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Phylogeography and biodiversity in an alpine leaf beetle genus

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Phylogeography and biodiversity in an alpine leaf-beetle genus

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Abstract

Changes in tectonic conformation and orbital parameters of the Earth (eccentricity, obliquity and axial wobble) caused drastic climatic oscillations during the Quaternary. These oscillations strongly influenced the genetic landscape of the contemporary European flora and fauna.

During cold periods the extending ice sheets and permafrost forced many temperate species to migrate into southern refugia. During interglacial periods these species recolonized northern Europe following the retreating ice shelf. The resulting patterns have been well demonstrated for temperate and lowland species, whereas much less is known about the effect on arctic or high altitude species.

In my study we investigated the phylogeography of four alpine leaf beetles to uncover the influence of Quaternary climatic changes on high altitude species. We used AFLP (amplified fragment-length polymorphism) genotyping and single marker sequencing methods for the construction of phylogenies and the geographical distribution of lineages.

In the phylogeny of *Oreina elongata*, a species restricted to the Alps and Apennines, only three out of seven described subspecies form monophyletic clades. The basal clades are found in the central Swiss Alps and northern Italy. Applying a molecular clock, we found that the split of the major clades occurred long before the last glacial maximum suggesting that *O. elongata* persisted for many glacial cycles within or at the edges of the Alps and Apennines.

The phylogeny of *O. gloriosa*, a species with a uniquely alpine distribution, splits into three main clades, one in the western Alps, one in the central Alps (southern Switzerland) and one in the eastern Alps. The molecular clock estimates suggest that the divergence into the three main clades occurred 0.5 MYA and can be considered as recent, which concords with the lack of morphological variation across the distribution. The comparison of the phylogeography of *Peucedanum ostruthium* and its monophagous herbivore *O. gloriosa* based on AFLP genotyping suggests incongruence between the histories of these antagonistic species.

Oreina cacaliae and *O. speciosissima* are two closely related species occurring in sympatry and with ranges that cover most of the distribution of the genus. The analysis of a nuclear marker and morphological criteria clearly split the two species. The phylogeny of three mtDNA markers show paraphyly for both species but suggest that 10 out of 12 analysed subspecies are monophyletic. And incongruence between the makers may be a result of hybridisation or lineage sorting of the ancestral polymorphism.

Further I analyzed the phylogeography of two subspecies within *Oreina speciosissima* based on three mtDNA markers and AFLP genotyping. The phylogeny of the mtDNA markers show very little structure whereas the AFLP phylogeny showed clear structure, strongly correlated with the ecology and particularly with the habitat. The presence of intermediate morphotypes at the base of both subspecies clades suggests that the divergence between these two subspecies is not yet complete.

I tested if colour is involved in mate choice and could influence the colour pattern in *O. gloriosa* populations. Mate choice experiments between two colour morphs of *O. gloriosa* with origin in pure or mixed populations show no assortative mating and thus no preference for colour.

Finally, we carried out a field experiment to test if positive frequency-dependent selection by predators drives *O. gloriosa* populations to monomorphism. The survival of the rare morph was significant lower than the survival of the common morph and thus suggests that predation can influence the population colour.

Keywords: Quaternary, high altitude species, *Oreina*, DNA sequencing, AFLP, molecular clock, phylogeography, refugia, Alpine antagonism, ecological speciation, mate choice, positive frequency-dependent selection.

Résumé

Des changements dans la conformation tectonique et orbitale de la terre (excentricité, obliquité et la précession) ont entraîné des oscillations récurrentes du climat pendant le Quaternaire. Ces oscillations ont fortement influencé le paysage génétique de la flore et de la faune contemporaines, notamment en ce qui concerne le continent Européen. Pendant les périodes glaciaires, l'extension de la glace et du permafrost ont forcé un grand nombre d'espèces à migrer ou à survivre dans des refuges au sud de l'Europe, alors que pendant les épisodes interglaciaires, ces espèces ont recolonisé le nord de l'Europe au fur et à mesure que la glace se rétractait. Si ces processus ont été largement étudiés chez les espèces tempérées de basse altitude (pour lesquelles différents paradigmes ont été établis), on connaît beaucoup moins l'influence de cette migration cyclique des populations chez les espèces arctiques ou de haute altitude.

Pendant ma thèse, j'ai étudié la phylogéographie (c'est-à-dire l'étude des principes et processus qui gouvernent la distribution des lignées généalogiques) de quatre espèces de chrysomèles alpines du genre *Oreina* (caractérisé par des espèces présentant une grande variété de couleurs) afin de mettre en évidence le rôle joué par les oscillations climatiques du Quaternaire sur des taxons de haute altitude. J'ai utilisé différentes méthodes moléculaires comme le génotypage par AFLP (amplified fragment-length polymorphism) et le séquençage de marqueurs cytoplasmiques et nucléaires afin de reconstruire la phylogénie et l'histoire évolutive des lignées.

J'ai tout d'abord abordé la phylogénie d'*Oreina elongata*, une espèce avec une distribution restreinte aux Alpes et aux Apennins. Les résultats montrent que seules trois des sept sous-espèces décrites ont formé des clades monophylétiques. Les clades basaux se trouvent dans les Alpes de Suisse centrale et en Italie du nord. En utilisant une horloge moléculaire calibrée sur la vitesse moyenne d'évolution des gènes mitochondriaux des insectes, j'ai montré que la séparation des clades majeurs au sein de cette espèce a eu lieu bien avant le dernier maximum glaciaire, il y a environ 5 Ma. Cela indique clairement qu'*Oreina elongata* a persisté à la périphérie ou même à l'intérieur des Alpes et des Apennins pendant plusieurs cycles glaciaires.

Ensuite, j'ai analysé la phylogénie d'*Oreina gloriosa*, une espèce qui présente une distribution strictement restreinte aux Alpes. Les analyses ont permis de mettre en évidence une séparation en trois clades principaux: un dans les Alpes de l'ouest, un dans les Alpes centrales et un dans les Alpes de l'est. L'estimation des temps de divergence à l'aide de l'horloge

moléculaire suggère que la séparation des trois clades majeurs a débuté il y a 0.5 Ma, ce qui peut être considéré comme récent, en regard des résultats précédents sur *Oreina elongata*. Ce résultat est confirmé par le fait qu'il n'y a aucune variation morphologique chez ce taxon, d'un bout à l'autre de son aire de distribution. Afin d'étudier les conséquences des oscillations climatiques sur les interactions trophiques, j'ai également comparé le patron obtenu pour *Oreina gloriosa* avec celui de sa plante-hôte exclusive, l'impératoire *Peucedanum ostruthium*, en inférant la structuration génétique spatiale des deux espèces à l'aide d'un génotypage par AFLP. Les résultats montrent une incongruence partielle dans l'histoire de ces deux espèces antagonistes, ce qui suggère que la plante et l'insecte n'ont pas systématiquement emprunté les mêmes voies de dispersion.

Puis, je me suis intéressé aux espèces génétiquement proches, *Oreina cacaliae* et *Oreina speciosissima*, deux taxons qui se rencontrent fréquemment en sympatrie sur leur aire de distribution (englobant la plupart des massifs montagneux européens). Alors que l'analyse d'un marqueur nucléaire – de même que des critères morphologiques – séparent nettement les deux espèces, la phylogénie basée sur trois marqueurs mitochondriaux montre une paraphylie réciproque marquée des deux espèces. Ces résultats mettent cependant en évidence la monophylie de 10 parmi 12 sous-espèces échantillonnées. L'incongruence entre la morphologie et l'information nucléaire et mitochondriale peut être expliquée par l'hybridation des lignées, éventuellement en combinaison avec un polymorphisme ancestral important.

J'ai par la suite analysé la phylogéographie de deux sous-espèces d'*Oreina speciosissima*, sur la base de trois marqueurs d'ADN mitochondriaux et d'un génotypage AFLP. Tandis que les marqueurs mitochondriaux n'ont démontré qu'un très faible niveau de variation, la phylogénie basée sur les AFLP a permis de mettre en évidence une structuration très fortement corrélée à l'écologie, et notamment au type d'habitat. Néanmoins, la présence de morphes intermédiaires à la base des clades des deux sous-espèces indique que la divergence entre ces deux sous-espèces n'est pas encore terminée.

Enfin, dans un volet d'écologie comportementale, j'ai testé si la couleur pouvait être impliquée dans le choix du partenaire et si cela pouvait influencer les densités relatives des différentes couleurs au sein des populations d'*Oreina gloriosa*. Les résultats des expériences (au cours desquelles on a testé le choix du partenaire entre deux morphes de couleurs et d'origines populationnelles différentes) n'a pas permis de conclure à des différences significatives entre les différents types d'accouplements, et par conséquent à des préférences dans le choix du partenaire liées à la couleur.

Finalement, on a réalisé une expérience de terrain pour tester si la sélection positive ″fréquence-dépendante″ des prédateurs est une force évolutive suffisante pour modifier les proportions des différentes couleurs au sein des populations d'*Oreina gloriosa* et maintenir le polymorphisme. Les résultats montrent que la survie du morphe le plus rare est significativement plus faible que celle du morphe le plus fréquent, indiquant par là que la prédation peut influencer le patron de coloration d'une population.

Mots clés: Quaternaire, espèces de haute altitude, *Oreina*, séquençage d'ADN, AFLP, horloge moléculaire, phylogéographie, refuge, antagonisme alpine, spéciation écologique, choix du partenaire, sélection positive ″fréquence-dépendante″.

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Introduction

The Quaternary, the youngest era in earth history, is characterized by multiple glacial and interglacial periods. The pace maker of these cycles are the orbital parameters of the Earth (axial tilt, precession and axial wobble), which changed the intensity and distribution of insolation reaching the Earth (Hays *et al.* 1976; Imbrie *et al.* 1992). Analyses of Arctic and Antarctic ice cores and its gas inclusions provide information about the atmospheric composition and thus about the predominant climate at a certain time. With this method, the Earth's climate can be traced back and suggests four glacial cycles over the last 420'000 years (Stauffer 1999). Fossil records of pollen and analyses of tree rings, peat bogs and lake sediments provide much information about the influence of climate change on species distribution (Stauffer 1999; Hewitt 2000). Modern molecular methods such as AFLP (amplified fragment-length polymorphism) genotyping or sequencing of individual DNA regions allows the measurement of genetic diversity as single-base changes. The combined analysis of genetic data and the geographic distribution of organisms rise to the field of phylogeography.

Climatic oscillations during the last 2.6 MY between glacial and interglacial cycles strongly affected the pattern of population regression and expansion of many European organisms (Hewitt 1996; Williams *et al.* 1998). During glacial cycles, when the growing ice shelf and permafrost covered wide areas of northern Europe, many temperate and lowland species became extinct over much of their distribution, dispersed to new terrain or survived in southern refugia, as the Iberian, Italian and Balkan Peninsulas (Coope & Wilkins 1994; Hewitt 1996; 2000; Schmitt & Seitz 2001; Widmer & Lexer 2001; Hewitt 2004). During interglacial ages, recolonization of northern Europe appears to have taken place rapidly (Hewitt 2001) and started from these refugia. For several expanding species mountain ranges such as the Alps or Pyrenees formed strict barriers and in regions where recolonizing lineages

met, hybrid zones arose (Taberlet *et al.* 1998; Hewitt 2000). Thus lineages from separate refugia could contribute differently to the genetic diversity of recolonized areas (Bennett 1990). The three paradigm species for different recolonization patterns in northern Europe are the grasshopper (*Chortippus parallelus*), the hedgehog (*Erinaceus europeus*) and the bear (*Ursos arctos*) (Fig. 1) (Hewitt 2000).

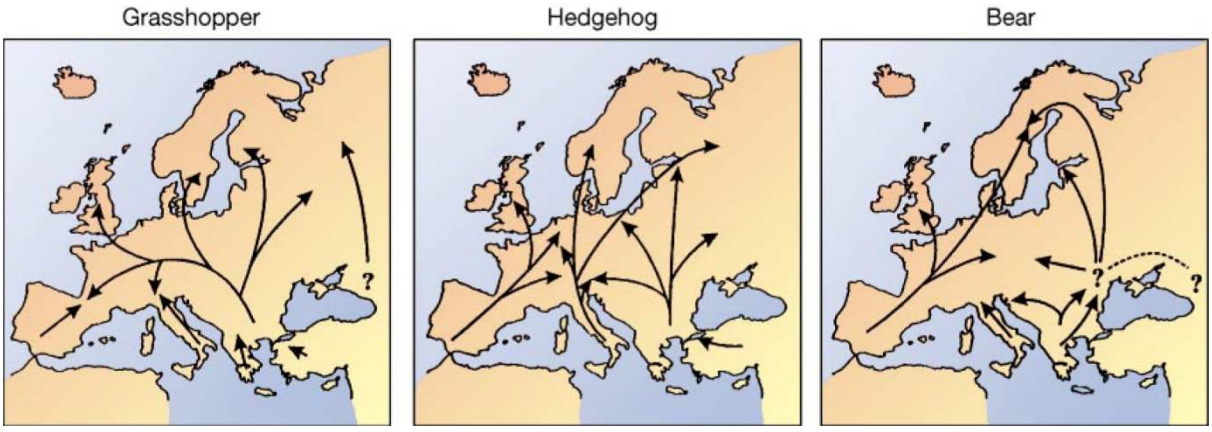

Figure 1. Three paradigm recolonization patterns from southern Europe for the grasshopper, hedgehog and bear. The main refugia contribute differently to the recolonization of northern Europe. Source: Hewitt 2000, Nature.

The grasshopper shows a pattern with one strong lineage, recolonizing nearly the whole of northern Europe from a refugium in the Balkans. The Pyrenees and the Alps seem to act as strong barriers against a spread northwards of lineages in the Iberian and Italian refugium.

A much more balanced recolonization of northern Europe is shown by the hedgehog where lineages from three refugia are equally involved. The lineage from the Iberian Peninsula recolonizes north-western Europe, the lineage from the Italian Peninsula spread to central and northern Europe, whereas eastern Europe is dominated by the lineage spreading from the Balkans.

The recolonization of northern Europe by the bear is strongly influenced by two lineages, one from the Iberian refugia and one from the Balkans and Caucasus. The Alps did not act as a barrier for the lineage in the Italian refugia, but its extinction due to human activity stopped this lineage from further dispersal.

Whilst such patterns for temperate and lowland species have been well documented (Garnery *et al.* 1992; Taberlet & Bouvet 1994; Demesure *et al.* 1996) there is much less known about the influence of ice ages on arctic or high altitude animals (Pauls *et al.* 2006; Schmitt *et al.* 2006; Haubrich & Schmitt 2007). They provide a contrast with the lowland species, since the current warm climate represents a period in which their range has probably declined. Recent molecular studies of Alpine plants suggest mountain refugia within the Alps, and at their northern, southern and eastern borders (Fig. 2) (Stehlik *et al.* 2001; Stehlik *et al.* 2002; Schönswetter *et al.* 2005).

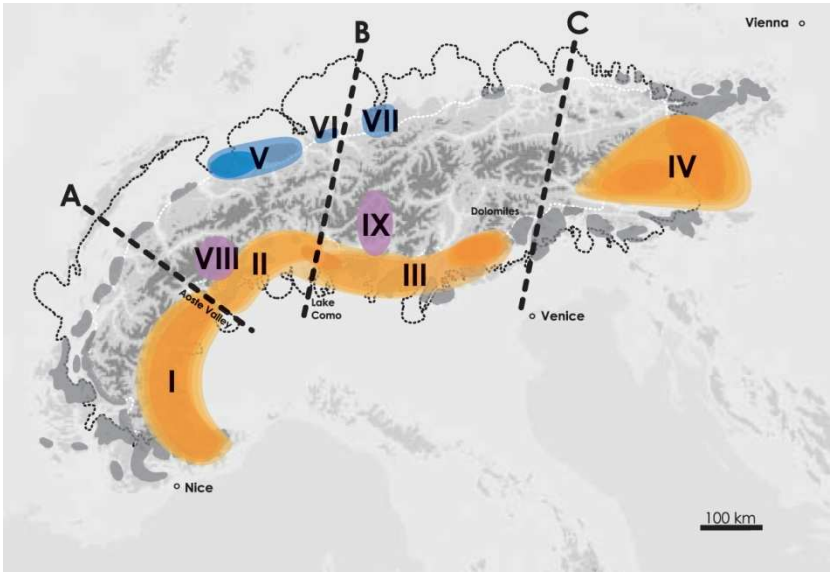

Figure 2. Map of glacial refugia of eleven mountain plant species in the Alps. Source: Schönswetter et al. 2005, Mol. Ecol.

Oreina beetles

Within the genus *Oreina* CHEVROLAT (Coleoptera, Chrysomelidae) about 28 species are described (Kippenberg 1994; 2008; and pers. comm.), occurring in isolated populations throughout the European mountains with the centre of their distribution in the Alps. Some incursions into the lowlands (*O. caerulea*) and into western Siberia (*O. sulcata* and *O. redikortzewi*) are known (Kippenberg 1994; Mikhailov 2008). *Oreina* beetles can be found in different habitat types. Many species live in moist high-forb plant communities, which are

often dominated by one or several of the following plant genus: *Adenostyles, Chaerophyllum, Heracleum, Petasites, Peucedanum* etc.. Other species, especially in higher altitudes, can be found in exposed stone runs with very scarce and patchy vegetation. A common host plant in such habitats is *Doronicum clusii. Oreina* living on high-forbs can be found on their host plants all day, whereas species living in stone run habitats are nocturnal and hide during the day underneath stones close to their host plant. Within the genus *Oreina* two different chemical defence strategies exist which are correlated with host plant use. Species feeding on Apiaceae or Cardueae produce cardenolides *de novo*, whereas species feeding on Senecioneae encounter pyrrolizidine alkaloid N-oxides (PAs) in several of their host plants. All species feeding on such plants are able to take up the alkaloids from their hosts and incorporate them into their defensive secretions (Pasteels & Rowell-Rahier 1991; Pasteels *et al.* 1995; Dobler *et al.* 1996).

