

TESTING PHYLOGEOGRAPHIC HYPOTHESES IN A EURO-SIBERIAN COLD-ADAPTED LEAF BEETLE WITH COALESCENT SIMULATIONS

Patrick Mardulyn,^{1,2} Yuri E. Mikhailov,^{3,4} and Jacques M. Pasteels^{1,5}

¹Evolutionary Biology and Ecology, Université Libre de Bruxelles, av FD Roosevelt 50, 1050 Brussels, Belgium

²E-mail: pmarduly@ulb.ac.be

³Urals State Forestry-Engineering University, Yekaterinburg, Russia

⁴E-mail: Yuri.Mikhailov@usu.ru

⁵E-mail: jmpastee@ulb.ac.be

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Few studies to date have investigated the impact of Pleistocene climatic oscillations on the genetic diversity of cold-adapted species. We focus on the geographic distribution of genetic diversity in a Euro-Siberian boreo-montane leaf beetle, *Gonioctena pallida*. We present the molecular variation from three independent gene fragments over the entire geographic range of this insect. The observed sequence variation identifies a genetic diversity hot spot in the Carpathian Mountains, in central Europe, which reveals the presence of (1) an ancestral refuge population or (2) a secondary contact zone in this area. Modeling of population evolution in a coalescent framework allowed us to favor the ancestral refuge hypothesis. These analyses suggest that the Carpathian Mountains served as a refuge for *G. pallida*, whereas the rest of the species distribution, that spans a large portion of Europe and Asia, experienced a dramatic reduction in genetic variation probably associated to bottlenecks and/or founder events. We estimated the time of isolation of the ancestral refuge population, using an approximate Bayesian method, to be larger than 90,000 years. If true, the current pattern of genetic variation in this cold-adapted organism was shaped by a climatic event predating by far the end of the last ice age.

KEY WORDS: Chrysomelidae, coalescent modeling, glacial refuge, *Gonioctena pallida*, phylogeography.

Numerous and severe global climate oscillations occurred during the Quaternary period, causing the geographic range of most animals and plants to greatly vary over time. For many organisms, today's geographic distribution of genetic diversity reflects this climate history (Avice 2000; Hewitt 2000; Hewitt 2003). In Europe, it is generally thought that the range of many species was, at the time of the last glaciation, restricted to three main isolated refugia in the South (Iberic peninsula, Italy, and the Balkans; Hewitt 2000), although some evidence also suggests the existence of additional northern refugia for several temperate species (e.g., Stewart and Lister 2001; Heckel et al. 2005; Kotlik et al. 2006;

Provan and Bennett 2008). Molecular genetic diversity studies, focusing on the contemporary geographic distribution of genes, have identified for several species (1) the postglacial recolonization routes of central and northern Europe from these southern refugia and (2) secondary contact zones, in which individuals that had been separated in different refuges for long periods of time meet again (Taberlet et al. 1998; Hewitt 2001).

So far, however, relatively little attention has been directed toward cold-adapted species, whose distribution is currently restricted to the north of Europe and to mountains in central and southern Europe (for exceptions, see Schonswetter et al. 2003;

Muster and Berendonk 2006; Skrede et al. 2006). In contrast with temperate-climate species, cold-climate species display a highly fragmented distribution during interglacial periods, as they do today, while they are likely to be characterized by a more panmictic distribution during glacial periods. The patterns of geographic diversity that characterize these organisms are therefore expected to be different from those highlighted for temperate species. First, because the species range fragmentation occurs at interglacial instead of glacial periods, and because glacial periods have lasted overall longer within the last 200 thousand years, the levels of differentiation characterizing separated lineages within a cold-adapted species should be lower than for a temperate climate species. Second, ancient refuges for cold-adapted species would be expected to be located in areas occupied during interglacial periods (when the range is fragmented), that is, areas in which the species is found today. Compared to the Mediterranean refuges that were inferred for many temperate species, ancient refuges for cold-adapted organisms are therefore likely to be located further north.

Here, we focus on the genetic diversity of a boreo-montane leaf beetle, *Gonioctena pallida*. It is restricted to montane habitats in the south and center parts of its range, that spans most of Europe (excluding the Mediterranean region) and Siberia, while it can be found in lowlands in the north. Like most chrysomelid beetles, it is a specialist herbivore, that is, its diet is restricted to a few host plant species (in this case, trees from the genera *Salix* and *Corylus*), and populations are structured because their spatial distribution follows the patchy distribution of their host plant. Its dispersal appears rather limited, and strong population differentiation was detected within a small mountain range such as the Vosges in France (Mardulyn 2001). We present the molecular variation from three independent loci, one mitochondrial, and two nuclear gene fragments, over the entire Euro-Siberian geographic range of *G. pallida*. The observed sequence variation identifies a genetic diversity hot spot in the Carpathian mountain range, in central Europe.

Coalescent theory concerns the design of population genetics models to simulate gene genealogies, and provides a powerful framework to test hypotheses about evolutionary history (e.g., Hudson 1990; Knowles and Maddison 2002; Rosenberg and Nordborg 2002; Knowles and Carstens 2007). We have developed coalescent models and performed simulations to test two competing hypotheses that could explain the pattern of genetic variation highlighted in this study. These analyses lead us to conclude that the Carpathian Mountains served as a refuge for *G. pallida*, while it experienced a dramatic reduction in genetic variation in all other parts of its geographic distribution. This reduced genetic variation was probably associated with bottlenecks and/or founder events, and occurred more than 90,000 years ago.

Materials and Methods

SAMPLES AND SEQUENCING

We collected *G. pallida* from 37 localities across its entire geographic range in Eurasia (Table 1). Note that this species is absent from the Pyrenees mountains (J. C. Bourdonné, pers. comm.). Genomic DNA was extracted using the Dneasy Tissue Kit from Qiagen (Hilden, Germany). Whole specimens were each ground in the Qiagen ATL buffer, and incubated 3 h with proteinase K at 55°C. The remaining DNA-extraction steps were conducted as described in the manufacturer's protocol. We sequenced 380 copies of a ~1300 base pair (bp) long fragment of the mitochondrial cytochrome oxidase 1 gene (COI) from all sampled localities. To confirm the phylogeographic pattern uncovered by the analysis of the COI sequences, we have additionally sequenced (1) 98 copies from a ~1200 bp fragment of the nuclear elongation factor-1 α gene (EF-1 α , including an intron that varied in length from 661 to 761 bp), and (2) 93 copies from a ~700 bp fragment of one copy of the nuclear actin gene family (actin). All fragments were PCR-amplified following the FastStart Taq DNA polymerase manufacturer's protocol (Roche Applied Science, Mannheim, Germany). The COI fragment was amplified (annealing temperature of 52°C) using primers TL2-N-3014 and C1-J-1751 (Simon et al. 1994), the EF-1 α fragment was amplified (annealing temperature of 64°C) using primers specifically developed for *G. pallida* (5'GTGGTGATGTTGGCTGGGCG3' and 5'CACTGGTACATCGCAAGCCG3'), and the actin gene fragment was amplified (annealing temperature of 52°C) using primers 5'ATGATYTTGATCTTGATGGTGG3' and 5'CAACGAAGTCCGTGTCGCT3'. For nuclear genes, when a heterozygote individual was detected, new PCR products were generated with the Long Expand Template PCR System Kit (Roche Applied Science; same PCR conditions as above), and were cloned in a no-background vector (StabyCloning kit, Delphi Genetics, Charleroi, Belgium). Five clones were sequenced and compared to the PCR product sequence to infer the two haplotypes of each heterozygote individual. All the haplotype sequences gathered for this project are available from GenBank under accession numbers FJ346829-FJ347029.

