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Genetic implications of phylogeographical patterns in the conservation of the boreal wetland butterfly *Colias palaeno* (Pieridae)

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The boreo-montane wetland butterfly species *Colias palaeno* has a European distribution from the Alps to northern Fennoscandia. Within its European range, the species' populations have shrunk dramatically in recent historical times. Therefore, detailed baseline knowledge of the genetic makeup of the species is pivotal in planning potential conservation strategies. We collected 523 individuals from 21 populations across the entire European range and analyzed nuclear (20 allozyme loci) and mitochondrial (600 bp of the cytochrome *c* oxidase subunit I gene) genetic markers. The markers revealed contrasting levels of genetic diversity and divergence: higher in allozymes and lower in mitochondrial sequences. Five main groups were identified by allozymes: Alps, two Czech groups, Baltic countries, Fennoscandia, and Poland. The haplotype mitochondrial network indicates a recent range expansion. The most parsimonious interpretation for our results is the existence of a continuous Würm glacial distribution in Central Europe, with secondary disjunction during the Last Glacial Maximum into a south-western and a north-eastern fragment and subsequent moderate differentiation. Both groups present signs of postglacial intermixing in the Czech Republic. However, even a complete extinction in this region would not considerably affect the species' genetic basis, as long as the source populations in the Alps and in northern Europe, comprising the most relevant evolutionary units for conservation, are surviving. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 1068–1081.

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INTRODUCTION

A comprehensive understanding of biogeographical patterns is an important prerequisite for the successful establishment of conservation measures. In this context, the biogeography of many animal and plant species has been studied intensively in Europe (Hewitt, 2004; Schmitt, 2007). Indeed, the biogeographical structures of Mediterranean species are particularly well known (Schmitt, 2007), and

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typical high mountain species (Schönswetter *et al.*, 2005; Schmitt, 2009) and continental species (Stewart & Lister, 2001; Stewart *et al.*, 2010; Schmitt & Varga, 2012) are also well studied. However, phylogeographical knowledge of species associated with comparatively cold wetland habitats, such as oligotrophic bogs, often exhibiting a boreo-montane distribution, is still rather limited (but see Šula & Spitzer, 2000). Our knowledge is mostly restricted to ecological analyses (Krogerus, 1960; Coulson & Butterfield, 1985) and some studies concerning local and regional genetic structures (Bernard & Schmitt, 2010; Buczkowska *et al.*, 2012; Rasic & Keyghobadi, 2012). Consequently, phylogeographical studies of European bog species, one of the pillars of conservation planning, are still scarce (Nève, 1994; Bernard *et al.*, 2011; Jermakowicz *et al.*, 2015).

Bogs with their unique flora and fauna are of particular biogeographical and conservation interest because most of them were already established in the late Pleistocene or the early Holocene and have existed continuously without subsequent major alterations (Spitzer & Danks, 2006). Peat bogs in particular are unique and vulnerable habitats that support many protected terrestrial and (semi-)aquatic animal and plant species (Barber, 1993; Barkham, 1993). Additionally, they play an important global role in the carbon cycle by exchanging CO_2 with the atmosphere, producing dissolved organic carbon or storage of carbon (Moore, Roulet & Waddington, 1998). However, bogs have suffered from massive peat extractions during the last few centuries (Stoneman & Brooks, 1997; Howie, 2002). More recently, many bogs have been lost to agriculture, urbanization, and forestry (Wheeler & Proctor, 2000; Stephens *et al.*, 2011). In Europe, the rate of this destruction has slowed down during the last few decades, although these habitats remain highly endangered, and newly-emerging threats include increased air and soil eutrophication (Spitzer & Danks, 2006), as well as climate change (Moore *et al.*, 1998; Weltzin *et al.*, 2003; Wu & Roulet, 2014). Consequently, their inhabitants are suffering from decreasing habitat areas and the subsequent loss of resources. The vulnerable situation of bogs and other wetland habitats prompted the international Ramsar Convention (www.ramsar.org). It is therefore highly important to study bog species in more detail, so that a better understanding of their distribution dynamics can be used to develop appropriate conservation management strategies.

In the present study, we investigated the population genetic structure of a boreal species, the endangered moorland clouded yellow, *Colias palaeno* (L., 1761), a character species of well preserved and interconnected bog habitat complexes (Bolotov, 2004; Spitzer & Danks, 2006). This species is distributed from Central Europe throughout temperate Asia to North America and has to be considered as a polytypic and polycentric species with several described subspecies, mostly from boreal East Asia and North America (Varga, 1977; Tshikolovets, 2011). As a result of habitat loss for the larvae and imagoes, *C. palaeno* is highly endangered in most parts of Europe (van Swaay & Warren, 1999). We analyzed nuclear (allozymes) and mitochondrial (mt)DNA genetic information obtained from 523 individuals from 21 populations scattered over most of the species' distribution in Western and Central Europe, in a longitudinal transect between the Alps to northern Fennoscandia. We examined the genetic structure of *C. palaeno* at two different scales: at the continental level in a longitudinal transect from the Alps to Fennoscandia and at a regional scale by focusing on the Czech Republic populations. Here, the species had lost more than 40% of its pre-1950 populations by 1994. This severe loss of populations was not distributed equally over the country (Pavličko, 2002b; Vrba *et al.*, 2014) and the establishment of a policy of genetic conservation has been perceived to be of urgent need. Thus, we intend to address the following questions:

1. Is *C. palaeno* differentiated into several genetic lineages (i.e. evolutionary significant units) within Europe with geographical association?
2. If so, which of these are of major conservation value?
3. Did the species survive the last ice age in a single retreat in Europe, or did multiple but geographically less extensive refugia exist from which independent postglacial expansions took place?
4. Is habitat availability reflected in the local and regional genetic make-up of populations and the degree of differentiation among them?
5. What implications for the conservation of *C. palaeno* can be derived from our results?