Cardenolides are produced in small volumes but at high concentrations (Rowell-Rahier *et al.* 1995) and stored exclusively in the pronotal and elytral glands of adults (Pasteels & Rowell-Rahier 1991). The sequestered PAs from asteraceous host-plants are stored in larger quantities than cardenolides and are stored in the body as well as in the elytral glands. *Oreina* larvae store the defence compounds in the whole body (Pasteels *et al.* 1988; Ehmke *et al.* 1991; Rowell-Rahier *et al.* 1991).

A wide range of colour morphs within as well as between species exist, spanning a continuum from shiny green through metallic blue to black, with or without blue or red stripes. Beetles of the Subgenus *Protorina* WEISE are the only exception not possessing such shiny colouration.

The remarkable colour polymorphism within *Oreina* beetles, both within populations and across the geographical distribution of species, and the fact that they have well developed chemical defence mechanisms seems to represent a paradox. Learning by predators of unpalatable prey-morphs is expected to produce strong purifying selection on aposematic colouration since only recognized colour patterns are avoided by predators. *Oreina* beetles

can therefore be used to test if there is frequency dependent selection within species acting against rare colour morphs.

In the following four chapters we investigated the phylogeography of four *Oreina* species. The phylogenetic analyses are mainly based on sequence data from single markers and AFLP genotyping. The phylogeography of a species restricted to the Alps and Apennines, a comparative phylogeography between a host plant and its monophagous beetle, a comparative phylogeography between two closely related species sharing a wide distribution across the European mountains, as well as an example of an ecological speciation are described.

Further, preliminary work on two aspects of colour-selection and mate choice have been done (chapter V and VI).

Chapter I

Here we investigated the phylogeography of *Oreina elongata* covering its whole distribution, based on three mtDNA regions and one nuclear region. We analyzed the data with Bayesian inference analyses, maximum parsimony and maximum likelihood analyses. Further we applied a molecular clock to obtain information about the time of divergence within this species. To investigate the phylogenetic and haplotype relationships we performed statistical parsimony networks (SPN).

Chapter II

In this chapter we compare the phylogeography of two antagonistic species. For *Oreina gloriosa,* a monophagous beetle on *P. ostruthium,* we analyzed sequence data from three mitochondrial and one nuclear region by Bayesian inference analyses and maximum parsimony analysis. To investigate the phylogenetic and haplotype relationships within *O. gloriosa*, we performed SPN networks. To get an idea about divergence time, we applied a molecular clock with independent substitution rate for each mtDNA locus. To compare the

phylogeographies of the two antagonistic species, we used AFLP genotyping. The AFLP data were analyzed using STRUCTURE.

Chapter III

In this chapter we compared the phylogeography of *O. cacaliae* and *O. speciosissima*, two closely related, sympatric species sharing the same distribution across most of the European mountains. We analyzed sequence data from three mitochondrial and one nuclear region by Bayesian inference analyses and maximum parsimony analysis. To obtain information about divergence we applied a molecular clock on the mtDNA with independent substitution rate for each locus.

Chapter IV

Here we investigate an example of ecological speciation within *Oreina speciosissima* by analyzing three mtDNA markers and AFLP genotyping. Further we discuss possible mechanisms that could cause the observed divergence within this species.

Chapter V

Here we investigate the role of colour in mate choice in *Oreina gloriosa*. We tested for male and female mate choice in quartet lab experiments using individuals from pure and mixed populations in *Oreina gloriosa*.

Chapter VI

In this chapter we demonstrate positive frequency-dependent selection in *Oreina gloriosa*. To test if purifying selection by predators acts in the wild we compared the survival of blue and green individuals in blue- or green-dominated populations.

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Chapter I

The phylogeography of an alpine leaf beetle: divergence within *Oreina elongata* spans the Quaternary

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Abstract

The genetic landscape of the current European flora and fauna was shaped by the ebb and flow of populations in time with the expansion and contraction of ice sheets during Quaternary climate cycles. While this has been well demonstrated for lowland species, much less is known about high altitude taxa. Here we analyze the phylogeography of the alpine leaf beetle *Oreina elongata* from 20 populations covering the entire distribution across the Alps and Apennines. Three mitochondrial and one nuclear region were sequenced in 65 individuals. Within the resulting phylogeny, four of the seven subspecies form monophyletic clades. The species is chemically defended and aposematic, with geographic variation in warning colour and unexpected within-population polymorphism of green and blue forms. These colour patterns show pronounced east-west geographical structure in distribution, but both colours appear repeatedly in the phylogeography, suggesting repeated origin or loss. Basal clades in the phylogeny are found in the central Swiss Alps and northern Italy. Ancestors of the other clades probably survived across northern Italy and the northern Adriatic before separation of the eastern, southern and western edges of the distribution and then rapid spread through the western Alps. After reviewing calibrated gene-specific substitution rates in the literature, we use a weighted average molecular clock to date our phylogeography. It shows that separation of the major clades occurred long before the last glacial maximum, suggesting that *O. elongata* persisted many glacial cycles within or at the edges of the Alps and Apennines.

Introduction

The Quaternary has proved a turbulent time for the flora and fauna of northern Europe. This period of Earth's history, from around 2.6 million years ago, has witnessed alternating glacial and interglacial periods driven by interactions between tectonic and orbital forces (Webb and Bartlein 1992; Williams et al. 1998; EPICA community members 2004). The fluctuating climate would have had profound effects on population migration and survival, the results of which are still apparent in the community composition and genetic diversity of recent organisms (Bennett 1990). During glacial periods, when large areas of northern Europe were covered by the growing ice shelf and permafrost, most lowland species survived in southern refugia in the Iberian, Italian and Balkan Peninsulas (Hewitt 1996; Taberlet et al. 1998; Hewitt 2000, 2004). Recolonization of temperate regions was affected by mountain ranges such as the Pyrenees or the Alps acting as barriers to some species, so that the contributions of different refugia varied in different taxa. For example, all northern European populations of *Chorthippus parallelus* and *Alnus glutinosa* emerged from the Balkans, whereas in *Erinaceus* and *Quercus* species, western, central and eastern regions were colonised independently from the Iberian, Italian and Balkan refugia, respectively (Hewitt 1999). This periodic extinction and recolonization shaped the genetic landscape of Europe, determining the large-scale patterns of spatial genetic structure and diversity (Hewitt 1996, 2001).

Whilst these patterns have been well documented for many lowland species, much less is known about the influence of ice ages on high altitude animals (Pauls et al. 2006; Schmitt et al. 2006; Haubrich and Schmitt 2007; Galbreath 2009; Mardulyn et al. 2009). They provide a contrast with the lowland taxa, since the current warm climate represents a period in which their range has probably declined. For the alpine flora, mountain refugia within the Alps and at their northern, southern and eastern borders have been proposed on the basis of recent

molecular studies (Stehlik *et al.* 2001; Stehlik 2002; Schönswetter *et al.* 2005). Such results have important implications for the community ecology of montane habitats. If many animal and plant species survived *in situ* this suggests a long period for coevolution and local adaptation, which would not have been available to most of the more recently assembled communities of the lowlands.

Here we address the bias towards lowland taxa by presenting the phylogeography of the alpine leaf-beetle *Oreina elongata* (Suffrian, 1851) (Coleoptera: Chrysomelidae). This species is adapted to survival at high altitudes, with isolated populations found across the Alps and Apennines at altitudes of 1200-2500m above sea level (Margraf *et al.* 2003; 2007; Röder *et al.* 2008). Seven allopatric subspecies have been described based on differentiation of male genitalia (the aedeagus) and cuticle microstructure (Ruffo 1946; Franz 1949; Daccordi and Ruffo 1976, 1986). These herbivorous beetles feed on hosts from two tribes of the Asteraceae: when feeding on *Cirsium* (Cynareae) larvae and adults synthesize cardenolides, whereas individuals feeding on *Adenostyles* or *Senecio* (Senecioneae) encounter plant-produced pyrrolizidine alkaloid N-oxides (PAs) that they are able to sequester (Dobler et al. 1996; Hsiao and Pasteels 1999; Röder et al. 2007; Verdon et al. 2007). This chemical defense is accompanied by what appears to be warning coloration in bright metallic patterns, with blue, green and mixed populations known. Color pattern does not covary with the type of defense, and the within-population polymorphism is unexpected, because learning by predators would be expected to generate positive frequency-dependent selection and lead to monomorphism (Mallet and Joron 1999).

In the present study we analyzed the genetic structure of 20 populations of *O. elongata* from across the whole species distribution. Sequencing of regions of three mitochondrial genes and one nuclear gene was used to answer the following questions:

1. Do the subspecies of *O. elongata* represent genetically distinct groups?
2. What does the phylogeography suggest about the evolution of color pattern in the species?
3. Did *O. elongata*, as a representative of the high altitude fauna, survive the cold periods of the Quaternary *in situ* in the Alps and Apennines?

Other studies in Quaternary phylogeography, particularly those based on allele frequencies from allozyme, microsatellite and AFLP data, have given little information as to the timescale over which differentiation is likely to have arisen. Here, we make use of an approximate molecular dating method based on a review of published gene-specific mtDNA substitution rates to answer a further question:

4. Was divergence within *O. elongata* a product of the last glacial cycle or is the differentiation more ancient?

Materials and Methods

Sampling

Between 2001 and 2007, *Oreina elongata* were collected from 20 populations covering the whole distribution (Alps and Apennines), including most of the sites where the species is known to exist, and all of the seven described subspecies (Ruffo 1946; Franz 1949; Daccordi and Ruffo 1976, 1986; Kippenberg 1994) (Fig. 1 & Table 1). Samples were preserved in pure ethanol and stored at -20°C, apart from the individuals from GLE that were dried specimens from a collection. For most populations, three individuals were chosen for the phylogeographic analysis, using only males to be sure of accurate identification based on genitalia (except for PDC, the holotype location for *O. elongata zoiai*, for which only larvae

could be obtained). Trees were rooted using two individuals of the closely related species, *Oreina virgulata* (Hsiao and Pasteels 1999).

Molecular methods

Total genomic DNA was extracted from four legs of each individual using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Three regions of mtDNA and one nuclear region were amplified using universal insect primers: a fragment of 16S ribosomal RNA [modLR-J-12887 (5'-CACCGGTTTGAACTCAGATC-3') with LR-N-13398 (Simon *et al.* 1994)]; cytochrome oxydase subunit I (COI) [C1-J-1751 with C1-N-2191 (Simon *et al.* 1994)]; cytochrome oxydase subunit II (COII) [modTL2-J-3037 with modC2-N-3661 (Mardulyn *et al.* 1997)]; and part of the nuclear region ITS2 [ITS3 with ITS4 (Gomez-Zurita and Vogler 2003)]. Fragments were amplified using a standard 30 µl PCR mix including: 3 µl of extracted DNA, 3 µl of 10X PCR buffer (Promega, Madison, USA), 3 µl of $MgCl_2$ solution (25 mM), 3 µl of dNTPs (1.5 mM), 0.5 µl of forward and reverse primer (Microsynth, Balgach, Switzerland), 0.3 µl of Taq DNA polymerase (Promega, Madison, USA), all made up to a final volume of 30 µl with purified MilliQ water. The PCR were run in a Biometra TGradient thermocycler (Biometra, Goettingen, Germany) using the following programs: for 16S and COI, initial denaturation for 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 45°C, 1.5 min at 72°C), then final elongation of 8 min at 72°C; for COII, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 53°C, 2 min at 72°C), then final elongation of 8 min at 72°C; for ITS2, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 48°C, 1 min at 72°C), then final elongation of 8 min at 72°C. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. Sequencing by Macrogen Inc. (Seoul, South Korea) was carried out with both forward and reverse primers under BigDye TM terminator cycling

conditions, purifying the products using ethanol precipitation and running them using an Automatic Sequencer 3730xl (Applied Biosystems, Foster City, USA).

Sequence alignment and phylogenetic analysis

Sequences (forward and reverse) were assembled and manually corrected using the software CHROMAS PRO version 1.34 (Technelysium, Helensvale, Australia). The protein coding nucleotide sequences of COI and COII were checked for reading frame errors and termination codons in MEGA 4 (Tamura *et al.* 2007). Alignment was carried out using CLUSTALW Multiple Alignment (Thompson *et al.* 1994) within the software BIOEDIT version 7.0.5.3, followed by minor manual correction.

Phylogenetic relationships within *Oreina elongata* were investigated as follows: i) "classical" phylogenetic algorithms were applied [based on Bayesian inference, maximum likelihood (ML) and maximum parsimony (MP) criteria] and subsequently the phylogeographic patterns were corroborated by analysing ii) mtDNA haplotype networks [using statistical parsimony networks (SPN) and median-joining networks (MJN)].

For i) partitioned Bayesian inference analysis was performed using MRBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with six runs of two chains of 30×10^6 generations, discarding a burn-in of 5×10^6 generations. Every 1000 generations one tree was saved and used to create a final majority rule consensus tree. The best fit substitution model for each partition was inferred using MRMODELTEST (Nylander 2004). ML analysis was carried out using RAxML version 7.0.0 (Stamatakis 2006) through the CIPRES portal in San-Diego, USA (http://www.phylo.org/portal/Home.do), with 500 rapid bootstraps (Stamatakis *et al.* 2008) followed by the search of the best-scoring ML tree in one single run. The MP analysis was performed using PAUP* version 4.0b10 (Swofford

2003) under the heuristic search option (tree-bisection-reconnection, branch swapping, random sequence addition, MaxTrees=500) with 1000 random addition replicates.

For ii) mitochondrial DNA haplotypes were used as raw data to perform single and multi-locus SPN and MJN networks, as implemented respectively in TCS version 1.21 (Clement *et al.* 2000) and SPLITSTREE version 4.10 (Huson and Bryant 2006). The SPN analyses were carried out by applying a 95% connection limit and gaps were treated as a fifth state.

Divergence time estimation

Bayesian divergence time analyses were performed with BEAST version 1.4.7 (Drummond and Rambaut 2007) and analyzed using TRACER version 1.4 (Rambaut and Drummond 2007). The three mtDNA regions were represented as separate partitions in the analysis, with substitution models previously estimated by MRMODELTEST (Nylander 2004) and four estimated alpha categories for the gamma parameter (Yang 1994). Three independent runs of 20×10^6 generations were performed, sampling one tree every 1000 generations. A maximum clade credibility tree was generated after removing a burn-in of 5×10^6 generations of each run.

Due to the lack of fossils for *Oreina* beetles, direct calibration of the tree topologies was not possible. Instead, branch lengths and node ages were estimated by applying an arthropod mtDNA clock of 0.0061 substitutions per site per MY (1.22% pairwise sequence divergence per MY). This value was based on a review of published gene-specific mtDNA substitution rates in diverse arthropod taxa (Table 2), which suggests average pairwise divergence rates of 1.73% for COI, 1.38% for COII and 0.61% for 16S. Calculating an average of these rates weighted by the length of sequence that we use for each gene region gives an overall value of 1.22%. Divergence time estimations were performed based on two molecular clock models: a

strict clock and a relaxed clock with log-normal branch length distribution (Drummond *et al.* 2006).

Results

Sixty-five specimens representing all seven described subspecies were collected from the 20 populations and amplified for the three mtDNA and the nuclear ITS2 region. Sequences are available at Genbank under accession numbers GQ220057 to GQ220295.

The 589 bp of ITS2 contained only three polymorphic sites and was not analyzed phylogenetically. However, two of the three sites were unique to one clade in the mtDNA phylogeography, being found exclusively in *O. e. siparii*. The other divides the species into eastern and western regions, separated by a line running to the west of MTT and FEN (Fig. 1). The concatenated mtDNA matrix included 1555 bp: 515 bp for 16S [nine parsimony-informative (pi) sites among nine polymorphic sites], 411 bp for partial COI (45 pi sites among 54 polymorphic sites) and 629 bp for partial COII (31 pi sites among 94 polymorphic sites). The relatively low proportion of polymorphic sites, ranging from 1.75% (16S) to 14.94% (COII), is typical for this taxonomic (intraspecific level) and geographic scale (spanning 800 km in north-south and east-west directions) (Simon *et al.* 1994). The best fit substitution models and parameter values suggested by MRMODELTEST (Nylander 2004) are given in Table 3. Amplification failed in a few cases (for COI, single individuals from VAL, LAF, MTT, and three from PAS; for COII, two from PAS and AFR, and three from BOG, MTT, MLS, GLE and PDC; for 16S all amplifications were successful; for ITS2, four individuals from GLE). This missing data does not seem to have adversely affected the results, for these populations have not been pulled together in the phylogenetic analyses, nor do individuals with a locus missing fall away from other members of their population.

