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Conflicting mitochondrial and nuclear phylogeographic signals and evolution of host-plant shifts in the boreo-montane leaf beetle *Chrysomela lapponica*

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ABSTRACT

We conducted a phylogeographic study on the cold-adapted leaf beetle *Chrysomela lapponica*, that feeds on willow or birch, by sampling several populations throughout most of the geographic distribution of the species, and by sequencing for each individual one mitochondrial and two nuclear DNA fragments. Patterns of DNA sequence variation from the mitochondrial and nuclear loci, as displayed in the median-joining networks, appear to display contradicting historical signal: a deep genealogical divergence is observed with the mitochondrial genome between the Alpine population and all other populations found in the Euro-Siberian distribution of the species, that is completely absent with both nuclear loci. We use coalescence simulations of DNA sequence evolution to test the hypothesis that this apparent conflict is compatible with a neutral model of sequence evolution (i.e., to check whether the stochastic nature of the coalescence process can explain these patterns). Because the simulations show that this is highly unlikely, we consider two alternative hypotheses: (1) introgression of the mitochondrial genome of another species and (2) the effect of natural selection. Although introgression is the most plausible explanation, we fail to identify the source species of the introgressed mitochondrial genome among all known species closely related to *C. lapponica*. We therefore suggest that the putative introgression event is ancient and the source species is either extinct or currently outside the geographic range of *C. lapponica* explored in this study. The observed DNA sequence variation also suggests that a host-plant shift from willow to birch has occurred recently and independently in each of the three birch-feeding populations. This emphasizes further the relative ease with which these beetles can escape their ancestral host-plant specialization on willow, but shows at the same time that host-plant shifts are highly constrained, as they only occur between willow and birch.

1. Introduction

An increasing number of published phylogeographic studies are collecting multilocus nuclear DNA sequences to complement the more traditional mitochondrial or chloroplastic sequence data, including studies of non-model organisms, despite the difficulties associated with the sequencing of nuclear gene fragments in heterozygote individuals (e.g., Villablanca et al., 1998; Knowles and Carstens, 2007; Peters et al., 2008; Chen et al., 2009; Nevado et al., 2009; Li et al., 2010). This relatively recent trend is certainly welcome, as it is now well established that a single locus is of limited use for inferring population history (Edwards and Beerli, 2000; Hare, 2001; Zhang and Hewitt, 2003; Hey and Nielsen, 2004; Edwards and Bensch, 2009), first because it is often impossible to disentangle the effect of selection and demographic history on the observed pattern of sequence variation in a single locus, and second, because the stochastic nature of the coalescence process tends to produce large confidence intervals around estimated values of historical parameters.

Because unlinked or partially linked loci experience different histories of transmission across generations, and because this process is largely stochastic, the resulting pattern of intra-specific sequence variation can vary greatly among loci (Hudson, 1990; Hein et al., 2005; Wakeley, 2009). When analyzing multilocus DNA sequence data in a phylogeographic context, the challenge becomes to decide whether the observed differences among loci is only caused by the randomness of the coalescence process, or if another evolutionary force, such as selection, is also responsible. In the latter case, extreme caution should be used when inferring demographic history from genetic variation. Here, we report a case where the comparison of the phylogeographic signal from multiple DNA fragments has revealed one major discrepancy between the mitochondrial and nuclear loci. We use coalescence simulations

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of DNA sequence evolution to test the hypothesis that this apparent conflict is compatible with a neutral model of sequence evolution, i.e., we test whether the stochastic nature of the coalescence process can explain the observed difference between the mitochondrial and nuclear patterns of variation, and then explore two alternative hypotheses, namely the possibility (1) that the observed pattern of sequence variation at this locus is caused by a past introgression event of the mitochondrial genome from another related species and (2) that the evolution of the mitochondrial DNA fragment is driven at least partially by selection.

The species studied is a chrysomelid beetle that, like most of its kind, is specialized on a few host plant species, in this case, trees either of the genus *Salix* (willow) or of the genus *Betula* (birch). It is distributed across the Palearctic region, spanning Europe and Siberia, and reaching Japan to the east, Mongolia and Northeast China to the southeast (Bukejs, 2010). It is adapted to cold climate, and currently displays a highly fragmented distribution: while distributed all over Lapland, i.e. north of the Arctic Circle, it is found only in a few scattered populations located at mid or high elevations (450–2000 m above sea level) elsewhere. Given this geographic distribution, these populations can probably be considered as relict populations from a much more widespread distribution that occurred when climate conditions were more favorable, i.e. during glacial periods.

Phylogeographic studies in Europe have largely focused on temperate-climate species, and much less on cold-adapted organisms. One previous study of another cold-adapted Euro-Siberian leaf beetle, *Gonioctena pallida*, (Mardulyn et al., 2009) has highlighted unique features in the pattern of genetic variation compared to what is observed for other temperate species, notably (1) the absence of one or more deep genealogical split in the inferred allele network and (2) the presence of a diversity center, interpreted as an ancestral refuge area, in central Europe (Carpathian mountains region). Before any general conclusion can be inferred however, on the impact of past climate history on the distribution of genetic variation in cold-adapted herbivorous insects, phylogeographic data on other species should be collected.

Molecular data on genetic variation within this species has also the potential to shed some light on the evolution of the interaction with its host plant. Major differences in host plant preferences are indeed observed among populations: while in most populations, larvae and adults feed exclusively on willow (*Salix)*, in a few others, they only feed on birch (*Betula)* (Gross et al., 2004). Laboratory tests show that in many cases, the beetles develop normally only if fed on the host plant on which they are feeding in the wild (Hilker and Schulz, 1994; Fatouros et al., 2006). Moreover, the plant diet influences larval chemical defense: larvae feeding on *Salix* secrete salicyl aldehyde derived from host plant phenolglucosides as major toxin, whereas those feeding on *Betula* secrete mainly isobutyrates and 2-methylbutyrates of mixed plant–insect origin (Schulz et al., 1997). In the latter case, the acid moieties are neo-synthesized by the insects from amino acids and the alcohol moieties are derived from the food plant. Individuals from at least two populations feeding on *Betula*, one in the Ore mountains (Hilker and Schulz, 1994), the other in South Altaï (Kirsch et al., 2011), are unable to produce salicyl aldehyde when fed with *Salix*.

Strong genetic differences among populations collected from different regions (Lapland and different mountain ranges in Europe) were previously reported for *Chrysomela lapponica* using allozyme data, suggesting the absence of migration among them (Machkour-M'Rabet et al., 2008). This is easily explained by the combination of a highly fragmented geographic distribution, with the poor capabilities of dispersal characterizing many species of leaf beetles (Knoll et al., 1996). The complete isolation of populations may have favored the observed local adaptations to the two different host plant genera. In this context, molecular sequence variation has the potential to help discriminate between two alternative hypotheses: Did the switch from willow to birch occur only once (assuming *Salix* is the ancestral host genus as suggested by Termonia et al. (2001)), with all populations specialized on birch derived from a single ancestral population feeding on that same host plant? Or did the switch from willow to birch occur several times independently?

To investigate these questions, a phylogeographic study was conducted on *C. lapponica*, by sampling several populations throughout the entire geographic range of the species, and by sequencing for each individual one mitochondrial and two nuclear DNA fragments.

