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Phylogeography of *Parnassius apollo*: hints on taxonomy and conservation of a vulnerable glacial butterfly invader

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Parnassius apollo (Linnaeus, 1758) is probably the most renowned Eurasian montane butterfly. Its specialized ecology makes it very sensitive to habitat and climate changes, so that it is now experiencing range contraction and local extinction across most of its range. We sequenced 869 bp of the mitochondrial DNA (mtDNA) *cytochrome oxidase I* gene in 78 *P. apollo* populations (201 individuals) in order to: (1) assess the phylogeographic pattern of the species; (2) shed light on the historical biogeographic processes that shaped the distribution of the species; and (3) identify geographic population units of special value for the conservation of the species' genetic diversity. Our analyses revealed a very strong phylogeographic structure in *P. apollo*, which displays a number of distinctive mtDNA lineages populating geographically distinct areas. Overall sequence divergence is relatively shallow, and is consistent with a recent (late Pleistocene) colonization of most of the range. We propose that *P. apollo* is best viewed as an atypical glacial invader in southern and western Europe, the isolated, montane populations of which, threatened by climate warming, retain a large fraction of the species evolutionary heritage. *Biological Journal of the Linnean Society*, 2010, **101**, 169–183.

ADDITIONAL KEYWORDS: butterflies – mtDNA – Pleistocene.

INTRODUCTION

Climate change is a new and potent risk to biodiversity. The species most threatened are the most ecologically demanding, as they require special conditions for their survival. This is particularly true for those species inhabiting high-altitude and high-latitude environments. Insects, especially butterflies, are highly sensitive to environmental change, as a result of their specialized ecology and coarse-grained perception of habitats. Butterflies are among the groups of organisms in which distribution has been studied most across time, so that an extensive and detailed volume of data is available (Parmesan *et al*., 1999; Kudrna, 2002). For this reason, they are particularly suited to serve as indicators of ecosystem response to climate variation (Parmesan *et al*., 1999; Araújo & Luoto, 2007).

Biogeographic and phylogeographic information may be essential in developing models of past and future response of species and biota to climate change (e.g. DeChaine & Martin, 2005; Schmitt & Hewitt, 2004). Despite its high potential relevance, the phylogeography of European butterflies is still not thoroughly known. With a few exceptions (e.g. Wahlberg & Saccheri, 2007; Gratton, Konopiński & Sbordoni, 2008), most available data consist of allozyme polymorphisms surveyed over a portion of the range of a species (e.g. Cassel & Tammaru, 2003; Habel, Schmitt & Müller, 2005; Schmitt, Röber & Seitz, 2005; Schmitt, Hewitt & Müller, 2006; Schmitt, 2007; Schmitt & Haubrich, 2008). Though these data are certainly informative about genetic diversity and geographic structure of populations, they need to be complemented by range-wide surveys of DNA variation, which can offer more detailed views on the evolutionary and historical significance of geographic patterns. Moreover, established models of phylogeographic patterns associated with climate oscillations mostly focus

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on temperate species, the biogeographic history of which reflects the retreat and advance of forests and woodlands (Hewitt, 1996, 1999, 2000, 2001, 2004; Taberlet *et al*., 1998). On the other hand, steppic and alpine species are expected to have been influenced conversely, and to have actually experienced range expansion and long-distance connectivity through cold/arid periods (DeChaine & Martin, 2004; Schmitt, 2007; Varga & Schmitt, 2008).

Papilionid butterflies of the genus *Parnassius* might be regarded as the invertebrate epitome of the conservation of mountain habitats, and may serve as one of the 'flagship species' for the whole montane environment. *Parnassius apollo* (Linnaeus, 1758) is the most representative species of the genus, and has a high priority for conservation. *Parnassius apollo* is presently included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) lists, categorized by the International Union for Conservation of Nature (IUCN) as vulnerable by meeting criterion A1cde, and enlisted in annex IV of the Habitats Directive 92/43/EEC. *Parnassius apollo* is decreasing in 12 out of 28 countries, and is extinct in three countries (Collins & Morris, 1985; Van Swaay & Warren, 1999). The primary causes of the observed decline fall into two general categories: (1) change in land management (afforestation of former pastures and meadows); and (2) global climate change. Climate change in particular, has been cited as the main cause of extinctions of many of the most marginal populations, occupying low-latitude and low-altitude sites (Descimon, 1995).

Parnassius apollo is a widely distributed Eurasian butterfly, usually represented by small, local populations. It inhabits diverse open, rocky, subalpine–montane habitats across all main ranges from Sierra Nevada (Spain) to Altai (Mongolia and Russia), and at low-altitudes sites scattered through north-eastern Europe and Siberia (Fig. 1A).

Both the appealing beauty of the butterfly, greatly appreciated by lepidopterists, and the occurrence of isolated and/or localized populations, often differing in morphological and ecological features (mostly wing pattern and larval food plant), encouraged specialists to name more than 200 subspecies, and many more forms (Bryk, 1935; Kostrowicki, 1969; Eisner, 1976; Capdeville, 1979–1980; Glassl, 1993; Dietz, 2000; Möhn, 2005; Weiss, 2005). However, colour and pattern variation, including polymorphism, is quite common in butterflies, and its taxonomic value has been severely disputed, as it may, at least in part, result from phenotypic plasticity (Napolitano, Descimon & Vesco, 1990; Brakefield & Gates, 1996; Rivoire, 1998). In fact, discordant results from wing pattern descriptors and molecular markers suggest that the first might be subjected to different evolutionary trajectories and rates, because of their particular adaptive significance, and might not represent reliable tracers of evolutionary relationships (Cesaroni *et al*., 1994; Lukhtanov *et al*., 2005).

Although several studies have shed light on aspects of ecology (Deschamps-Cottin, Roux & Descimon, 1997; Brommer & Fred, 1999) and conservation (Descimon, 1995; Fred & Brommer, 2003; Fred, O'Hara & Brommer, 2006; Nakonieczny, Kedziorski & Michalczyk, 2007) of *P. apollo*, very little effort has been put towards using molecular methods to make inferences about the evolutionary history and subspecific taxonomy of this highly relevant butterfly.