Bayesian inference, ML and MP analyses produced highly congruent topologies (with the same major nodes and branching order) and only the phylogenetic tree obtained with Bayesian inference methods in BEAST is shown (Fig. 2). All basal nodes in this tree have very high support except one (node 4 in Fig. 2), with a Bayesian posterior probability of 0.86. Four of the subspecies form monophyletic clades (*O. e. styriaca*, *O. e. siparii*, *O. e. zoiai* and *O. e. zangherii*) while the others are paraphyletic or polyphyletic. Within the phylogenetic tree, individuals of *Oreina e. ruffoi* and *O. e. elongata* are found basally, with one population of *O. e. elongata* forming a distinct clade. The subspecies *O. e. styriaca* and *O. e. siparii* then split off as well-defined individual clades. Finally, the subspecies *O. e. occidentalis* is paraphyletic with respect to *O. e. zangherii* and *O. e. zoiai*. However, given the weak support (0.86) for one of these nodes this could also be considered as a polytomy between *zangherii-zoiai* and two *occidentalis* clades.

To investigate the phylogenetic signal expressed per marker, single mtDNA haplotype networks were carried out based on SPN and MJN criteria. Both methods showed highly congruent topologies and only SPN results are discussed hereafter. Haplotypes are given in Table 1. Although the markers differ in their level of polymorphism and percentage of missing data, the three mtDNA haplotype networks display highly congruent patterns (Supporting information Fig. S1). They were therefore united to perform a combined network analysis, but to avoid potential misleading relationships produced by missing data and because the algorithm implemented in TCS does not handle missing data, this analysis was based on the 16S and COI datasets (the COII dataset contains 28.4% of missing data). The combined analysis defines five unconnected networks based on a 95% connection limit (Fig. 3). The main network includes five haplotypes in *O. e. occidentalis* (OC1-5) linked closely to single haplotypes in *O. e. zoiai* (ZO) and *O. e. zangherii* (ZA), together with two more

distantly connected haplotypes in *O. e. siparii* (SI1 and 2). When the connection limit is increased to 18 steps the three previously unconnected haplotypes EL, RU1 and RU2 connect to SI1-2. At 20 steps, haplotypes ST1-3 connect near to the SI1-2 cluster. Finally, the outgroup joins after increasing the connection limit to 52 steps. The network analyses therefore attest to the isolation and distinctness of most subspecies, with their separation by long chains of extinct haplotypes. Most subspecies include one to three haplotypes, but the pattern within *O. e. occidentalis* corroborates its paraphyly with respect to *O. e. zoiai* and *O. e. zangherii* and its division into small and large clades in the Bayesian analysis. Furthermore, the LAU and GAL populations include individuals with haplotypes typical of both clades, suggesting some migration. The two clades are each dominated by a single haplotype, and in the larger *O. e. occidentalis* clade in particular, the OC1 haplotype is found across the entire north-south range suggesting relatively rapid expansion across that region of the western Alps.

Divergence time estimates

To establish a temporal framework for evolution within *Oreina elongata*, we applied a weighted average of published arthropod mtDNA substitution rates (1.22% pairwise sequence divergence per MY). The analysis suggests that all the major clades were formed long before the last glacial period (Table 4 and Fig. 2), with divergence spanning almost the last 5 million years. The subspecies from the central Alps, *O. e. elongata* and *O. e. ruffoi*, are located in a basal position. Separation of *O. e. styriaca* (Fig. 2, node 1), a subspecies found on the eastern edge of the Alps, took place 1.78 or 1.48 MYA depending on the clock model used (each with relatively wide confidence intervals; Table 4). The subspecies *O. e. siparii*, with its distribution in the central Apennines, diverged 1.43 or 1.14 MYA (Fig. 2, node 2), followed by individuals from four populations in the western Alps (PAL, AFR, LAU, and GAL) that

formed a small *O. e. occidentalis* clade 0.53 or 0.58 MYA (Fig. 2, node 3). Finally, two subspecies in the northern Apennines, *O. e. zoiai* and *O. e. zangherii*, separated from the larger *O. e. occidentalis* clade 0.39 or 0.42 MYA (Fig. 2, node 4).

Color pattern evolution

There is strong geographical structure in the distribution of color variation, with green populations in the eastern part of the species range, and with blue and mixed populations in the west (Fig. 1, based on personal observations). When mapped onto the phylogeography, however, the pattern is much less clear (Fig. 2). Blue and green morphs are present in the basal group, then two clades consisting of green populations diverge, followed by a large clade that includes all possible color combinations.

Discussion

Do the subspecies of O. elongata *represent genetically distinct groups?*

Microsatellite data suggests very strong genetic differentiation of populations within *O. elongata* (with an overall F_{ST} of 0.381 and high pairwise values everywhere) and hence little migration (Margraf *et al.* 2007). This high level of isolation is confirmed here, as attested by haplotype network, the clustering of individuals from the same populations in the phylogeny and by the differentiation of the nuclear locus in *O. e. siparii*. Of the seven subspecies, four (*styriaca*, *siparii*, *zoiai* and *zangherii*) form monophyletic clades within the phylogeny. However, each of these is represented within the analysis by a single population. This is unavoidable because some subspecies are only known from single localities, but it means that distinctness at the level of subspecies is confounded with population-level differentiation. The phylogeny therefore does not provide strong evidence that the subspecies represent

genetically distinct groups. In addition, the subspecies *O. e. elongata* and *O. e. ruffoi* are not monophyletic. However, analysis of microsatellites clusters the four populations into two distinct subspecies (Margraf *et al.* 2007), so the mtDNA data may represent incomplete lineage sorting of ancestral polymorphism in the 16S locus. Finally, *O. e. occidentalis* is paraphyletic with respect to *O. e. zoiai* and *O. e. zangherii*. For these three subspecies, analysis of morphological data and perhaps the addition of more variable genetic sequences would be needed to determine whether *O. e. occidentalis* should be split and then the groups treated as four separate subspecies or as forms within *O. e. occidentalis*.

What does the phylogeography suggest about the evolution of color pattern in O. elongata*?*

The distribution of color pattern variation shows strong geographic structure, with green populations in the eastern Alps and Apennines, blue in southern Switzerland, and both blue and mixed populations scattered throughout the western Alps. However, this would be misleading if taken as a marker of large-scale phylogeographic structure, as shown by comparison with the mtDNA phylogenetic hypothesis. The outgroup is known to be polymorphic (Kippenberg 1994), and both green and blue populations are found in the basal *O. elongata* clade of the phylogenetic tree, later giving rise to green, blue and mixed populations. This suggests two possible evolutionary scenarios: polymorphism at the base of the phylogeography but with loss of one or the other morph in most populations, or independent origin of blue morphs at the base and within the *occidentalis* subspecies (perhaps twice). Population size is small in most subspecies except *occidentalis* so the species would seem predisposed to lose diversity through genetic drift, and purifying selection by predators would be expected to reinforce this effect, but both scenarios must be considered plausible. Two potential explanations for within-population polymorphism can be excluded, for it is clear that the two color morphs do not represent coexisting distinct species or subspecies, and

neither is there a pattern that would suggest that polymorphism is maintained by current dispersal from monomorphic source populations. The evolutionary forces that maintain this unexpected color polymorphism in a chemically defended species therefore remain to be elucidated.

Did O. elongata*, as a representative of the high altitude fauna, survive the cold periods of the Quaternary* in situ *in the Alps and Apennines?*

The phylogenetic analysis and molecular clock hypothesis, even if only providing an approximate dating method, show that separation of the major clades in *Oreina elongata* occurred long before the last glacial maximum. In fact, divergence within the species spans the entire Quaternary, and the repeated glacial and interglacial periods may have been the engine for this differentiation. In contrast to lowland taxa, these beetles are likely to have occupied a larger area during cold periods, for although they may have been forced to retreat from the highest altitudes by the enlargement of glaciers within the Alps, a greater area of lower habitats would have become available. During warm spells like the present they therefore display a reduced distribution, separated into more isolated, higher altitude populations. The general pattern in the phylogeography is that the populations from the central Swiss Alps and northern Italy are basal, with subsequent separation of the eastern, southern (Apennine) and western extremes of the distribution, and finally a second colonization of the Apennines. The basal populations of *O. e. elongata* and *O. e. ruffoi* separate early and must have survived many glacial cycles in isolation from the rest of the species, perhaps in refuges within the Alps. The ancestors of the other clades remained in contact elsewhere, and may have inhabited an expanded range during repeated cold periods south of the Alps across northern Italy and the northern part of the Adriatic (which would have been exposed at such times by falling sea levels). Populations from this region seem to

have given rise to the subspecies *O. e. styriaca* in the eastern Alps around 1.78 MYA, and *O. e. siparii* in the central Apennines around 1.43 MYA. The remaining populations may have survived colder periods along the edge of the western Alps. A small clade within *O. e. occidentalis* arose in the far west around 0.53 MYA. This geographical structuring within the subspecies is associated with the north-south orientated Guisane and Durance valleys, with the populations PAL and AFR found to the south and in the north the populations LAU and GAL (both containing individuals from the small and large clades of *O. e. occidentalis*, suggesting some migration). The remaining populations form the larger clade of *O. e. occidentalis*, together with a second invasion into the Apennines around 0.39 MYA, forming the subspecies *O. e. zoiai* and *O. e. zangherii*. The haplotype network suggests that this large clade of *O. e. occidentalis* appears to have spread through the western Alps relatively recently and rapidly.

The structuring within *Oreina elongata* is very similar to that seen in plants in the Alps. The contemporary distributions of alpine populations correspond strikingly with the four refugia proposed for alpine plants along the southwestern, southern and eastern edges of the Alps (Schönswetter *et al.* 2005; Margraf *et al.* 2007). Peripheral refugia in the southwestern and southern Alps have also been suggested for the mountain ringlet, *Erebia epiphron* (Schmitt *et al.* 2006), and the caddisfly, *Drusus discolor* (Pauls *et al.* 2006). *Oreina elongata* therefore joins a growing list of high altitude plant and animal species that appear to have survived glacial periods close to their current distribution (Schmitt 2009). The phylogeography presented here indicates that divergence of the major clades occurred between 5 and 0.4 million years ago, suggesting that *Oreina elongata* survived the Quaternary within or at the edge of the Alps and Apennines.

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Table 1. Sampled populations of *Oreina elongata* with their geographical coordinates, subspecies, number of treated individuals, year of collection and haplotype identification. Numbers in brackets show individuals per haplotype in populations with several haplotypes or those with missing loci.

Code	Population	Altitude (m a.s.l.)	Geographical coordinates	Subspecies	Sample size	Year	haplotype			
							16S and COI	16S	COI	COII
LAF	La Fouly (CH)	2184	45°56'N, 07°04'E	*occidentalis*	3	2006	OC1(2)	OCZO	OC1(2)	OC1
EMO	Lac Emosson (CH)	1970	46°03'N, 06°55'E	*occidentalis*	3	2007	OC1	OCZO	OC1	OC1
PSB	Col du Petit St. Bernard (F)	2188	45°40'N, 06°52'E	*occidentalis*	3	2001	OC1	OCZO	OC1	OC1
MCE	Mont Cenis (F)	2085	45°15'N, 06°54'E	*occidentalis*	6	2005	OC1	OCZO	OC1	OC1
GAL	Col du Galibier (F)	1999	45°05'N, 06°26'E	*occidentalis*	3	2001	OC1(2)	OCZO	OC1(2)	OC1(2)
							OC2(1)		OC2(1)	OC2(1)
LAU	Col du Lautaret (F)	1811	45°00'N, 06°22'E	*occidentalis*	3	2001	OC2(1)	OCZO	OC2(1)	OC1(2)
							OC4(2)		OC4(2)	OC2(1)
AFR	Ailefroide (F)	1800	44°53'N, 06°26'E	*occidentalis*	4	2001	OC2	OCZO	OC2	OC2(2)
PAL	Lac Palluel (F)	2479	44°43'N, 06°24'E	*occidentalis*	3	2001	OC2	OCZO	OC2	OC2
FEN	Col de Fenestre (F)	2470	44°06'N, 07°21'E	*occidentalis*	3	2007	OC1(2)	OCZO	OC1(2)	OC1(2)
							OC5(1)		OC5(1)	OC4(1)
VAL	Terme di Valdieri (I)	2340	44°12'N, 07°15'E	*occidentalis*	3	2001	OC1(2)	OCZO	OC1(2)	OC1
NCE	L'Authion (F)	2080	44°00'N, 07°26'E	*occidentalis*	3	2001	OC3	OCZO	OC3	OC1
MON	Monte Mongioie (I)	1920	44°10'N, 07°47'E	*occidentalis*	3	2001	OC1	OCZO	OC1	OC3
PDC	Pania della Croce (I)	1718	44°02'N, 10°19'E	*zoiai*	3	2007	ZO	OCZO	ZOZA	-
PLC	Passo la Calla (I)	1260	43°51'N, 11°44'E	*zangherii*	3	2007	ZA	ZA	ZOZA	ZA
ABB	Pizzone (I)	-	41°41'N, 13°57'E	*siparii*	3	2001	SI1(2)	SI1(2)	SI1(2)	SI1(2)
							SI2(1)	SI2(1)	SI2(1)	SI2(1)
MLS	Giazza (I)	1510	45°41'N, 11°06'E	*elongata*	3	2001	EL	EL1	EL	-
PAS	Monte Pasubio (I)	2110	45°47'N, 11°10'E	*elongata*	3	2001	-	EL2(2)	-	EL(1)
								ELRU(1)		
GLE	Glein (A)	1570	47°13'N, 15°03'E	*styriaca*	4	2004	ST1(2)	ST	ST1(2)	ST(1)
							ST2(1)		ST2(1)	
							ST3(1)		ST3(1)	
BOG	Bosco Gurin (CH)	1835	46°18'N, 08°27'E	*ruffoi*	3	2001	RU1	ELRU	RU1	-

MTT	Mattmark (CH)	2239	46°01'N, 07°58'E	*ruffoi*	3	2001	RU1(1) RU2(1)	ELRU	RU1(1) RU2(1)	-

A, Austria; CH, Switzerland; F, France; I, Italy; m a.s.l., metres above sea level.

Table 2. Published substitution rates (pairwise divergence per million years) of three mtDNA gene regions for arthropods.

Gene region	Taxon	Substitution rate (% MY^{-1})	Calibration (fossil or biogeographic)	Reference
COI	*Alpheus* (Decapoda: Alpheidae)	1.40	Uplift Isthmus of Panama (3 MYA)	(Knowlton and Weigt 1998)
COI	*Sesarma/Sesarmoides* (Decapoda: Sesarmidae/Grapsidae)	1.66	Uplift Isthmus of Panama (3.1 MYA)	(Schubart *et al.* 1998)
COI	*Ovobathysciola/Patriziella/Speonomus* (Coleoptera: Cholevidae)	2.50	Corsica-Sardinia plate separation (29 MYA)	(Caccone and Sbordoni 2001)
COI	*Tetraopes* (Coleoptera: Cerambycidae)	1.50	Origin of habitats (1-20 MYA)	(Farrell 2001)
COI	*Plateumaris* (Coleoptera: Chrysomelidae)	1.60	Fossils and biogeography (0.5 - 10 MYA)	(Sota and Hayashi 2007)
16S	*Sesarma/Sesarmoides* (Decapoda: Sesarmidae/Grapsidae)	0.65	Uplift Isthmus of Panama (3.1 MYA)	(Schubart *et al.* 1998)
16S	*Petrolisthes/Pachycheles* (Decapoda: Porcellanidae)	0.53	Uplift Isthmus of Panama (3 MYA)	(Stillman and Reeb 2001)
16S	*Timarcha* (Coleoptera: Chrysomelidae)	0.45	Opening of Gibraltar strait (5.3 MYA)	(Gomez-Zurita *et al.* 2000)
16S	*Iurus* (Scorpiones: Iuridae)	0.79	Separation Crete-Peloponnisos (5.33 MYA)	(Parmakelis *et al.* 2006)
COII	*Timarcha* (Coleoptera: Chrysomelidae)	0.76	Opening of Gibraltar strait (5.3 MYA)	(Gomez-Zurita *et al.* 2000)
COII	New Zealand cicadas (Hemiptera: Cicadidae)	2.00	Geological calibrations (9.3 MYA)	(Arensburger *et al.* 2004)

Table 3. Best supported models of molecular evolution and estimated parameter values for the analyzed genes.

		Nucleotide frequencies					
Gene region	Model	A	T	C	G	α	Tr/Tv
16S	HKY	0.40	0.36	0.15	0.09	-	3.517
COI	HKY+G	0.28	0.35	0.20	0.17	0.0158	3.708
COII	HKY+G	0.35	0.38	0.16	0.11	0.0996	4.330
ITS2	JC	0.26	0.28	0.22	0.24	-	-

α is the shape parameter for the gamma distribution and Tr/Tv is the transition-transversion ratio.

Table 4: Ages (in millions of years) of four nodes in the *O. elongata* phylogeography, based on strict and log-normal relaxed clock models.

	Strict		Lognormal	
Node	Mean	95% C.I.	Mean	95% C.I.
1	1.78	1.31-2.31	1.48	0.60-3.08
2	1.43	1.00-1.88	1.14	0.48-2.33
3	0.53	0.32-0.78	0.58	0.22-1.37
4	0.39	0.22-0.59	0.42	0.17-0.92

Figure legends

Figure 1. Distribution of the 20 sampled populations of *O. elongata* in Italy and neighboring countries, showing their subspecies (symbols) and color morphs present (shading). Topographic shading in the Alps and Apennines shows altitudes above 1000m.

