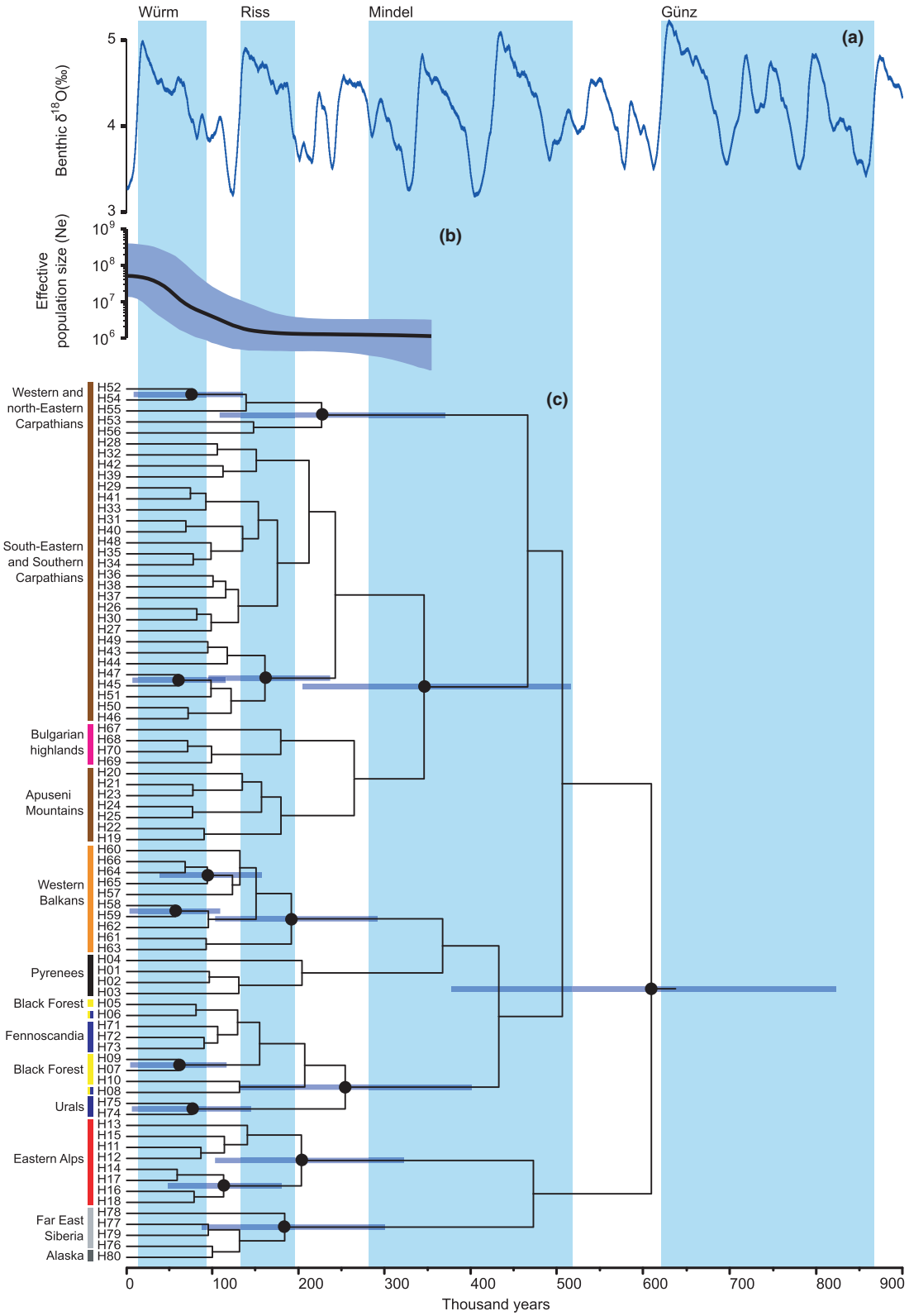

Figure 4 Haplotype ancestries of *Arcynopteryx dichroa* determined using BEAST. (a) Benthic oxygen isotope stages. (b) Demographic changes of *A. dichroa* populations, presented by a Bayesian skyline plot. Mean (black line) and 95% confidence intervals (grey) indicate effective sample size (N_e) estimates. The demographic changes reflected by the graphs are estimated back in time to the mean timing of the coalescent event. (c) Bayesian phylogenetic tree of the 80 identified *A. dichroa* haplotypes. Posterior probabilities $\geq$ 0.95 are marked by black circles. Grey bars represent 95% highest probability densities of the inferred mean divergence dates. Colours of the main clades correspond to sampled mountain ranges.

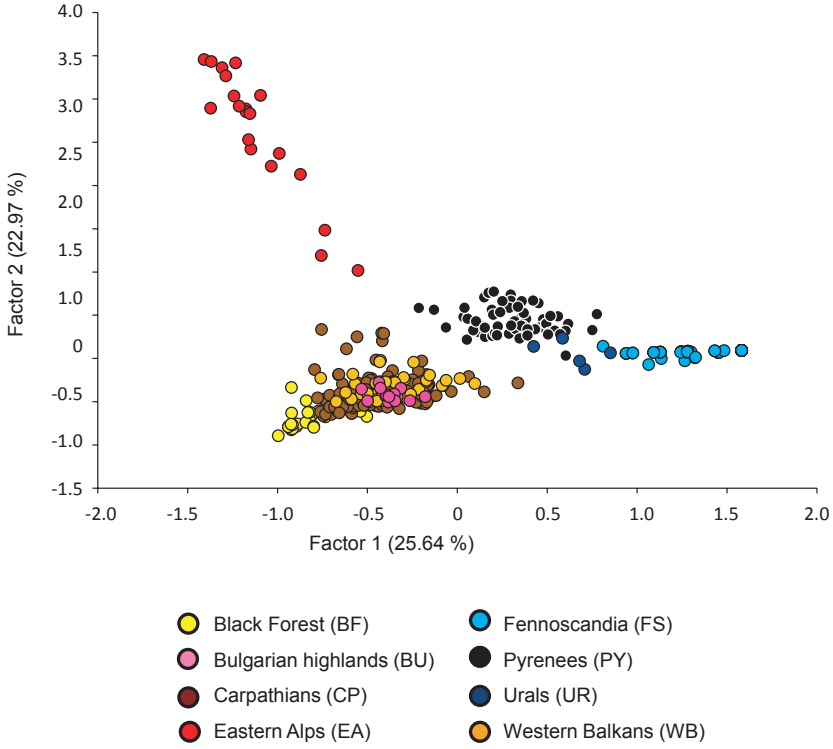

Figure 5 Factorial correspondence analysis determined using GENETIX showing the degree of similarity of the 365 *Arcynopteryx dichroa* individuals across six microsatellite loci based on the first two dimensions (factors), which explain the major part of genetic variation (48.61%). The program graphically projects the individuals on the factor space defined by the similarity of their allelic states. Each dot indicates an individual. The closer the dots the more related the individuals. Colours correspond to sampled mountain ranges.

mountain range-specific lineages (Figs 2 & 3), monophyly of most regional haplogroups (Fig. 4) and clear geographical structuring among sampled mountain ranges (Fig. 3a). Microsatellites also indicated that most sites were genetically unique (Fig. 3b). Moreover, we detected high population pairwise F_{ST} and R_{ST} values within most mountain ranges (Appendix S3), suggesting geographical barriers even at intra-regional scales. These patterns are consistent with the low dispersal capability of *A. dichroa*.