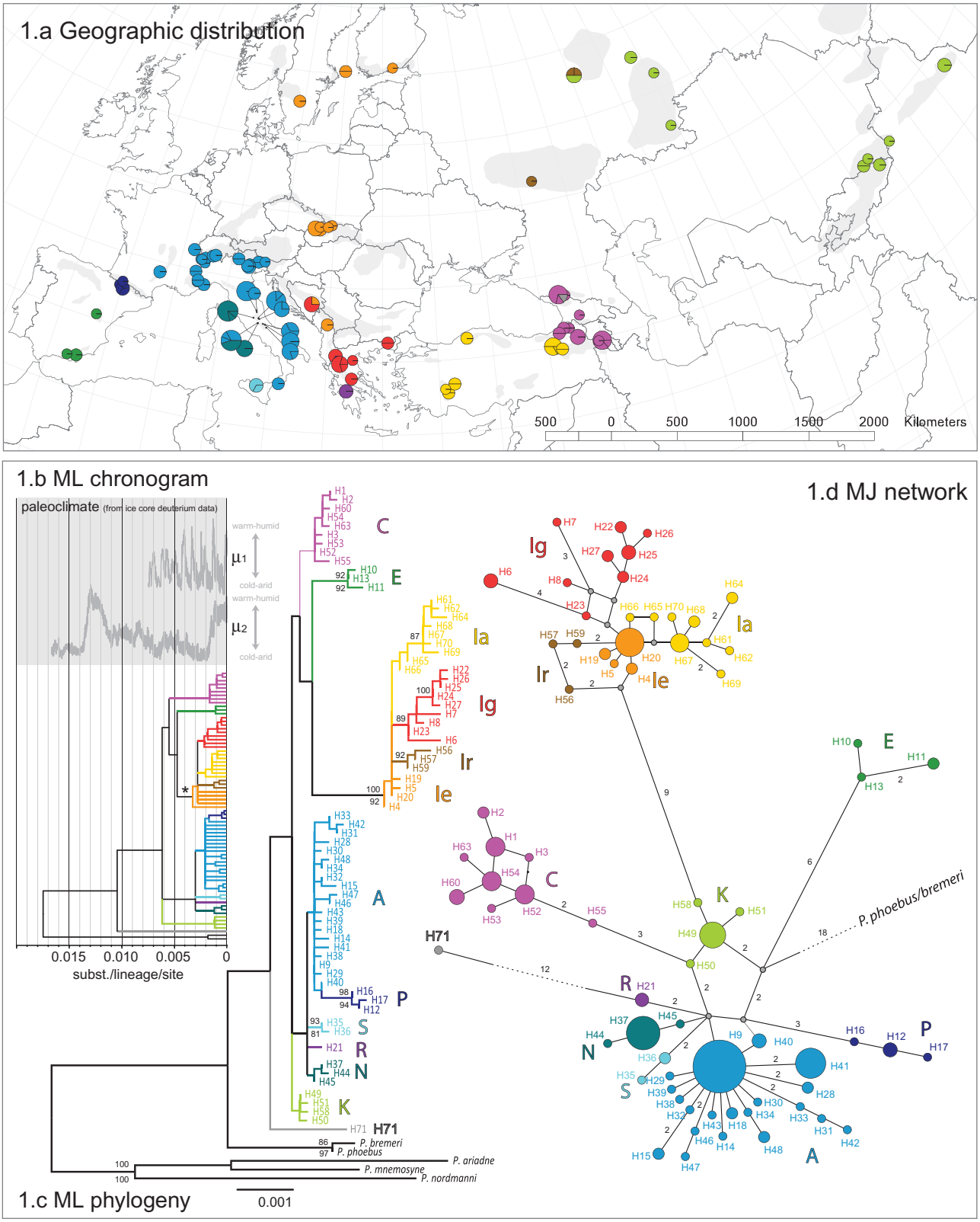
In this study, we present an analysis of the geographical patterns of mitochondrial DNA (mtDNA) variation in *P. apollo*. DNA sequence variation at the mitochondrial *cytochrome oxidase I* (*COI*) gene was analysed in 201 individuals from the whole range of the species in order to: (1) shed light on the historical biogeographic processes that determined the present-day distribution of the species; (2) provide a first genetic basis for a revision of subspecific taxonomy; and (3) identify evolutionarily significant geographic population units for conservation.

MATERIAL AND METHODS

Samples and molecular techniques

In this study we analysed 78 population samples, distributed across 17 countries, totalling 201 individuals (Appendix; Fig. 1A). Three to nine dried specimens, conserved in private collections or directly collected by the authors (authorization of the Italian Ministry of Environment DPN/2D/2005/21020; Appendix), were

Figure 1. Reconstructed evolutionary relationships and geographical distribution of the 71 mitochondrial DNA haplotypes sampled in *Parnassius apollo*. Main haplogroups are highlighted and shown in different colours. A, geographical distribution: pie charts show the frequency of haplogroups in each sample; circled areas are proportional to sample size; shaded areas indicate approximate range of occurrence of *P. apollo*. B, maximum likelihood (ML) chronogram of *P. apollo* haplotypes according to a local molecular clock model: 'long' branch with separate rate parameter is indicated by *; palaeoclimatic data from the Antarctic Ice Core (EPICA community members, 2004) scaled according to mutation rate μ_1 (0.01 substitutions per site per Ma) and μ_2 (0.096 substitutions per site per Ma). C, maximum likelihood (ML) phylogeny under GTR + Γ + I model of evolution; numbers above and below branches represent LR-ELW and bootstrap support above 80%, respectively. D, median-joining network: circled areas are proportional to haplotype frequency; number of nucleotide substitutions indicated along connections, except for single substitution.


1.a Geographic distribution
500 0 500 1000 1500 2000 Kilometers
1.b ML chronogram
paleoclimate (from ice core deuterium data)
warm-humid
μ1
cold-arid
warm-humid
μ2
cold-arid
0.015 0.010 0.005 0
subst./lineage/site
C
E
Ia
Ig
Ir
Ie
A
P
S
R
N
K
H71
P. bremeri
P. phoebus
P. ariadne
P. mnemosyne
P. nordmanni
1.c ML phylogeny
0.001
1.d MJ network
P. phoebus/bremeri

analysed for each population. Samples of *Parnassius ariadne* (Lederer, 1853), *Parnassius bremeri* (Bremer, 1864), *Parnassius mnemosyne* (Linnaeus, 1758), *Parnassius nordmanni* (Ménétriés, 1850), and *Parnassius phoebus* (Fabricius, 1793) (one individual each) were included as out-groups.

DNA was extracted from two legs of each individual, using a GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St Louis, MO, USA), resuspended in 100 μL of sterile water, and stored at –40 °C.

Novel specific primers pairs (see Table S1) were developed from conserved regions and used in polymerase chain reaction (PCR) amplifications (see Table S1 for PCR conditions) to obtain two overlapping fragments covering 970 bp of the *COI* gene. PCR products were purified by ExoSAP (Amersham Biosciences 800 Centennial Avenue, PO Box 1327 Piscataway, NJ 08855-1327) exonuclease reaction and sequenced in both directions using BigDye terminator ready-reaction kit (Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington, WA3 7QH, UK), and resolved on an ABI 3100 Genetic Analyzer (Applied Biosystems), following the manufacturer's protocols.

DNA polymorphism and phylogenetic analyses

Sequence data were edited and aligned using SEQUENCHER 4.1 (1999–2000; Gene Codes Corporation, Ann Harbor, MI, USA). All sequences of *P. apollo* were submitted to GenBank (accession numbers are listed in the Appendix).

Haplotype and nucleotide diversity were calculated using DNASP 4.10.9 (Rozas *et al*., 2003). Average distances between groups of haplotypes were calculated using MEGA 4 (Tamura *et al*., 2007). The software package TREEFINDER (Jobb, 2008) was used to select the best-fitting model of evolution by likelihood ratio test (LRT) and Akaike's information criterion (AIC), to determine the maximum likelihood (ML) phylogeny of mtDNA haplotypes, and to calculate patristic (tip-to-tip) nucleotide distances among haplotypes. The robustness of phylogenetic inference was assessed by bootstrap procedure and by the expected likelihood weights of local rearrangements (LR-ELW) approach in TREEFINDER (Jobb, 2008) on 500 replicates. Finally, NETWORK 4.5 (Bandelt, Forster & Röhl, 1999) was employed to calculate a median joining (MJ) network representing the genealogical relationships among mtDNA haplotypes.

Tests of demographic equilibrium and measures of evolutionary time

Demographic equilibrium in different sets of sequences (selected on a geographical basis and taking into account the results of previous phylogenetic analysis) was tested by calculating F_s (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) statistics, which have been shown to be the most powerful tests of population expansions (Ramos-Onsins & Rozas, 2002). ARLEQUIN 3.0 (Excoffier, Laval & Schneider, 2005) and DNASP 4.0 (Rozas *et al*., 2003) were employed to compute F_s and R_2, respectively, and to test their statistical significance by simulating random samples (10 000 replicates) under the null hypothesis of selective neutrality and constant population size, using coalescent algorithms (both modified from Hudson, 1990). *P* values for the two statistics were obtained as the proportion of simulated values smaller than or equal to the observed values (critical value = 0.05).