Figure 2. Phylogenetic reconstruction for *O. elongata* produced by the software BEAST with tree calibration based on 1.22% pairwise mtDNA divergence per million years. Details for the four numbered nodes are given in Table 4 and discussed in the text. Values at the nodes are Bayesian posterior probabilities and ages in million years (italics). Terminal clades have been collapsed. Population codes are the same as in Figure 1 and Table 1, with the color patterns of individuals indicated by the squares: unfilled green, filled blue.

Figure 3. Statistical parsimony network (SPN) for the combined 16S and COI haplotype data. Symbol area is proportional to the number of specimens sharing the haplotype, and they are named according to the subspecies in which they are found (see Table 1 for details). Lines represent one mutational step, while small black circles indicate missing haplotypes that were either not sampled or have become extinct.

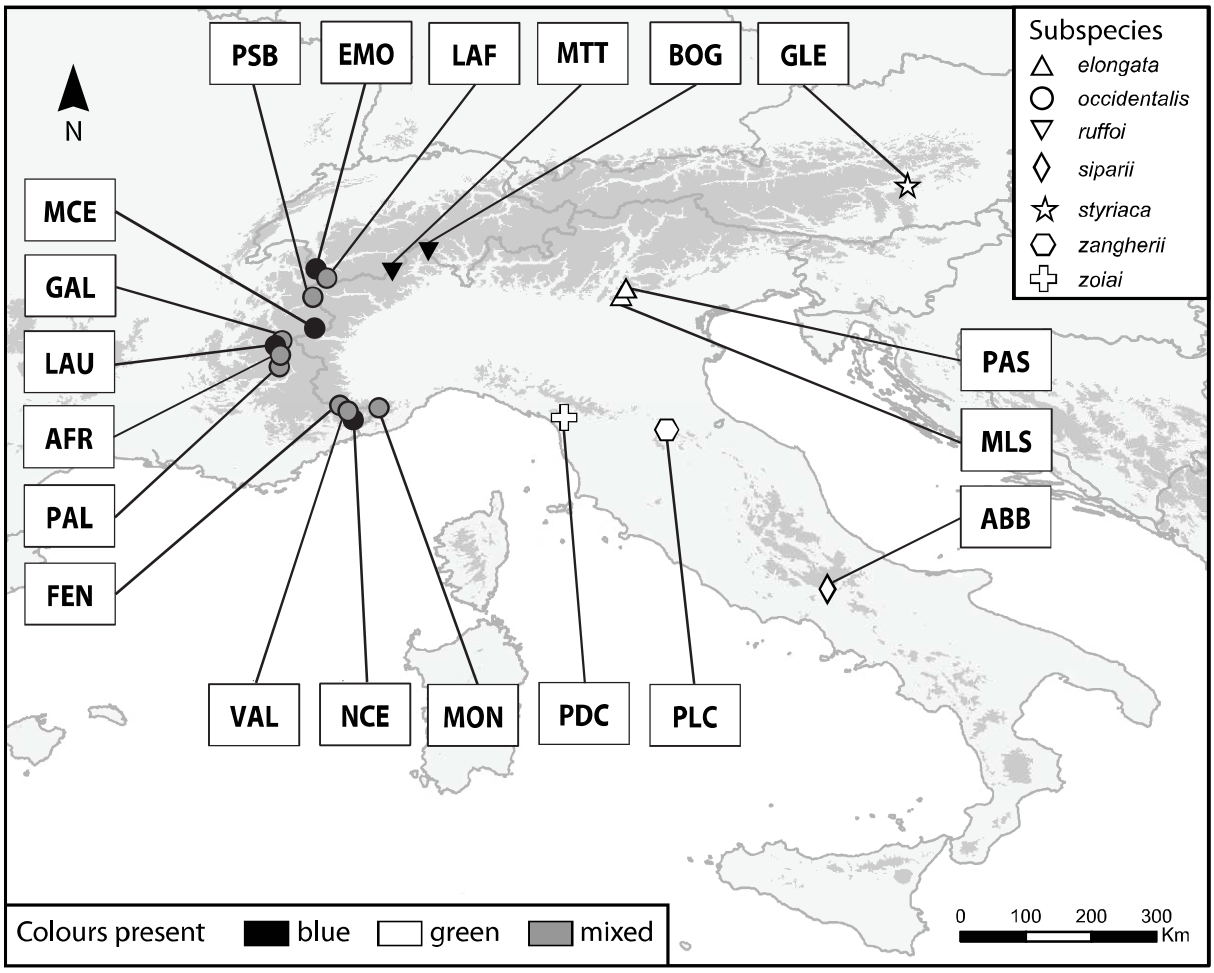
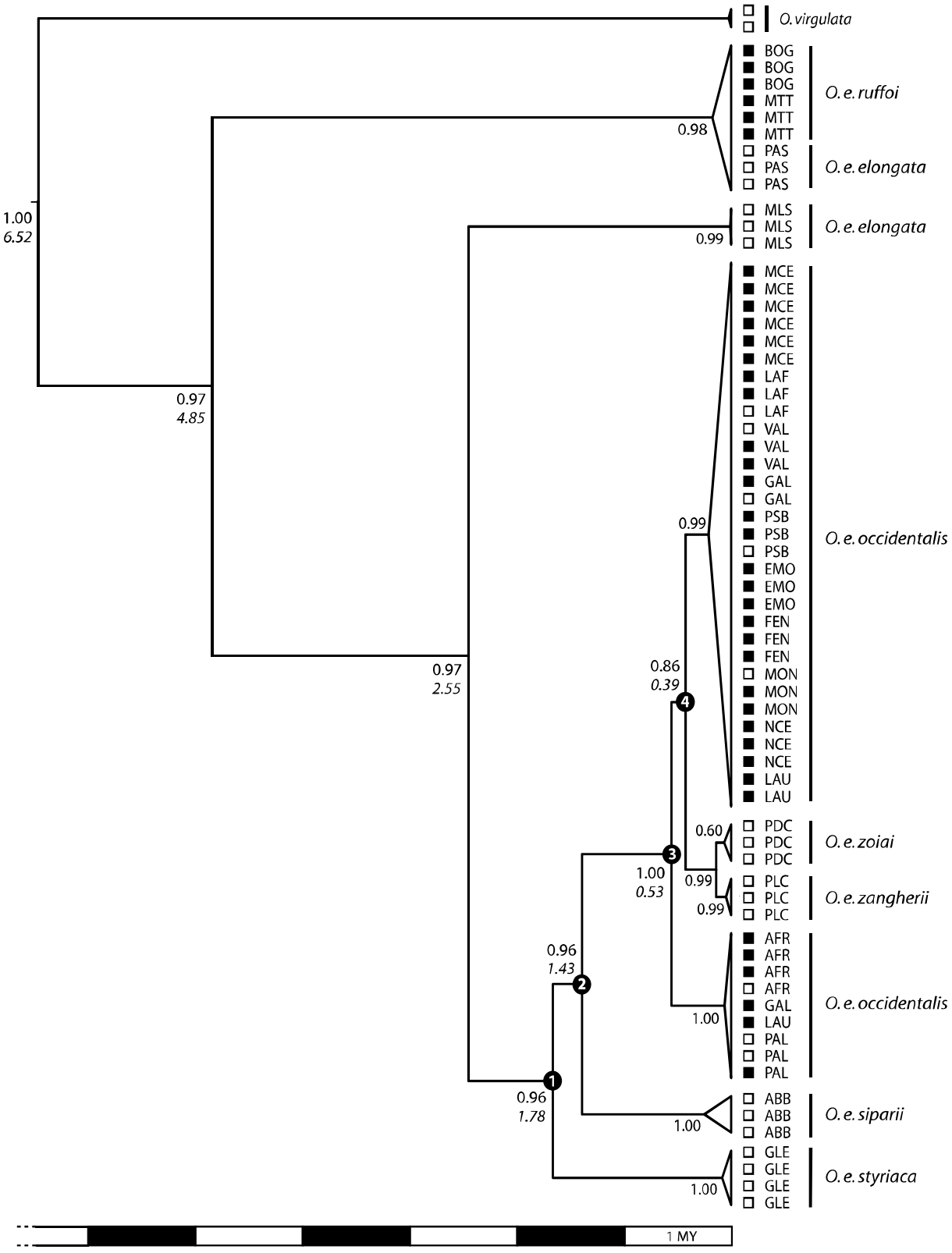

PSB
EMO
LAF
MTT
BOG
GLE
N
MCE
GAL
LAU
AFR
PAL
FEN
PAS
MLS
ABB
VAL
NCE
MON
PDC
PLC
Subspecies
elongata
occidentalis
ruffoi
siparii
styriaca
zangherii
zoiai
Colours present
blue
green
mixed
0
100
200
300
Km

Figure 1


O. virgulata
BOG
BOG
BOG
MTT
MTT
MTT
O. e. ruffoi
PAS
PAS
PAS
O. e. elongata
MLS
MLS
MLS
O. e. elongata
MCE
MCE
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MCE
MCE
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LAF
LAF
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VAL
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PSB
EMO
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FEN
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MON
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MON
NCE
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LAU
LAU
O. e. occidentalis
PDC
PDC
PDC
O. e. zoiai
PLC
PLC
PLC
O. e. zangherii
AFR
AFR
AFR
AFR
GAL
LAU
PAL
PAL
PAL
O. e. occidentalis
ABB
ABB
ABB
O. e. siparii
GLE
GLE
GLE
GLE
O. e. styriaca
0.98
1.00
6.52
0.97
4.85
0.99
0.97
2.55
0.99
0.86
0.39
4
0.60
3
1.00
0.53
0.99
0.99
0.96
1.43
2
1.00
1
0.96
1.78
1.00
1.00
1 MY

Figure 2

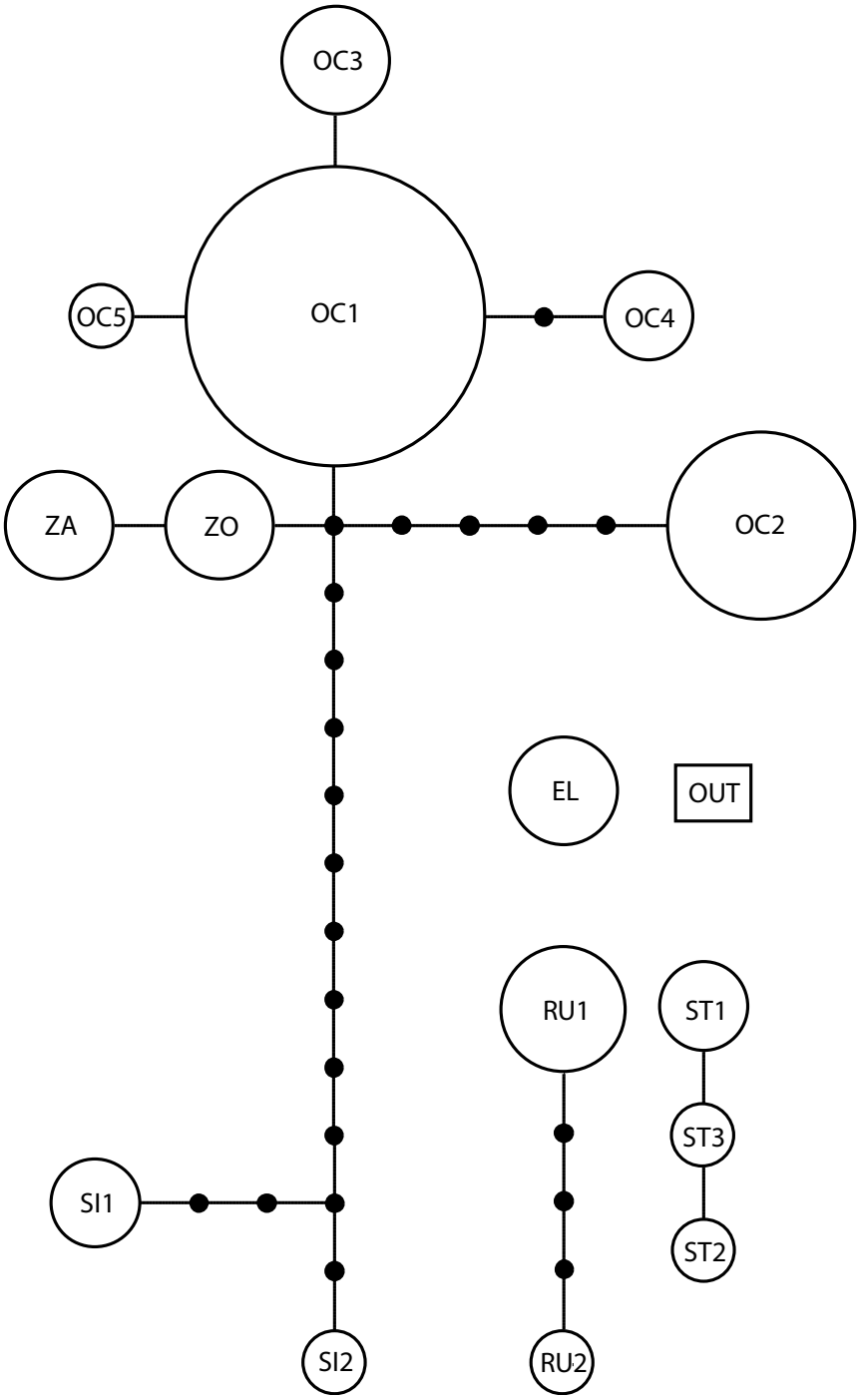

Figure 3

Supplementary information

Single mtDNA haplotype network

Despite the low level of pi sites within 16S (nine pi sites), this marker (without missing data) provided nine mtDNA haplotypes (Fig. S1a). The haplotype OCZO (including all specimens of *O. e. occidentalis* and *O. e. zoiai*) is one step apart from the ZA haplotype (*O. e. zangherii*). SI1 and SI2 clustered together and belong to *O. e. siparii*. The haplotype ST includes all specimens of *O. e. styriaca*. Analyzed specimens of *O. e. elongata* are distributed in three distinct haplotypes: ELRU (shared with specimens of *O. e. ruffoi*), EL1 and EL2. The outgroup is separated by seven steps from the closest related haplotype, ELRU. The haplotype network inferred from COI (containing 45 pi sites and six missing samples; see Table 1) results in six unconnected networks when the 95% connection limit is applied. Haplotypes OC1-5 and ZOZA form one main network, whereas the remaining haplotypes are connected as follows: RU1-2, SI1-2, ST1-3, EL and OUT (Fig. S1b). The COII marker (including 31 pi sites and 19 missing samples) shows four unconnected networks when the 95% connection limit is applied (Fig. S1c). The main network includes three very closely related haplotypes (OC1, OC3, OC4) together with ZA and CO2. Haplotypes SI1 and SI2 complete the main network with a distance of respectively nine and 11 steps. EL, ST and the outgroup form single unconnected networks (Fig. S1c).

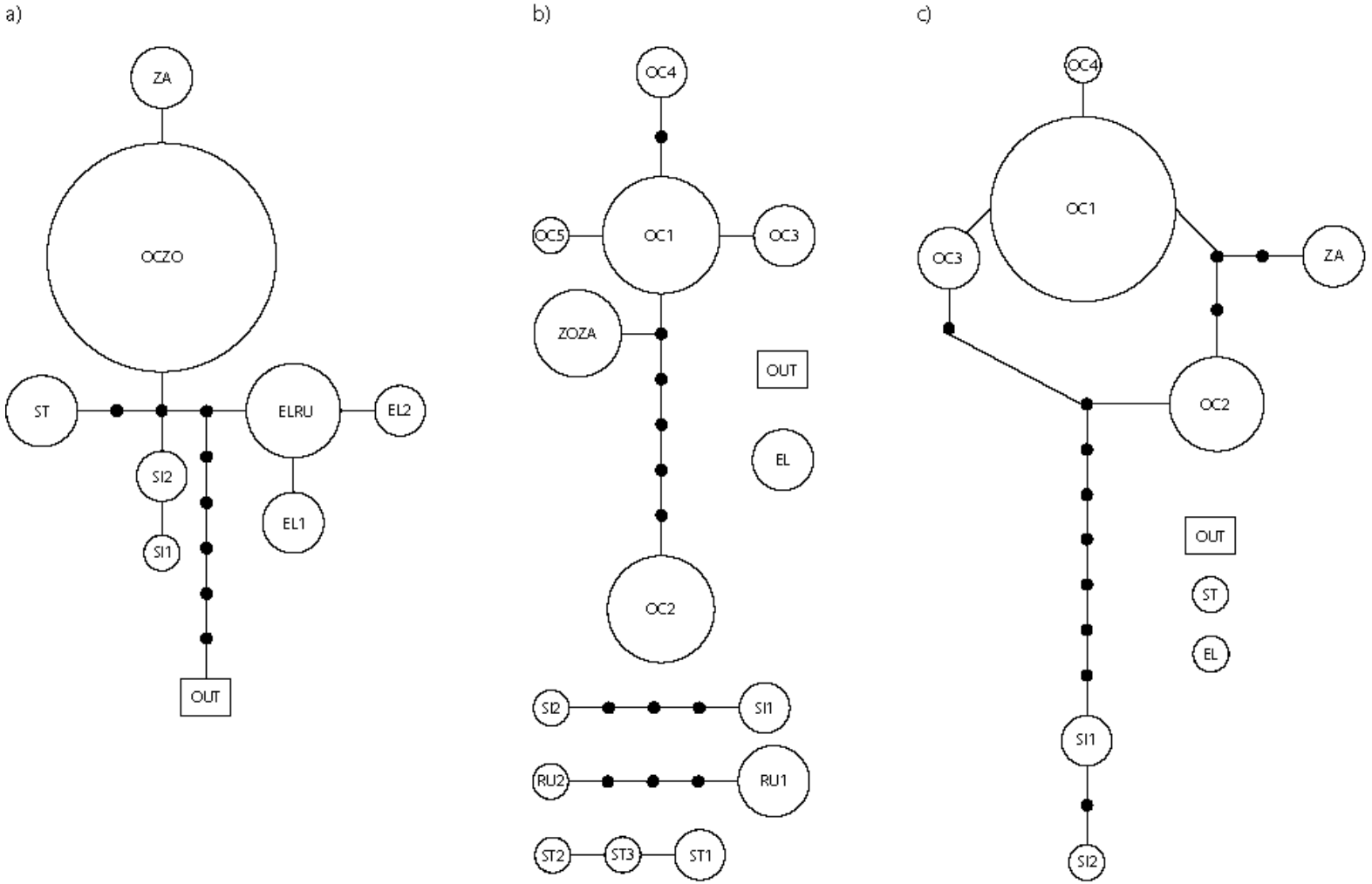

Fig. S1. Statistical parsimony networks (SPN) for the a) 16S, b) COI and c) COII haplotypes. Symbol area is proportional to the number of specimens sharing the haplotype, and they are named according to the subspecies in which they are found (see Table 1 for details). The lines between haplotypes represent one mutational step. The small black circles indicate missing haplotypes which were either not sampled or have become extinct.