2. Materials and methods

2.1. Samples and sequencing

C. lapponica samples were collected from 15 populations throughout most of its geographic range, across Europe and Siberia. Table 1 provides precise geographical locations, sample sizes, and host-plant information. 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 (1) 92 copies of a ~1300 base pair (bp) long fragment of the mitochondrial cytochrome oxidase 1 gene (COI), (2) 99 copies from a ~500 bp fragment of the nuclear elongation factor-1α gene (EF-1α), and (3) 165 copies from a ~ 500 bp fragment of the nuclear 60S acidic ribosomal protein P0 gene (RpP0), from these samples. Different publications have reported the transfer of mitochondrial DNA fragments to the nuclear genome in a wide range of animal species (e.g., Collura and Stewart, 1995; Bensasson et al., 2001). Therefore, in order to test that all COI fragments sequenced were of mitochondrial origin, we sequenced 10 copies of a ~550 bp fragment of another mitochondrial gene, the large subunit (16S) ribosomal RNA. These 10 copies were amplified and sequenced from individuals sampled in the Alps (2 from both locations), the Ural mountains (2), the Massif Central (2) and Finland (2). Our goal was to check whether the overall pattern of COI variation, which displayed one striking difference with those of the nuclear fragments, was similar to that of another, independently amplified, mitochondrial gene fragment. All fragments were PCR-amplified following the FastStart Taq DNA polymerase manufacturer's protocol (Roche). 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-1α fragment was amplified (annealing temperature of 54 °C) using primers 5′GGTATCACCATTGATATTGCHTTDTGGAA3′ and 5′ACCAGCAACATAACCACGACG3′, the RpP0 gene fragment was amplified (annealing temperature of 56 °C) using primers 5′ATGGGTAGGGAGGACAAIGCIACITGG3′ and 5′GCDATIGCICCIGIACGRGCYGGIG3′ (Gomez-Zurita et al., 2004), and the 16S ribosomal RNA gene fragment was amplified (annealing temperature 53 °C) using primers LR-J-12887 and LR-N-13398 (Simon et al., 1994). For nuclear genes, when a heterozygote individual was detected with more than one polymorphic site, new PCR products were generated with the Long Expand Template PCR System Kit (Roche; same PCR conditions as above), and were cloned in a no-background vector (*StabyCloning™ kit*, Delphi Genetics; 23 such heterozygotes cloned for RpP0, none for EF-1α). Five clones were sequenced and compared to the PCR product sequence to infer the two alleles of each heterozygote individual. All the allele sequences gathered for this project are available from GenBank under accession numbers JN543185–JN543224.

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

Region	Geographical coordinates	Host-plant genus	No. of indiv.	Alleles (no. of copies) COI	No. of indiv.	Alleles (no. of copies) EF-1α	No. of indiv.	Alleles (no. of copies) RpP0
Ore Mountains, Czech Republic	50°00′N, 12°40′E	Betula	7	8 (7)	5	9 (10)	9	1 (6), 3 (2), 4 (7), 10, 13
Massif Central, France	45°40′N, 2°24′E	Salix	6	1 (6)	3	3 (2), 4 (4)	6	1 (3), 8 (8), 9
Pyrenees, France	42°53′N, 0°58′E	Salix	5	7 (5)	4	4 (4), 6 (4)	5	1 (6), 8 (4)
Alps, France	44°20′N, 6°50′E	Salix	18	13 (18)	10	2 (13), 3 (6), 5	10	1 (5), 5 (3), 6, 7 (9), 9, 12
Alps, Italy	44°33′N, 7°07′E	Salix	8	13 (8)	3	2 (6)	8	6 (10), 7 (2), 9 (4)
Black Forest, Germany	48°18′N, 8°12′E	Salix	2	1 (2)				
Finland	67°14′N, 28 23′E	Betula	9	2 (9)				
Finland	69°45′N, 27°01 E	Salix	8	1 (2), 2 (6)	5	1 (10)	7	1 (10), 2, 15 (3)
North Ural, Russia	59°37′N, 59°17′E	Salix, Betula	5	2 (3), 8 (2)	2	7 (2), 8 (2)	5	1 (8), 3, 11
Polar Ural, Russia	67°50′N, 64°00′E	Salix	3	9, 11, 12	4	1 (7)	3	1 (2), 2 (4)
West Sibera, Russia	61°05′N, 69°26′E	Salix	5	6 (5)	4	1 (7), 8	8	1 (11), 2 (3), 9 (2)
South Altai, Kazakhstan	49°08′N, 86°01′E	Betula, Salix	6	2 (6)	10	1 (20)	10	1 (14), 14 (5), 15
South Altai, Kazakhstan	49°28′N, 83°23′E	Salix	3	10 (3)			3	1 (5), 10
South Altai, Kazakhstan	50°05′N, 87°40′E	Salix	1	5				
Mountains of East Tuva, Russia	51°41′N, 96°58′E	Salix	5	2, 4 (4)			9	1 (9), 16 (4), 17 (4), 18

2.2. Data analyses

Sequences were aligned manually with Se-Al (Rambaut, 2002). They were pruned at both 5′- and 3′- ends to ensure that no trailing gaps were present in the final dataset. A median-joining network (Bandelt et al., 1999) was inferred for each separate DNA fragment using the program Network (available at http://www.fluxus-engineering.com/sharenet.htm), with parameter epsilon = 0. A maximum likelihood tree was also inferred, for each locus separately, with the program PhyML version 3.0 (Guindon and Gascuel, 2003; Guindon et al., 2010). The most appropriate model of nucleotide substitution was previously identified for each locus using the Akaike information criterion, as implemented in the program jModeltest version 0.1.1 (Posada, 2008): HKY + I for the COI sequences, F81 for the RpP0 sequences, and HKY + I for the EF-1α sequences.

Sampled populations were pooled into broader geographic entities roughly corresponding to isolated regions (such as mountain ranges): Alps, Pyrenees, Massif Central, Schwarzwald, Ore Mountains, Scandinavia, Urals, Siberia, Altaï, mountains of East Tuva. Genetic differentiation among regions was then assessed by an analysis of molecular variance (AMOVA) (Excoffier et al., 1992) with the program Arlequin version 3.11 (Excoffier et al., 2005), separately for each gene fragment. The same program was used to compute pairwise F_{ST}'s between regions. A second median-joining network was inferred from COI sequences, for a smaller fragment (569 nucleotides), but with additional sequences from all other closely-related *Chrysomela* species available from GenBank: *C. aeneicollis* (AY027628), *C. falsa* (AY027618), *C. interna* (AY027619), *C. interrupta* (AY027610), *C. knabi* (AY027627), *C. littorea* (AY027614), *C. mainensis* (AY027617), *C. sp* (AY027625), *C. walshi* (AY027615). A maximum likelihood analysis using PhyML was also conducted on this sequence data set, with the best nucleotide substitution model (HKY + I) identified using the Akaike information criterion with the program jModeltest. Branch support was assessed with a bootstrap analysis (500 replicates). We used the phylogeny of Termonia et al. (2001), derived from the same COI mitochondrial gene, to determine which *Chrysomela* species were closest to *C. lapponica*.