The BEAST analysis (Fig. 4) suggested a long-term persistence of alpine *A. dichroa* populations over numerous climatic cycles in Europe. The BSP (Fig. 4b) indicated a continuous population growth over the past 150 kyr, which only stagnated in the late Würm. The relationship among several haplogroups was not resolved (Fig. 4c), reflecting the star-like topology of the MJ network (Fig. 2). However, the haplotype phylogeny dated the deepest lineage split back to the beginning of the oscillation phase before the Mindel ice age (*c.* 600 ka). This split significantly separates the eastern Alps from the remaining European populations. The eastern Alps exhibited an exceptionally high degree of genetic diversity (Figs 2 & 5, Appendix S2) indicating the importance of this area as a Pleistocene refugium in the alpine ecoregion, as previously shown for other freshwater invertebrates (e.g. Malicky, 2006; Taubmann *et al.*, 2011; Weiss *et al.*, 2012) and vascular plants (Tribsch & Schönswetter, 2003).

Besides the eastern Alps, high levels of genetic diversity were detected in the Carpathians (Figs 2 & 4, Appendix S2). This is an important extra-Mediterranean refugium for temperate (e.g. Kotlík *et al.*, 2006) and cold-adapted species (e.g. Ujvárosi *et al.*, 2010; Bálint *et al.*, 2011), as the Carpathians, with the exception of the highest elevations, remained ice-free during the LGM (Reuther *et al.*, 2007). Based on *mtCOI* sequence data, we detected a connection between the Apuseni and the Bulgarian highlands (H19, H20; Figs 2 & 4), a pattern previously observed for montane caddisflies (Pauls *et al.*, 2009; Lehrian *et al.*, 2010). Within the Carpathian range we discovered at least two distinct *mtCOI* lineages (Fig. 4), separating the Apuseni and the south-eastern Carpathian bow from the north-western Carpathians. These lineages probably correspond to independent glacial refugia (Bálint *et al.*, 2011).

Our SDM is largely congruent with our genetic data (Appendix S1). For the LGM, our SDM projected large areas of high probability of occurrence in the western and central European highlands and disconnected areas of suitable climatic conditions in all mountain ranges inhabited by the species today except Fennoscandia (Fig. S1b in Appendix S1). This is consistent with mountain system-specific lineages and the clear geographical structuring among sampled mountain ranges. Therefore we conclude that *A. dichroa* is a glacial relict that survived glacial cycles probably through elevational shifts in isolated populations in the Pyrenees, the central European highlands, the eastern Alps, the Carpathians and the western Balkans.

Recolonization of Fennoscandia

In Europe, Fennoscandia was the last region to become free of ice (Lundqvist & Mejdahl, 1995) and was thus probably recolonized more recently than the southern range (Pamilo

& Savolainen, 1999). Fennoscandia exhibited very low genetic diversity (Figs 2 & 5, Appendix S2), suggesting a single refugial source. Moreover, there were shared and closely related *mtCOI* haplotypes between Fennoscandia and the Black Forest (Fig. 2). These two sites formed a significant group with the Ural haplotypes, with the most recent common ancestor 250 ka (Fig. 4c).

The microsatellite data, however, did not support the connection of the Black Forest and Fennoscandia (Figs 3 & 5). This incongruence of mitochondrial and microsatellite data is a common issue in phylogeographical studies (Zink & Barrowclough, 2008), highlighting the need to use various marker types with different modes of inheritance to sufficiently understand phylogeographical histories (Godinho *et al.*, 2008). Here, the discrepancies can be explained in two ways. First, ancestral polymorphism could be conserved in the *mtCOI* data while the microsatellites diverged more quickly due to random mutations under strong genetic drift. In this scenario the two populations either became disjunct due to habitat vicariance, or there was historical long-distance dispersal between the disjunct northern and southern periglacial populations. Another plausible explanation is female-biased dispersal, leading to a weaker genetic signal of the maternally inherited mtDNA (Zink & Barrowclough, 2008). Unfortunately, there were insufficient ecological data to examine sex-biased dispersal. However, considering the large-scale recolonization into Fennoscandia, directional larval dispersal seems unlikely to be the underlying dispersal mechanism in this recolonization. Moreover, the species' Holarctic distribution and the limited divergence among *mtCOI* sequences from different regions and continents (Fig. 2) make it plausible that long-distance dispersal is primarily maintained by winged females, rather than brachypterous males.