Chapter II

Comparative phylogeography in an Alpine antagonism: the monophagous leaf beetle *Oreina gloriosa* (Coleoptera; Chrysomelidae) and its host plant *Peucedanum ostruthium* (Apiaceae)

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Keywords: phylogeography, *Oreina gloriosa, Peucedanum ostruthium*, DNA sequencing, AFLP

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Running title: Comparative phylogeography in an Alpine antagonism

Abstract

Climatic oscillations during the Quaternary strongly influenced the genetic structure of many plants and animals in Europe. Numerous phylogeographic studies have shown the resulting patterns in the genetic structure of lowland species due to the extension and retraction of ice sheets during glacial and interglacial periods. Much less is known about the effect on arctic and high altitude species, and in both cases, usually only single species have been investigated.

Here we compare the phylogeography of two species involved in an alpine antagonism, the monophagous leaf beetle *Oreina gloriosa* and its host plant *Peucedanum ostruthium.* For the beetle three mitochondrial regions were sequenced and analyzed. In addition, AFLP genotyping for both species was done in order to compare the phylogeographies of the two species. For the beetle, the phylogeographies based on AFLP and mtDNA analyses show similar results, with lineages in the eastern, central and western Alps. When comparing the two species, the patterns are broadly similar, but with differences that suggest different histories of dispersal and sub-division.

We applied a molecular clock with an individual substitution rate for each mtDNA marker, to date the divergence in the beetle. It shows that divergence in *O. gloriosa* started long before the last glacial maximum, with differentiation spanning half a million years.

Introduction

During the Quaternary, climatic oscillations strongly influenced the genetic structure of organisms in the Palaearctic (Bennett 1990; Hewitt 1996; 2001). Such effects were particularly exemplified in species occurring in harsh habitats, such as those with current alpine distributions. Numerous evidence suggests that in the past, these species were much more broadly distributed than they are today, especially during the ice age maxima when they occupied the extensive European steppe (Schmitt 2009). Recent molecular studies of the alpine flora and insects have even proposed mountain refugia within the Alps (nunataks) as well as on their northern, southern and eastern edges (Borer *et al.* ; Stehlik *et al.* 2001; Stehlik 2002; Schönswetter *et al.* 2005; Pauls *et al.* 2006; Schmitt *et al.* 2006; Haubrich & Schmitt 2007).

However, it can be expected that climatic oscillations not only influence single-species evolutionary histories, but also ecological interactions and species coevolution as a whole (Jablonski & Sepkoski 1996; Mouritsen & Poulin 2002). From one glacial cycle to another, the species composition of alpine communities should differ, according to the ability of populations to recolonise suitable habitats, but also due to many stochastic processes in their population dynamics (Jackson & Overpeck 2000). Therefore, the evolution of specific interactions between European taxa is threatened during every glacial cycle and emerging coevolutionary processes could stop brutally. If one species strongly depends on another, its history is completely linked to that of its partner. After addressing the biogeographic histories of single species, the next step is therefore to understand how glaciation cycles modify the pattern of specific interactions among such species. So far no study has investigated the influence of trophic interactions on the phylogeography of the dependent species in two high altitude species.

Here we investigate and compare the phylogeographies of the alpine leaf beetle *Oreina gloriosa* (FABRICIUS, 1781) (Coleoptera: Chrysomelidae), and its exclusive host plant, the masterwort *Peucedanum ostruthium* (Apiaceae). These beetles are found in isolated populations, with a distribution restricted to the Alps (Kippenberg 1994). *Peucedanum ostruthium* is a subalpine to alpine, perennial herbaceous plant. Its distribution spans Western, Central and Northern Europe, covering parts of Sweden, Scotland, the Massif Central, the northern Apennines and the Alps. *Oreina gloriosa* is one of the few *Oreina* species that is highly specialized on one host plant (Dobler *et al.* 1996; Verdon *et al.* 2007), although it has not spread throughout the entire distribution of its host. In the present study we analysed and compared the genetic structure of *O. gloriosa* and its host plant *P. ostruthium* from 22 populations covering the entire distribution of the betle (i.e., the Alps). Sequencing of one nuclear and three mitochondrial regions (in *O. gloriosa*) and AFLP genome fingerprinting (in both *O. gloriosa* and *P. ostruthium*) were performed to address whether or not the monophagous beetle shares the phylogeographic pattern with its host plant. As one of the two species is independent of the other, we could expect that the plant could disperse freely, whereas the other is strongly constrained in its migration pathways; as a consequence, the phylogeographies would not necessarily be congruent. The alternative hypothesis would be that despite this unbalanced dependence, the two species disperse in parallel, the insect following the footsteps of the plant. In such a scenario, the phylogeographies would be congruent. Finally, in order to include a time framework in our understanding of the evolutionary histories of the two species, we also investigated the time span over which the divergence processes occurred within *O. gloriosa*.

Materials and Methods

Sampling

Between 2004 and 2008, *Oreina gloriosa* were collected from 22 populations (three beetles per site) covering the whole Alpine distribution (Table 1). Samples were preserved in pure ethanol and stored at -20°C. For the phylogeographic analysis only males were chosen, in order to allow accurate species identification based on genitalia (Kippenberg 1994). Trees were rooted using two individuals of the closely related species, *Oreina speciosa* (Hsiao & Pasteels 1999). *Peucedanum ostruthium* was sampled between 2007 and 2008 (one to three individuals per site) at the same sites as *O. gloriosa*. The freshly collected leaf material was put into plastic bags filled with silica gel for rapid desiccation.

Oreina gloriosa

DNA extraction, sequencing and alignment

Total genomic DNA was extracted from four legs of each individual using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Three regions of mtDNA and one nuclear region were amplified using universal insect primers: a fragment of *16S* ribosomal RNA (hereafter *16S*) [modLR-J-12887 (5'-CACCGGTTTGAACTCAGATC-3') with LR-N-13398 (Simon *et al.* 1994)]; cytochrome oxydase subunit I (*COI*) [C1-J-1751 with C1-N-2191 (Simon *et al.* 1994)]; cytochrome oxydase subunit II (*COII*) [modTL2-J-3037 with modC2-N-3661 (Mardulyn *et al.* 1997)]; and part of the nuclear region *ITS2* [ITS3 with ITS4 (Gomez-Zurita & Vogler 2003)]. Fragments were amplified using a standard 30 µl PCR mix including: 3 µl of extracted DNA, 3 µl of 10X PCR buffer (Promega, Madison, USA), 3 µl of $MgCl_2$ solution (25 mM), 3 µl of dNTPs (1.5 mM), 0.5 µl of forward and reverse primer (Microsynth, Balgach, Switzerland), 0.3 µl of Taq DNA polymerase (Promega, Madison, USA), all made

up to a final volume of 30 µl with purified MilliQ water. The PCR were run in a Biometra TGradient thermocycler (Biometra, Goettingen, Germany) using the following programs: for *16S* and *COI*, initial denaturation for 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 45°C, 1.5 min at 72°C), then final elongation of 8 min at 72°C; for *COII*, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 53°C, 2 min at 72°C), then final elongation of 8 min at 72°C; for *ITS2*, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 48°C, 1 min at 72°C), then final elongation of 8 min at 72°C. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. Sequencing by Macrogen Inc. (Seoul, South Korea) was carried out with both forward and reverse primers under BigDye TM terminator cycling conditions, purifying the products using ethanol precipitation and running them using an Automatic Sequencer 3730xl (Applied Biosystems, Foster City, USA).

Sequences (forward and reverse) were manually corrected using the software CHROMAS PRO 1.34 (Technelysium, Helensvale, Australia). The protein coding nucleotide sequences of *COI* and *COII* were checked for reading frame errors and termination codons in MEGA 4 (Tamura *et al.* 2007). Alignment was carried out using CLUSTALW Multiple Alignment (Thompson *et al.* 1994) within the software BIOEDIT 7.0.5.3, followed by minor manual correction.

Phylogenetic analyses

Individual and combined phylogenetic analyses were performed using the maximum parsimony (MP) and Bayesian Markov chain Monte Carlo (MCMC) criteria. Each partition and the combined data set (including the three mitochondrial markers) were analyzed using parsimony ratchet (Nixon 1999) as implemented in PAUPrat (Sikes & Lewis 2001). Based on recommendations by Nixon (1999), ten independent searches were performed with 200 iterations and 15% of the parsimony informative characters perturbed. The shortest equally

most parsimonious trees were combined to produce a strict consensus tree. Branch supports were calculated using the Bremer support (also known as decay index; Bremer, 1988) as implemented in TreeRot (Sorenson & Franzosa 2007). The Bremer support measures the number of extra steps in tree length required before a node collapses (Bremer 1988; Baker & DeSalle 1997). This method compares, for each node, the length of the most-parsimonious tree(s) (containing all the nodes) and the length of the shortest tree(s) not containing a specific node. For example, if the most-parsimonious tree containing a node composed by specimens ABC has 138 steps, and the shortest tree that lacks this node has 143 steps, the Bremer support for that node would be 143-138=5.

To investigate phylogenetic and haplotype relationships we performed mtDNA haplotype networks [using statistical parsimony networks (SPN) and median-joining networks (MJN)]. Mitochondrial DNA haplotypes were defined and used as raw data to perform single and multi-locus SPN and MJN networks, as implemented respectively in TCS version 1.21 (Clement *et al.* 2000) and SPLITSTREE version 4.10 (Huson & Bryant 2006). Analyses for the SPN network were carried out by applying a 95% connection limit and gaps were treated as a fifth state.

Divergence time estimation

To estimate phylogenetic relationships among individuals, partitioned Bayesian analysis was performed with BEAST 1.4.7 (Drummond & Rambaut 2007) and analyzed using TRACER v1.4 (Rambaut & Drummond 2007). The three mtDNA regions were represented as separate partitions in the analysis, with substitution models as estimated by MRMODELTEST (Nylander 2004) and four estimated alpha categories for the gamma term (Yang 1994). Three independent runs of 30 x 10^6 generations were performed, sampling one tree every 1000

generations. A maximum clade credibility tree was generated after removing a burn-in of 10 x 10^6 generations of each run.

Due to the lack of fossils for *Oreina* beetles, direct calibration of the tree topologies was not possible. Instead, branch lengths and node ages were estimated by applying an arthropod mtDNA molecular clock based on a review of published gene-specific mtDNA substitution rates in diverse arthropod taxa (Borer *et al.*), which suggests average pairwise divergence rates of 1.73% for COI, 1.38% for COII and 0.61% for 16S. We dated six relevant nodes using a relaxed clock with log-normal branch length distribution (Renner 2005; Drummond *et al.* 2006).

Congruence among mtDNA markers

Before combining mtDNA markers and applying a total evidence approach (Kluge 1989), we investigated topological congruence among markers. Pairwise incongruence length difference (ILD; (Farris *et al.* 1994)) tests were performed following the snowball procedure (Planet & Sarkar 2005) as implemented in the program mILD (Planet & Sarkar 2005). The main advantage of this approach compared to the classical ILD test relies on its ability to pinpoint putative incongruent markers that might be removed in subsequent analyses. This procedure fits with the total evidence approach (Kluge 1989; Lecointre & Deleporte 2005).

AFLP procedure

Reactions were conducted by using 96-well plates in which samples were randomly distributed. The restriction of extracted DNA was performed using a standard 30 µl mix including: 7.5 µl of extracted DNA, 3 µl of NEbuffer 2, 0.3 µl of 100 x BSA (10 mg/ml), 0.06 µl of MseI), 0.6 µl of EcoRI, all made up to a final volume of 30 µl with purified MilliQ water (all reagents by New England BioLabs, Ipswich, Massachusetts). The restriction was

run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) for two hours at 37°C followed by inactivation of the restriction enzymes at 65°C for 20 min.

To perform ligation of the adapters, 20 µl of the restriction mix was added to a standard 20 µl mix including: 4 µl of 10 x T4 ligase buffer (Promega, Madison, USA), 1.44 µl of EcoRI adapter (5'-AATTGGTACGCAGTCTAC-3' and 5'-CTCGTAGACTGCGTACC-3'), 1.44 µl of MseI adapter (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'), 0.2 µl of T4 ligase (3 U/µl, Promega, Madison, USA), all made up to a final volume of 20 µl with purified MilliQ water. The ligation was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) for two hours at 37°C.

The amplification process was started with a preamplification, in which only one selective basepair was added to the primer, followed by a selective amplification with two additional basepairs. This two-step procedure avoids the occurrence of nonspecific bands and reduces background noise (Vos *et al.* 1995). For the preamplification, 2 µl of the ligation mix was added to 28 µl of a PCR mix consisting of: 6 µl of 5 x GoTaq flexi buffer (Promega, Madison, USA), 2.4 µl of $MgCl_2$ (25mM), 0.75 µl of dNTPs (10mM), 0.75 µl of EcoRI primer + A (10 µM, 5'-GACTGCGTACCAATTCA-3'), 0.75 µl of MseI primer + A (10 µM, 5'-GATGAGTCCTGAGTAAA-3'), 0.15 µl of Taq DNA polymerase (Promega, Madison, USA) and mad up to a final volume of 28 µl with purified MilliQ water. The preamplification was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) using the following program: initial denaturation for 2 min at 94°C, 29 cycles (45 s at 94°C, 45 s at 56°C, 1 min at 72°C), then final elongation of 10 min at 72°C.

For the selective amplification, 3 µl of the 20 times diluted preamplification mix was added to 17 µl of a PCR mix including: 6 µl of 5 x GoTaq flexi buffer (Promega, Madison, USA), 2.4 µl of $MgCl_2$ (25mM), 0.75 µl of dNTPs (10mM), 1.2 µl of EcoRI primer + ACA (10 µM, 5'-

GACTGCGTACCAATTCACA-3'), 1.2 µl of MseI primer + AXX (10 µM, 5'-GATGAGTCCTGAGTAAAXX-3'), 0.15 µl of Taq DNA polymerase (Promega, Madison, USA) and made up to a final volume of 17 µl with purified MilliQ water. We used the following MseI primer + AXX (AGC, ACG and AAC) each with 5-FAM fluorescently labelled EcoRI primer + ACA. The selective amplification was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) using the following program: initial denaturation for 2 min at 94°C, 14 cycles (30 s at 94°C, 30 s at 65°C, 1 min at 72°C) in which the 65°C annealing temperature was decreased by 0.7°C each cycle, followed by 24 cycles (30 s at 94°C, 30 s at 56°C, 1 min at 72°C), completed by a final elongation of 5 min at 72°C.

AFLP analysis

PCR products were analysed using the GeneScan technology with a capillary sequencer (ABI 3730XL, Applied Biosystems, Foster City, CA; the service was provided by Macrogen Inc. Seoul, South Korea). Resulting electropherograms were analysed with PeakScanner (ABI, peak detection parameters: default parameters with the addition of a light peak smoothing) in order to detect and calculate the size of AFLP bands. The scoring was performed using an automated scoring R CRAN package, RawGeno (Arrigo *et al.* 2009). The library was set as follows: scoring range = 100 – 300 bp, minimum intensity = 50 rfu, maximum bin width = 1 bp and removal of closely sized bins (5%). Finally, the matrices of the three scored primer pairs were concatenated into one binary matrix where individuals and bands were stored as lines and columns, respectively. Fifteen individuals were amplified twice in order to determine the overall reproducibility rate.

Genetic structure analyses based on AFLP

The biogeographical structure of *O. gloriosa* was investigated for the AFLP matrix, through two independent approaches. (i) A model-based Bayesian inference was run using STRUCTURE 2.2 [(Pritchard *et al.* 2000; Falush *et al.* 2007), version adapted for dominant datasets] to assign individuals into a user-defined number of genetic groups (hereafter *K*), defined in a way that maximizes the Hardy-Weinberg equilibrium within the *K* groups. We used the "admixture" model and set the MCMC algorithm with 100,000 generations of burn-in and 400,000 generations for data acquisition. Here, *K* values ranged from one to ten, with five replicates for each tested value. For each K value, the replicate that obtained the highest likelihood was considered for further analyses. Individuals were assigned to groups if their respective assignment probability was higher than 0.5 (majority-rule criterion). (ii) A nonhierarchical K-means clustering (Hartigan & Wong 1979) was performed using R CRAN (R Development Core Team 2009; script available from the first author). A total of 10,000 independent runs were carried out for each value of *R* clusters assumed. For each *R* value, ranging from one to ten, only the first run yielding a positive value for the second derivative of the inter-cluster inertia was considered (Kergoat & Alvarez 2008).