To investigate the evolution of host-plant shifts in *C. lapponica*, we have estimated the host-plant species (*Salix* or *Betula*) of the ancestral node separating *C. lapponica* from other *Chrysomela* species, using a parsimony criterion, with the program MacClade version 4.08 (Maddison and Maddison, 2005). The maximum likelihood tree inferred from the small COI fragments (Fig. 4) was used for this analysis. The host-plant affiliation of the different *Chrysomela* species was assumed as described in Termonia et al. (2001). For *C. lapponica*, we used two separate branches, one leading to the alpine population, associated with *Salix*, and a second branch leading to all other *C. lapponica*, for which a dual plant association (*Salix* and *Betula*) was assumed (polymorphic state). Because we suggest in this article that the sequences from the alpine populations could be of foreign origin (hypothesis of introgression), we performed the same analysis after discarding the alpine lineage of *C. lapponica*, thus represented by a single branch associated with the two host-plant genera. Finally, the significance of the non-monophyly of the sequences from individuals feeding on *Betula*, suggested by all three loci separately, and the significance of the non-monophyly of the *Betula*-feeding populations, suggested by the RpP0 data set, were tested using a Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) in PAUP (Swoford, 2003; SH test using RELL bootstrap, 1000 replicates).

2.3. Coalescent simulations

Coalescent simulations were conducted with the program Simcoal 2.1.2 (Laval and Excoffier, 2004), to evaluate the probability that the apparent conflict in the observed phylogeographic signal between the mitochondrial and nuclear DNA is simply due to the stochastic component of the coalescence process. In other words, we aimed at testing whether the genealogical discrepancy observed between the mitochondrial and nuclear loci could have emerged under neutral sequence evolution.

To that end, we simulated backward the evolution of the sampled sequences (coalescent model) within a structured population framework mirroring the fragmented distribution of *C. lapponica*. The structured coalescent model used is depicted in Fig. 1. The sampled sequences are distributed among 10 populations (corresponding to the 10 regions defined above) that are completely isolated from each other today, but that were exchanging migrants sometimes in the past. All populations except one (the Alps population) start to exchange migrants (going backward in time) at time t_1. The Alps population starts to exchange migrants with the other populations only at time t_2. When a population exchange migrants, it does so only with adjacent populations (assuming *C. lapponica*, like many other leaf beetle species, is characterized by poor capabilities of dispersal), and migration rates are proportional to the geographic distance separating two adjacent populations. The delay between t_1

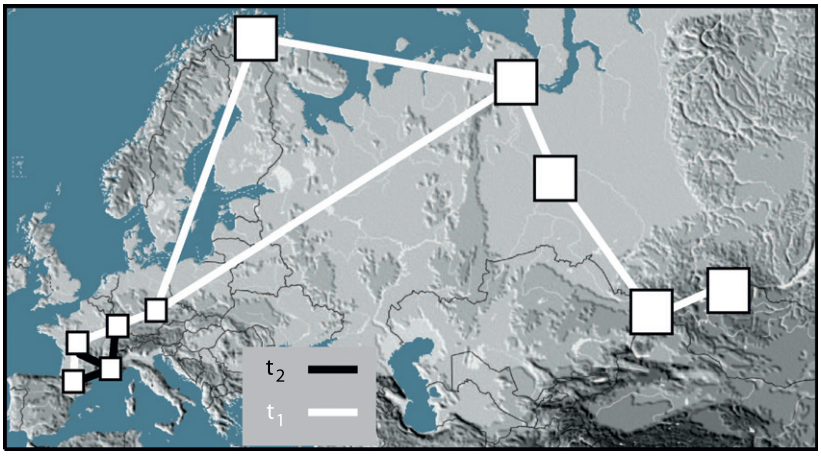

Fig. 1. Description of the structured coalescent model used to test whether the apparent conflicting phylogeographic signal observed between the mitochondrial and nuclear loci could have arisen under a strictly neutral model of sequence evolution. Squares represent sampled populations. At t_0, all populations are isolated from each other. At t_1, all adjacent populations, with the exception of the Alpine population, are connected by gene flow (white lines). Finally, at t_2, the Alpine population is connected to its most adjacent populations (black lines). See text for a complete description of the model.

and t_2 was essential to obtain a phylogeographic break similar to the one observed with the COI sequence data. A longer delay δt (i.e., $t_2 - t_1$) increased the probability that the simulation generated a phylogeographic break similar in strength to the one observed with the real COI sequence data, but decreased the probability of obtaining a network without such phylogeographic break, similar to the ones observed with the nuclear loci. We therefore attempted to find the value of δt that would maximize the probability of finding both the COI and nuclear phylogeographic patterns under the same demographic model. This was achieved by simulating sequences under a range of δt values. More specifically, t_1 was held constant at 10,000 generations (assuming one generation per year) in all simulations, while t_2 was varied from 30,000 to 1000,000 generations. The time t_1 roughly corresponds to the end of the last glaciation, when we can be reasonably confident that all the sampled populations in this study became completely isolated from each other. For each value of t_2, we simulated 200 data sets for each gene fragment, mirroring the specific (different) sampling of each of the three empirical data sets (same number of sequences sampled from the same populations). All population sizes were arbitrarily set to 1000 for the nuclear loci and to 250 for the mitochondrial locus (assuming a 1:1 sex ratio, because the mitochondrial genome is haploid and is transmitted through females only). The mutation rate was previously adjusted separately for each set of 200 simulations, and for each gene fragment, in the following way: we conducted several sets of 10 preliminary simulations, with a different mutation rate for each set, until a mutation rate was found for which the average value of the number of differences between the Alps sequences and all other sequences from the data set was close enough (less than 5% difference) to the corresponding value calculated from the observed data. Note that the value of the parameter θ (i.e., 4 Nμ) was therefore set identical for each population. We did not explore the possibility of having different values of θ for the different populations, to limit the size of the space of hypotheses to explore, and because it is unlikely that it would make it easier to reconcile the observed nuclear and mitochondrial DNA patterns of variation.

For each simulated data set, we then used the program Arlequin version 3.11 (Excoffier et al., 2005) to estimate (1) the nucleotide diversity (Nei, 1987) π_{Alps} within the Alps population, (2) the nucleotide diversity π_{rest} within the rest of the distribution (i.e., the nucleotide diversity of all sequences sampled elsewhere), and (3) the average number of differences D (Nei and Li, 1979) between the sequences from the Alps population and the sequences from all other populations. The ratio π_{rest}/D was then used as summary statistic to compare the empirical and simulated data. For each value of t_2 and each gene fragment, we then calculated the proportion of simulated data sets for which the calculated summary statistic was larger than, or equal to, the one calculated from the empirical data. Assuming that the model used takes all important parameters into account, this proportion was taken as an estimate of the probability that the empirical sequence data were generated under that specific t_2 value. In the end, the probability that the sequence data for all three loci were generated under the same model of population evolution, including the same t_2 value and in the absence of selection, was estimated by multiplying the probabilities estimated for each gene fragment separately.

The model depicted in Fig. 1 assumes that each population has the same geographic position throughout the entire simulation. This is probably not realistic, given that the species range is likely to have changed across different glacial and interglacial periods. The modification of the geographic position of populations would result in different migration rates among population pairs. In an attempt to test the impact of geographic range modifications on our results, we have also simulated sequence data in a similar fashion, but following a second model, identical to the first up to t_1 (going backward in time), at which time all nine non-Alpine populations are gathered into a single large population (10 times larger). At t_2, the Alpine population is transferred into this unique large population. Again, sequences were simulated for several values of t_2, and the probability that the sequence data for all three loci were generated under this model of population evolution was evaluated as described above. While the second model is not necessarily more realistic than the first, it is assumed here that the real evolution of populations from *C. lapponica* lies somewhere between these two extremes (static populations in the first model, all populations move into a single ancestral geographic location in the second model), and that conclusions that can be inferred from both models at the same time will be valid.