DATA ANALYSES

Sequences were aligned manually with SE-AL (Rambaut 1996). They were pruned at both 5'- and 3'- ends to ensure that no trailing gaps were present in the final dataset. Gaps present in the EF-1 α dataset were considered as missing data for the purpose of inferring a haplotype network, but were recoded as single characters irrespective of their length and added to the end of the nucleotide dataset. When gaps of different lengths overlapped, each size class was considered a different character state. A median-joining network (Bandelt et al. 1999) was inferred

Table 1. Sampling localities, and distribution of haplotypes for the three gene fragments used in this study.

Region	Geographical coordinates	<i>n</i> (COI)	Haplotypes (COI) (no. of copies)	<i>n</i> (EF-1 α)	Haplotypes (EF-1 α) (no. of copies)	<i>n</i> (actin)	Haplotypes (actin) (no. of copies)
Scandinavian mnts, Sweden	68°06'N, 23°16'E	6	105(2), 111, 112, 113, 119	6	36(2), 37(4)	8	1(5), 5(3)
Scandinavian mnts, Finland	69°02'N, 20°48'E	5	105, 119(2), 120, 121	4	29, 31, 37(2)	10	1(3), 6, 14(2), 16(3), 18
Finland	65°58'N, 29°10'E	9	93(4), 94, 95(2), 96(2)				
Vosges (France)	47°48'N, 06°49'E	8	1(2), 3, 30(2), 31, 78, 81				
Vosges (France)	48°20'N, 07°11'E	8	1(2), 72, 73(2), 74, 83, 85				
Vosges (France)	47°58'N, 07°01'E	7	1(3), 3(4)				
Vosges (France)	48°06'N, 07°01'E	9	1(3), 75(5), 76				
Schwarzwald (Germany)	47°55'N, 07°58'E	6	2(2), 20, 28, 30, 33				
Schwarzwald (Germany)	47°58'N, 07°54'E	9	1(3), 2, 32, 40, 58(2), 63	6	1(3), 2, 12(2)	2	4(2)
Bayerischerwald (Germany)	49°07'N, 13°09'E	16	1, 2(6), 33, 36, 44(3), 67, 68(3)				
Rhön (Germany)	50°31'N, 10°04'E	17	1(4), 11(2), 24, 25, 38(3), 39, 56(3), 62, 88				
Rhön (Germany)	50°37'N, 10°50'E	13	1, 2(3), 7(2), 43, 44(4), 87(2)	9	1(5), 3, 6, 48(2)	5	4, 9, 12, 16(2)
Ore mnts (Czech rep)	50°31'N, 13°22'E	19	2(6), 4(8), 55(4), 89	18	1(13), 5, 7, 8, 10, 11	12	16(6), 17(6)
Ore mnts (Czech rep)	50°01'N, 12°39'E	16	2(5), 6, 32(6), 41, 44, 45, 55				
Ore mnts (Czech rep)	50°03'N, 12°47'E	12	1(2), 2(8), 36, 44				
Carpathians (Slovak rep)	48°58'N, 18°37'E	18	12, 14, 18, 48(2), 49, 50, 51, 52, 53, 57, 117(4), 122, 123(2)				
Carpathians (Slovak rep)	48°49'N, 21°32'E	23	100(20), 103(3)	4	1(3), 14	4	11(4)
Carpathians (Slovak rep)	48°53'N, 19°39'E	16	9, 14(2), 15(2), 16, 17(2), 48(2), 54, 117, 118(2), 123, 125	7	25(2), 26, 27, 30, 39, 40	8	1, 2(2), 3, 4, 5(2), 7
Carpathians (Slovak rep)	49°13'N, 20°21'E	21	14, 93, 97, 98, 102(2), 107(2), 115(3), 133(2), 123(2), 126(3), 127, 128, 132	20	1(2), 4, 13, 15, 16, 17, 24, 28, 33, 34, 35, 36, 44, 45, 46, 47(3), 50	2	4, 15
Carpathians (Slovak rep)	49°16'N, 19°06'E	16	13(2), 19(4), 86, 116, 123(3), 124, 129(2), 130, 131				
Alps (Austria)	47°15'N, 10°34'E	9	2(2), 5(7)				
Alps (Switzerland)	46°49'N, 09°36'E	10	69(9), 82				
Alps (Austria)	47°13'N, 14°06'E	13	1(3), 8(2), 21(3), 26, 27, 34, 35, 90	2	19, 49	12	1(6), 4(2), 8(4)
Alps (Austria)	47°27'N, 13°50'E	11	1(4), 10, 14, 46(3), 61, 77				
Alps (Austria)	47°20'N, 13°56'E	5	22(3), 42, 44				
Alps (France)	44°55'N, 05°13'E	6	1(2), 37, 65, 66(2)				
Alps (France)	45°13'N, 06°05'E	9	1(6), 47(2), 71				
Alps (France)	45°01'N, 05°58'E	8	1(3), 22, 33, 70, 91, 92				
Alps (France)	44°53'N, 05°56'E	2	1(2)				
Massif Central (France)	45°55'N, 03°43'E	5	1(2), 3, 60, 84				
Massif Central (France)	45°09'N, 02°49'E	9	3(7), 29, 64				
Massif Central (France)	45°04'N, 02°44'E	8	3(7), 23	8	1(6), 9, 32	12	10(3), 17(9)
Massif Central (France)	44°27'N, 03°44'E	7	1(4), 3, 79, 80				
North Ural (Russia)	59°37'N, 59°17'E	1	101	2	32(2)		
Polar Ural (Russia)	66°56'N, 65°25'E	7	105(4), 59(3)				
Altai (Rep. of Altai, Russia)	50°05'N 85°26'E	6	104, 105(2), 108, 109, 110	6	37(6)	8	1(5), 5, 16(2)
Altai (Rep. of Altai, Russia)	50°03'N 85°21'E			2	38, 41		
Mnts of East Tuva (Russia)	51°41'N 96°58'E	10	99, 105(6), 106(2), 114	4	32, 42(2), 43	10	1(6), 13(2), 14(2)

for each gene fragment using the program NETWORK (available at <http://www.fluxus-engineering.com/sharenet.htm>), with parameter $\epsilon = 0$. Sampled populations were pooled into broader geographic entities roughly corresponding to isolated regions (such as mountain ranges). Nucleotide diversity within each region was computed using ARLEQUIN version 3.0 (Excoffier et al. 2005).