The results of the two different clustering analyses were displayed on geographical maps using ARCGIS 9.1 (ESRI), representing each population as a pie-chart showing the number of individuals assigned to each AFLP group.

Congruence between mtDNA and AFLP data sets

Although the mILD test allows the investigation of character congruence, it was not designed to compare sequence and AFLP (composed of 1/0) data. In this study, the congruence between mtDNA and AFLP data sets was investigated by comparing phylogeographic patterns. Decay indices were used to define well-supported mtDNA clades, by collapsing

clades with a Bremer support inferior to 1. Maps showing the mtDNA clades were compared with those derived from the AFLP analysis (for both STRUCTURE and K-means algorithms), and the level of congruence quantified using contingency tables.

Peucedanum ostruthium

DNA extraction

Fifty-three specimens were collected across the 22 sampled populations. DNA was extracted from 10 mg of silica-dried leaves using the DNeasy Plant Kit (Qiagen, Hilden, Germany).

AFLP and genetic structure analyses

Since no variation was apparent when sequencing cpDNA fragments (data not shown), we relied on a genomic screening approach to unravel the spatial genetic structure of *P. ostruthium*. All amplification, scoring and analyses were identical to those used for O. gloriosa, except that a scoring range of 100 – 500 bp was used.

Results

Oreina gloriosa

Phylogenetic reconstruction of mtDNA data sets

From 66 sequenced individuals, 49 resulted in satisfactory AFLP amplification and only these were used for all further analyses. Exceptions are populations CHA and BOR, in which our sampling yielded two mismatches (Table 1). Whereas *ITS2* sequences revealed no variation at all in our sampled specimens, the three mtDNA fragments were polymorphic. Total alignment length of the mtDNA region was 1626 bp: 529 bp for *16S*, 462 bp for *COI* and 635 bp for *COII*. Among 50 variable characters, 29 were potentially parsimony informative (hereafter PPIc; representing 3.1% of the total number of characters) excluding the outgroup. The three

mtDNA regions contribute as follows: *16S* (3 PPIc among 8 polymorphic sites), *COI* (10 PPIc among 20 polymorphic sites) and *COII* (16 PPIc among 22 polymorphic sites). The best substitution models suggested by MRMODELTEST were: HKY for *16S* and HKY+G for *COI* and *COII*. Amplification failed in six cases for the *COII* region (all individuals from GAL and BRC).

The mILD analysis showed no incongruence among the three mtDNA markers (*COI* and *COII*, *P* value = 0.78; *COI* and *16S*, *P* value = 0.48; *COII* and *16S*, *P* value = 1.00). Consequently we applied the total evidence approach and combined the three mtDNA regions into a single matrix for the following analyses.

Maximum parsimony analysis resulted in 1991 trees with 115 steps, with Bremer supports ranging from one to eight (Supporting Information Fig. S1). In the strict consensus topology, *O. gloriosa* builds a well defined ingroup with Bremer support = 6. One individual (995_BRC) splits first (Bremer support < 1), whereas all other individuals are included in a wide polytomy. We defined three strongly supported clades (with Bremer supports of eight, four and three) for later analyses, leaving five individuals (359_ISE, 406_BOR, 1132_GAL, 1133_GAL and the early-branching 995_BRC) assigned to none of the three groups.

When analyzing haplotype networks for each marker individually, the SPN and MJN algorithms showed highly congruent patterns. To avoid redundancy only SPN networks are discussed hereafter. Since several individuals possessed identical sequences, only distinct haplotypes were considered (see Table 2). Single-marker analyses showed compatible patterns supporting the same groups, although the *16S* network was less resolved than the other markers (due to the low number of variable sites) (Supporting information Fig. S2). To avoid potential misleading relationships produced by missing data and because the algorithm implemented in TCS does not handle missing data, we implemented the mtDNA haplotype

network analysis after excluding the *COII* data. The haplotypes of *16S* and *COI* were therefore merged to perform a combined network analysis which defines one network based on a 95% connection limit (Fig. 1). The resulting network is highly compatible with the MP analysis, in showing the same three main groups with 359_ISE, 406_BOR, 1132_GAL, 1133_GAL and 995_BRC in intermediate or outlier positions.

Divergence time estimates

The Bayesian Inference analysis yielded a fully-resolved topology highly congruent with the MP analysis. For each parameter, convergence of the three independent runs was confirmed by the visual examination of their respective distributions in TRACER v1.4. The estimated sampling size was >400 for all parameters. To obtain a half-compatible topology, nodes with supports < 0.5 were polytomized. The main six nodes of interest are indicated on Figure 2. The basal node (node one) and node five have a very high support with a Bayesian posterior probability (hereafter bpp) of 1. Nodes two and three have lower support (0.73 and 0.65 bpp), whereas nodes four and six support clades with 0.9 and 0.95 bpp respectively (Fig. 2). Nodes five, four and six define the same clades (clades I, II and III) that are produced by the MP approach, with specimens 359_ISE, 406_BOR, 1132_GAL and 1133_GAL and 995_BRC falling in low-supported clades or in polytomies. The dating analysis suggests that those three major clades were formed before the last glacial period (Table 3 and Fig. 2), with divergence spanning the last 0.5 million years. Clade I, representing specimens from the eastern and central Alps diverged 0.5 MYA (at node 1), followed approximately 300’000 YA (at node 2) by divergence of the enlarged clade III containing specimens from ten populations from the western and south western Alps and clade II represented by individuals of four populations from the central Alps. About 300’000 YA (node 3) clade III diverged followed by a split of one individual from GAL 90’000 YA (node 6).

AFLP data

The AFLP analysis produced a total of 497 bands (154, 156 and 187 for EcoRI-ACA/MseI-AGC, EcoRI-ACA/MseI-ACG and EcoRI-ACA/MseI-AAC, respectively) with an average of 257 bands per individual. Among 457 variable bands, 397 were potentially parsimony informative. When comparing band patterns in replicates, we computed an overall reproducibility rate of 88.7%.

Genetic structure analyses based on AFLP

The best results in the STRUCTURE analysis were obtained when six groups were considered (log likelihood (K=5) =-10656.4 < log likelihood (K=6) = -10606 > log likelihood (K=7) =-10762.2). Generally, populations contained individuals from one single group, with the exception of three admixed Swiss populations that comprised specimens from two lineages (CDM, GLT, SAG), un-assigned individuals (white colour) were not defined as group (Fig. 3). Evaluation of the inter-cluster inertia in the K-means method allowed selection of the best number of clusters and accordingly, the best K-value was R = 7. The resulting pattern was similar to that produced by STRUCTURE, with a majority of non-admixed populations, with the exception of the Swiss CDM, GLT, GON and SAG (Supporting Information Fig. S3). Globally, the main groups were congruent in the two methods with three quarters of the groups matching at ≥ 80% (see Table 4).

Congruence between mtDNA and AFLP data sets

Since the results of the K-means and STRUCTURE algorithms were highly congruent, we will only consider the latter to evaluate the level of compatibility of the AFLP dataset with the mtDNA topology. When considering the specimens that were attributed both to a mtDNA clade and a STRUCTURE group (i.e., 39 of 48 specimens), a high level of compatibility was

seen (Table 5): mtDNA clade I matched the purple STRUCTURE group at 76.9 %, clade II matched the yellow group at 55.6 % and clade III matched the green group at 85 %. When considering the other STRUCTURE groups comprising less specimens, they also match fully with a given mtDNA clade: the light blue group with clade I, the red group with clade III and the black group with clade II. As an illustration of the compatibility between the two approaches, mtDNA clades are shown on the geographical map with STRUCTURE clusters on Figure 3.

Peucedanum ostruthium

AFLP analyses

The AFLP analysis produced a total of 113 bands (42, 29 and 42 for EcoRI-ACA/MseI-AGC, EcoRI-ACA/MseI-ACG and EcoRI-ACA/MseI-AAC, respectively) with an average of 39 bands per individual. Among 100 variable bands, 89 were potentially parsimony informative.

Phylogenetic analyses applying the maximum parsimony and Bayesian Markov chain Monte Carlo (MCMC) criteria resulted in one larg polytomy without any structure (data not shown) and were therefore excluded.

According to Alvarez *et al.* (2009), the best *K* value across the Alps for *P. ostruthium* in STRUCTURE runs was obtained when five groups were considered (log likelihood (K=5) = -14448.2). The eastern Alps are split into a northern and southern group, both with pure genotypes (un-assigned individuals = white colour, were not treated as genotype), while the other three groups fall into pure and admixed populations (Fig. 4): one group dominates southern Switzerland, one the western Alps and the third occurs in the Alpes Maritimes and western Alps. Evaluation of the inter-cluster inertia in the K-means method allowed selection of R=5 as the best number of clusters. The resulting spatial genetic structure also demonstrates a high number of admixed populations over a large part of the species

distribution in all but one cluster (Supporting Information Fig. S4). Although the algorithms concur in revealing the same numbers of clusters, the level of congruence was not high (Table 6): only one group is highly congruent (80 %) and two others are somewhat compatible (with a match of respectively 50 % and 61.5 %). Since the spatial distribution of the groups is much more aggregated and because populations are globally less admixed in the STRUCTURE analysis than in the K-means calculations, we chose to consider the former method for further analyses of comparative phylogeography.

Comparison between the phylogeographic patterns of O. gloriosa and P. ostruthium

Visual inspection of the AFLP-based phylogeographic patterns of *O. gloriosa* and *P. ostruthium* (Fig. 3 & 4) indicates that the two species demonstrate some level of congruence in their biogeographic histories. This comparison will serve as a base to discuss the comparative refugial survival and post-glacial dispersal in our two antagonistic species.

Discussion

Was divergence within O. gloriosa caused by the last glacial cycle or was it a consequence of multiple cycles?

The phylogenetic analysis and divergence time estimation, even if providing only an approximate timescale, show that *O. gloriosa* diverged into a west- and east alpine group 0.5 MYA. On the eastern edge of the west alpine group the next divergence took place 300'000 YA. The resulting three groups form the main structure detectable by mtDNA analyses within *O. gloriosa* and indicate that divergence within *O. gloriosa* spanned several glacial cycles. Thus the present genetic structure is not only a product of the last glacial maximum 18'000 YA (COHMAP 1988). Compared to *O. elongata*, a species sharing a very similar distribution

and in which divergence spanned the whole Quaternary (Borer *et al.*), divergence in *O. gloriosa* can be considered as recent. The fact that morphological criteria such as cuticle microstructure and male genitalia (aedeagus) do not allow the definition of subspecies within *O. gloriosa* (Kippenberg 1994) reinforces this conclusion.

Several orphan mtDNA sequences (separated by four and six steps in the haplotype network; haplotypes 7 and 16 in the haplotype network representing respectively 406_BOR and 995_BRC) might reveal an even more ancient history. In the haplotype network (Fig. 3) specimen 1132_GAL and 359_ISE (haplotype 21 and 6) are placed in a central position, connecting the three groups. This placement might be an indication to the origin of *O. gloriosa* located in the Alpes Maritimes.

Do the monophagous beetles share the phylogeographic pattern with their host plant?

To understand the phylogeographic pattern of this plant-herbivore antagonism two important facts have to be considered. First, in this system the plant is independent whereas *O. gloriosa*, as a monophagous insect on *P. ostruthium*, is highly dependent on its host. And second, the dispersal abilities of these two species are different. While *P. ostruthium* possesses very mobile seeds and pollen, beetles of the genus *Oreina* are known to have very limited dispersal abilities (Margraf *et al.* 2007).

The purple populations (OBE and GKO) of the plant seem to represent a refugium for both *Peucedanum ostruthium* (independent species) and *Oreina gloriosa* (dependent species), whereas the plant's blue group (HEI, PES and VDD) in the south eastern Alps seems to have been an *Oreina*-free area that was recolonized by two lineages from the north east and central Alps.

In the Alpes Maritimes, the red group (ISO and BOR) formed a refugium for both the plant and insect. Perhaps due to its greater dispersal abilities, *P. ostruthium* has dispersed

northwards (BRC and part of CHA and BRE), whereas the insect lineage is only found in the south.

Another refugium is likely to have sheltered both species, represented by the green colour. But in contrast to the red group, *O. gloriosa* has dispersed further than its host plant. In this case the plant lineage may have been displaced by populations from the central Alps.

The central and western Alps are dominated by the yellow groups in both antagonistic species. *Peucedanum ostruthium* is represented by only one lineage in contrast to three lineages of *O. gloriosa*. In that group an identical spatial scale has different effects on the isolation of refugial populations. For *P. ostruthium* only weak barriers are present and due to the good dispersal capabilities no divergence into different lineages occurs. In contrast, in *Oreina*, a genus with limited dispersal abilities (Margraf *et al.* 2007), barriers have started to isolate populations into diverging, micro-endemic lineages.

Molecular studies of interacting species are beginning to accumulate. Many of these treat parasite-host interactions at the level of cospeciation, such as in bats and their ectoparasites or pocket gophers and their lice (Hafner & Page 1995; Itino *et al.* 2001; Desdevises *et al.* 2002; Bruyndonckx *et al.* 2009). Fewer studies take into account the geographical structure of such interacting species (phylogeography) (Nieberding *et al.* 2004; Hayward & Stone 2006; Crandall *et al.* 2008). As a result, it is too early to determine if the two interacting species typically disperse together, or if there is pursuit of the host by its parasite.

Within the *Oreina* system there are many other antagonistic interactions that would benefit from study in a phylogeographic context, including other beetle-plant pairs, between pairs of beetles that share hosts, and in the tripartite interaction between a host plant, a highly specialized rust fungus and *Oreina* beetles (Röder *et al.* 2007). This would open the way to a community level phylogeography of this alpine system.

Conclusion

Even though there is some congruence between the phylogeographies of this alpine antagonism, the two species do not share identical histories. This is perhaps seen most clearly by comparing the five groups of *P. ostruthium* defined by the STRUCTURE analysis, which each have a different story concerning their herbivore antagonist. These include an apparently *O. gloriosa*-free refugium in the south-eastern Alps (blue group) a group where the plant dispersed further than the beetle (red group), the reverse situation (green group), a homogenous plant lineage with three diverging beetle lineages (the yellow group) and one group where the beetle and its host plant share identical histories (purple group). Further, the western Alps seem to be a hotspot of diversity in both, plants and insects.

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Table 1. Sampled populations of *Oreina gloriosa* and *Peucedanum ostruthium* with their geographical coordinates, number of individuals and the year of sampling.

Country	Code	Population	Altitude (m a.s.l.)	Geographical coordinates	Sample size			Year of sampling
					Oreina sequencing	Oreina AFLP	Peucedanum AFLP	
A	GKO	Graukogel	1647	47°06’N, 13°09’E	3	3	2	2007
CH	BOG	Bosco Gurin	1858	46°18'N, 08°27'E	1	1	3	2004
CH	CDM	Col-des-Mosses	1843	46°23’N, 07°07’E	2	2	2	2007
CH	CHA	Chandolin	1945	46°14'N, 07°36'E	2	1	3	2007
CH	EMO	Lac Emosson	1949	46°03'N, 06°55'E	3	3	2	2007
CH	GLT	Gletsch	1634	46°33'N, 08°21'E	2	2	1	2007
CH	GON	Gondo	1554	46°09’N, 80°06’E	3	3	3	2007
CH	LAF	La Fouly	1571	45°56'N, 07°05'E	3	3	2	2007
CH	LDT	Lai da Tuma	1997	46°39'N, 08°40'E	2	2	3	2007
CH	PES	Alp Pesca	1900	46°13'N, 10°04'E	2	2	3	2007
CH	SAG	Saas Grund	1639	46°06'N, 07°56'E	3	3	1	2007
D	OBE	Oberstdorf	1496	47°24’N, 10°20’E	2	2	3	2007
F	AFR	Ailefroide	1700	44°53'N, 06°26'E	3	3	3	2005
F	BOR	Le Boréon	1795	44°06’N, 07°18’E	1	2	2	2007
F	GAL	Col du Galibier	1658	45°05'N, 06°26'E	3	3	2	2007
F	ISE	Col de l’Iseron	1818	45°23'N, 07°02'E	2	2	3	2007
F	ISO	Isola 2000	1855	44°11'N, 07°08'E	2	2	1	2007
F	PAL	Lac Palluel	1855	44°44'N, 06°26'E	2	2	3	2007
I	BRC	Breuil-Cervinia	2149	45°55’N, 07°37’E	3	3	3	2006
I	BRE	Vallon du Breuil	1996	45°42’N, 06°52’E	2	2	2	2005
I	HEI	Madonna di Senale	1559	46°43’N, 10°51’E	2	2	3	2007
I	VDD	Valle di Daone	1319	46°01'N, 10°30'E	1	1	3	2007

A, Austria; CH, Switzerland; D, Germany; F, France; I, Italy; m a.s.l., meters above sea level.

Table 2. Sampled populations of *Oreina gloriosa* with the number of treated individuals and haplotype identification. Numbers in brackets show individuals per haplotype in populations with several haplotypes.