2.4. Additional test of selection

The coalescent simulations of a structured population described above offer a parametric approach to test departure from a neutral pattern of evolution. In addition, we have implemented a McDonald–Kreitman (MKT) test, which compares the patterns

of synonymous and non-synonymous substitutions within and between species, and has thus the potential for providing independent evidence that one gene fragment is under positive or negative selection. We have performed this test on the COI sequences using the standard and generalized MKT website (http://mkt.uab.es; Egea et al., 2008). Two different standard MKT tests were implemented. First, all *C. lapponica* sequences were compared to a single *C. aeneicollis* sequence, and second, all *C. lapponica* COI sequences sampled from the Alps were compared to all other *C. lapponica* COI sequences collected for this study.

3. Results and discussion

3.1. Overall pattern of genetic variation

The three complete data sets of aligned sequences (COI, EF-1α, and RpP0) contain 1059, 431, and 471 nucleotides, respectively. Among these, 32, 8, and 23 were found polymorphic. No insertions/deletions were observed in the data sets. The allele frequencies for each sampling locality are reported in Table 1.

C. lapponica is a cold-adapted leaf beetle, mostly found in mountain habitats in the central and southern part of its range. Even in those habitats that appear suitable, as determined by the appropriate climatic conditions and the presence of its host plants, populations of this species are scarce (personal observations; Bukejs, 2010). As a result, its geographic distribution is highly fragmented. If we assume that this species, like most leaf beetles, do not migrate over long distances, it is probably safe to consider that populations of this insect have been isolated from each other, at least for the last 10,000 years, i.e. since the end of the last glacial period. At the time, climatic conditions were more favorable to this species, probably allowing a more widespread distribution.

High levels of population differentiation among regions were indeed calculated: the fixation index F_{ST}, estimated by an AMOVA to assess the global level of population differentiation among regions, was 0.932, 0.830, and 0.347 respectively for the COI, Elongation Factor, and RpP0 DNA fragment. All three values were highly significant (p value $< 10^{-5}$). In addition, most pairwise F_{ST}'s calculated between regions were large and statistically significant (supplementary information).

Evolutionary relationships among allelic sequences are shown for each DNA fragment in the median-joining networks of Fig. 2, in association with their geographic location. Evolutionary relationships among alleles as inferred under the maximum likelihood criterion (not shown) were very similar to those of the median-joining networks. While a quick examination of the networks confirms the strong level of population structure calculated by the AMOVA in all three networks (e.g., in the RpP0 and EF-1α networks, sequences from the Alps or from the Ore mountains appear closer to each other than they are to sequences from other regions), for the most part (see exception for the COI network, below), no clear phylogeographic break (defined here as a strong genealogical subdivision, characterized by a relatively high number of mutations, associated with a geographic separation) among regions can be identified. That is, sequences collected from one geographic region can seldom be separated from all other sequences by cutting one branch in the network, which means they cannot form a monophyletic group. When they do have the potential to form a monophyletic group (i.e., if the network was rooted), they are separated from the rest of the network by only one (e.g., Ore mountains sequences on EF-1α network or Pyrenees sequences on the COI network) or two (e.g., West Siberia sequences on the COI network) mutations. For the most part, the fragmentation of the species range, as strong as it is today, has not occurred long enough to allow for reciprocal monophyly of each studied region.

This is in contrast with many temperate organisms in Europe, for which phylogeographic breaks were identified (e.g., Taberlet et al., 1998; Hewitt, 2000, 2001; Kotlík et al., 2006). These are often interpreted as a consequence of past climatic variation: during glacial periods, the geographic range of temperate organisms is restricted to a few small isolated refuges, in which climate conditions are favorable. These episodes of isolation seem to be sufficiently long to cause the appearance of strong phylogeographic breaks among glacial refugia that remain visible after the recolonization of Europe during interglacial periods. In other words, the strong geographic isolation of populations that occurred during glacial periods allowed the maintenance of ancestral polymorphisms within temperate species. A similar combination of a strong genetic differentiation among populations with the absence of phylogeographic break was already observed for another cold-adapted leaf beetle, *G. pallida* (Mardulyn et al., 2009), and could be related to the fact that glacial periods have lasted overall longer than interglacial periods within the last 200 thousand years (e.g., Petit et al., 1999). Episodes of strong range fragmentation for cold-adapted species occur during interglacial periods that may be too short to have generated patterns similar to the ones observed for temperate species.

3.2. Conflict between mitochondrial and nuclear loci

One important exception to the absence of phylogeographic break concerns the Alps sequences on the COI network: the unique allele found in the Alpine population (two sampled locations in the Alps) is exclusively present in this region, and its sequence has considerably diverged (17 mutations) from other sequences of the same species. We believe this pattern of variation was unlikely caused by the combination of true mitochondrial sequences with COI copies of nuclear origin (so called Numts), because (1) we found no sign of having amplified more than one COI allele per individual on the sequencing chromatograms (double peaks resulting from the co-amplification of a mitochondrial and a nuclear copy would be expected for at least some individuals) and (2) the presence of a phylogeographic break on the COI network is confirmed by the sequences obtained for another mitochondrial gene, 16S, for only 10 individuals: two alleles were found, separated by two mutations, one characterizing the four Alpine individuals, the other belonging to all other individuals (from Massif Central, Urals and Finland).

This pattern of variation suggests a long history of geographic isolation of the sampled Alpine population from the rest of the geographic distribution. It is surprising, given the small geographic distances separating the Alpine population from the neighboring mountain ranges (Massif Central, Pyrenees, Black Forest, Ore Mountains), compared to the much larger distances separating these central and western European mountain ranges from the Ural mountains, the Altaï mountains, or Siberia. Moreover, this phylogeographic break does not appear on the nuclear fragments networks. How can this apparent conflict between mitochondrial and nuclear DNA be explained?

3.2.1. Hypothesis 1: neutral variation

Before discussing potential explanations, we wished first to make sure that these signals are truly in conflict. Because the coalescence of lineages that generates gene genealogies is a stochastic process, unlinked loci are expected to display different genealogical patterns. Also, the mitochondrial genome effective population size can be considered to be four times smaller than that of the corresponding nuclear locus populations (because it is haploid and transmitted through maternal lines only; e.g., Avise, 2000), which could explain in part the phylogeographic signal discrepancy between them (in an ideal finite-size population, assuming neutrality, the coalescence time for mitochondrial sequences should be,