The expected mismatch distribution and parameter of sudden expansion $\tau = 2\mu t$ were calculated using ARLEQUIN 3.0 by a generalized least-squares approach (Schneider & Excoffier, 1999), under both models of pure demographic expansion and spatial expansion (Ray *et al*., 2003; Excoffier, 2004). The probability of the data according to the given model has been assessed by a goodness-of-fit test implemented in ARLEQUIN 3.0. Parameter confidence limits were calculated in ARLEQUIN 3.0 through a parametric bootstrap (1000 simulated random samples).

The application of molecular clocks is historically a controversial and even frustrating task. Recent approaches stress the importance of modelling both the stochasticity of the coalescent process and demographic effects on the shape of gene genealogies when estimating evolutionary dates from sequence data (e.g. Drummond & Rambaut, 2007). Moreover, a growing body of evidence (e.g. Ho *et al*., 2005, 2007b; Burridge *et al*., 2008; Gratton *et al*., 2008) questions the validity of evolutionary time estimates based on extrapolating interspecific substitution rates (based on calibrated phylogenies) into intraspecific and population molecular data sets. Providing a correct time frame for several evolutionary events, spanning across a relatively long interval (from recent events to several hundred Ka), and involving multiple populations and lineages (as it is the case in most range-wide phylogeographic studies), may therefore represent a hopeless enterprise, unless a huge number of independent calibrations is available, and fully-parameterized evolutionary models may be tested.

Thus, we are aware that a fully dated reconstruction of the *P. apollo* population history can hardly be obtained from mtDNA data. Nonetheless, maintaining that some useful, though approximate, indications on the timing of the most relevant evolutionary processes can be obtained from our data, we applied a relatively simple ML approach. Ultrametric trees were generated by applying global

and local molecular clock models to our ML haplotype phylogeny, using PAML (Yang, 2007), and LRT was used to determine the fit of clock and non-clock models.

All demographic and evolutionary parameters were converted in absolute times using two alternative published rates, providing useful higher and lower bounds for interpreting our results: (1) a slow, 'traditional' interspecific insects rate ($\mu_1 = 0.01$ substitutions per site per lineage per Ma; Brower, 1994; Caccone & Sbordoni, 2001; Farrell, 2001); (2) the fast, 'time-dependent' rate ($\mu_2 = 0.096$ substitutions per site per lineage per Ma) proposed by Gratton *et al*. (2008). The latter rate was estimated for European populations of the congeneric *P. mnemosyne* through a coalescent-based analysis calibrated at 11–12 Ka, and has recently been applied in a phylogeographic study of Pleistocene-related phylogeography of *Parnassius smintheus* Doubleday (1847) (Schoville & Roderick, 2009). It may therefore represent a reasonable yardstick for recent evolutionary events in *P. apollo*. We chose not to consider the extremely slow substitution rate obtained by Michel *et al*. (2008), by calibrating a phylogeny of the genus *Parnassius* based on divergence of Papilionid butterflies 100 Ma, as the authors casted doubt on its potential utility in lower-level divergence within the genus.

RESULTS

A complete alignment of 869 bp was obtained. The 201 individual sequences characterized a total of 71 haplotypes, of which 42 were found only in one individual (Appendix; Fig. 1D). Global haplotype diversity (h) was 0.941 (± 0.010) and nucleotide diversity (π) was 0.011 (± 0.00048). Estimates of haplotype diversity for each sampled population are reported in the Appendix.

Haplotype H_9 (Appendix) is widespread across sample localities, being found throughout the Alpine range, in Central Apennines (Italy), and in the Massif Central (France). The greatest geographical separation between identical haplotypes was shown by haplotype H_{49}, found from the Kirov region and Urals (Russia) across Kazakhstan and Kyrgyzstan, up to Xinjiang (China), and by haplotype H_{20}, found in Slovakia, Finland, and Sweden.

Phylogenetic analyses and network analysis

The ML analysis was used to reconstruct phylogenetic relationships of the mtDNA haplotypes. GTR + Γ + I (Rodríguez *et al*., 1990) was selected as the preferred model of evolution according to both the hierarchical LRT and AIC ($\alpha = 0.73$; proportion of invariants = 0.67), and the resulting tree was rooted by using out-group sequences of *P. mnemosyne*, *P. ariadne*, *P. nordmanni*.

The ML tree (Fig. 1C) confirms the monophyletic status of the species and the close relatedness to the *P. phoebus–P. bremeri* complex (Omoto *et al*., 2004; Katoh *et al*., 2005), with an average patristic nucleotide distance (*P. apollo* vs. *P. phebus–P. bremeri*) of 0.034 (SD = 0.004).

Our analysis highlighted a strong phylogeographic structure in *P. apollo*, concordant with the fragmented distribution of the species: a number of distinctive mtDNA lineages were identified that occupy geographically distinct areas, frequently corresponding to a single mountain range (Fig. 1A–C). Although only a few of the nodes show strong robustness, all clades display a well-defined geographical distribution (Fig. 1C), thus corroborating the phylogeographical relevance of inferred haplogroups. A highly distinctive and strongly supported lineage (I), includes all sequences from Anatolia (except the extreme north-eastern region), Greece (excluding Peloponnesus), and eastern Europe (Balkans, Carpathians, Scandinavia, and European Russia). Within this clade, lower level unique lineages characterize Anatolian (Ia), Southern Balkan Peninsula (Ig), and European Russian (Ir) samples, respectively. Sequences from Carpathian, Scandinavian, and northern Balkan samples (group Ie), although closely related, occupy a basal position, and are not recognized as a monophyletic clade. Samples from central and southern Spain are also markedly divergent, and form a robust monophyletic clade (E). All mtDNA sequences from the remaining European mountain ranges (Alps, Apennines, Pyrenees, Massif Central, Sicilian, and Peloponnesian heights) are included in a large set of closely allied lineages. Lineage A occupies the Alps, the French Massif Central, and stretches across the eastern slopes of the Apennines to the Aspromonte in southern Italy. All other haplogroups are confined to individual ranges: lineage P to the Pyrenees, lineage S to Madonie (Sicily), lineage R to Mount Erimanthos (Peloponnesus, Greece), lineage N to the Central Apennines, where it coexists with lineage A (ITMAG sample). Populations from Central Asia display another distinctive haplogroup (K) spreading up to the Urals, where it mixes with haplogroup Ir. Caucasian, Armenian, and north-eastern Turkish samples share the exclusive lineage C, and a highly divergent haplotype (H_{71}) occurs in a single Caucasian sample (RUAKS). It is worth mentioning that, although deep phylogenetic relationships among *P. apollo* mtDNA haplotypes could not be satisfactorily resolved because of very low divergence along internal branches, Asian haplotypes occupy a basal position in our ML reconstruction.

The MJ network analysis (Fig. 1D) is fully consistent with the ML phylogenetic analysis. Eleven mutational steps join haplogroup I to the rest of the network. Nonetheless, the rooting of the network with *P. phoebus–P. bremeri* does not indicate a sister relationship of this divergent clade with the other *P. apollo* sequences. Indeed, the whole network shows a star-like structure, with all major haplogroups connecting to a central unresolved loop, with no evident substructuring. The analysis also highlights the marked star-like configuration of haplogroup A, the ancestral haplotype (H_9) of which is widely distributed from the Massif Central through the Alps and the Apennines. Similarly, a central haplotype (H_{20}) widely distributed from the Scandinavian Peninsula to the Carpathians, is ancestral to the subgroup Ie, which includes three closely related (one mutational step) haplotypes distributed in the same area.