HYPOTHESIS TESTING

Given the genetic diversity hot spot uncovered for *G. pallida* in the Carpathian mountains (see Results), two competing hypotheses were identified as likely historical scenarios: the presence of an ancient refuge (1), or of a secondary contact zone (2), in this area. To discriminate between (1) and (2), we have conducted computer simulations of population evolution with the program SIMCOAL 2.1.2 (Laval and Excoffier 2004), following two models of coalescence, each describing one of the hypotheses being evaluated.

In a first step, we performed several simulations under each model, spanning a large range of parameter values (see below), to roughly explore the space of variable parameters and to identify regions of that space that appear compatible with the empirical data. In a second step, we performed additional simulations under each model, within the regions of the parameter space defined in step 1, to compare the simulated data to the empirical data through a criterion that we identified as useful to discriminate between the two hypotheses: the ratio of nucleotide diversities between the Carpathian samples and the samples from the rest of the species distribution, which appeared remarkably high in our samples. The simulated datasets in step 2 were used to generate a null distribution of this parameter, against which to compare the empirical value. These simulations were done for all three gene fragments, mirroring the specific (different) sampling of each of the three empirical datasets. When comparing the ratios of nucleotide diversities from the empirical and simulated datasets, it is indeed important to ensure that these ratios were calculated from two datasets of identical structure (i.e., the same number of sequences sampled from the same populations).

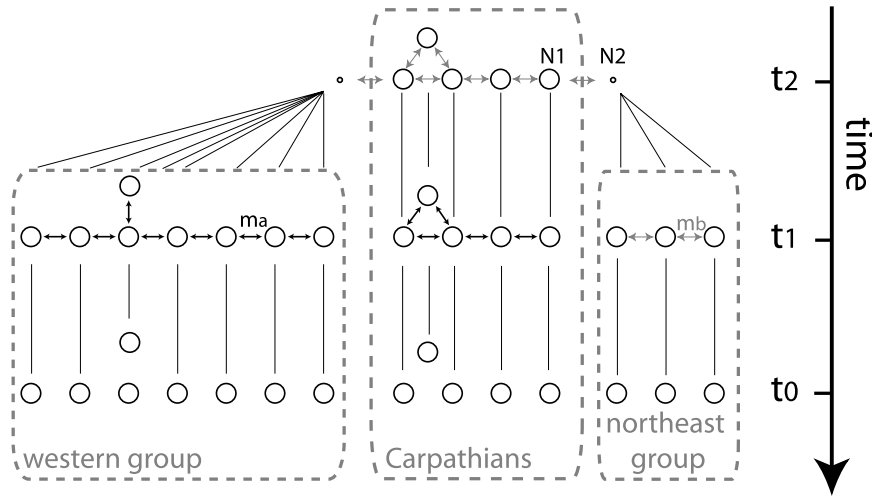
Description of the coalescent models

In the first hypothesis, an area in or near the Carpathian Mountains served as a refuge for the ancestral population of *G. pallida*. The level of genetic variation within this area remained thus mostly unchanged (ancestral refuge hypothesis). The rest of the contemporary Eurasian distribution was either (1) colonized from parts of the ancestral population that remained outside the Carpathian refuge, but were subject to a severe bottleneck that considerably reduced its genetic variation or (2) colonized from the populations inside the hypothesized Carpathian refuge, but associated with a strong founder effect. The coalescence model designed to test

this hypothesis is as follows (although coalescent simulations are performed backward in time, the following description proceeds forward in time, for clarity; see Fig. 1): in the most ancestral stage (t_2), seven populations (corresponding to the five sampled Carpathian populations in our study plus two ancestral source populations for the colonization of the rest of the Eurasian geographic distribution) are connected by gene flow (see below). The populations located at both extremes of the spatial distribution will serve as the source populations for the colonization of the entire Eurasian distribution, one of them for the colonization of western and central Europe, the other for the colonization of Asia and Scandinavia. It was determined in step 1 that these two populations needed to be reduced in size compared to the other five ancestral populations, which correspond to the five Carpathian populations that were sampled in our study. In a second stage (still going forward in time; t_1), one of the source populations is used to create (by transferring individuals) eight new western + central European populations (corresponding to the eight regions that were sampled in this study and defined by a different color in Fig. 2; referred hereafter as the western group), and the other to create three new eastern and northern populations (corresponding to the Urals, the Altai and mountains of east Tuva, and Scandinavian populations sampled in this study; referred hereafter as the northeast group). At this stage, all populations are of equal size and migration occurs within the western group, the northeast group, and the group of ancestral populations, but not among these groups. In both t_2 and t_1 , gene flow follows a two-dimensional stepping-stone model in which populations are ordered according to their geographic position. Migration is implemented between pairs of contiguous regions on the map of Eurasia. In the migration matrix, nonzero migration rates are restricted to only two different values, (1) to reduce the parameter space to explore, and (2) because the distances between contiguous regions within the northeast group or between contiguous regions within the rest of the distribution is of the same order of magnitude. In the northeast group, migration rates are 10 times higher than elsewhere, to reflect the greater geographic distances separating the sampled regions. Gene flow between nonadjacent regions was always set to zero, because *G. pallida* is believed to be virtually incapable of long-distance dispersal. In other words, the probability of migration between two adjacent regions should be so much higher than between nonadjacent regions, that the latter is considered to be negligible. Finally, in the most recent stage (t_0), gene flow between any pair of populations is equal to zero, reflecting the most fragmented state of the species distribution, as it is known today. At the beginning of the coalescent simulation (going backward in time), the sample size of each population equals the sample size of the empirical data.

In the second hypothesis (Fig. 1; secondary contact hypothesis), the distribution range of *G. pallida* was split in a western and

A ancestral refuge model



B secondary contact model

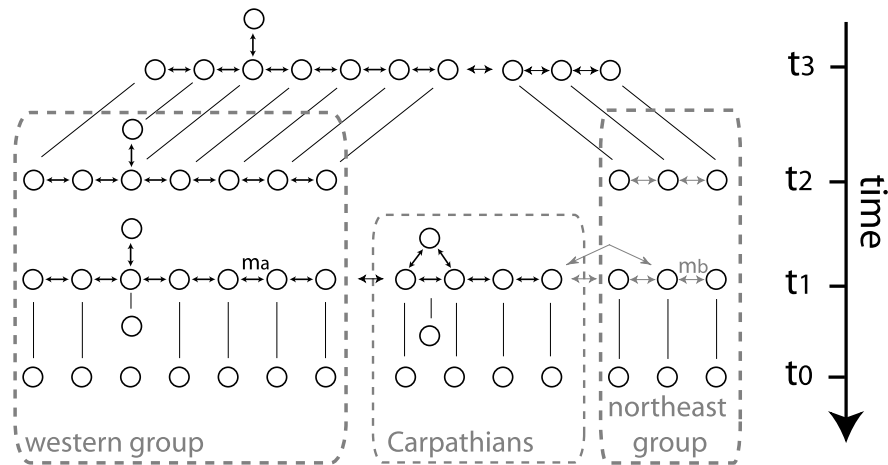


Figure 1. Description of (A) the ancestral refuge model and (B) the secondary contact model developed to compare the two historical hypotheses that could explain the pattern of genetic diversity uncovered in this study. Each circle represents a population of leaf beetles, and a double arrow indicates ongoing gene flow between two populations. m_a and m_b denote two different migration rates. Each line in the model corresponds to a change in population structure occurring at a specific time (t_0 , t_1 , t_2 , and t_3). The range of parameter values investigated, as well as the values used to generate the distribution of the simulated ratio of nucleotide diversities, can be found in the text.