MATERIAL AND METHODS

Study species

The moorland clouded yellow *C. palaeno* (Pieridae) has a Holarctic distribution, reaching from the French Jura and the Alps in the West through parts of Central, Northern, and Eastern Europe, to Siberia, Korea, Japan, and North America (Huemer, 2004). At the southern edge of its range, the species is montane-subalpine with a vertical range from approximately 900–2500 m a.s.l. (Huemer, 2004), whereas it is a lowland species in Northern Europe (Henriksen & Kreutzer, 1982).

Colias palaeno is not only a characteristic species of bogs and other oligotrophic wetlands (Erhardt, 1985), but also can be found in dwarf shrub heaths of the Central Alps (Huemer, 2004). Moderately disturbed (i.e. eutrophic and/or drying) bog sites offer especially favourable conditions, if sufficient sun-exposed bog bilberry shrubs (*Vaccinium uliginosum*), the only larval food plant, are present and if, at the same time, the supply of nectar plants is sufficient for the imagoes. Where such sites change into heath or pioneer trees stands as a result of further succession or draining (e.g. as a consequence of peat extraction), *C. palaeno* rapidly disappears (Settele *et al.*, 2009). Conservation measures may therefore require the restoration of former bog hydrology. Occasional mowing at the edges of the marsh areas may have a positive effect because this increases the variety of nectar plants in the vicinity of the larval habitats (Rüetschi & Scholl, 1985; Ebert & Rennwald, 1991).

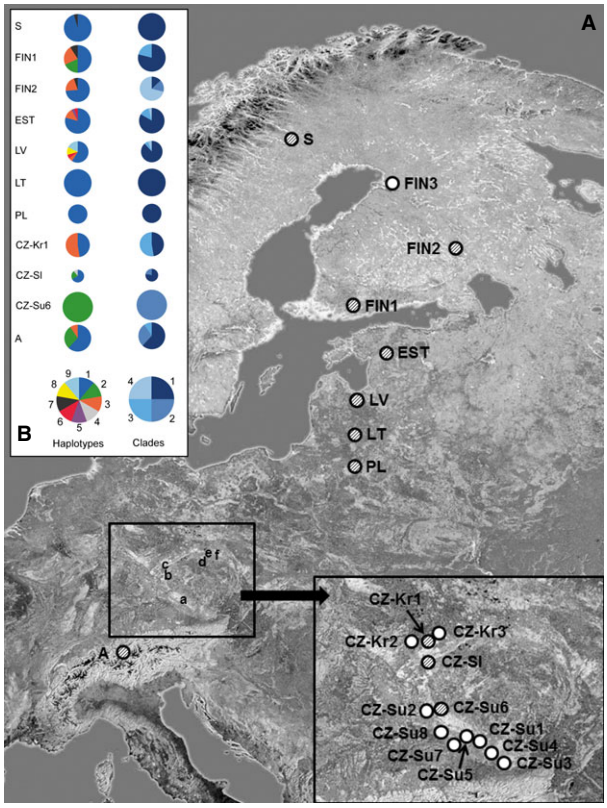

Figure 1. A, geographical distribution of the 21 studied populations of *Colias palaeno* and relevant Czech mountain ranges [a, Šumava (= Bohemian Forest). b, Slavkovský les (= Kaiserwald). c, Krušné hory (= Ore Mountains). d, Hrubý Jeseník (= High Ash Mountains). e, Jizerské hory (= Jizera Mountains). f, Krkonoše (= Giant Mountains)]. Populations with hatched circles were used for allozyme electrophoresis and mtDNA sequencing. B, distribution of the nine cytochrome *c* oxidase subunit I (COI) haplotypes and four clades found in the present study. Additional details and abbreviations are found in the online version of this article.

Sampling

We sampled 523 *C. palaeno* individuals from 21 populations in Central and Northern Europe (Austria, Czech Republic, Estonia, Finland, Lithuania, Latvia, Poland, and Sweden) (Fig. 1A; see also Supporting information, Appendix S1). In the Czech Republic, sampling was more intensive (12 sites and 258 individuals), covering most large populations of the butterfly occurring in the country, in three separate mountain ranges. In the Baltic and Nordic countries, much less intensive sampling was carried out along an approximate South–North transect from 53° to 66°N.

Individuals were frozen in liquid nitrogen immediately after capture and stored in this medium until genetic analyses were performed. For allozyme electrophoresis, all of the individuals were analyzed, whereas only 213 individuals from eleven populations were used for mtDNA sequencing.

Allozyme electrophoresis

Half of the abdomen of each individual was homogenized in PGM buffer (Harris & Hopkinson, 1976) by ultrasound and centrifuged at 8000 ***g*** for 5 min. Electrophoresis was run on cellulose acetate plates (Hebert & Beaton, 1993). We analyzed 16 different enzyme systems representing 20 allozyme loci. The electrophoresis conditions are given in the Supporting information (Appendix S2).

mtDNA sequencing

For mtDNA analyses, DNA was extracted from the heads of the samples using the standard Chelex 100 protocol (Walsh, Metzger & Higuchi, 1991). A partial sequence of the cytochrome *c* oxidase subunit I (COI) gene was amplified by a polymerase chain reaction (PCR) with the forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGGC-3′) and reverse primer HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). PCR amplifications were performed in total volume of 20 μL, using 5 μL of 5× PCR Colorless buffer (pH 8.5), 2 mM (2 μL of a 25 mM $MgCl_2$ solution), 0.2 mM (0.5 μL of a 20 mM dNTP stock), 0.2 μL of 5 μ μL^{-1} 1 U GoTaq DNA polymerase (Promega), and 0.2 μM (0.4 μL of a 10 μM stock) of each primer.