The genetic differentiation among main lineages, excluding the singular haplotype H_{71}, estimated as the average pairwise distances (Kimura two-parameter distance) between groups of haplotypes, ranges from 0.0046 (±0.0020) to 0.0236 (±0.0048). However, excluding pairwise comparisons involving haplogroup I, the maximum distance lowers to 0.0152 (±0.0042) between haplogroups E (Spain) and P (Pyrenees).

Tests of demographic equilibrium and evolutionary time frames

According to F_s and R_2 statistics, calculated for nine sequence sets with at least 15 sequences (Table 1), the null hypothesis of constant population size could be rejected for two phylogeographic units (set in bold in Table 1): haplogroup Ia (Anatolia) and haplogroup A (including all sequences from the Alps and the Massif Central, and most Apennine sequences). Mismatch distribution of these groups was examined according to the sudden-expansion model (Table 1), and goodness of fit tests did not show significant deviations from expected distributions, so that parameter $\tau = 2\mu t$ could be used to estimate the time (t) elapsed from population expansion: estimated values of τ and their 5 and 95% confidence limits are shown in Table 1. According to our two 'benchmark' mutation rates ($\mu_1 = 0.01$ and $\mu_2 = 0.096$ substitutions per site per lineage per Ma), demographic expansion of the Anatolian group (Ia) could be traced back to $t_1 = 151$ (64–231) Ka and $t_2 = 16$ (7–24) Ka, respectively. A similar estimate was obtained for haplogroup A: $t_1 = 145$ (29–277) Ka; $t_2 = 15$ (3–29) Ka.

The software package PAML (Yang, 2007) was used to calculate ultrametric trees by applying molecular clock models to our ML phylogeny. The LRT rejected a global clock model against a model with an independent rate (no clock) for each branch (LR = 57.9, d.f. = 71, $P < 0.01$), even when out-groups (all but *P. phoebus*) were removed from the analysis (LR = 57.9, d.f. = 74, $P < 0.001$). We hypothesized that deviation from a molecular clock could derive from a divergent substitution rate along the anomalous 'long' branch connecting haplogroup I to the rest of the tree (Fig. 1C). Indeed, LRT showed that a local-clock model, where this branch was given a separate rate parameter, was significantly better than a global-clock model (LR = 6.2, d.f. = 1, $P < 0.001$), although the no-clock model was still preferred over the local-clock model (LR = 51.7, d.f. = 70, $P < 0.01$). These results indicate that, although faster evolution in a single 'anomalous' branch cannot account for all the observed rate variation, nonetheless it is a significant source of inequality. Therefore, we chose to present results from the local-clock model (Fig. 1B),

Table 1. Tests of demographic equilibrium and mismatch analysis in phylogeographic groups with at least 15 sequences

Groups	*N* (haplotypes)	*N* (sequences)	F_s	*P*	R_2	*P*	τ	τ (5%)	τ (95%)
I	25	52	–12.96	0.00	0.06	0.08	–	–	
Ie	4	18	–1.54	0.13	0.10	0.06	–	–	–
Ia	**9**	**15**	**–3.83**	**0.00**	**0.093**	**0.01**	**2.63**	**1.11**	**4.02**
Ig	9	16	–1.32	0.26	0.13	0.41	–	–	–
C	9	25	–3.58	0.01	0.09	0.17	–	–	–
A	**20**	**77**	**–14.31**	**0.00**	**0.033**	**0.00**	**2.52**	**0.50**	**4.81**
Apennines (all sequences)	11	63	–1.73	0.24	0.09	0.37	–	–	–
Apeninnes (group A only)	9	46	–2.53	0.08	0.06	0.09	–	–	–
N	3	18	–1.74	0.02	0.15	0.36	–	–	–

Groups for which the null hypothesis of constant population size was rejected are shown in bold.

which sets the deepest divergence among all haplotypes at 0.11 substitutions per site, implying a maximum age for the origin of current mtDNA diversity in *P. apollo* later than 1.5 Ma (μ_1), and a 'fast' estimate of about 100 Ka (μ_2). Moreover, except for the highly divergent haplotype H_{71}, most lineages coalesce within a short evolutionary time frame, with about 0.005–0.006 substitutions per site.

DISCUSSION

A RECONSTRUCTION OF *P. APOLLO* POPULATION HISTORY

Our phylogenetic analysis (Fig. 1C, D) highlighted a strong phylogeographic structure of *P. apollo* populations, with a number of distinctive mtDNA lineages populating geographically distinct areas. However, global genetic divergence among mtDNA lineages in *P. apollo* is rather shallow, compared with Eurasian temperate butterflies surveyed with the same marker (Gratton, 2006; Wahlberg & Saccheri, 2007), and no robust phylogenetic structure was recovered among the geographically recognizable haplogroups. Indeed, a similar degree of global divergence has been reported by Albre , Gers & Legal (2008) in the whole alpine *Erebia tyndarus* (Esper, 1781) species complex, which displays a European distribution that is highly congruent with *P. apollo*.

The basal position of the Central Asia populations in the ML reconstruction, although not statistically robust, seems to support the hypothesis that *P. apollo* had its origin in Central Asia, from where the species spread across Europe. In fact, Central Asia is believed to be the radiation geographic centre of the genus *Parnassius* (Omoto *et al*., 2004; Nazari , Zakharov & Sperling, 2007).

The LRT rejected the general validity of a clock-like evolution of *COI* sequences in *P. apollo*. Non-clock-like evolution in *P. apollo* mtDNA may be related to the survival of the species as a set of several demographically independent lineages for most of its past existence, each experiencing different demographic and selective dynamics. However, if some hypothesis on the timing of evolutionary events is to be drawn, a local-clock model is to be preferred. In fact, setting a separate rate for the 'anomalous' long branch connecting haplogroup I with the rest of the tree provided a significantly better evolutionary model than a global clock, thus suggesting that this mtDNA lineage may have evolved at a faster rate. However, all of the 11 substitutions unique to haplogroup I are synonymous, so that direct positive selection can be ruled out as causing its apparent faster rate.

Applying the time-dependent rate (μ_2) to the local-clock model (Fig. 1B), calculated in PAML, indicates that *P. apollo* may have reached its present range limits not earlier than 60 Ka, during the Würm glaciation. The alternative 'phylogenetic' rate (μ_1) would, instead, indicate a middle-Pleistocene origin, about 500–600 Ka. In the absence of an external calibration point, it is not possible to pick a single time frame. However, we argue that the early 'phylogenetic' date is less likely. In fact, the ultrametric tree, as well as the star-like network topology, suggests the almost simultaneous origin of all main lineages, consistent with a fast expansion from a single centre of origin. A middle-Pleistocene expansion would be expected to generate a much more structured phylogeographic pattern, caused by several episodes of range expansion and contraction (not less than five complete glacial–interglacial cycles have occurred in the last 600 000 years). A late-Pleistocene origin is, on the other hand, perfectly consistent with the relatively simplified phylogeographic pattern of *P. apollo*.