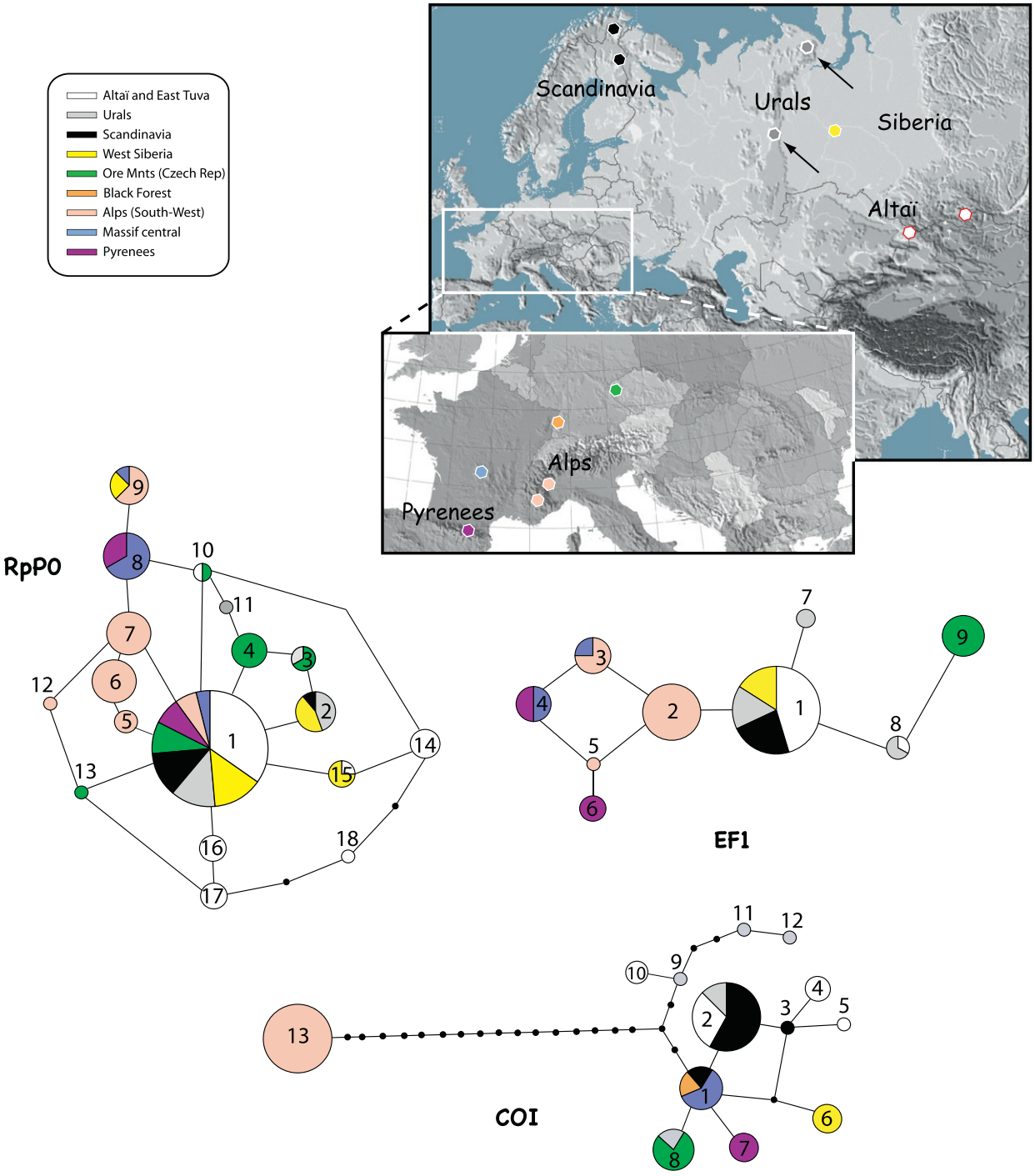

Fig. 2. Median-joining networks for the three gene fragments (Cytochrome oxydase I, COI; Elongation Factor 1-α, EF-1α; nuclear 60S acidic ribosomal protein P0, RpP0) sequenced in this study. Each sequenced allele is represented by a circle, the size of which is proportional to its overall frequency, and is identified by a unique number (see also Table 1). Each line in the network represents a single mutational change. Black dots without numbers indicate intermediate alleles that are not present in the sample, but are necessary to link all observed alleles 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 allele networks to show geographic distribution of alleles among regions.

on average, 4 times smaller than for nuclear sequences). In order to formally test the possibility that the strikingly incongruent patterns that we observe between the nuclear and mitochondrial loci concerning the Alpine sequences is simply caused by the stochasticity of the genealogical process, in combination with the smaller effective size of the mitochondrial genome population, we have simulated the evolution of sequences following a structured coalescent model that mirrors the specific sampling conditions of each of our three gene fragments and the fragmented distribution of this leaf beetle species. In order to achieve this, we have assumed that the phylogeographic break highlighted with the COI allele network had been caused by a longer period of geographic isolation for the Alps population. We have performed a series of simulations (two different structured coalescent models, see Material and Methods) with different times of isolation for this population, and have each time estimated the probability of observing simultaneously a

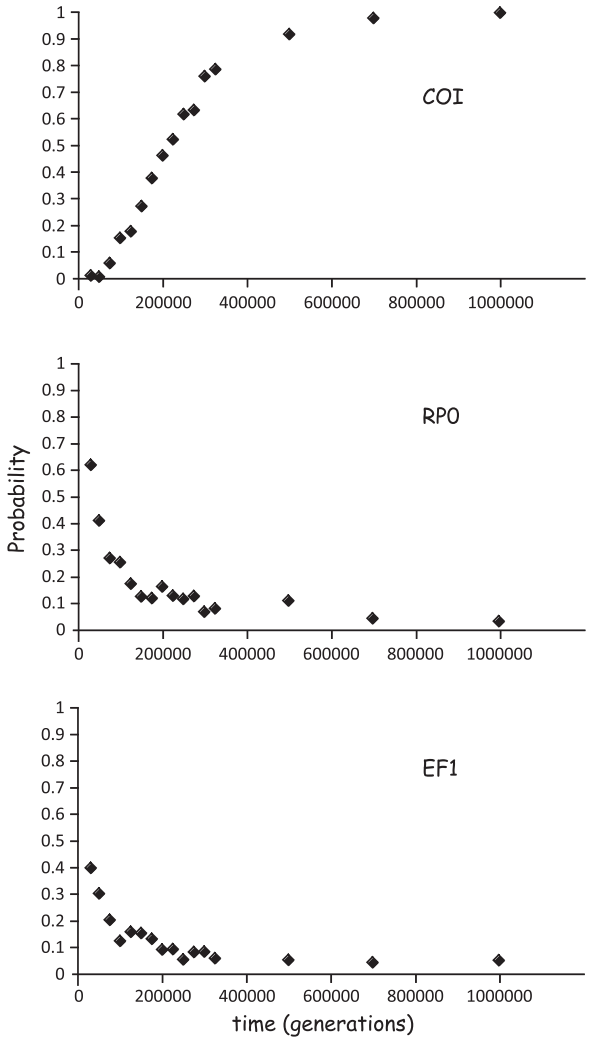

Fig. 3. Estimated probability, separately for each gene fragment, that the observed phylogeographic pattern observed was generated by the same coalescent model (see text for description) under various values of t_2.

strong phylogeographic break with the mitochondrial gene fragment and the absence of such break for both nuclear gene fragments. Fig. 3 shows the estimated probabilities that the variation displayed by each gene fragment was generated under the first model for various values of t_2 ranging from 30,000 to 10^6 generations. The combined probabilities that this model generated the observed mitochondrial and nuclear patterns shown here, under the same t_2 value, are never above 0.0065. Simulations performed according to the second model resulted in very similar values, with a combined probability that is never above 0.0197. In other words, if we assume that the models used here are appropriate to capture the essential features of the evolution of the *C. lapponica* populations, the probability of population history alone generating simultaneously the phylogeographic patterns observed for all three gene fragments is below 2%. It seems safe to conclude that it is unlikely (although not impossible) that demographic history alone has caused the apparent conflict between the mitochondrial and nuclear gene fragments.