Country	Code	Population	Sample size	haplotype			
				16S_COI	16S	COI	COII
A	GKO	Graukogel	3	12(1), 13(2)	8(1), 9(2)	1	7(2), 9(1)
CH	LAF	La Fouly	3	2, 3, 4	2(2), 3(1)	2(2), 3(1)	2
CH	EMO	Lac Emosson	3	2, 5(2)	2	2(1), 4(2)	2
CH	SAG	Saas Grund	3	1	1	1	1
CH	CDM	Col-des-Mosses	2	2	2	2	2(1), 3(1)
CH	GLT	Gletsch	2	18(1), 19(1)	7(1), 12(1)	10	12(1), 13(1)
CH	GON	Gondo	3	17(2), 18(1)	7	10(1), 12(2)	11
CH	PES	Alp Pesca	2	12	8	1	7
CH	CHA	Chandolin	2	11	7	2	2
CH	LDT	Lai da Tuma	2	9(1), 10(1)	6	7(1), 8(1)	7
CH	BOG	Bosco Gurin	1	18	7	10	11
D	OBE	Oberstdorf	2	12	8	1	7(1), 8(1)
F	GAL	Col du Galibier	3	21(2),22(1)	7	5(2),14(1)	-
F	ISE	Col de l'Iseron	2	2(1), 6(1)	2	2(1), 5(1)	4(1), 5(1)
F	AFR	Ailefroide	3	2	2	2	2
F	PAL	Lac Palluel	2	11(1), 20(1)	7	2(1), 13(1)	2
F	ISO	Isola 2000	2	2	2	2	2
F	BOR	Le Boréon	1	7	4	6	6
I	BRC	Breuil-Cervinia	3	14(1),15(1),16(1)	7(1),10(1),11(1)	9(1),10(1),11(1)	-
I	BRE	Vallon du Breuil	2	2(1), 8(1)	2(1), 5(1)	2	2
I	VDD	Valle di Daone	1	12	8	1	10
I	HEI	Madonna di Senale	2	12(1), 13(1)	8(1), 9(1)	1	7(1), 9(1)

A, Austria; CH, Switzerland; F, France; I, Italy; m a.s.l., metres above sea level.

Table 3. Ages (in millions of years) of relevant nodes in the *O. gloriosa* phylogeography, based on a log-normal relaxed clock model.

	Lognormal	
Node	Mean	95% C.I.
1	0.5	0.25-0.83
2	0.3	0.17-0.48
3	0.15	0.07-0.27
4	0.14	0.05-0.26
5	0.1	0.07-0.34
6	0.09	0.04-0.18

Table 4. Contingency table comparing the grouping of *O. gloriosa* individuals by STRUCTURE and K-means. Group names refer to color codes in Figures 5 and S3. The supernumerary K-means group is represented with shaded cells. Groups in bold show a perfect match in specimen assignments; groups in italics match at ≥ 80%; groups with an "*" correspond to one STRUCTURE group split into two K-means groups. NA refers to non assigned samples.

	STRUCTURE groups							
K-means groups	**green (1)**	**black (6)**	**light blue (4)**	*purple (3)*	*red (5)*	yellow (2)*	NA	Total
green (1)	**20**							20
black (6)		**1**						1
light blue (7)			**1**					1
purple (2)				*9*			1	10
red (3)					*4*	1		5
yellow (4)*						3	1	4
dark blue (5)*				1		5	2	8
Total	20	1	1	10	4	9	4	49

Table 5. Contingency table comparing the grouping of *O. gloriosa* individuals by STRUCTURE and mtDNA results for samples that were comprised both in a defined mtDNA clade and in an assigned STRUCTURE group. Group names refer to color codes in Figures 1, 2 and 4. Matching percentages for the three mtDNA clades and the main three STRUCTURE groups (in bold) are respectively ≥75%, ≥55% and ≥85%.

	STRUCTURE groups						
mtDNA clade	**purple (3)**	**yellow (2)**	**green (1)**	light blue (4)	red (5)	black (6)	Total
purple (clade I)	**10**	2		1			13
yellow (clade II)		**5**				1	6
green (clade III)		1	**17**		2		20
Total	10	8	17	1	2	1	39

Table 6. Contingency table comparing the grouping of *P. ostruthium* between STRUCTURE and K-means results. Group names refer to color codes in Figures 6 and S4. Groups in bold match at ≥ 80%; groups in italics match at ≥ 50%; NA refers to non assigned samples.

	STRUCTURE groups						
Kmeans groups	**purple (4)**	*red (1)*	*green (2)*	dark blue (3)	yellow (5)	NA	Total
purple (1)	**4**					**1**	5
red (3)		*8*	3		3	2	16
green (6)			*8*			1	9
dark blue (5)			1	5	6	2	14
yellow (4)				3	4	2	9
Total	4	8	12	8	13	8	53

Figure legends

Figure 1. Statistical parsimony network (SPN) for the combined 16S and COI haplotype data. Lines between the haplotypes represent one mutational step, while small black circles indicate missing haplotypes that were either not sampled or have become extinct.

Figure 2. Half-compatible topology of the BEAST analysis, with the corresponding bpp values and definition of three well supported clades. Specimens marked with an * did not successfully amplify for COII. Their removal from the analysis results in an identical topology (not shown) with a substantial increase in node supports, as indicated by the bpp values in brackets. Dates for the numbered nodes are given in Table 3 and discussed in the text. Branches illustrated with \\ are reduced by 0.8 MY.

Figure 3. Geographical distribution of the groups obtained in the STRUCTURE (K=6) analysis of *O. gloriosa*. Lines indicate the geographical distribution of the three clades obtained in the MP and BEAST analyses, white = unassigned individuals.

Figure 4. Geographical distribution of the groups obtained by the STRUCTURE (K=5) analysis of *P. ostruthium*, white = unassigned individuals.

Supporting Information

Figure S1. Strict consensus tree of the MP analysis. Three well defined clades are defined and node supports are given by Bremer supports (decay index).

Figure S2. Statistical parsimony networks (SPN) for the a) 16S, b) COI and c) COII haplotypes. Lines between haplotypes represent one mutational step and small, black circles indicate missing haplotypes which were either not sampled or have become extinct. Colours represent the three clades (purple = group I, yellow = group II, green = group III and white = unassigned individuals) obtained with MP and Bayesian analysis with BEAST.

Figure S3. Geographical distribution of the groups obtained by the Kmeans (K=7) analysis of *O. gloriosa.*

Figure S4. Geographical distribution of the groups obtained by the Kmeans (K=6) analysis of *P. ostruthium.*

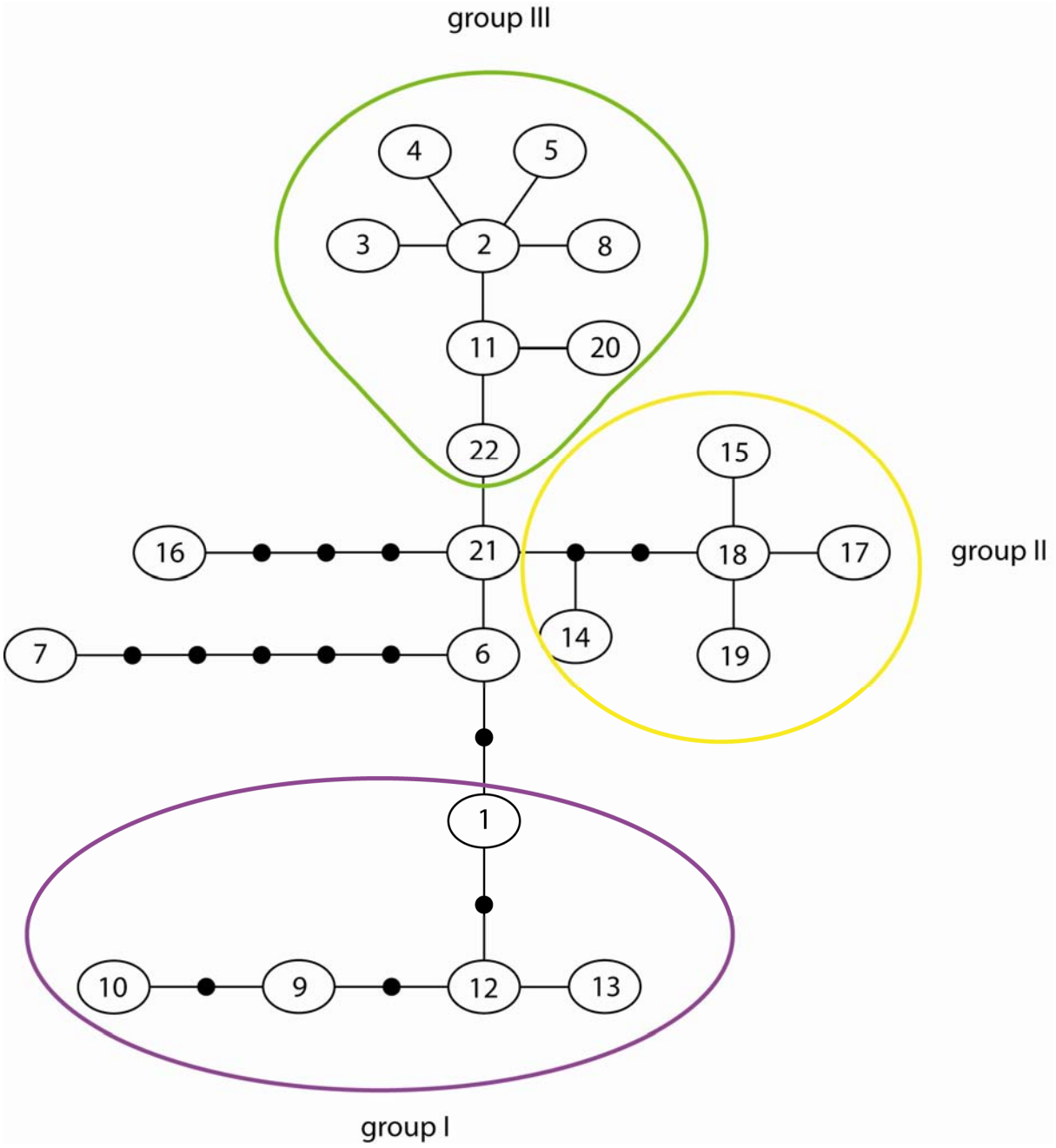

group III
4
5
3
2
8
11
20
22
15
16
21
18
17
group II
14
7
6
19
1
10
9
12
13
group I

Figure 1

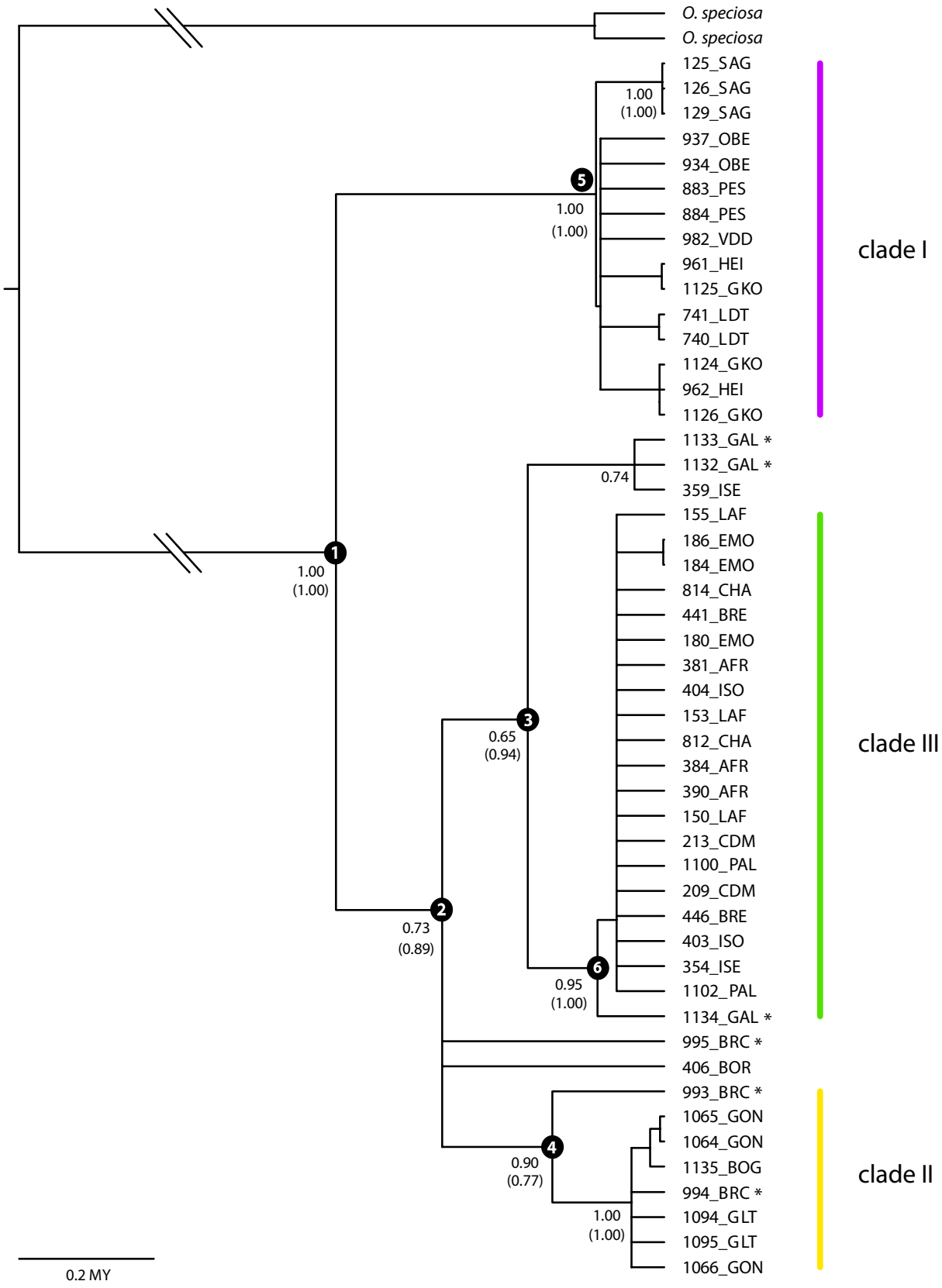

O. speciosa
O. speciosa
125_SAG
126_SAG
129_SAG
1.00
(1.00)
937_OBE
934_OBE
883_PES
884_PES
982_VDD
961_HEI
1125_GKO
741_LDT
740_LDT
1124_GKO
962_HEI
1126_GKO
5
1.00
(1.00)
clade I
1133_GAL *
1132_GAL *
0.74
359_ISE
155_LAF
186_EMO
184_EMO
814_CHA
441_BRE
180_EMO
381_AFR
404_ISO
153_LAF
812_CHA
384_AFR
390_AFR
150_LAF
213_CDM
1100_PAL
209_CDM
446_BRE
403_ISO
354_ISE
1102_PAL
1134_GAL *
1
1.00
(1.00)
3
0.65
(0.94)
2
0.73
(0.89)
6
0.95
(1.00)
clade III
995_BRC *
406_BOR
993_BRC *
1065_GON
1064_GON
1135_BOG
994_BRC *
1094_GLT
1095_GLT
1066_GON
4
0.90
(0.77)
1.00
(1.00)
clade II
0.2 MY