The COI PCR profile consisted of 2 min at 95 °C, 35 cycles of 30 s at 94 °C, and 30 s at 53 °C, followed

by an extension for 1 min at 72 °C and a final extension for 5 min. Negative controls were included in each set of reactions. The PCR results were afterwards checked by electrophoresis in a 1% GelRed stained agarose gel and purified by ethanol precipitation (Sambrook & Russell, 2001). Sequencing was performed at the Centre of Marine Sciences (CCMAR, Portugal) on an ABI 3130xl (Applied Biosystems) capillary sequencer using the forward primer employed in the PCR.

Statistical analysis

Allozyme alleles were labelled according to their relative mobility, starting with '1' for the slowest. Allele frequencies and parameters of genetic diversity [mean number of alleles per locus (A), expected and observed heterozygosity (H_E, H_O), total percentage of polymorphic loci (P_{tot}) and percentage of polymorphic loci with the most common allele not exceeding 95% (P_{95})] were computed using GSTAT, version 3.2 (Siegismund, 1993). Weighted F statistics were calculated using the estimators described by (Weir & Cockerham, 1984). Their values and significances were estimated in FSTAT, version 2.9.3 (Goudet, 1995) after 10 000 randomizations. To allow for differences in population sizes (N = 11–42), allelic richness (A_R) was calculated for 11 and 20 diploid individuals with in HP-RARE, version 1.1 (Kalinowski, 2005), which uses the technique of 'rarefaction', so that the number of alleles in large samples can be compared with the number of alleles in smaller samples (Hurlbert, 1971). The estimator of actual differentiation (D_{est}) (Jost, 2008) was calculated using SMOGD, version 1.2.5 (Crawford, 2010).

Conventional F statistics, analysis of molecular variance (AMOVA), hierarchical genetic variance analyses, tests of Hardy–Weinberg equilibrium and linkage disequilibrium were calculated with ARLEQUIN, version 3.5.2.2 (Excoffier & Lischer, 2010). Significance was obtained after 10 000 permutations.

Additionally, we determined the number of private alleles. Nei's standard genetic distances (Nei, 1972) were calculated and Neighbour-joining phenograms were constructed with PHYLIP, version 3.6 (Felsenstein, 2005), including bootstrap values (based on 10 000 iterations).

To disentangle the population structure, we used the Bayesian multilocus assignment method *sensu* Corander & Marttinen (2006) as implemented in BAPS, version 6. BAPS requires an expectation for the maximum number of groups (K) as a prior. For each K value, BAPS attempts to determine the optimal partitions, stores them internally, and, after all the runs have been processed, it merges the stored results according to the log-likelihood values. In our analysis, values of K ranging from one to 21 (the number of studied populations) were explored with ten replicates for each value of K. Additionally, we tested the validity of the assumed genetic groups with a population assignment test implemented in GENALEX, version 6.5 (Peakall & Smouse, 2006) using the default settings.

All COI sequences were aligned using CLUSTAL X, version 2.0 (Larkin *et al.*, 2007) with default settings, implemented in GENEIOUS, version 5.4 (Kearse *et al.*, 2012) and double-checked manually. To reduce sequences to haplotypes, we used the online toolbox FABOX, version 1.4.1 (Villesen, 2007). The number of individuals (N), frequency (f), number of haplotypes (n), number of private haplotypes (n_P), and haplotype (h) and nucleotide diversities (Θ) for each location were calculated in ARLEQUIN.

An mtDNA haplotype network was constructed with the median-joining algorithm using NETWORK, version 4.5 (Bandelt, Forster & Röhl, 1999). Corrected genetic distances between clades were estimated in MEGA, version 6 (Tamura *et al.*, 2013).

The same software was used to calculate a maximum likelihood tree. We used the general time reversible (GTR) + Gamma (G) + Invariable substitution (I) model and performed 1000 bootstrap replicates to obtain measures of branch support (Felsenstein, 1985). The data set contained our haplotypes of *C. palaeno* and all COI sequences of the genus *Colias* found in GenBank (Benson *et al.*, 2009; Sayers *et al.*, 2009). For convenience of presentation, we collapsed identical sequences.

RESULTS

Allozymes

Eighteen of the 20 allozyme loci analyzed were polymorphic. Only GAPDH and HBDH were monomorphic for all individuals. The number of alleles per polymorphic locus varied from two to 12 (PGI) with a mean ± SD of 3.85 ± 2.04. No general linkage disequilibrium was observed for any locus (all $P > 0.05$). We calculated several population genetic parameters, based on allele frequencies. Details for all populations are given in Table 1.

Most of the pairwise F_{ST} values were significant ($P < 0.05$), apart for four pairs of populations. The maximum pairwise F_{ST} was 0.447 between the populations Slavkovský les (CZ-Sl) and Poland (PL) ($P = 0.001$). The unbiased genetic distances (Nei, 1972) between all 21 populations ranged from 0.006 to 0.124, with a mean ± SD of 0.034 ± 0.023. Isolation-by-distance analysis yielded no significant correlation ($R = 0.03$; Mantel test: $P = 0.087$).