a northeastern group, sometime in the past. After being isolated from each other for a substantial amount of time, individuals from both groups meet again inside the Carpathian range, thereby creating a secondary contact zone. The following coalescence model (described hereafter going forward in time) was designed to test this hypothesis: In the most ancestral stage (t_3), eight populations from the western group and three populations from the northeast group (corresponding to the populations sampled in this study) are connected by gene flow in a two-dimensional stepping-stone

fashion (same conditions as in model 1). Then, in a second stage (t_2), the western and northeast groups are isolated from each other. In a third stage (t_1), one region from the western group and two regions from the northeastern group (those that are closest to the Carpathian mountains) are each allowed to colonize one of the five populations from the Carpathian range. These five populations are connected by gene flow in a one-dimensional stepping-stone fashion. The two previously isolated groups are therefore connected again through migration across the Carpathian Mountains.

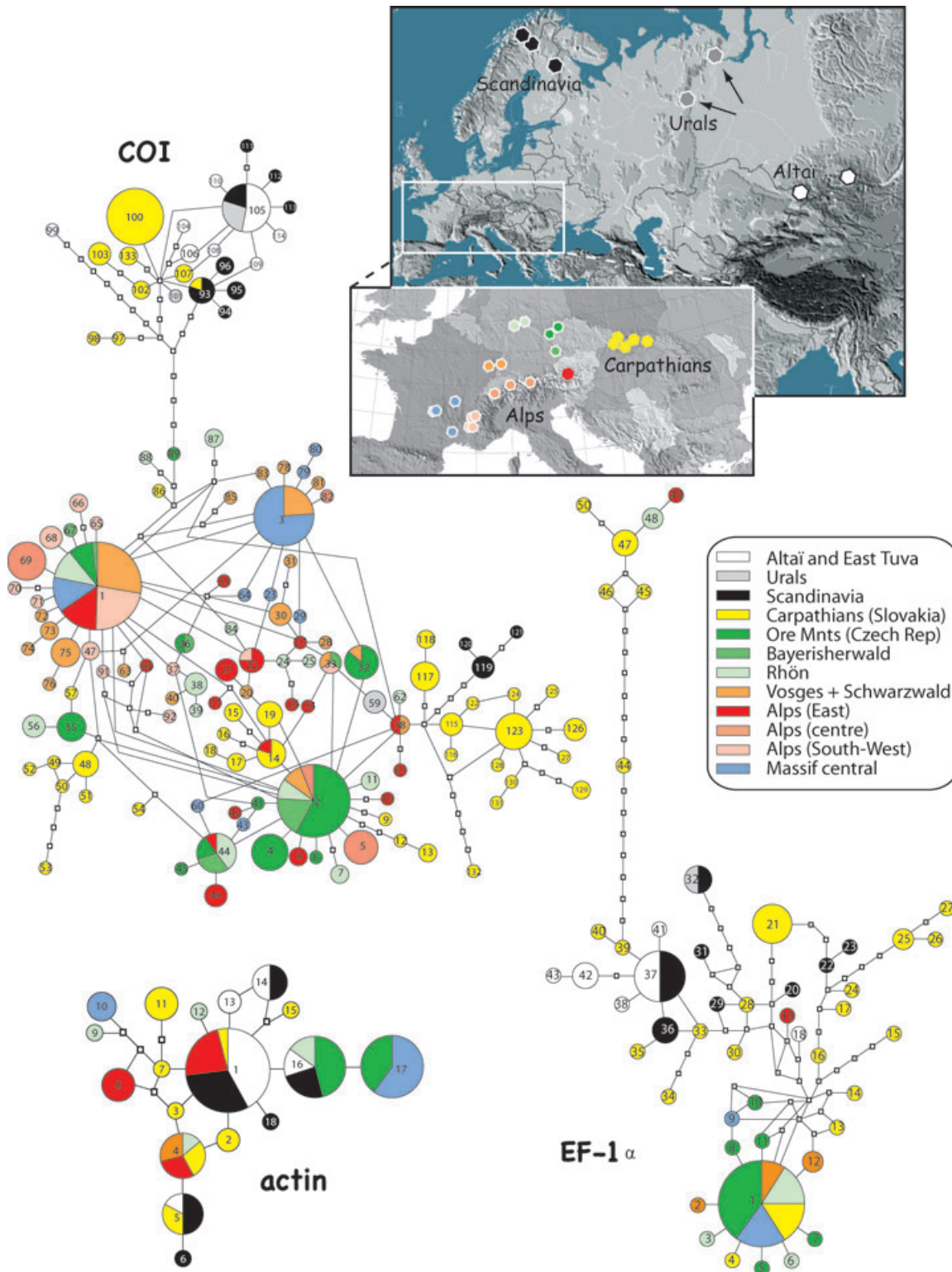


Figure 2. Median-joining networks for the three gene fragments (Cytochrome oxidase I, COI; Elongation factor 1- α , EF-1 α ; actin) sequenced in this study. Each sequenced haplotype is represented by a circle, the size of which is proportional to its overall frequency, and identified by a unique number (see also Table 1). Each line in the network represents a single mutational change. Small squares indicate intermediate haplotypes that are not present in the sample, but are necessary to link all observed haplotypes to the network. Sampling sites are shown on a map of Eurasia and were pooled into broader geographic entities roughly corresponding to isolated regions, such as mountain ranges, that are identified by a specific color. These same colors are used directly on the haplotype networks to show the geographic distribution of haplotypes among regions.

Finally, in the most recent stage (t_0), gene flow between any pair of populations is equal to zero, reflecting the most fragmented state of the species distribution, as it is known today. As in the ancestral refuge model, at the beginning of the coalescent simulation (going backward in time), the sample size of each population equals the sample size of the empirical data.