3.2.2. Hypothesis 2: introgression

The most likely hypothesis that comes to mind to explain the observed conflicting patterns between the nuclear and mitochondrial markers related to the Alpine sequences is the past introgression of a foreign mitochondrial genome (i.e. the Alpine mitochondrial genome could have been imported from another unidentified species into *C. lapponica*, following hybridization). Indeed, many cases of mitochondrial DNA introgression have already been observed in animals (e.g., Ballard and Whitlock, 2004; Chen et al., 2009; Nevado et al., 2009), including insects (e.g., Ballard, 2000; Sota, 2002; Zhang and Sota, 2007; Polihronakis, 2010) and, more specifically, leaf beetles (e.g., Gomez-Zurita and Vogler, 2003, 2006). This hypothesis seems congruent with our observation of a highly divergent mitochondrial DNA sequence present in a single population of an otherwise rather genetically homogeneous and geographically widespread species. Identification of the source species of the putatively introgressed mitochondrial genome would support it further. We have attempted to identify the source species by comparing the *C. lapponica* COI sequences with those of closely related *Chrysomela* species, available in public sequence databases. While *C. lapponica* was traditionally classified in the subgenus Strickerus, along with *C. cuprea* and *C. vigintipunctata* (e.g., Warchalowski, 2003), a recent phylogenetic study (Termonia et al., 2001) has clearly shown that it belongs to the "interrupta group" (as defined by Brown, 1956) that otherwise include exclusively North American species. Fig. 4 presents the allele network and the maximum likelihood tree combining all sequences from this interrupta group. It is worth noting that the phylogenetic analysis of Termonia et al. (2001) did include a COI sequence from *C. vigintipunctata* and *C. cuprea*, both previously believed sister species to *C. lapponica*, and that they were found to fall well outside the interrupta group and are therefore clearly distant from any *C. lapponica* COI sequences. In both graphs of Fig. 4, the Alpine COI allele is more distant from the other *C. lapponica* alleles than from sequences of other species (e.g., *C. littorea*, *C. walshi*, etc.). This observation suggests the possibility that the Alpine COI allele belongs to another species, and thus appears to favor the hypothesis of a past introgression event. On the other hand, this analysis fails to identify the source species of the putative introgression, as all *C. lapponica* sequences are several mutations away from sequences of any other species. If introgression has taken place, we suggest the source species is either extinct, or belongs to a species located outside Europe and North America. In both cases, the introgression has not occurred recently, as the source species is most probably absent from the vicinity of the Alps.

Most other suggested cases of mitochondrial introgression come from phylogenetic studies (e.g., Nevado et al., 2009; Sota, 2002), in which the mitochondrial sequence of one individual falls with a different species than the nuclear sequences of the same individual. In these cases, the source of the introgressed mitochondrial genome is clearly identified. In the tree of Fig. 4, all COI sequences of *C. lapponica*, including those from the Alps, form a clade (albeit with a weak bootstrap support), and we cannot identify an obvious source species for the Alpine COI sequences. The support for introgression is based here on a somewhat different evidence: a very divergent allele found, for the mitochondrial locus, in a small portion of the range of an otherwise homogeneous widespread species, a pattern of variation that is not corroborated by the nuclear loci.

3.2.3. Hypothesis 2: selection

Although mitochondrial DNA has been treated as a neutral marker by many phylogeographic studies, some authors have recently discussed evidences challenging this assumption (Ballard and Whitlock, 2004; Bazin et al., 2006). Bazin et al. (2006) argued in particular that when compared to variation found in nuclear loci, mitochondrial variation shows some signs of recurrent adaptive evolution. In our study, because genetic drift alone is unlikely to be responsible for the co-occurrence of the phylogeographic patterns found for the three loci, it could be suggested that selection

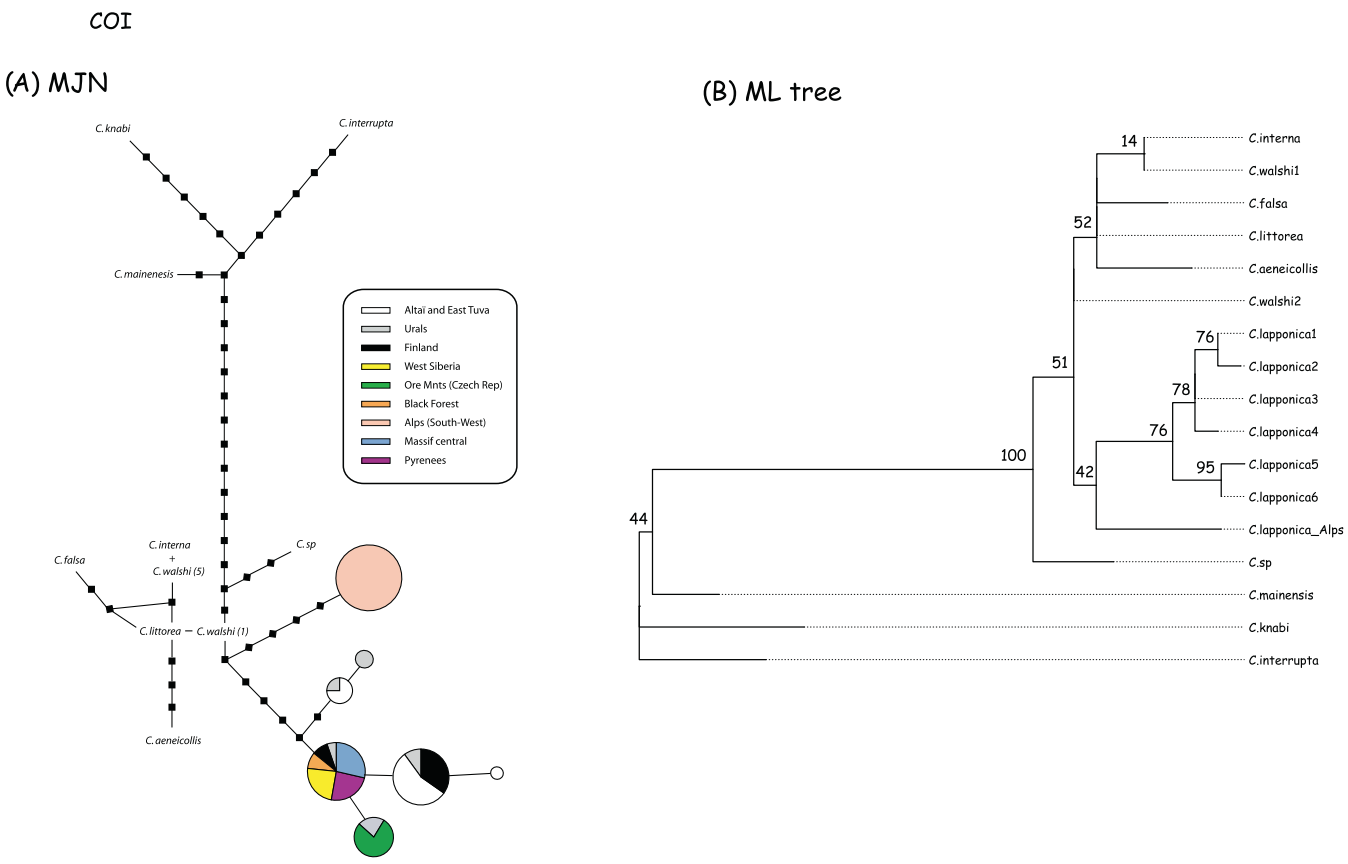

Fig. 4. Median-joining network (A) and maximum likelihood (ML) tree (B) of a partial COI fragment for all individuals sequenced in this study, with the addition of sequences of other closely-related *Chrysomela* species available in GenBank (represented by species name). On the median joining network, black squares are missing alleles. On the ML tree, numbers above branches are bootstrap values (500 replicates).

on the mitochondrial genome has caused the observed ancestral polymorphism to be maintained until today.