For Bayesian clustering analysis using BAPS (Fig. 2), the highest posterior probability (0.9999)

Table 1. Parameters of genetic diversity of allozymes averaged over loci for all 21 *Colias palaeno* populations analyzed: number of individuals (N), expected (H_E) and observed heterozygosity (H_O), total number of alleles (A), allelic richness (A_R) based on eleven and 20 individuals, total percentage of polymorphic loci (P_{tot}), percentage with the most common allele not exceeding 95% (P_{95}), inbreeding coefficient (F_{IS}), deviations from Hardy–Weinberg equilibrium (HWE), and number of private alleles (PA)

PopID	N	H_E (%)	H_O (%)	A	A_{R11}	A_{R20}	P_{tot} (%)	P_{95} (%)	F_{IS}	HWE	PA
A	23.0	21.0	15.7	1.95	1.69	1.94	55.0	50.0	0.244	***	0
CZ-Kr1	25.9	19.7	15.8	2.10	1.62	2.03	55.0	50.0	0.198	*	1 (PK)
CZ-Kr2	15.0	20.1	15.0	(1.70)	1.60	–	55.0	55.0	0.253	**	0
CZ-Kr3	18.9	21.3	15.4	(1.85)	1.66	–	60.0	60.0	0.280	***	0
CZ-Sl	15.0	19.0	17.0	(2.00)	1.63	–	65.0	55.0	0.098	NS	0
CZ-Su1	13.1	17.6	17.8	(1.70)	1.56	–	55.0	50.0	−0.003	NS	1 (MDH1)
CZ-Su2	11.0	19.1	15.9	(1.95)	1.63	–	65.0	50.0	0.189	**	0
CZ-Su3	19.5	22.7	17.8	2.10	1.72	2.10	65.0	65.0	0.217	***	1 (MDH2)
CZ-Su4	22.4	25.4	18.7	2.15	1.74	2.20	80.0	70.0	0.047	NS	0
CZ-Su5	29.0	19.8	16.4	2.00	1.62	1.92	70.0	55.0	0.173	**	1 (GOT1)
CZ-Su6	34.0	17.7	15.2	2.25	1.62	2.07	75.0	55.0	0.140	**	0
CZ-Su7	18.9	20.1	15.8	(1.95)	1.64	–	60.0	55.0	0.228	**	1 (G-6-PDH)
CZ-Su8	31.0	18.9	15.6	2.20	1.64	2.06	70.0	55.0	0.171	***	2 (MPI, PGI)
EST	34.0	16.3	13.5	2.30	1.61	2.12	60.0	45.0	0.170	***	1 (PK)
FIN1	22.0	17.0	11.4	2.25	1.61	2.22	65.0	50.0	0.309	***	0
FIN2	38.3	19.4	17.2	2.45	1.66	2.22	75.0	65.0	0.118	***	0
FIN3	26.9	22.1	19.5	2.45	1.78	2.34	60.0	55.0	0.118	**	0
LT	41.5	17.2	13.6	2.45	1.66	2.23	70.0	50.0	0.204	***	2 (IDH2, PGI)
LV	30.8	17.5	13.2	2.35	1.69	2.22	50.0	50.0	0.248	***	1 (PGI)
PL	15.0	16.7	17.4	(1.83)	1.61	–	61.1	44.0	−0.031	NS	1 (FUM)
S	28.9	17.5	15.3	2.25	1.63	2.12	70.0	55.0	0.123	**	1 (GOT2)
Mean	24.5	19.3	15.9	2.23	1.65	2.13	63.9	54.3	0.166		
SD	8.4	2.2	1.9	0.16	0.05	0.11	7.7	6.3	0.086		

For A, populations with < 20 individuals analyzed (given in parenthesis) are excluded from the calculation of the mean as a result of an insufficient sample size.
NS, not significant ($P > 0.05$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

was obtained for $K = 5$: (1) Austrian Alps (A); (2) Czech Republic (except CZ-Sl and CZ-Kr2-3); (3) CZ-Sl and CZ-Kr2-3; (4) Baltic populations (EST, LT, LV), and (5) Poland (PL) and Fennoscandia (S, FIN1-3). A population assignment test assigned most of the individuals to their respective BAPS group. The greatest probability had group 1 (78.3%), followed by group 2 (71.4%) and group 3 (69.4%). Groups 4 and 5 had a lower probability, although still more than half of the individuals were assigned to their respective group (see Supporting information, Appendix S3).

A Neighbour-joining phenogram of the 21 populations (Fig. 3) based on Nei's (1972) genetic distances had a low resolution, with low bootstrap values. Nevertheless, the five geographical clusters distinguished showed striking similarity with the BAPS analysis: (1) Austrian Alps (A); (2) Ore Mountains and Slavkovský les; (3) Šumava; (4) Baltic populations (EST, LV, LT); and (5) a northern group (S, FIN1-3), including Poland (PL).

The genetic variance among all European populations was moderate (locus-by-locus AMOVA: variance component = 0.193; $F_{ST} = 0.092$, $P < 0.001$). The D_{est} value amounted to 0.035. The genetic variance among individuals within populations was higher than among populations (variance component = 0.322, $F_{IS} = 0.169$, $P < 0.001$). However, the highest proportion of the genetic variance (i.e. 75.49%) was within individuals (variance component = 1.586) (see Supporting information, Appendix S4). A hierarchical AMOVA applying the groupings of BAPS for group definition distributed the variation among the five BAPS groups as: 7.42% among groups, 4.14% among populations within groups, and 88.44% within populations.