Simulations in step 1

We performed several simulations under each model for testing different values of the following parameters (10 replicates per parameter value combination): migration rates (both models: 0.01, 0.001, 0.0001), population size (both models: 1000–10,000,000), time of historical events (the time at which a new stage of the model is reached; ancestral refuge model: time 1 = 10,000–50,000 generations, time 2 = 10,000–100,000 generations; secondary contact model: time 1 = 10,000–50,000 generations, time 2: two generations after time 1, time 3: 10,000–100,000), extent of bottleneck (ancestral refuge model—expressed as a proportion of the initial population size: 0.01, 0.0001, 0.000001), and mutation rate (10^{-7} – 10^{-9} substitutions/site/generation). For each simulated dataset, a median-joining network was inferred and was compared to the median-joining network inferred from the empirical data. More specifically, we checked (1) whether the overall number of mutations and of haplotypes (both measures quickly accessible in NETWORK) were similar (less than 10% difference), (2) the presence of a phylogeographic break (only for COI and EF-1 α) between haplotypes of the western group and haplotypes from the northeastern group, which are separated by a reasonable number of mutations (1–10 mutations), and (3) the presence of Carpathian haplotypes at least among the western group haplotypes and the northeast group haplotypes. If these three conditions were met by at least one simulated dataset of ten, the combination of parameter values associated with the simulation was accepted.

Simulations in step 2

Within the parameter space identified in step 1, different combination of parameter values was used to simulate 100 datasets under both models. These datasets were analyzed with ARLEQUIN, to infer nucleotide diversity within the Carpathian region and within the rest of the species distribution. The distribution of the ratio between these two values was generated and compared among different sets of parameter values. In the end, 200 datasets were generated under each model with the tested set of parameter values that generated the highest calculated ratios of nucleotide diversities (ancestral refuge hypothesis: $m_a = 0.001$, $m_b = 0.0001$, time 1 = 20,000 generations, time 2 = 20,500 generations; COI: $N_1 = 1,000,000$, $N_2 = 20$, $\mu = 2 \times 10^{-8}$; EF-1 α : $N_1 = 10,000,000$, $N_2 = 10$, $\mu = 2 \times 10^{-8}$; actin: $N_1 = 1,000,000$, $N_2 = 20$, $\mu = 17 \times 10^{-9}$; secondary contact hypothesis: $m_a = 0.001$, $m_b =$

0.0001, time 1 = 20,000 generations, time 2 = 20,010 generations, time 3 = 50,000 generations; COI: N at t_0 and $t_1 = 100,000$, N at t_2 and $t_3 = 500$, $\mu = 5 \times 10^{-8}$; EF-1 α : N at t_0 and $t_1 = 30,000$, N at t_2 and $t_3 = 150$, $\mu = 7 \times 10^{-8}$; actin: N at t_0 and $t_1 = 50,000$, N at t_2 and $t_3 = 250$, $\mu = 35 \times 10^{-9}$). Note that increasing the size of the populations over time in the secondary contact hypothesis was necessary to meet the three criteria in the first test. A null distribution of the summary statistic considered (i.e., the ratio of nucleotide diversities) was generated against which the value calculated from the empirical data was compared.

Alternative ancestral refuge model

Finally, we have also simulated 200 datasets for each gene fragment under an ancestral refuge model slightly different from the one described above. This was done only once, with the parameter values selected for the first ancestral refuge model at the end of step 2. In this second ancestral refuge model, all populations outside the Carpathians are subject to a severe size reduction in t_2 , as was already the case for the first version of the model (see Fig. 1), but this time are not reunified (going backward in time) in a single western and a single northeast population. Instead, each sampled population is continuously occupied at each stage, with migration rates among populations from the western or the northeast group in t_2 unchanged from t_1 . Migration between the Carpathian populations and the two other groups is implemented as in the first version of the model (with migration occurring only between contiguous regions). The aim of these simulations was to explore the possibility that more than two ancestral bottlenecked populations are the source of the contemporary populations outside the Carpathians (see Results and Discussion).

ESTIMATION OF THE TIME OF COLONIZATION

Although there is no specific *Goniocetena* molecular clock calibration available for any of the gene fragments sequenced, we have attempted, with the COI sequences, to get a rough estimate of the time of colonization of the current species range, from an ancestral population, assuming the first hypothesis described above (i.e., the ancestral refuge hypothesis) is correct. We have used a model of coalescence, similar to the one used to test this hypothesis (see above), to estimate the times of the two defined historical events (the time since all regions are isolated from each other [time t_1 in Fig. 1], and the time of colonization of the distribution from two bottlenecked populations [time t_2 in Fig. 1]).

Molecular clock calibrations are available for some mitochondrial genes in a variety of insects (Zakharov et al. 2004), including some beetles, and range from 2.8×10^{-9} (for ND5 in carabid beetles; Su et al. 1998) to 4.9×10^{-8} (for COII, vs. 4.3×10^{-8} for COI, both in honey bees; Crozier et al. 1989) substitutions per site and per generation. For the purpose of estimating the

time of colonization, we have considered that the COI mutation rate in *G. pallida* was included within this range, thereby making a rather conservative assumption.

As noted by Hudson (1990), both the coalescence of lineages (going backward in time) and the accumulation of mutations along the branches of the genealogy are stochastic processes contributing to the uncertainty of a time estimate. Modeling of these processes can serve to infer the variance associated with this estimate (e.g., Griffiths and Tavaré 1994; Tavaré et al. 1997). The use of approximate methods based on summary statistics has been proposed to estimate population evolution parameters such as the time of divergence (Tavaré et al. 1997; Pritchard et al. 1999; Beaumont et al. 2002). Replacing the full data by a limited number of summary statistics greatly reduces the calculations and thus allows one to investigate a much wider range of model parameters and to implement more complex coalescence models. We have used such an approach, based on the calculation of three summary statistics: the number of mutations on the genealogy (M_n , easily measured from the observed data by counting the number of mutations on the inferred most parsimonious tree, as already suggested by Fu [1997]), the number of allele types in the sample (K_n), and the s parameter of Slatkin and Maddison (1989), which is used here to characterize the geographic distribution of the haplotypic variation (the more scattered on the genealogy are gene copies from each delimited region, the higher the s value). Our hope is that these three statistics capture most of the information in the data. A modified version of the program Trees Sifter (Mardulyn 2007; <http://ueg.ulb.ac.be/treesSifter/>) was created to allow the use of the additional s statistic, and was used to perform these analyses. A total of 120,000 genealogies were simulated following a model similar to the one described above for the first hypothesis (parameter values: time of historical event randomly chosen at each simulation between 10,000 and 400,000 generations, mutation rate randomly chosen between 2.8×10^{-9} and 4.9×10^{-8} mutations/generation/site, population size: equal number of simulations run with all populations of size 100,000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, and 800,000 except for populations that are subject to a founder event, which were 100 times smaller, migration rate of 0.001 between populations exchanging migrants). Each simulated genealogy was then accepted or rejected depending on the size of the differences between the simulated and empirical values (i.e., the values calculated from the DNA sequence data) of the three summary statistics considered (Pritchard et al. 1999; $\Delta = 0.1$). In the end, the accepted genealogies were used to construct an estimation of the probability density function of several variables, for example the time to the most recent common ancestor (TMRCA). Since the time of colonization (the parameter we seek to estimate) is varied between each simulation (chosen randomly within a range of 10,000 to 400,000 generations), an estimate

of the probability density function of this parameter could be derived.