Our favoured hypothesis is that *P. apollo* experienced a rapid westward expansion between 100 and 70 Ka (Fig. 2), corresponding with the initial spread of open habitats in Europe after the Riss–Würm interglacial (Velichko *et al*., 2002; Müller, Pross & Bibus, 2003; Varga, 2010). However, as the actual evolutionary rate remains largely uncertain, we cannot rule out that this species met its primary expansion during the Riss glacial. Later, full glacial conditions would have prevented further contact through central and northern Europe, and diversification took place around southern ranges (see Schmitt, 2009). A second wave of range expansion in southern Europe (Fig. 2) is suggested by almost contemporary coalescence of sublineages within group I and haplogroups A, P, R, N, and S (mountains in central Mediterranean areas). Under our hypothesis, this wave may have matched a re-expansion of cold–arid landscapes in southern Europe at the onset of the last glacial peak (*c.* 30 Ka). Genetic traces of demographic expansion in haplogroups Ia (Anatolia) and A (Alps and Massif Central, and most Apennine sequences) point to the last glacial maximum (LGM; about 18 Ka), when scaled by μ_2, and can be consistently interpreted as a consequence of enhanced dispersal in southern peninsulas during a cold phase. In fact, further support for a recent time frame for *P. apollo* comes from Gratton, Todisco & Sbordoni (2006), who analysed part of the present data from the Italian Peninsula in a comparative study of *P. apollo* and *P. mnemosyne*. The authors showed that, by scaling parameters of three different models by the μ_2 rate, genetic signals were congruent with ecological requirements of the two butterflies. Indeed, although *P. mnemosyne* showed range and demographic expansions congruent with the rise of the forest pollen

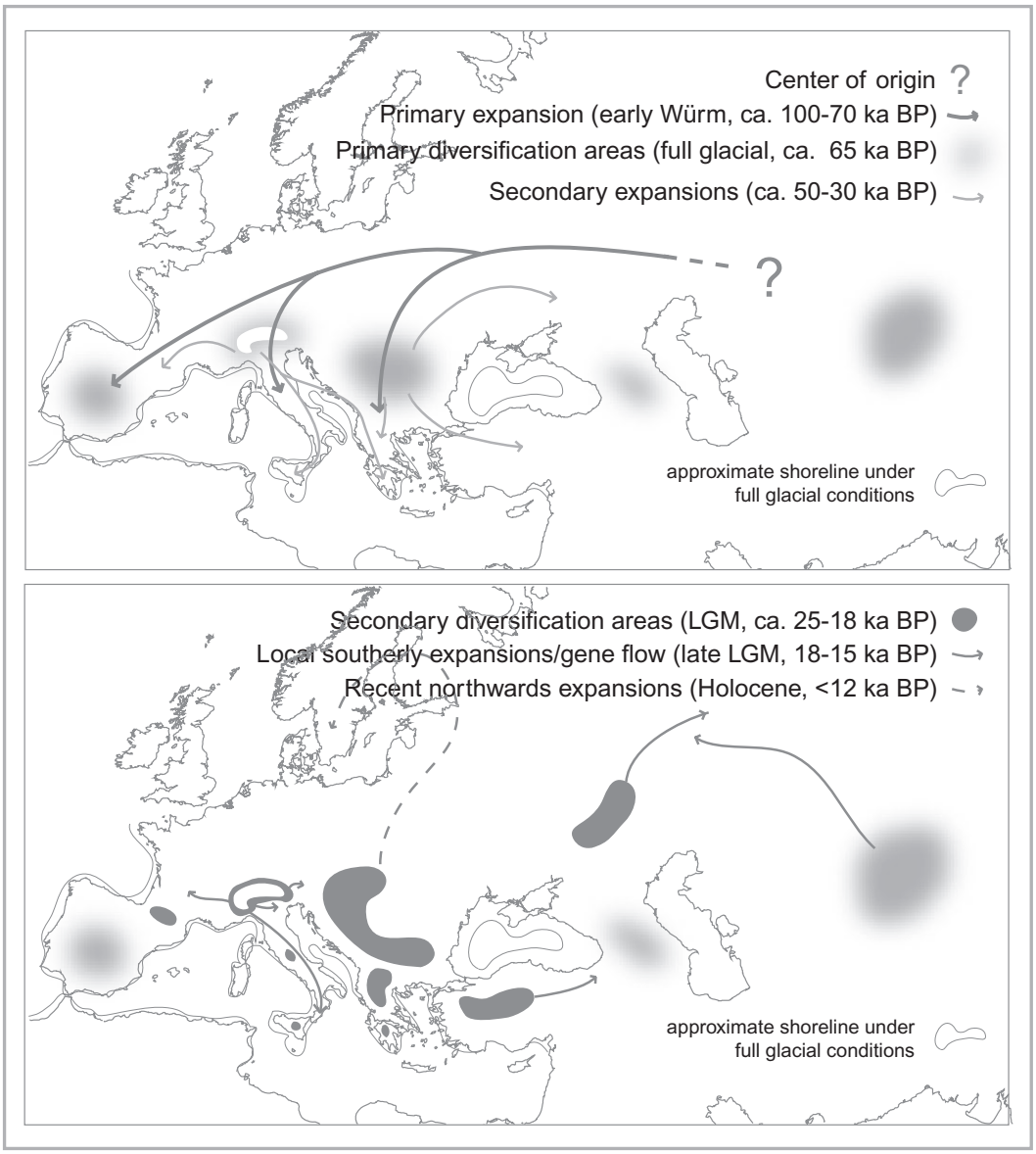


Figure 2. Hypothetical reconstruction of the evolution of *Parnassius apollo* range, consistent with mitochondrial DNA data. Diversification areas are defined according to the occurrence of exclusive lineages. Colonization routes reflect the reconstructed evolutionary relationship of lineages. Note that locations of primary diversification areas in the Balkan Peninsula and around the Alps should be considered as particularly tentative, as they are defined as geographic centres of widely distributed lineages.

record (after 15 Ka), *P. apollo* parameters pointed to the earlier steppic phase (LGM).

As a general point, we argue that the adoption of time-dependent fast molecular rates could offer a convincing interpretation of other distribution patterns in temperate butterflies (see Schoville & Roderick, 2009). In this perspective, the shallow molecular divergence found in the *E. tyndarus* complex (Albre *et al*., 2008), which shares the same montane steppic habitat as *P. apollo*, could be redirected to expansion events that occurred in the last glacial.

Certainly, the last northward expansion of *P. apollo* occurred later than 10–11 Ka (Fig. 2), when the Scandinavian region became available with the retreat of the ice cap. Consistently, Scandinavian samples share most haplotypes with populations from the Carpathian and Balkan regions, from which this late colonization originated.

Samples from central Urals harbour two different lineages: K, also present in Central Asia, and Ir, related to eastern European haplogroups. This may be the only suggestion of secondary contact of largely

allopatric lineages in *P. apollo* (Fig. 2). On the other hand, sharing of haplotypes between the Urals and Central Asia clearly shows that genetic contact existed across presently unoccupied areas in south-west Siberia and Kazakhstan until quite recently (see Varga, 2010).

The striking divergence of haplotype H_{71}, found in a single individual from the Caucasian region, highlights this area as the most genetically diverse, and a possible centre of origin for present mtDNA diversity. However, the Caucasus is strongly over-represented in our sample compared with other candidate areas (Central Asia), and further sampling is needed to corroborate this suggestion. Similar remarks apply to populations from the Balkans (East Carpathians and Bulgarian Stara Planina), which could shed light on the connection between eastern Europe colonization waves.

Subspecific taxonomy and conservation remarks

More than 200 subspecies of *P. apollo* have been described, based on fragmented distribution and a few morphological characters, and several of them have been recognized as scarcely relevant (Weiss, 2005). Our mtDNA analyses allowed the recognition of a total of twelve *P. apollo* distinctive lineages (Fig. 1A) that are confined to different geographical areas and characterized by pools of strictly related haplotypes. Subspecific taxonomy is commonly taken into account in the definition of conservation targets in European butterflies (Witkowsky *et al*., 1994). Subspecies serve as a useful benchmark for conservation, especially to the extent that they are the product of significant evolutionary processes, as reflected by phylogeographic patterns.