The SDM displayed no suitable climatic conditions for *A. dichroa* within the northern glaciated habitats during the LGM (Fig. S1b in Appendix S1), indicating that the species should have recolonized northern European sites in the post-glacial. Based on the species' limited dispersal capabilities, this recolonization was most likely to have been a relatively slow process that was driven by rare long-distance dispersal events. SDM did indicate that the central and western European highlands (Fig. S1b) were suitable source areas from which recolonization could have initiated. Considering that the species is currently not found in the western European highlands and that our sequence data exhibited a connection between Fennoscandia and the Black Forest, the refugial population probably persisted along the glacial fringe in the central European highlands over numerous glacial cycles. This ancestral and potentially large periglacial population declined in the post-glacial and is currently restricted to the Black Forest in the south and shifted northward with the retreat of the glaciers. Whether the source population was located directly or in the vicinity of the Black Forest remains unknown, but other studies of cold-tolerant aquatic insects indicate that *in situ* persistence in Black Forest streams was possible (Malicky, 1983, 2006; Pauls *et al.*, 2006).

A close relationship between central and northern European populations has often been suggested by biogeographers (e.g. de Lattin, 1967). In arctic–alpine plant species, separations between the northern populations and the central European highlands are most often post-glacial phenomena (e.g. Alsos *et al.*, 2009). There is evidence that some arctic plant species survived the LGM at high latitudes or north of the extensive alpine ice shields (Westergaard *et al.*, 2011). Schmitt *et al.* (2010) summarized several examples of this connection between central and northern Europe in plant species, which all colonized the North Atlantic region from source populations in the Alps. Additionally, the cold-adapted fish *Cottus gobio* (Linnaeus, 1758) is thought to have recolonized the north from central European populations (Englbrecht *et al.*, 2000).

European-wide molecular studies on arctic–alpine invertebrates are rare, but include the wolf spiders *Pardosa saltuaria* (L. Koch, 1870; Muster & Berendonk, 2006), the ground beetle *Nebria rufescens* (Ström, 1768; Schmitt *et al.*, 2010), the butterfly *Erebia pandrose* (Borkhausen, 1788; Schmitt *et al.*, 2010) and the mayfly *Ameletus inopinatus* (Eaton, 1887; Theissinger *et al.*, 2011). Sequence data on *P. saltuaria* exhibited three clades of deep mitochondrial divergence, a Pyrenean clade, a Balkan clade, and a 'northern clade' including Fennoscandia, the Alps, the Carpathians, the Giant Mountains, and the Bohemian Forest (Muster & Berendonk, 2006). Schmitt *et al.* (2010) compared these findings with preliminary sequence results of *N. rufescens* and *E. pandrose* and found similar genetic patterns among these species. This suggests a late glacial connection of Fennoscandian and central European populations. In sharp contrast, Theissinger *et al.* (2011) showed that in the mayfly *A. inopinatus*, Fennoscandian populations formed a highly diverged and structured group and did not descend from a central European refugial source. Instead, Theissinger *et al.* (2011) proposed that *A. inopinatus* extended its northern range during the early Pleistocene from multiple lineages, probably of north-eastern Siberian or central Asian origin. These differences in recolonization patterns emphasize the fact that species with similar current distribution patterns do not necessarily share the same phylogeographical history. Clearly, there is a continued need to examine the current distributions and population genetics of arctic–alpine species to better understand the processes and mechanisms of range expansion, regression and lineage diversification in Europe's high-latitude and high-elevation biota.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Species distribution modelling: methods and results.

Appendix S2 Sampling localities and summary of genetic results.

Appendix S3 Population pairwise F_{ST} and R_{ST} values.

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BIOSKETCH

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