The persistence of an old polymorphism within a population or species is sometimes attributed to balancing selection, i.e., selection in favor of heterozygote individuals (e.g., Hedrick and Thomson, 1983; Ferguson et al., 2008). However, because the mitochondrial genome can be considered as haploid, balancing selection can be reasonably excluded as an explanation. The only other possibility for selection to have maintained such an old polymorphism is by exerting different selection pressures in different environments (adaptive selection). It is possible that one or more habitat differences between the Alps populations and all others populations have maintained a different selection pressure on the mitochondrial genome, which would account for the observed pattern. In other words, it would mean that the two sampled Alpine populations share a peculiar habitat that favors the strongly divergent allele, and that all other populations sampled are located in habitats that favor the other allelic variants sequenced.

One possible clue of the occurrence of natural selection is the presence of a single COI allele in the Alpine samples (26 individuals). The low genetic variation found in this region contrasts with the level of variation found with the two nuclear loci, with 6 (RpP0) and 3 (EF-1α) alleles observed, and could result from a selective sweep. Note however that the low COI variation observed in the Alps is also compatible with introgression, as it is likely that the transfer of the mitochondrial genome from one species to the other occurred through a limited number of introgression events, thereby imposing a severe founder effect. Nonetheless, to look for further signs of selection in the COI pattern of variation, we conducted two standard McDonald-Kreitman tests, comparing patterns of synonymous and non-synonymous changes within the *C. lapponica* sequences and between *C. lapponica* and the related species *C. aeneicollis*. Both tests returned non-significant *p* values, $p = 0.820$ and $p = 0.327$, respectively. Thus, this test provided no additional evidence that the COI fragment is under selection in this species. Of course, the non rejection of a hypothesis of neutrality does not guarantee that selection does not influence variation in COI *C. lapponica* sequences, but at least, no evidence that it does was found.

Also, the habitat characteristics of the Alpine population are very similar to those of the Altaï population sampled in this study. In fact, much more pronounced habitat differences are found among other populations, that are associated with much less genetic differentiation. For example, the Ore Mountains (Czech Republic) population was found at a relatively low elevation (820 m) on *Betula*, while the Polar Urals population is located much more to the north, and feeds on Salix, yet, the unique allele found in the Urals population was also present in the Ore mountains population. It is therefore difficult to imagine what habitat feature specific to the Alpine population could have favored one mitochondrial allele over another.

Perhaps more problematic for this hypothesis is the high number of mutations (17) separating the Alpine COI allele from the rest of the network. It shows that this polymorphism is really old. The earth has experienced several episodes of profound climate change over the quaternary period, and, as a result, the geographic range of most organisms in Europe have each time undergone major changes (e.g., Hewitt, 2000). Thus, we cannot assume that the current geographic distribution of *C. lapponica* has been maintained for a period of time sufficiently long to account for the observed

ancestral polymorphism. This is even more obvious when we compare the *C. lapponica* COI sequences to those in other related *Chrysomela* species (Fig. 4). The large genetic distance separating the Alpine alleles from the others shows that this divergence most probably predates the speciation events leading to the differentiation of the group of closely related *Chrysomela* species to which *C. lapponica* belongs. Moreover, all of these related *Chrysomela* species are located in North America, while *C. lapponica* is found in Eurasia. It is thus clear that the maintenance of this polymorphism must have occurred in a different geographic setting than the one we see today. If differential adaptive selection is indeed responsible for its maintenance, it must be universal enough to have persisted for a long time, going through several episodes of profound population structure and geographic range modifications.

3.3. Host-plant shifts

A previous phylogenetic study of Chrysomelina leaf beetles (that includes the genus *Chrysomela*), combined with data on the chemistry of the defensive secretions produced by their larvae, has suggested that this group has shifted early on from an autogenous synthesis of their defensive secretion to a host-plant derived strategy (deriving salicyl aldehyde from the salicin found in their host plant), rendering it highly dependent to Salicaceae (Termonia et al., 2001). Later in the history of the genus *Chrysomela*, the "*interrupta* group", that includes *C. lapponica*, has escaped this dependency by evolving a dual defense strategy in which they are able either (1) to derive salicyl aldehyde from Salicaceae hosts or (2) to produce their defensive secretion through a mixed metabolism, combining the de novo synthesis of butyric acids with the esterification of these with alcohols taken from their host-plant. Because alcohols are commonly found in plants, this mixed metabolism offers the potential to shift to other host plants. Indeed, several species of the *interrupta* group have shifted independently from Salicaceae to Betulaceae (Termonia et al., 2001).

Reconstructing the history of host-plant shifts using MacClade on the maximum likelihood tree of Fig. 4 resulted in *Salix* being the most parsimonious ancestral host plant for *C. lapponica*, whether the alpine lineage was included or not. Two lines of evidence further confirm this ancestral host-plant association: (1) for one out of three populations feeding on *Betula* (South Altaï), we also found individuals feeding on *Salix*. Insects from this last population, but also from the birch-feeding population from Finland, accepted willow leaves as food in the laboratory. In contrast, all willow-feeding populations tested in the laboratory (French Alps, Finland, Black Forest, and Massif Central) declined to feed on birch. If today's populations feeding on birch have recently evolved from populations exclusively feeding on willow, some individuals from the former would indeed be expected to still be able to accept willow leaves as food. (2) Kirsch et al., 2011 have