MTDNA

A total of 213 individuals from eleven locations were analyzed. The alignment was 600-bp long and

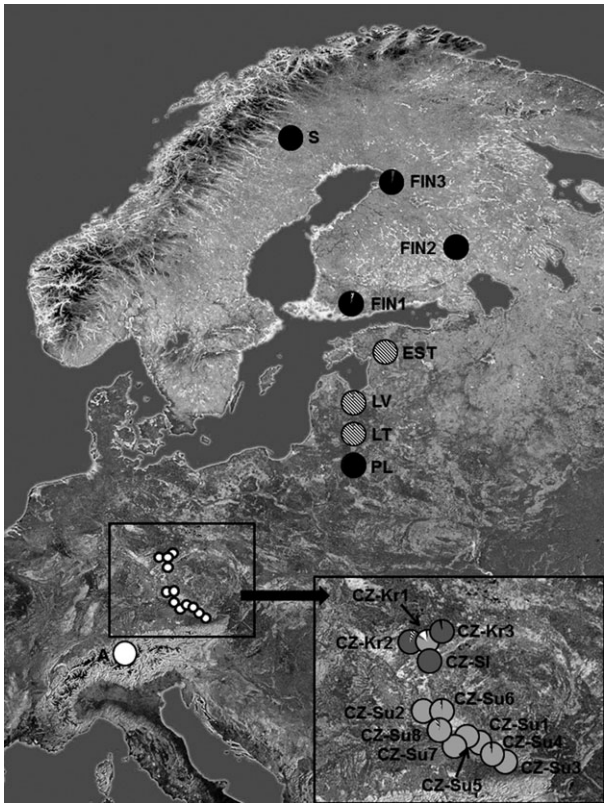

Figure 2. Genetic clustering analyses of *Colias palaeno* genotypes as calculated using BAPS. Different hatchings represent the proportional memberships of a population to the respective genetic clusters, averaged over individual membership proportions for $K = 5$.

comprised 58 (9.7%) polymorphic sites of which 54 (9.0%) were parsimony-informative. Overall mean ± SD haplotype diversity was low (35.1 ± 7.3%), ranging from 0.000 to 0.688, with haplotype numbers varying from one to three in each location. The mean ± SD nucleotide diversity was moderate (1.2 ± 0.7%), with values ranging from 0.0% to 4.3% (see Supporting information, Appendix S5).

The haplotype network (Fig. 4) contained nine haplotypes arranged in four clades: clade 1, the most abundant, consisted of six different haplotypes (1, 4, 5, 7, 8, and 9), whereas all other clades consisted of a single haplotype each (2, 3, and 6, respectively). Clades 1 and 4 were separated by 50 mutational steps. Genetic divergences among the other clades were much smaller; clade 1 and 2 amounted to eight mutational steps, whereas clade 1 and 3 were separated by 13 mutational steps. The genetic distance, calculated with the Kimura-two parameter model for comparative purposes, ranged from 0.17% to 0.67% within clades and from 1.17% to 9.17% between clades.

The four COI groups showed different geographical distributions (Fig. 1B). Clade 1 was found throughout the geographical range (except CZ-Su6), whereas the other clades are geographically more restricted: clade 2 was restricted to Austria, CZ-Sl, CZ-Su6 (the only 100% clade 2 location), and Finland; clade 3 was found only in the Baltic populations LV and EST; and clade 4 was found in Finland, Estonia, Latvia, Ore Mountains, and Austria. Although four sites (S, LT, PL, and CZ-Su6) only had haplotypes belonging to a single clade, no geographical replacement of one clade by another one was detectable (Fig. 4).

The large divergence between clade 1 and 4 prompted us to perform a NCBI BLASTn search to ensure the correct taxonomic identity of these clades. The most closely-related available GenBank *C. palaeno* sequences belonged to clades 1 and 2, which were also the most closely related clades in our dataset, whereas both clades also had a high maximum identity of at least 99% with other *Colias* species, namely the arctic species *Colias tyche*, *Colias hyperborea*, and *Colias hecla* (clade 1) or the northern North American species *Colias gigantea* and *Colias chippewa* (clade 2), with the latter often synonymized with *C. palaeno*. Clade 3 was equally similar to *Colias phicomone* (southern European high mountain systems), *Colias ladakensis* (Tibet) or the Canadian species *Colias christina* and *Colias occidentalis*, whereas clade 4 sequences were most similar to *Colias alfacariensis* (but only with 94% maximum identity).

DISCUSSION

Both genetic markers displayed distinct signatures. Allozymes have relatively high diversity compared to butterflies in general (Schmitt, Röber & Seitz, 2005; Habel *et al.*, 2010; Habel & Schmitt, 2012). However, the mean allozyme diversity of the analyzed populations of *C. palaeno* is lower than for other *Colias* species (e.g. *Colias crocea* and *Colias erate*) (Milovanov & Simchuk, 2008). Furthermore, the genetic diversity is mostly equally distributed over our study area, with no latitudinal gradients and no major regionally or locally impoverished populations. These findings indicate constantly high population sizes and the general absence of major genetic bottlenecks in space and time.

Genetic differentiation among populations was moderately low for the mtDNA data. COI sequences are represented by nine haplotypes, with most individuals sharing one common haplotype, which was found in all populations, except in CZ-Su6. This central haplotype had five satellite haplotypes of low overall frequency, with four of them only