Our data highlighted several instances of populations ascribed to different subspecies (Glassl, 1993; Dietz, 2000; Möhn, 2005; Weiss, 2005) that share similar or identical mtDNA sequences, indicating very recent evolutionary divergence. The most evident example occurs in the western Carpathians, where all of the ten samples from five localities (three subspecies *sensu* Weiss, 2005) share an identical haplotype, consistent with the likely postglacial origin of the Carpathian populations (< 10 Ka). Similarly, Weiss (2005) suggested the occurrence of eleven subspecies within the Alps, retained from more than eighty subspecies previously described (Glassl, 1993; Dietz, 2000; Möhn, 2005), whereas mtDNA analyses show that a single haplotype (H_9) and its descendants are widespread across the whole region, and in the neighbouring ranges of the Massif Central and Apennines. Mismatch analysis indicated a recent geographic expansion throughout the whole area, which can be reasonably dated close, or slightly after, the LGM (about 18 Ka). Our results are corroborated by an early investigation of allozyme polymorphism (Racheli, Cianchi & Bullini, 1983), which revealed a very low level of genetic differentiation between a few populations from the Alps and Apennines.

Similar examples of detectable morphological differentiation contrasting with mtDNA homogeneity are commonly reported in butterflies (e.g. Sperling & Harrison, 1994; Kato & Yagi, 2004; Vandewoestijne *et al*., 2004). As a very few loci may influence characters of the wing pattern in butterflies (Beldade & Brakefield, 2002; Gross, 2006), some morphological differentiation can evolve rapidly as a consequence of genetic drift in small, isolated populations, so that divergence cannot be revealed by mtDNA markers. Differences in food-plant preferences (Nakonieczny & Kedziorski, 2005) may also evolve quickly, as variation of host plant in butterflies is usually under strong selection, and at the same time reflects the evolutionary potential of the species and the availability of possible host plants (Singer, Ng & Moore, 1991; Singer, Thomas & Parmesan, 1993; Radtkey & Singer, 1995).

Our analyses revealed a major, abrupt phylogeographic divide across eastern Turkey (Appendix; Fig. 1A). Sequences from Anatolian populations, which show a clinal morphological differentiation from east to west, and ascribed to several subspecies (included in *Parnassius apollo graslini* Oberthür, 1891 by Weiss, 2005), form the unique haplogroup Ia, related to eastern European lineage Ie, whereas more eastern samples (including subspecies *Parnassius apollo tkatshukovi* Sheljuzhko, 1935 and *Parnassius apollo tirabzonus* Sheljuzhko, 1924) bear the Caucasian lineage C. Four described subspecies distributed across Anatolia have been recently gathered in the single subspecies *P. a. graslini* (Weiss, 2005). However, in spite of his reviewing efforts, Weiss was unable to discriminate between the two main clades that reflect at least two major subspecies.

Our results indicate that an intensive sampling effort within restricted areas could reveal cores of differentiated haplotypes, not taxonomically distinct, such as the haplogroup N in the central Apennines, which is currently in (at least partial) sympatry with the widespread haplogroup A. The distribution of these haplogroups has been interpreted (Gratton *et al*., 2006) as a result of a recent (late glacial) dispersal from the Alps that overlapped, perhaps only partially, with pre-existing populations.

Finally, our data also evidenced the distinctiveness and evolutionary value of the highly divergent mitochondrial lineage E in Iberian populations, previously recognized as two well-differentiated subspecies (*Parnassius apollo nevadensis* Oberthür,

1891 and *Parnassius apollo hispanicus* Oberthür, 1883, *sensu* Weiss, 2005). However, as there are gaps in the sampling, especially in the Iberian peninsula, different haplotypes could be expected to occur, particularly in the Cordillera Cantabrica in northwestern Spain.

Examples of small, isolated populations, the mtDNA divergence of which highlights their significance for biodiversity, are presented by *Parnassius apollo siciliae* Oberthür, 1899, from the Madonie mountains (Sicily), and *Parnassius apollo atrides* (van der Poorten & Dils, 1986), from Peloponnesus (Greece), that was pronounced extinct, until some individuals were newly found in 1983 (Casale & Cecchin, 1990; Bollino *et al*., 1996). This last one bears a unique mtDNA haplotype closely related to Italian lineages A, N and S (Fig. 1C, D), and probably represents a relict of a colonization process independent from those originating the northern Greek populations. A large fraction of the mitochondrial genetic variation of *P. apollo* is therefore concentrated in the southernmost populations in the highest mountains of Spain, Sicily, and southern Greece.

Although this study provides useful hints to simplify the controversial subspecific classification of *P. apollo*, a taxonomical review of this species is beyond the scope of this paper, as it would require a thorough nomenclatorial revision, as well as some additional geographic sampling. However, the twelve major haplogroups identified in this study (Appendix) seem to offer a starting point that will be useful to both taxonomists and conservation biologists.

Our results are largely consistent with the hypothesis that *P. apollo* populations expanded their southern ranges within, or close to, glacial episodes, and fragmented into alpine patches during interglacial periods, when forested habitats expanded. Southernmost and geographically isolated populations are therefore the most threatened, as small populations are particularly vulnerable to genetic erosion and negative demographic trends, and because in southern regions the impact of climate change might be more pronounced.

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SUPPORTING INFORMATION

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

Table S1. Sequence primers, thermal cycle and polymerase chain reaction conditions.

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APPENDIX

Taxon[1] = *P. apollo* subspecies according to Glassl (1993), Dietz (2000), Möhn (2005) and Taxon[2] = *P. apollo* subspecies according to Weiss (2005), haplogroups, collection locality, number of samples (*N*), population and haplotype code, haplotype diversity (*h*), ands GenBank accession numbers.