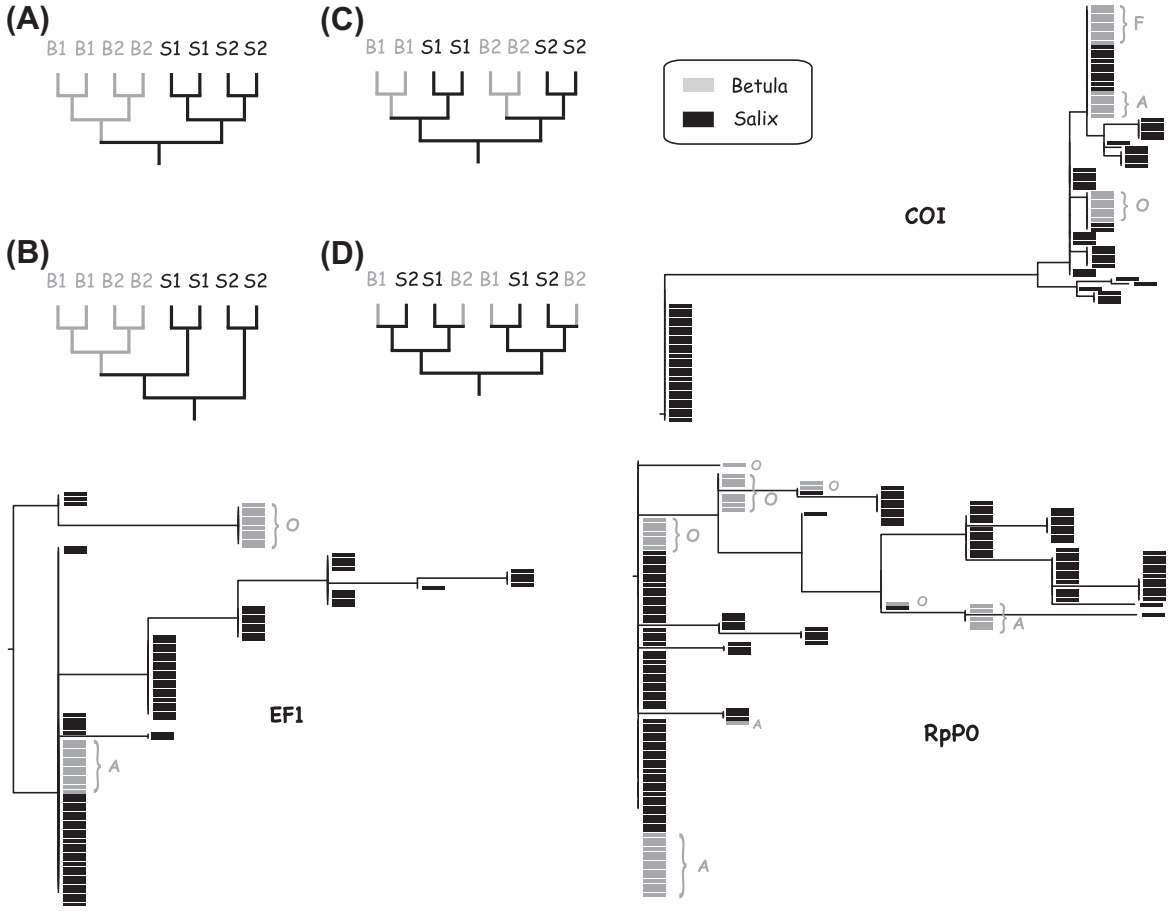

Fig. 5. Schematic of four alternative phylogenetic hypotheses (A–D) concerning the evolution of populations of *C. lapponica*. Labels indicate the population from which a sequence was sampled: B1 and B2 are two populations feeding on *Betula* (gray branches), S1 and S2, two other populations feeding on *Salix* (black branches). It is assumed that *Salix* is the ancestral host plant. The unrooted maximum likelihood trees for each locus are also presented, associated with the information on the host-plant genus on which the individuals feed. Horizontal bars to the right of the trees represent sequences, either from individuals feeding on *Betula* (gray), or *Salix* (black). A different letter (A, Altaï; F, Finland; O, Ore mountains) identifies each one of the three birch-feeding populations. Note that while the insects of the North Ural population were collected on both willow and birch trees (see Table 1), we did not test the plant diet in the laboratory. For the purpose of the discussion on host-plant shifts, we take the conservative view that this population feeds on willow only.

highlighted the loss of function of the enzyme ensuring the production of salycil aldehyde (Salicyl Alcohol Oxidase) in the individuals from the Altaï birch-feeding population. This loss of function also points towards a shift from *Salix* to *Betula*.

Fig. 5 presents four possible alternative phylogenetic hypotheses (A–D) related to the host-plant affiliation of the populations collected. Hypotheses A and B would suggest a common origin for all populations feeding on *Betula* (assuming *Salix* is the ancestral host plant), and thus a single ancient shift from *Salix* to *Betula*. Hypothesis C suggests that each population has specialized on either *Salix* or *Betula*, with more than one host-plant shift between *Salix* and *Betula* occurring during the evolution of the species. It also assumes a relatively long history of adaptation of each population to its host-plant. Finally, in hypothesis D, alleles found in one population do not form a monophyletic group, suggesting thus a recent origin of contemporary populations. It would reveal that the populations that specialized on *Betula* (still assuming *Salix* is the ancestral host plant of the species) had relatively little time to adapt to its new host plant. ML trees inferred from each locus are also shown in Fig. 5, in association with the host-plant affiliation (*Salix* or *Betula*) of individuals. For each locus, we find no clear separation between the alleles of the willow-feeding beetles and those from the birch-feeding beetles, which allows us to reject hypotheses A and B. Performing a new ML analysis while constraining all sequences from Betula-feeding individuals to be monophyletic resulted indeed in ML trees that are significantly different from the unconstrained ML trees for the COI and RpP0 data sets (SH test, $p = 0.047$ for COI, $p = 0.047$ for RpP0, $p = 0.090$ for EF-1α).

Moreover, on the RpP0 tree, the alleles from the Ore mountains or from the Altaï birch-feeding population are intermixed with those of willow-feeding individuals, suggesting rejection of even hypothesis C. Performing a new ML analysis while constraining sequences from each birch-feeding population to be monophyletic resulted in two ML trees, one being significantly different from the unconstrained ML tree (SH test, $p = 0.047$ and $p = 0.095$). In all three ML trees of Fig. 5, individuals from birch-feeding populations often share one allele with those from one or more willow-feeding populations. This points towards a recent origin of the sampled populations, possibly the result of the fragmentation of a large continuous population present during the last glacial maximum, when climatic conditions were presumably more favorable for this insect. Moreover, given the large geographic distances separating the birch-feeding populations, we suggest that the shift from willow to birch has occurred independently in each of them. This is further corroborated by observations suggesting that the three birch-feeding populations are not at the same stage of adaptation to their new host plant: larvae collected in the Ore Mountains on *Betula* cannot be grown on *Salix* (Fatouros et al., 2006), but those collected on *Betula* in the Altaï can easily be reared on different *Salix* species, without the ability to derive salicyl aldehyde from that host plant however. In fact, as already mentioned above, the enzyme ensuring the production of salycil aldehyde (Salicyl Alcohol Oxidase) is believed to have lost its function in the latter population (Kirsch et al., 2011). If the three birch-feeding populations had recently originated from a common ancestral population, they would be expected to show a similar level of adaptation to their host plant.

These data on the intra-specific variation of *C. lapponica* emphasize even further the relative ease with which these beetles can escape their host-plant specialization on willow, but show at the same time that host-plant shifts are highly constrained, as they only occur between willow and birch. Another case of constrained host-plant shifts among unrelated plant families was reported for the leaf-beetle genus *Gonioctena* (Mardulyn et al., 1997), by highlighting several convergent host-plant shifts that had occurred during its evolutionary history. Some possible causes for these constrained host-plant shifts were discussed in this last paper, including the presence of some unknown similar plant compounds in both host-plants, mutational constraints affecting the insect's chemosensory system, and/or simply the presence of the two host-plants in their environment. In favor of this latter hypothesis, Termonia et al. (2001) noted that species from the genera *Salix* and *Populus* (Salicaceae) and from the genera *Alnus* and *Betula* (Betulaceae), were the dominant trees at the time where the *interrupta* group originated.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.09.001.

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