Results and Discussion

DATASETS

The three complete datasets of aligned sequences (COI, EF-1 α , and actin) contain 994, 1180 (including gaps and a 768 bp-long intron), and 654 nucleotides, respectively. Among these, 107, 34, and 14 were found polymorphic. In addition, 15 polymorphic characters corresponding to the coding of gaps were added to the EF-1 α dataset.

OBSERVED GENETIC VARIATION AND HYPOTHESIS TESTING

A haplotype network, in other words a graphical representation of the evolutionary relationships among gene copies, is presented in Figure 2 for the three gene fragments sequenced. Two main features of the data, important for the interpretation of the evolutionary history of this species, are highlighted by this figure. First, gene copies that are found in one geographic region do not form a monophyletic group, but are instead scattered in different places of the network. Isolation among mountain ranges has therefore not been sufficiently long to allow for the completion of lineage sorting among them.

Second, genetic diversity within populations seems much higher in the Carpathian region than in any other region of the species distribution: Carpathian haplotypes (in yellow) are scattered over the entire networks of Figure 2. This was confirmed by the calculation of the nucleotide diversity within each region (Table 2). Moreover, the nucleotide diversity calculated for all individuals sampled in the Carpathians was higher than the nucleotide diversity calculated for all other collected individuals for this study. That is, the genetic variation present in the Carpathian Mountains alone, which represents a fairly small portion of the entire species range, was higher than the genetic variation uncovered in the rest of the species distribution, which spans a large portion of Europe and Siberia. This is true for all three gene fragments taken separately. Indeed, the ratio of the nucleotide diversity calculated in the Carpathian region to the nucleotide diversity calculated in the rest of the species distribution (i.e., calculated for all individuals collected, minus the Carpathian individuals) was 1.80, 3.20, and 1.31 for the COI, EF-1 α and actin gene fragments, respectively.

The presence of such a genetic diversity hot spot in the Carpathian Mountains can be interpreted in two alternative ways (e.g., Petit et al. 2003): either (1) this area is located in or near an ancient refuge in which the species has survived during less favorable times, for example during a severe glacial period, or (2) the same area is a secondary contact zone (or hybrid zone, in the case

Table 2. Nucleotide diversity (mean pairwise differences, in bold) within each sampled region, with standard deviations.

	Massif central	Alps (south-west)	Alps (center)	Alps (east)	Vosges+ BF	Bayerischerwald	Ore mnts	Rhön	Carpathians	Scandinavia	Urals	Altai
COI	0,0010	0,0015	0,0018	0,0029	0,0022	0,0014	0,0017	0,0035	0,0089	0,0068	0,0054	0,0024
SD	0,0008	0,0010	0,0012	0,0018	0,0014	0,0010	0,0011	0,0021	0,0046	0,0037	0,0032	0,0015
EF-1 α	0,0025	-	-	0,0619	0,0021	-	0,0015	0,0301	0,0423	0,0082	0,0000	0,0057
SD	0,0017	-	-	0,0623	0,0015	-	0,0010	0,0165	0,0208	0,0045	0,0000	0,0032
actin	0,0025	-	-	0,0028	0,0000	-	0,0008	0,0049	0,0044	0,0029	-	0,0017
SD	0,0018	-	-	0,0019	0,0000	-	0,0008	0,0035	0,0028	0,0019	-	0,0013

in which the individuals from both origin mate and reproduce) in which individuals from two areas, which have been isolated from each other for a long period of time, meet again. This second hypothesis is plausible because, in the COI haplotype network, the haplotypes found in western and central Europe, excluding the Carpathian haplotypes, are separated by several mutations from a group of haplotypes that were observed in the Altai and mountains of East Tuva, the Ural mountains, and Scandinavia. A similar pattern of variation can be found in the EF-1 α network as well, although the northeast/southwest differentiation is less pronounced in that case. It could therefore easily be hypothesized that the species distribution was at some point separated in a north-east and a southwest area, and that today's Carpathian range is a secondary contact zone in which both previously isolated groups are again connected.

Yet, if the secondary contact zone hypothesis is correct, we would intuitively expect that the level of genetic diversity inside this contact zone would be smaller, or at most could equal the genetic diversity found in the rest of the distribution. Indeed, the haplotypes found in a secondary contact zone are made up of the addition of haplotypes coming from the previously isolated areas. On the other hand, the level of genetic variation encountered in an ancient refuge area can be expected to be higher than in the rest of the species distribution, as is the case here, because populations outside the refuge area were likely subject to strong reduction in size (bottleneck or founder events) that severely decreased their level of genetic variation.

To formally discriminate between the two competing historical explanations described above, we have conducted computer simulations following two models of structured coalescent, each designed to simulate one of the two evolutionary scenarios. Figure 3 shows the null distribution of the nucleotide diversity ratio, generated by computer simulation under both hypotheses. Although this ratio was sometimes higher than 1 for the data simulated under the secondary contact scenario, it was never as high as the ratio calculated for the empirical data, in the case of the COI and EF-1 α gene fragments (for actin, only 4% of the simulated ratios were equal or higher than the ratio calculated from the empirical data). In other words, we were not able to replicate the observed pattern of genetic diversity in our simulations under the secondary contact hypothesis, at least within the framework of the model used and with the parameters values explored. On the contrary, the ratio calculated for the empirical data was compatible with the distribution generated under the Carpathian refuge scenario for all three gene fragments. Our simulation study therefore favors the Carpathian refuge hypothesis over the secondary contact scenario.

Because the multidimensional space of all possible combinations of parameter values for each model is infinite, it was obviously possible to explore only a small portion of it. The above