Taxon[1]	Taxon[2]	Haplogroup	Collection locality	*N*	Population code	Haplotype code	*h*	GenBank Accession
P. a. apollo	*P. a. apollo*	**Ie**	Sweden: Schereden 30 km von Huskuarna	2	SESCH	H_{20}	0.000 ± 0.000	GU947601-2
P. a. finmarchicus	*P. a. apollo*	**Ie**	Finland: Etela-Suomi	1	FINFIN	H_{19}	–	GU947470
P. a. fennoscandicus	*P. a. apollo*	**Ie**	Finland: Åland Islands	2	FINSWA	H_{19},H_{20}	1.000 ± 0.500	GU947471-2
P. a. nevadensis	*P. a. nevadensis*	**E**	Spain: Sierra Nevada	1	ESNEV	H_{13}	–	GU947458
P. a. filabricus	*P. a. nevadensis*	**E**	Spain: Sierra de los Filabres	2	ESFIL	H_{11}	0.000 ± 0.000	GU947455-6
P. a. hispanicus	*P. a. hispanicus*	**E**	Spain: Fries de Albarracin,Valle del Guado	1	ESALB	H_{10}	–	GU947454
P. a. aragonicus	*P. a. aragonicus*	**P**	Spain: Pyrenees, Huesca, Panticosa, 1700 m a.s.l.	1	ESPAN	H_{12}	–	GU947457
P. a. pyrenaicus	*P. a. pyrenaicus*	**P**	France: Pyrenees, Lac D'Estaing (42° 54′N 00° 13′W), 1100 m a.s.l.	3	FRLAC	H_{16},H_{17},H_{12}	1.000 ± 0.272	GU947465-7
P. a. pyrenaicus	*P. a. pyrenaicus*	**P**	France: Pyrenees, Super Bareges (42° 54′N 00° 06′W), 1600 m a.s.l.	1	FRSBA	H_{12}	–	GU947469
P. a. lozerae	*P. a. lozerae*	**A**	France: Causse Méjean, Hures	2	FRHUR	H_9	0.000 ± 0.000	GU947463-4
P. a. nivatus	*P. a. nivatus*	**A**	France: Gex, Col della Faucille, 1200 m a.s.l.	2	FRGEX	H_{15}	0.000 ± 0.000	GU947461-2
P. a. valdieriensis	*P. a. valdieriensis*	**A**	Italy: Piemonte, Bosco Navette (CN)	2	ITBOS	H_9	0.000 ± 0.000	GU947494-5
P. a. valdieriensis	*P. a. valdieriensis*	**A**	Italy: Piemonte, Alpi Cozie meridionali, Sambuco, Moriglione, 1500 m a.s.l.	2	ITSAM	H_9,H_{18}	1.000 ± 0.500	GU947556-7
P. a. substitutus	*P. a. geminus*	**A**	France: Hautes Alpes, L'Argentière-la-Bessée, 900–1200 m a.s.l.	1	FRLAB	H_{18}	–	GU947468
P. a. substitutus	*P. a. geminus*	**A**	France: Briançon, Gramom Mt, 2300 m a.s.l.	2	FRBRI	H_9, H_{14}	1.000 ± 0.500	GU947459-60
P. a. pedemontanus	*P. a. geminus*	**A**	Italy: Valle D'Aosta,Val di Cogne,Lillaz (AO), 1600 m a.s.l.	1	ITCOG	H_{31}	–	GU947499
P. a. pedemontanus	*P. a. geminus*	**A**	Italy: Valle D'Aosta, Dint. Courmayeur,Val Vèny, La Visalle, 1663 m a.s.l.	1	ITCOU	H_{32}	–	GU947500
P. a. pedemontanus	*P. a. geminus*	**A**	Italy: Valle D'Aosta,Valgrisanche, 1400 m a.s.l.	1	ITGRI	H_{33}	–	GU947509
P. a. pedemontanus	*P. a. geminus*	**A**	Italy: Valle D'Aosta,Morgex, 1558 m a.s.l.	1	ITMOR	H_{42}	–	GU947539
P. a. valesiacus	*P. a. geminus*	**A**	Switzerland: Gr.St.Bernard, Fontaine Dessous, 1000 m a.s.l.	2	CHSTB	H_9	0.000 ± 0.000	GU947452-3
P. a. caloriferus	*P. a. redivivus*	**A**	Switzerland: Laggintal, Sempione, 1600–1800 m a.s.l.	2	CHLAG	H_9,H_{34}	1.000 ± 0.500	GU947513-4
P. a. redivivus	*P. a. redivivus*	**A**	Italy: Piemonte,Varzo, San Domenico, 1300 m a.s.l.	2	ITVAR	H_{48}	0.000 ± 0.000	GU947574-5
P. a. tonalensis	*P. a. rubidus*	**A**	Italy: Trentino,Passo Tonale, 1500 m a.s.l.	2	ITTON	H_{46},H_{47}	1.000 ± 0.500	GU947572-3
P. a. victorialis	*P. a. rubidus*	**A**	Italy: Veneto, Croce D'Aune	1	ITDAU	H_9	–	GU947508
P. a. victorialis	*P. a. rubidus*	**A**	Italy: Veneto, Loc. Lazzaretti (VI), 1300 m a.s.l.	2	ITLAZ	H_9	0.000 ± 0.000	GU947515-6
P. a. grappensis	*P. a. rubidus*	**A**	Italy: Veneto, Mt Grappa	3	ITGRP	H_9	0.000 ± 0.000	GU947510-2
P. a. friulanus	*P. a. rhea*	**A**	Italy: Friuli, Campone (PN), Lago Tramonti	2	ITCAM	H_9,H_{30}	1.000 ± 0.500	GU947496-7
P. a. friulanus	*P. a. rhea*	**A**	Italy: Friuli, Sella Canizza, 1000 m a.s.l.	1	ITCAR	H_9	–	GU947498
P. a. euapenninus	*P. a. italicus*	**A**	Italy: Marche, Mt Rotondo (MC), 1450 m a.s.l.	8	ITMRT	H_{43},H_9	0.250 ± 0.180	GU947540-7
P. a. euapenninus	*P. a. italicus*	**A**	Italy: Marche, Mt Argentella (MC), Mt Palazzo Borghese,1900–2100 m a.s.l.	2	ITPAB	H_9	0.000 ± 0.000	GU947548-9
–	*P. a. italicus*	**N**	Italy: Lazio, Mt Terminillo, 1600–2200 m a.s.l.	9	ITTER	H_{37},H_{44},H_{45}	0.417 ± 0.191	GU947563-71
P. a. civis	*P. a. italicus*	**A–N**	Italy: Abruzzo, Mt Magnola, 1500 m a.s.l.	9	ITMAG	H_9,H_{37},H_{38},H_{39}	0.750 ± 0.112	GU947520-8
P. a. civis	*P. a. italicus*	**N**	Italy: Abruzzo, Pizzo Ovindoli	5	ITOVI	H_{37}	0.000 ± 0.000	GU947558-62
P. a. romeii	*P. a. italicus*	**A**	Italy: Abruzzo, Gran Sasso, Monte Corvo (AQ), 1500–1600 m a.s.l.	7	ITCOV	H_9	0.000 ± 0.000	GU947501-7
P. a. romeii	*P. a. italicus*	**A**	Italy: Abruzzo, Assergi, Campo Imperatore	5	ITASS	H_9,H_{29}	0.400 ± 0.237	GU947489-93
P. a. italicus	*P. a. italicus*	**A**	Italy: Abruzzo, Mt Maiella, Lama Bianca, 1100–1500 m a.s.l.	4	ITMAI	H_{40},H_{41}	0.500 ± 0.265	GU947529-32
P. a. italicus	*P. a. italicus*	**A**	Italy: Abruzzo, Mt Maiella, Pizzoferrato (CH), 1370 m a.s.l.	6	ITPIZ	H_{41}	0.000 ± 0.000	GU947550-5
P. a. italicus	*P. a. italicus*	**A**	Italy: Abruzzo, Mt Morrone,Ghiaccio Rosso, 1400–1600 m a.s.l.	6	ITMMO	H_{40},H_{41}	0.533 ± 0.172	GU947533-8
P. a. pumilus	*P. a. pumilus*	**A**	Italy: Aspromonte, Montalto, 1800 m a.s.l.	2	ITALT	H_{28}	0.000 ± 0.000	GU947487-8
P. a. siciliae	*P. a. siciliae*	**S**	Italy: Sicily, Madonie, Pizzo Carbonara, 1680 m a.s.l.	3	ITMAD	H_{35},H_{36}	0.667 ± 0.314	GU947517-9
P. a. antiquus	*P. a. antiquus*	**Ie**	Slovakia: Carpathians, Stazovske Vrchy, Podmanin	3	SKSTR	H_{20}	0.000 ± 0.000	GU947610-2
P. a. antiquus	*P. a. antiquus*	**Ie**	Slovakia: Carpathians, Manin	1	SKMAN	H_{20}	–	GU947607