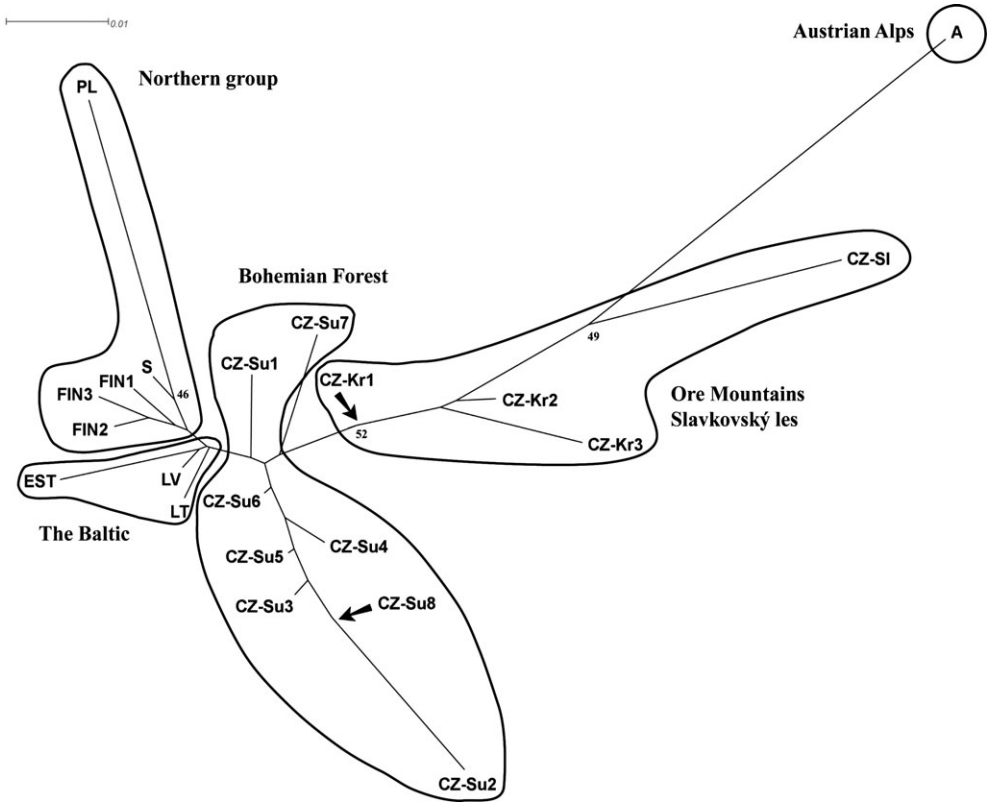

Figure 3. Neighbour-joining phenogram based on Nei's (1972) genetic distances of the 523 individuals from 21 populations of *Colias palaeno* analyzed by allozyme electrophoresis.

distinguished by one mutational step, a typical signal for a recent range expansion (Rogers & Harpending, 1992). However, our GenBank search and the resulting maximum likelihood tree revealed relatively similar patterns of low differentiation within species for a number of other *Colias* species (Fig. 5), thus highlighting a comparatively shallow phylogeographical COI structure within species of this pierid genus (Wheat & Watt, 2008). The other three haplotypes are distinguished by eight to fifty mutational steps, although these are likely the result of introgression from other *Colias* species. Hybridization and subsequent introgression between different *Colias* species has been reported frequently in the past (Hovanitz, 1963; Wang & Porter, 2004; Wheat & Watt, 2008). However, introgression is also known as a frequent phenomenon for other butterfly families, such as the Lycaenidae (Talavera *et al.*, 2013), and is a common phenomenon for many animal groups, with many examples not only reported in amphibians (Babik *et al.*, 2005; Komaki *et al.*, 2015), but also in fish species (Hayden, Coscia & Mariani, 2011).

The star-like mtDNA structure of clade 1 is compatible with postglacial range expansion from a single Würm ice age refugium (Paulo *et al.*, 2001; Pinceel *et al.*, 2005). In some contrast with this shallow mtDNA differentiation, allozymes reveal an F_{ST} of 0.09. Furthermore, mean genetic distances (Nei, 1972) among populations are not compatible with postglacial range expansion from one refugium and subsequent fragmentation. Nevertheless, the overall allozyme differentiation in *C. palaeno* is lower than that in most other mountain butterflies with populations separated for the entire Würm ice age or longer (Schmitt & Besold, 2010; Vila *et al.*, 2011; Schmitt *et al.*, 2014). As a compromise between the interpretation of mtDNA and allozyme information, a continuous and geographically extended distribution during most of the Würm ice age with late Würmian disjunction [maybe during the cryoxerotic Last Glacial Maximum (LGM), some 20 kya] is postulated as a likely scenario. Consulting the phylogeographical literature, the survival of boreo-montane species in more than one refugium, often also at the norther edge of the glaciated Alps (examples see below), is the common pattern. Postglacial expansion to Europe out of just one refugium in Asia (or the southern Urals) is relatively rare and only demonstrated for typical taiga forest species (Zink, Drovetski &