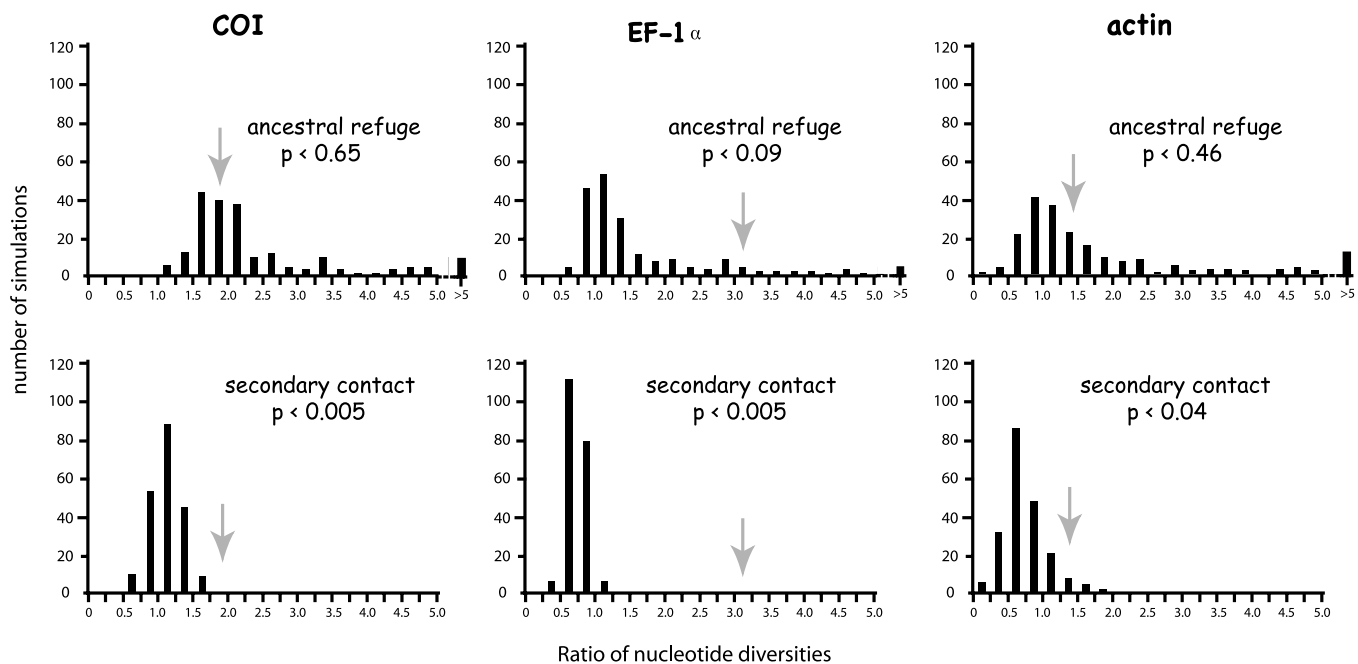


Figure 3. Null distribution of the ratio of nucleotide diversities (nucleotide diversity within the Carpathian Mountains samples divided by the nucleotide diversity calculated from all sequences of the rest of the distribution) generated by coalescent simulations (see text) under the two historical hypotheses considered in this study to explain the presence of a genetic diversity hot spot in the Carpathian Mountains: (A) the ancestral refuge hypothesis, in which the Carpathian Mountains host an ancient refuge population, whereas the rest of the species distribution experienced a dramatic reduction in size resulting in a much lower level of genetic variation, (B) the secondary contact hypothesis in which two areas of the species distribution have been isolated from each other for a long period of time, and meet again in the Carpathian Mountains. An arrow indicates the position of the ratio calculated from the empirical data. Probability values associated with each distribution are indicated.

conclusion therefore only applies to the combinations of parameter values that were tested. However, in our simulations analysis, we tried as much as possible to sample that space in a homogeneous fashion, to identify one or more regions that could generate patterns of genetic diversity compatible with the empirical observations. This analysis has shown that the crucial parameter to simulate data compatible with the observed genetic data (in the ancestral refuge model) was the size of the bottlenecked populations relative to the Carpathian populations. That is, the reduction in population size outside the Carpathians needed to be fairly strong to allow the simulated data to be in accordance with the observed ratio of genetic diversities. Changes in other parameters had much less impact on the difference in genetic diversity between the Carpathian populations and the rest of the *G. pallida* distribution. This formal investigation of the impact of several parameter changes on patterns of genetic diversity has thus confirmed our initial intuitive suggestion that the relatively high level of genetic variation observed in the Carpathian mountains can more easily be explained by historical events that caused strong reduction in genetic diversity (bottleneck or founder events) elsewhere, than by the occurrence of a secondary contact zone in this area. We suspect that, although exploring additional combinations of parameter values might slightly modify the distribution of the

ratio of nucleotide diversities presented in Figure 3, the ancestral refuge model will always have the potential to generate higher levels of nucleotide diversities ratios than the secondary contact model, because strong reductions in populations sizes outside the Carpathian region is only possible in the first model (allowing this to happen in the secondary contact model would in essence transform the secondary contact model in an ancestral refuge model).

TIMING OF HISTORICAL EVENTS

The observed pattern of genetic variation in *G. pallida* contrasts with the phylogeographic structure uncovered in many temperate climate species, in which well-differentiated evolutionary lineages were found to be geographically separated. It is then tempting to conclude that the fragmentation of the range of *G. pallida* occurred well after the fragmentation of the range of these temperate species, perhaps at the end of the last ice age (the geographic distribution of the cold-adapted *G. pallida* was likely to be much less fragmented during glacial periods).

However, the vast majority of the COI haplotypes found outside the Carpathian Mountains (i.e., in the rest of the species range) are absent from the Carpathians (see Fig. 2). Similarly, no EF-1 α haplotypes found in Scandinavia, the Ural Mountains, or

the Altai and mountains of east Tuva, in Siberia, are present in the Carpathian samples. Therefore, the isolation of the Carpathian refuge populations from the rest of the distribution must have occurred a long time ago. Had this separation occurred only recently, a higher proportion of haplotypes would be shared between the Carpathian populations and the other populations.

Using this principle, we have attempted to estimate the time of isolation of the Carpathian refuge from the COI data, for which a molecular clock calibration is available for a variety of insects. The probability distribution estimates of (1) the time when the defined regions (see Fig. 2) became isolated and (2) the time of the colonization of Eurasia from two bottlenecked ancestral populations, indicate that both events are estimated to have occurred well beyond the time of the last glacial maximum (Fig. 4), > 30,000 years ago (no accepted simulation below that date; assuming one generation per year) and >90,000 years ago (less than 5% of the accepted simulations below 90,000 years), respectively.

These time estimates are not compatible with the end of the last ice age. A period of 10,000–20,000 years of isolation among mountain ranges is not long enough to explain the inferred pattern of COI sequence variation. It is therefore likely that the species range of *G. pallida* was also fragmented, at least partially, during the last ice age. The second time estimate sets the severe size reduction of the ancestral population (except in the Carpathian refuge) 90,000 years ago at the earliest, i.e., at the beginning of the last glaciation event (Early Weichselian glaciation; Svendsen et al. 2004). The COI data are however compatible with an even older time, up to at least 400,000 years ago. If we are willing to assume that a major climatic event is responsible for the hypothesized restriction of the *G. pallida* range to a small refuge in or near the Carpathian Mountains some time in the past, it is probably worthwhile to look for possible climatic event candidates in the earth's known history. For example, it is thought that a huge ice sheet formed over northern Eurasia 130,000 to 160,000 years ago, more extensive (reaching the Carpathian Mountains) and probably longer lasting than those of the Weichselian (late Saalian glaciation, Svendsen et al. 2004). A plausible hypothesis would therefore suggest that the more severe glaciation that occurred at that time has forced even cold-adapted organisms as *G. pallida* to retreat within one or more refuge areas in central Europe.