P. a. interversus	*P. a. sztrecsnoensis*	**Ie**	Slovakia: Carpathians, Bile Karpaty,Vrsatec Pod.	4	SKBIL	H_{20}	0.000 ± 0.000	GU947603-6
P. a. liptauensis	*P. a. sztrecsnoensis*	**Ie**	Slovakia: Carpathians, Osobità	1	SKOSO	H_{20}	–	GU947608
P. a. liptauensis	*P. a. sztrecsnoensis*	**Ie**	Slovakia: Carpathians, Choc. Pohorie, Val. Dubova	1	SKPOH	H_{20}	–	GU947609
P. a. bosniensis	*P. a. bosniensis*	**Ie–Ig**	Bosnia-Herzegovina: Sarajevo, Mt Bjelasnica, 1400–1500 m a.s.l.	4	BABJE	H_5,H_6	0.500 ± 0.265	GU947446-9
P. a. dardanus	*P. a. bosniensis*	**Ie**	Albania: Tropoje Margegaj,Valbona, Mt Kollates, 1000–1800 m a.s.l.	2	SLTRO	H_4	0.000 ± 0.000	GU947444-5
P. a. rhodopensis	*P. a. rhodopensis*	**Ig**	Bulgaria: Rhodopi Pl.	2	BGROD	H_7,H_8	1.000 ± 0.500	GU947450-1
P. a. graecus	*P. a. graecus*	**Ig**	Greece: Ioannina, Mt Gramos, 1450–1600 m a.s.l.	3	GRGRA	H_{22},H_{23}	0.667 ± 0.314	GU947476-8
P. a. graecus	*P. a. graecus*	**Ig**	Greece: Pindo, Katara Pass, 1500–1700 m a.s.l.	5	GRKAT	H_{24},H_{25},H_{26}	0.700 ± 0.218	GU947479-83
P. a. graecus	*P. a. graecus*	**Ig**	Greece: Lamia, Oiti Oros, 1500–1700 m a.s.l.	2	GRLAM	H_{27}	0.000 ± 0.000	GU947484-5
P. a. olimpiacus	*P. a. graecus*	**Ig**	Greece: Mt Olympus	1	GROLI	H_{24}	–	GU947486
P. a. atrides	*P. a. atrides*	**R**	Greece: Peloponnesus, Erimanthos Oros, 1500–1600 m a.s.l.	3	GRERI	H_{21}	0.000 ± 0.000	GU947473-5
P. a. anatolicus	*P. a. graslini*	**Ia**	Turkey: Konia, Sultan Dagh 1900–2000 m a.s.l.	2	TRKON	H_{65},H_{66}	1.000 ± 0.500	GU947622-3
P. a. tauricus	*P. a. graslini*	**Ia**	Turkey: near Isparta,Tota Dagh, 2200 m a.s.l.	1	TRTOT	H_{67}	–	GU947636
P. a. tauricus	*P. a. graslini*	**Ia**	Turkey: near Isparta, Davras Dagh, 2000–2300 m a.s.l.	2	TRDAV	H_{61},H_{62}	1.000 ± 0.500	GU947616-7
P. a. paphlagonicus	*P. a. graslini*	**Ia**	Turkey: Ahmetusta, Nord Karabuk, 1650 m a.s.l.	2	TRKAR	H_{64}	0.000 ± 0.000	GU947620-1
P. a. dubius	*P. a. graslini*	**Ia**	Turkey: Kop Dagi Geçidi, 2100–2300 m a.s.l.	6	TRKOP	H_{67},H_{68},H_{69}	0.733 ± 0.155	GU947624-9
P. a. dubius	*P. a. graslini*	**Ia**	Turkey: Palandöken Dağlari (str.Erzurum-Tekman), 2500–3000 m a.s.l.	2	TRPAL	H_{67},H_{70}	1.000 ± 0.500	GU947630-1
P. a. dubius	*P. a. graslini*	**C**	Turkey: Kars, Sarikamis, 1900 m a.s.l.	4	TRSAR	H_{54}	0.000 ± 0.000	GU947632-5
P. a. tkatshukovi	*P. a. graslini*	**C**	Armenia: Ararat, Ararat Marz, Khosrov, 1800 m a.s.l.	1	AMARA	H_1	–	GU947613
P. a. tkatshukovi	*P. a. graslini*	**C**	Armenia: Kotayk prov., Agveran vill. env. (39° 48′N 44° 58′E), 1700 m a.s.l.	7	AMARM	H_1,H_2,H_3	0.667 ± 0.160	GU947437-43
P. a. tirabzonus	*P. a. tirabzonus*	**C**	Turkey: Yalnizçam Daglari, ArdanuçYalnizçan	1	TRYAL	H_{60}	–	GU947637
P. a. tirabzonus	*P. a. tirabzonus*	**C**	Turkey: Artvin	2	TRART	H_{60}	0.000 ± 0.000	GU947614-5
P. a. tirabzonus	*P. a. tirabzonus*	**C**	Turkey: Artvin, Kackar Dagi, 1700–1900 m a.s.l.	2	TRKAC	H_{54},H_{63}	1.000 ± 0.500	GU947618-9
P. a. caucasicus	*P. a. caucasicus*	**C**	Georgia: Mesketskiy, Azkuri, 1400 m a.s.l.	1	GECISa	H_{54}	–	GU947590
P. a. ciscaucasicus	*P. a. caucasicus*	**C**	Russia: Caucasus, MtElbrus	1	RUCISb	H_{55}	–	GU947591
P. a. ciscaucasicus	*P. a. caucasicus*	**C** (+H_{71})	Russia South: Caucasus Mts, Karachaevsk reg., Aksaut River, 1600 m a.s.l.	7	RUAKS	H_{52},H_{53},H_{71}	0.524 ± 0.209	GU947583-9
P. a. moscovitus	*P. a. moscovitus*	**Ir**	Russia: Voronezh,Volga Heights	1	RUVOR	H_{59}	–	GU947600
P. a. democratus	*P. a. democratus*	**K–Ir**	Russia: Kirov reg., Medvedov vill.	4	RUKIR	H_{49},H_{56},H_{57}	0.833 ± 0.222	GU947592-5
P. a. limicolus	*P. a. limicolus*	**K**	Russia: Urals Mts, Chusovaya River	2	RUURA	H_{49}	0.000 ± 0.000	GU947598-9
P. a. limicolus	*P. a. limicolus*	**K**	Russia: S.Ural Mts, Cheljabinsk, Itkul Lake	1	RULIMa	H_{58}	–	GU947596
P. a. limicolus	*P. a. limicolus*	**K**	Russia: SW Siberia, Cheljabinsk reg., Arkaim v.	1	RULIMb	H_{49}	–	GU947597
P. a. transiliensis	*P. a. mongolicus*	**K**	–	1	KZTRA	H_{49}	–	GU947580
P. a. transiliensis	*P. a. mongolicus*	**K**	Kazakhstan: Alma-Ata (Almaty)	2	KZALM	H_{49},H_{50}	1.000 ± 0.500	GU947578-9
P. a. transiliensis	*P. a. mongolicus*	**K**	Kyrgyzstan: Terskey Ala-Tau, Altyn, Arashan r., 2000 m a.s.l.	2	KGALA	H_{49}	0.000 ± 0.000	GU947576-7
P. a. transiliensis	*P. a. mongolicus*	**K**	China: Xinjiang reg., Zhaosu	1	CNZHA	H_{51}	–	GU947582
P. a. mongolicus	*P. a. mongolicus*	**K**	China: Xinjiang reg., Barkol Karlik Shan,N slopes, 2500 m a.s.l.	1	CNBAR	H_{49}	–	GU947581
P. phoebus	*P. phoebus*	**–**	Italy: Trentino, PN Stelvio (Peio), Rifugio Larcher, 2600 m a.s.l.	1	ITpPSP	–	–	GU947638
P. bremeri	*P. bremeri*	**–**	Far East of Russia: Amur reg., Skovorodino v.	1	RUbERU	–	–	GU947639
P. mnemosyne	*P. mnemosyne*	**–**	Italy: Portella di Calacudera, Nebrodi Mts, Sicily	1	ITNEB01	–	–	GU947642
P. ariadne	*P. ariadne*	**–**	Kazakhstan: Tarbagatai Mts	1	KZTRB02	–	–	GU947640
P. nordmanni	*P. nordmanni*	**–**	Russia: Krasnodar reg., Adygela dist.	1	RUKRS01	–	–	GU947641