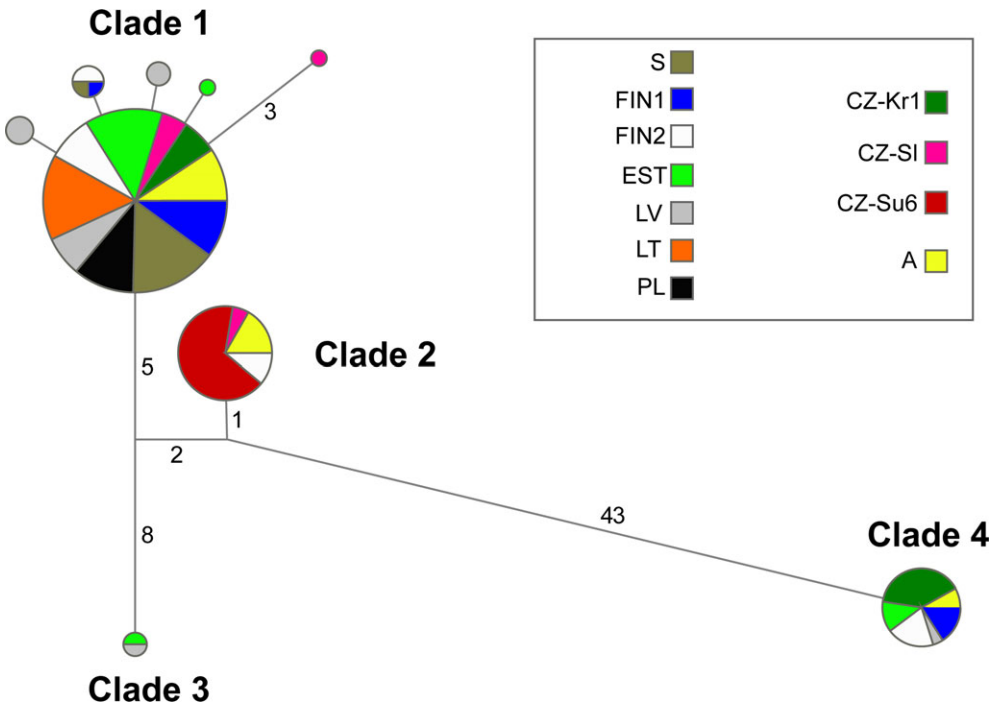

Figure 4. Median-joining cytochrome *c* oxidase subunit I (COI) haplotype network of *Colias palaeno*. Each pie chart represents a different haplotype, made up of collection sites labelled by colour in which that haplotype occurs. Haplotypes connected by a line differ in sequence by 1 bp unless otherwise indicated. A haplotype circle size denotes the number of sampled individuals.

Rohwer, 2002; Oshida *et al.*, 2005; Fedorov *et al.*, 2008; Zink *et al.*, 2008; Saitoh *et al.*, 2010). Thus, the more-than-one-refugium-scenorio is most likely also for *C. palaeno*.

These two putative retreats during the LGM might have been located north of the glaciers of the Alps and south of the Fennoscandian glacier in eastern Central Europe, or even further to the East. Survival of cold-adapted species in retreats in the cold steppe areas north of the Alps has long been postulated for species with arctic-alpine disjunctions (Holdhaus, 1954). However, recent studies demonstrate that, in particular, the more humid areas adjoining the Alps were of high relevance for the survival of mountain biota (Schmitt, 2009), even including the northern edge of the glaciated Alps, such as postulated for the butterflies *Erebia sudetica* (Haubrich & Schmitt, 2007) and *Erebia epiphron* (Schmitt, Hewitt & Müller, 2006); the chrysomelid beetles *Oreina alpestris/speciosa* (Triponez *et al.*, 2011) and *Gonioctena pallida* (Mardulyn, Mikhailov & Pasteels, 2009); and the plants *Meum athamanticum* (Huck *et al.*, 2009), *Cicerbita alpina* (Michl *et al.*, 2010), and *Polygonatum verticillatum* (Kramp *et al.*, 2009). The caddisfly *Drusus discolor* (Pauls, Lumbsch & Haase, 2006) and the stonefly *Arcynopteryx dichroa* (Theissinger *et al.*, 2012) even have endemic lineages in the mountains north of the Alps supporting their permanent survival in this region. Furthermore, the likely introgression from *C. phicomone*, a species restricted to western European mountain ranges including the Alps (Tshikolovets, 2011) and a possible donor of the clade 3 haplotype, might have taken place in a shared peri-Alpine refugium during glacial (Würm or earlier) sympatry. Both species even today co-occur in some parts of the Alps (T. Schmitt, personal observation).

The existence of a second late Würm glacial core area in eastern Central Europe is supported by the (recent) hybridization with *C. tyche* or *C. hecla*, which, in Europe, are both currently restricted to the Arctic (Tshikolovets, 2011). Therefore, introgression with *C. palaeno* (haplotypes of clade 1) might have occurred during a glacial co-occurrence of these species in this core area. However, the strong genetic divergence of haplotype 3 from all other so-far analyzed *Colias* individuals convincingly supports its origin in a much more ancient introgression event and indicates that continuous introgression from other *Colias* species into *C. palaeno* has taken place.

The intermediate genetic position of the Czech populations in the Ore Mountains, Slavkovský les and, to a lesser extent, Šumava Mountains might be the result of postglacial intermixing of the