REMAINING QUESTIONS

One important limitation of the so-called statistical phylogeography approach (Knowles and Maddison 2002) used here, in which historical hypotheses are explicitly defined and tested through coalescent modeling, is that strong assumptions about population history are made before the data are analyzed. The space of possible historical scenarios, which is much too large to be explored exhaustively, is considerably reduced a priori, based at least in part on external information (geographic location of contempo-

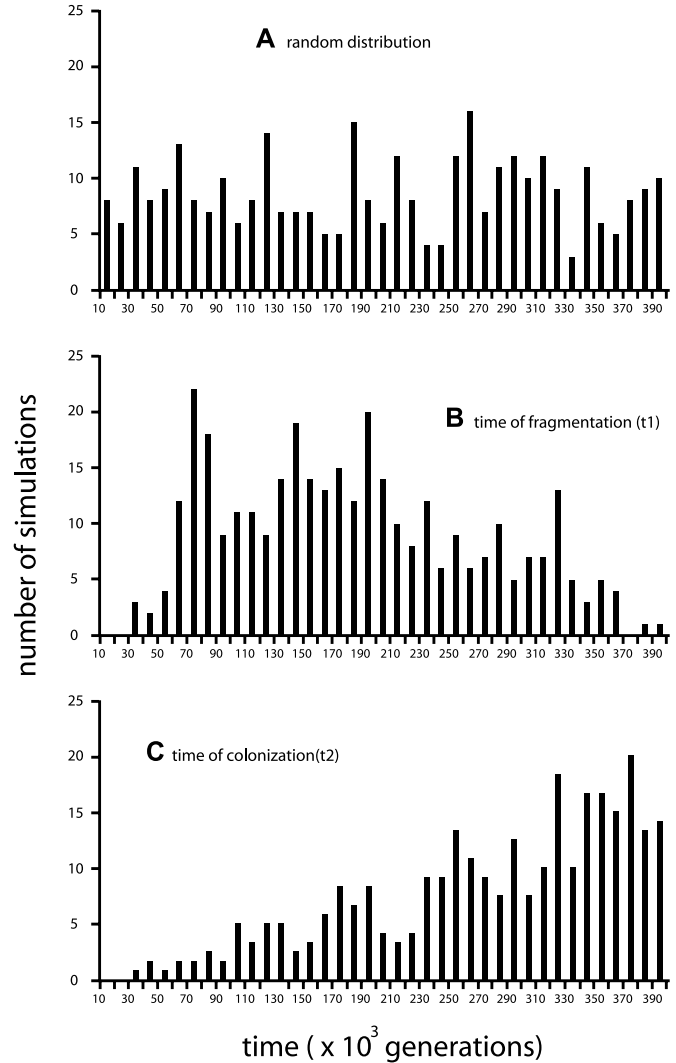


Figure 4. Estimate of the posterior probability distribution of the time of fragmentation t_1 (B) and of the time of colonization t_2 (C) of the ancestral refuge model using the COI DNA fragment. Coalescent simulations of 120,000 genealogies following the ancestral refuge model defined a priori, coupled with the rejection algorithm described in the Material and Methods section, resulted in 337 accepted genealogies, from which the distributions were derived. A distribution of 337 time values, chosen at random in the same time range as for the coalescent simulations, is shown in (A) for comparison. The distributions in (B) and (C) are wide, in part because of the large range of mutation rate values used for the simulations, but do offer a conservative lower bound for the estimated variables (the upper bound was constraint to 400,000 generations in our simulations, biasing the upper bound estimate for t_2).

rary populations, evolutionary history of past climatic conditions, and estimates of how it has affected the species geographic distribution, inability of *G. pallida* to disperse over long distances, . . .), excluding perhaps potentially interesting scenarios from statistical scrutiny. Hence, we cannot exclude the possibility that another

historical hypothesis that we have not considered here can explain better the observed molecular data. Nonetheless, an unusually high level of genetic diversity found in a small area (here the Carpathian region), is usually interpreted in the phylogeography literature (e.g., Avise 2000; Petit et al. 2003) as a case of ancestral refuge or secondary contact, and we cannot think of another hypothesis that could explain this pattern.

Although our analyses appear to exclude the possibility of the Carpathian mountains being a secondary contact zone for *G. pallida*, and rather suggest that it is an ancestral refuge area for that species, some important details of its evolutionary history remain open to question. In our coalescent ancestral refuge model, two ancestral source populations go through a severe size reduction before colonizing, respectively, the west and the east of the species distribution. However, the location of these two ancestral populations is unknown. It could be that the reduction in size reflects a founder event associated with the colonization of the current distribution of *G. pallida* from the Carpathian area. Alternatively, these two ancestral populations could have been located elsewhere, and been subject to a strong bottleneck event before colonizing the western and eastern part of the geographic distribution, respectively. Also, the number of bottlenecked ancestral populations colonizing Eurasia was set to two in our coalescent model for convenience, but could be larger in principle. In fact, we have performed additional simulations with a slightly different ancestral refuge model, in which each sampled population outside the Carpathian region has a distinct ancestral population that goes through a size reduction identical in amplitude to the size reduction of the western and northeast ancestral population in the first model (see Materials and Methods). In other words, in this model, all populations are continuously occupied, but populations outside the Carpathian region go through a severe bottleneck. For all three gene fragments, the distribution of the ratio of nucleotide diversities generated under this second version of the ancestral refuge model is slightly displaced toward the left compared to the ancestral refuge distribution shown in Figure 3. For the COI and actin fragment, these distributions are still largely compatible with the empirical value of the summary statistic, but not for the EF-1 α fragment ($P < 0.03$). Because these two versions of the ancestral refuge model implement two extreme case in terms of the number of ancestral bottlenecked populations (from one ancestral population for the entire western or northeast group, to one ancestral population for each sampled contemporary population), we cannot rule out the possibility that more than two ancestral bottlenecked populations are the source of the contemporary populations outside the Carpathians.

Is there any evidence that the Carpathian area is an ancestral refuge for other animals and plants as well? A Carpathian refuge was already suggested for several temperate-climate vertebrates (e.g., field vole, bank vole, adder; reviewed in Provan and

Bennett 2008) during the Pleistocene. In these cases, however, the Carpathian area is one of several glacial refuges that were suggested. In the case of *G. pallida*, this region is the only glacial refuge that we have identified (although some future additional sampling could highlight others). A few studies using molecular markers have investigated the phylogeographic patterns encountered in some arctic-alpine distributed species, that are thus also adapted to low temperatures. For example, AFLP data suggested the separation of the mountain avens in a southern and eastern lineage, possibly reflecting isolation and expansion from two glacial refugia (Skrede et al. 2006). An investigation of the phylogeography of the wolf spider using the mitochondrial ND1 gene showed that the haplotypes were separated in three deeply divergent clades, (1) a northern European clade, that includes Scandinavian, alpine, and Carpathian populations, (2) a Pyrenean clade, and (3) a Balkan clade (Muster and Berendonk 2006). Low pairwise genetic divergence was found among populations of the northern European clade, and the authors suggest its recent origin from a single source population. So far, to the best of our knowledge, no phylogeographic studies other than the present one have suggested that the Carpathian Mountains had been an ancestral refuge for cold-adapted species in the past. Heckel et al. (2005) did suggest that the cold-tolerant common vole, *Microtus arvalis*, colonized Europe from the east, before the last glacial maximum. Although they did not identify the population of origin of this expansion, it could have originated from the Carpathians. Data from more organisms, including other insects, are needed to find out whether the Carpathian range can be considered as a major refugial area for cold-adapted taxa, or if the pattern found in *G. pallida* is specific to that insect species.

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