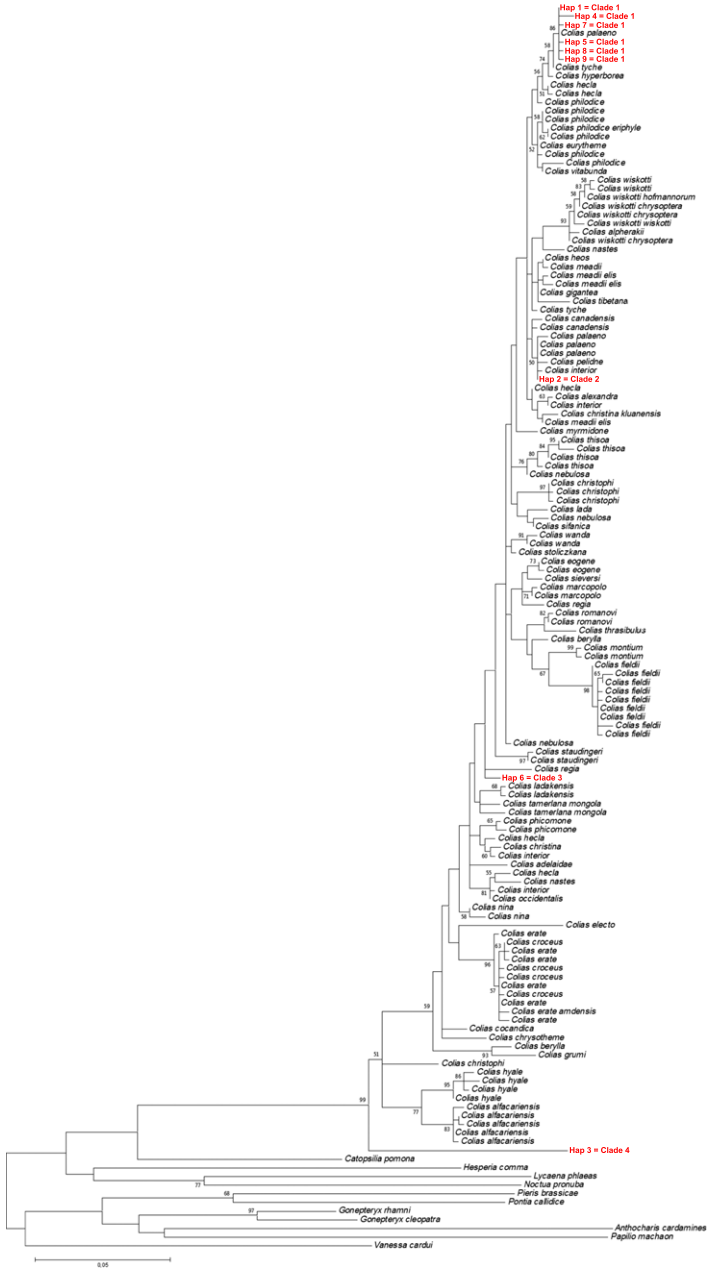

Figure 5. Maximum likelihood tree (GTR + G + I) for 137 sequences mined from GenBank, representing 51 putative species of *Colias* for mitochondrial partial COI, including the nine haplotypes we found in the present study. Bootstrap values > 50% are given. *Colias palaeno* haplotypes and corresponding clades identified in the red study are indicated in red.

populations expanding from the two LGM core areas located to the south-west and the north-east of these regions. These mountains between Bavaria, Saxony, and Bohemia are well known as important suture zones between biota that dispersed postglacially from different refuge areas (Hewitt, 1999, 2000) and there are several well-studied examples in other butterfly species (Schmitt & Müller, 2007; Schmitt & Zimmermann, 2012).

The colonization of Fennoscandia after its postglacial deglaciation most probably followed the eastern pathways via the Baltic countries through Finland to Sweden, and not the western route via Denmark. The lack of genetic differentiation and the high genetic diversity of the Fennoscandian populations provide good support for a phalanx-wise colonization (Ibrahim, Nichols & Hewitt, 1996) by a relatively high number of founder individuals (and

thus without loss of genetic diversity) (Hewitt, 1996). The complete absence of *C. palaeno* in the North German Plain (Settele *et al.*, 2009), as well as the mostly continuous distribution from north-eastern Poland to Fennoscandia, clearly favours the use of this eastern pathway, which has also been confirmed for many other species (Taberlet *et al.*, 1998; Hewitt, 1999).

CONCLUSIONS AND IMPLICATIONS FOR CONSERVATION

The two different genetic markers analyzed (i.e. nuclear and mitochondrial ones) display somewhat different levels of genetic diversity and divergence. At first sight, the genetic diversity of *C. palaeno* at the allozyme level appears to be markedly high for such a geographically restricted species. However, this species can reach quite high local abundances: mark-recapture study (Pavličko, 2002a) carried out in population CZ-Su4 indicated a population of approximately 10 000 adults (details in Pavličko, 2002b). Furthermore, it has apparently good dispersal ability, given that most *Colias* species are strong fliers (Watt, Han & Tabashnik, 1979; Rüetschi & Scholl, 1985) and occasional records of stray individuals of *C. palaeno* have been made far away from their habitats (Pavličko, 2002b). The good dispersal ability is also supported by our results for Šumava Mts, revealing one intermixed population across this spacious (100 km in length) mountain range.

By contrast to *C. palaeno*, most rare taxa show rather low levels of genetic diversity for their populations (Debinski, 1994; Gadeberg & Boomsma, 1997; Habel *et al.*, 2009). This genetic paucity is apparently a prerequisite for survival in complete isolation, whereas more genetically diverse species need to maintain gene flow among different populations to avoid the negative impact of inbreeding (Habel & Schmitt, 2012). In this context, the high susceptibility of *Colias* species to inbreeding referred by Wang *et al.* (2009) might be related to the high genetic diversity of these taxa. This might become a serious conservation problem for the often highly isolated and relatively small populations in Central Europe. The rapid decline in Europe of two other habitat specialist *Colias* species, *C. myrmidone* and *C. chrysotheme* (van Swaay *et al.*, 2010), also might be a result of the same phenomenon. This also accords with the unpredicted complete extinction of *C. palaeno* in the Jizerské Mountains, Krkonoše, Hrubý Jeseník, and other mountain systems in the northern Czech Republic, without apparent ecological causes (Pavličko, 2002b). Therefore, even without further habitat fragmentation and deterioration, many of the still existing populations might become lost simply because of genetic degradation, as also documented for most of the populations of the Hermit butterfly *Chazara briseis* in the Czech Republic (Kadlec *et al.*, 2010).

However, the loss of these populations in Central Europe might be regarded as less severe if we take into account the evolutionary history of the species: apparently, even a complete loss of the Central European populations would not eradicate any unique genetic lineage, providing that populations survive in the Alps and in northern Europe. Currently, the populations in the Alps are not threatened (Stettmer *et al.*, 2007) and the northern lineage also should be safeguarded by the presence of more continuous and more extensive populations in the Baltic countries and Fennoscandia (van Swaay & Warren, 1999).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Sampling locations (with abbreviations and coordinates), region, sample sizes (*N*), and altitude for all *Colias palaeno* populations analyzed by allozyme electrophoresis.

Appendix S2. Electrophoresis conditions for the enzyme systems analyzed for *Colias palaeno.*

Appendix S3. Assignment test for the five genetic groups of *Colias palaeno* calculated using BAPS. All values are expressed as percentages.

Appendix S4. Locus-by-locus analysis of molecular variance (AMOVA) (averaged over 18 polymorphic loci) based on the total data set of the allozyme data for *Colias palaeno*.

Appendix S5. Sampling locations, haplotypes per site, and diversity values for the eleven *Colias palaeno* populations used for cytochrome *c* oxidase subunit I (COI) analyses.