Figure 2

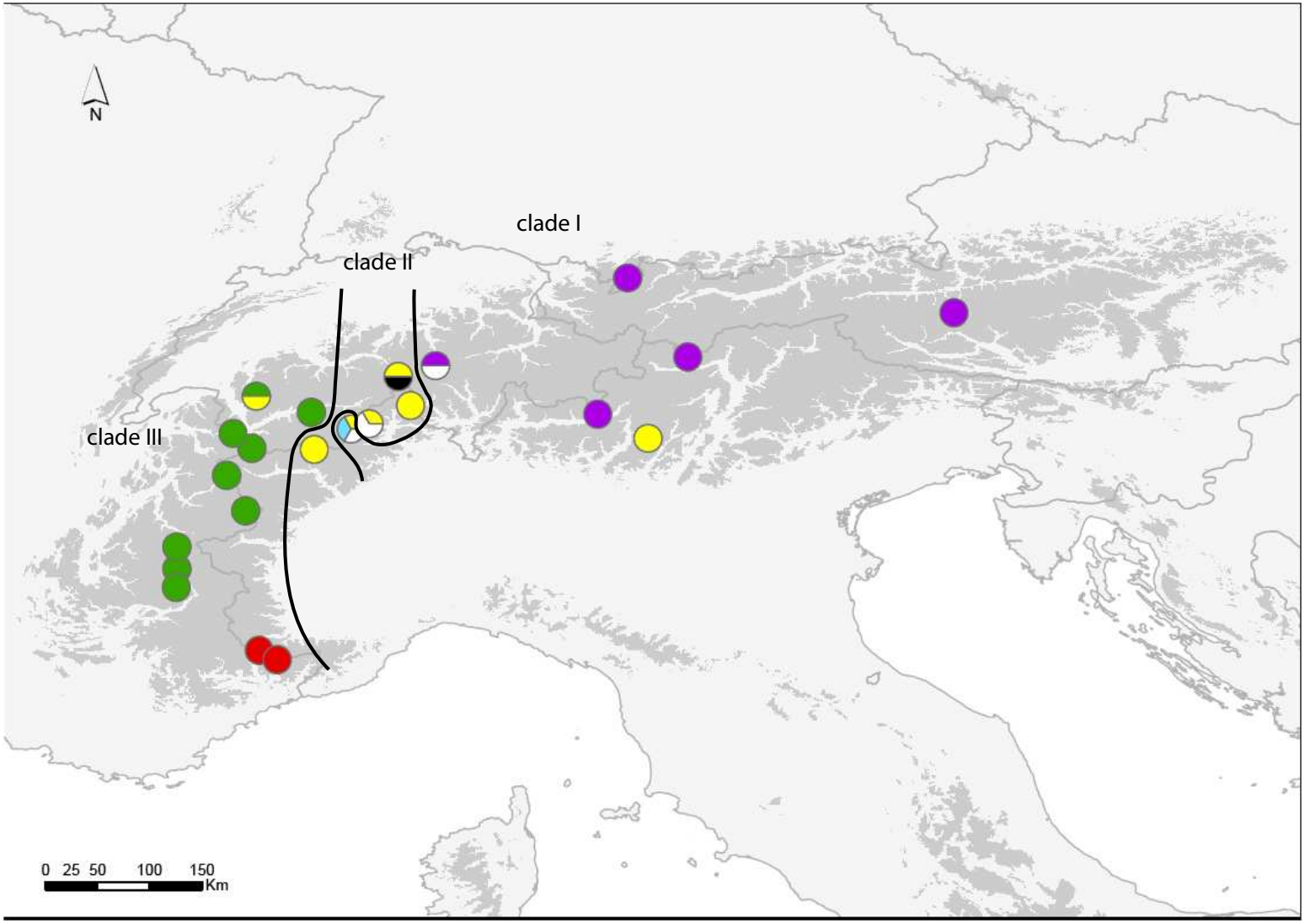


Figure 3

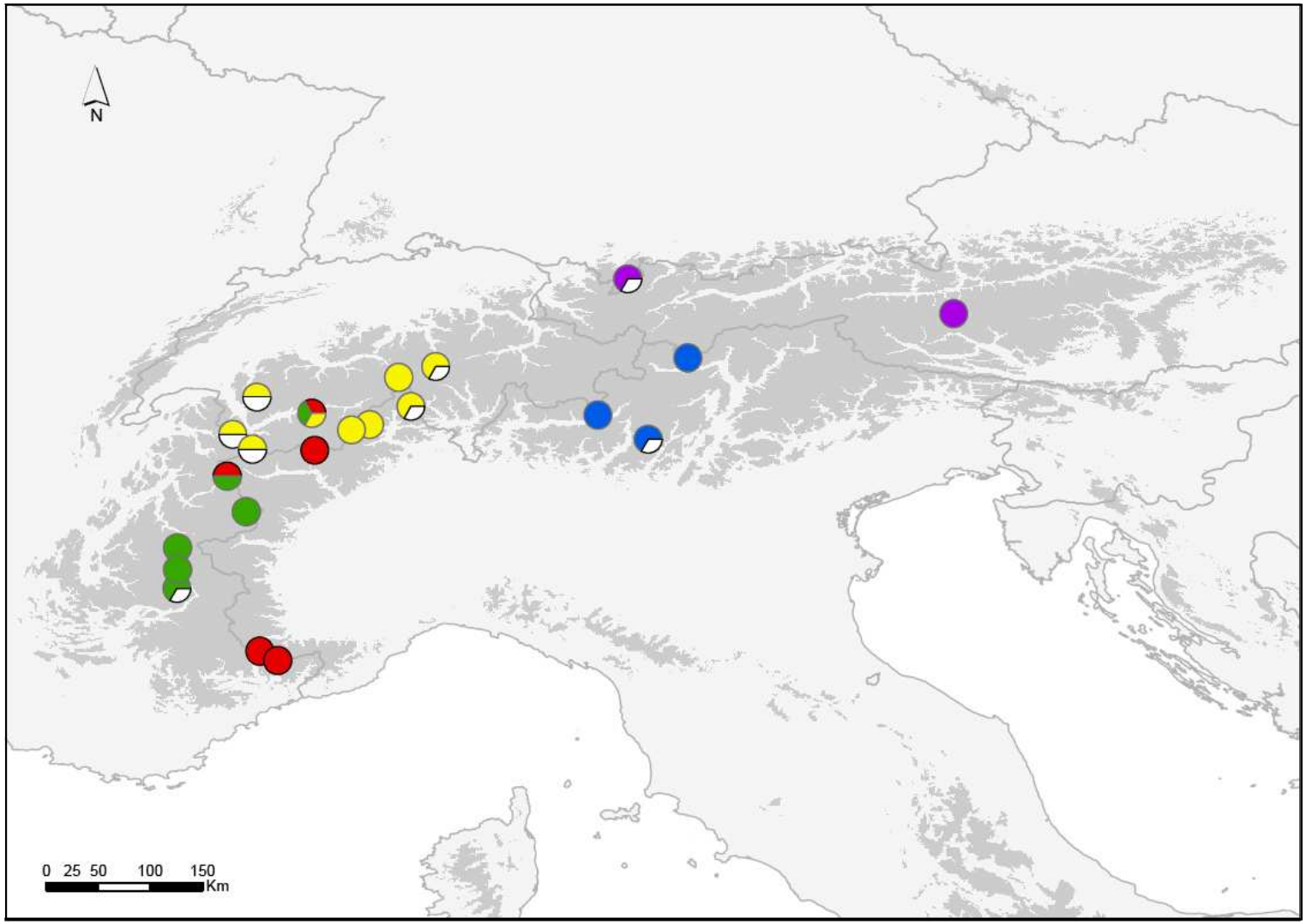
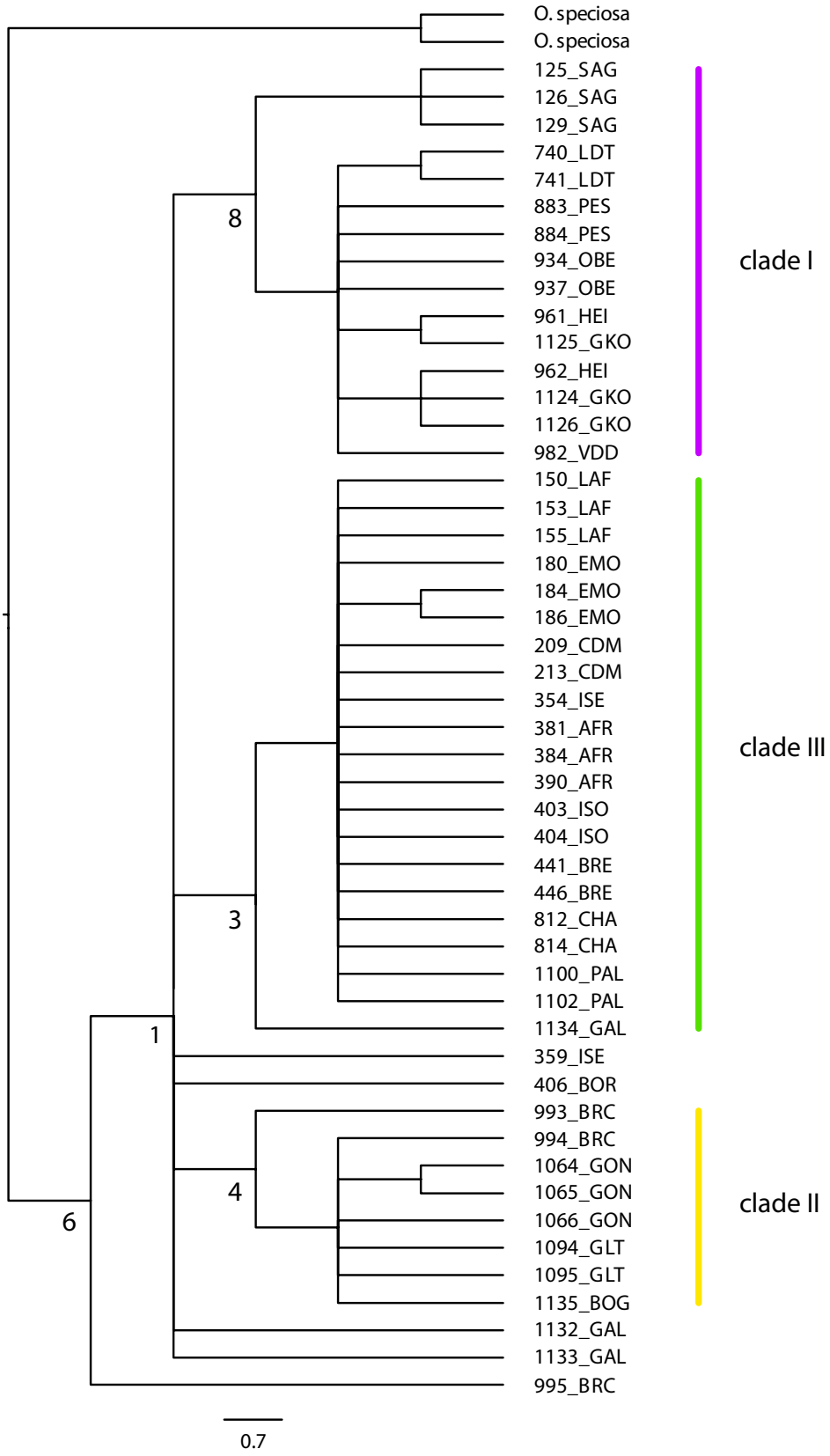


Figure 4


O. speciosa
O. speciosa
125_SAG
126_SAG
129_SAG
740_LDT
741_LDT
883_PES
884_PES
934_OBE
937_OBE
961_HEI
1125_GKO
962_HEI
1124_GKO
1126_GKO
982_VDD
150_LAF
153_LAF
155_LAF
180_EMO
184_EMO
186_EMO
209_CDM
213_CDM
354_ISE
381_AFR
384_AFR
390_AFR
403_ISO
404_ISO
441_BRE
446_BRE
812_CHA
814_CHA
1100_PAL
1102_PAL
1134_GAL
359_ISE
406_BOR
993_BRC
994_BRC
1064_GON
1065_GON
1066_GON
1094_GLT
1095_GLT
1135_BOG
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clade I
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clade II
8
3
1
4
6
0.7

Figure S1

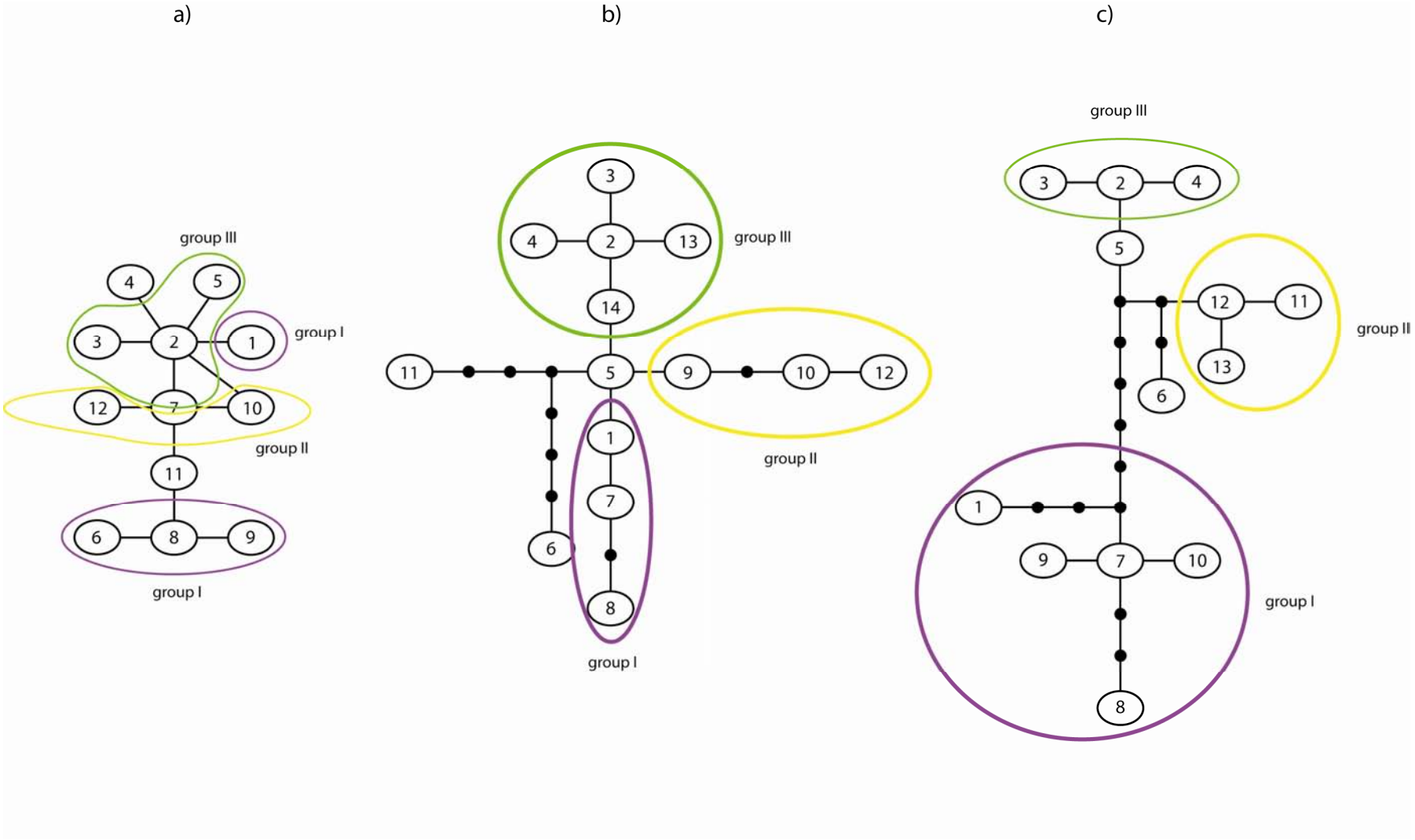
a)
b)
c)
group III
group I
group II
group III
group II
group I
group III
group II
group I

Figure S2

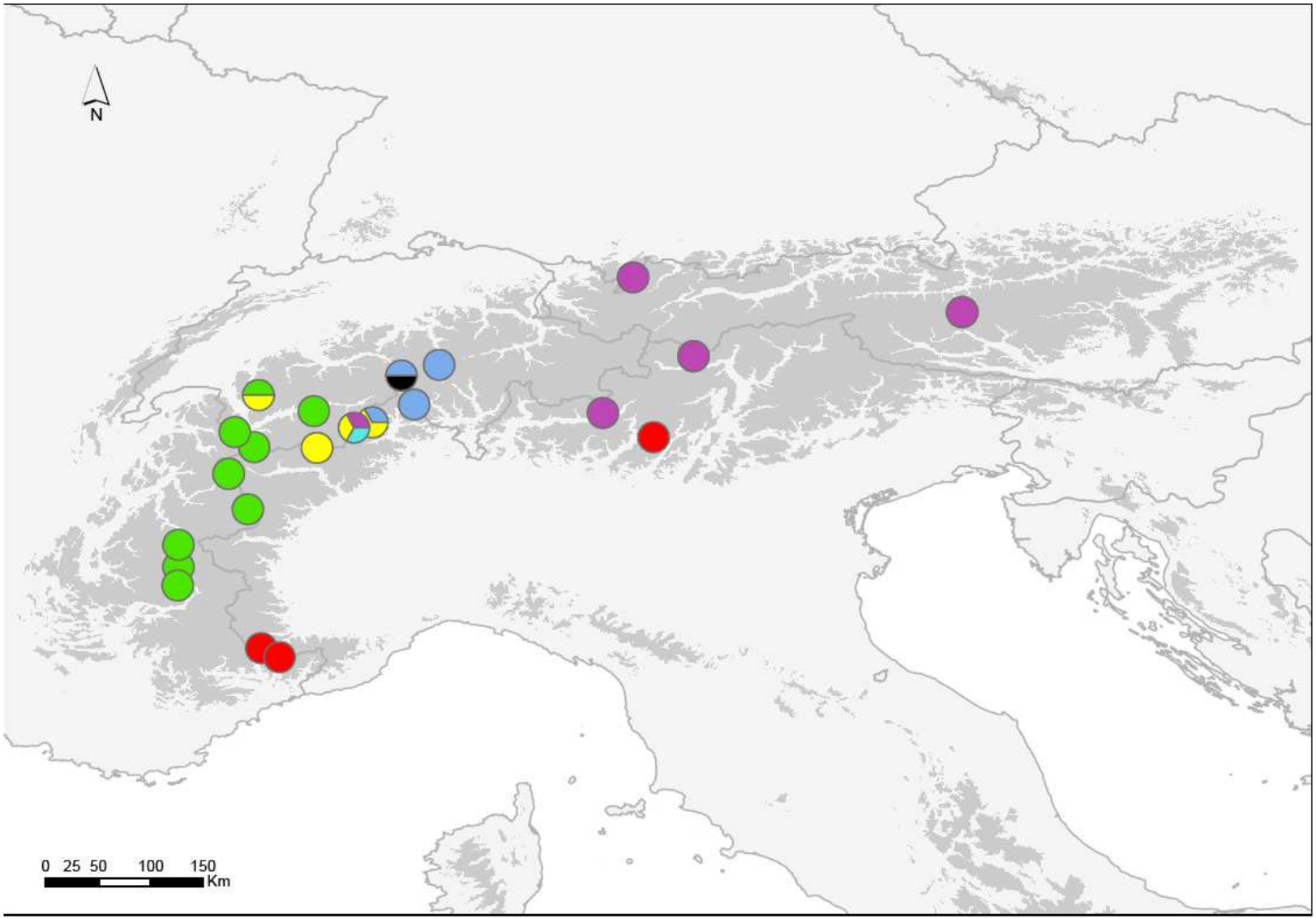

Figure S3

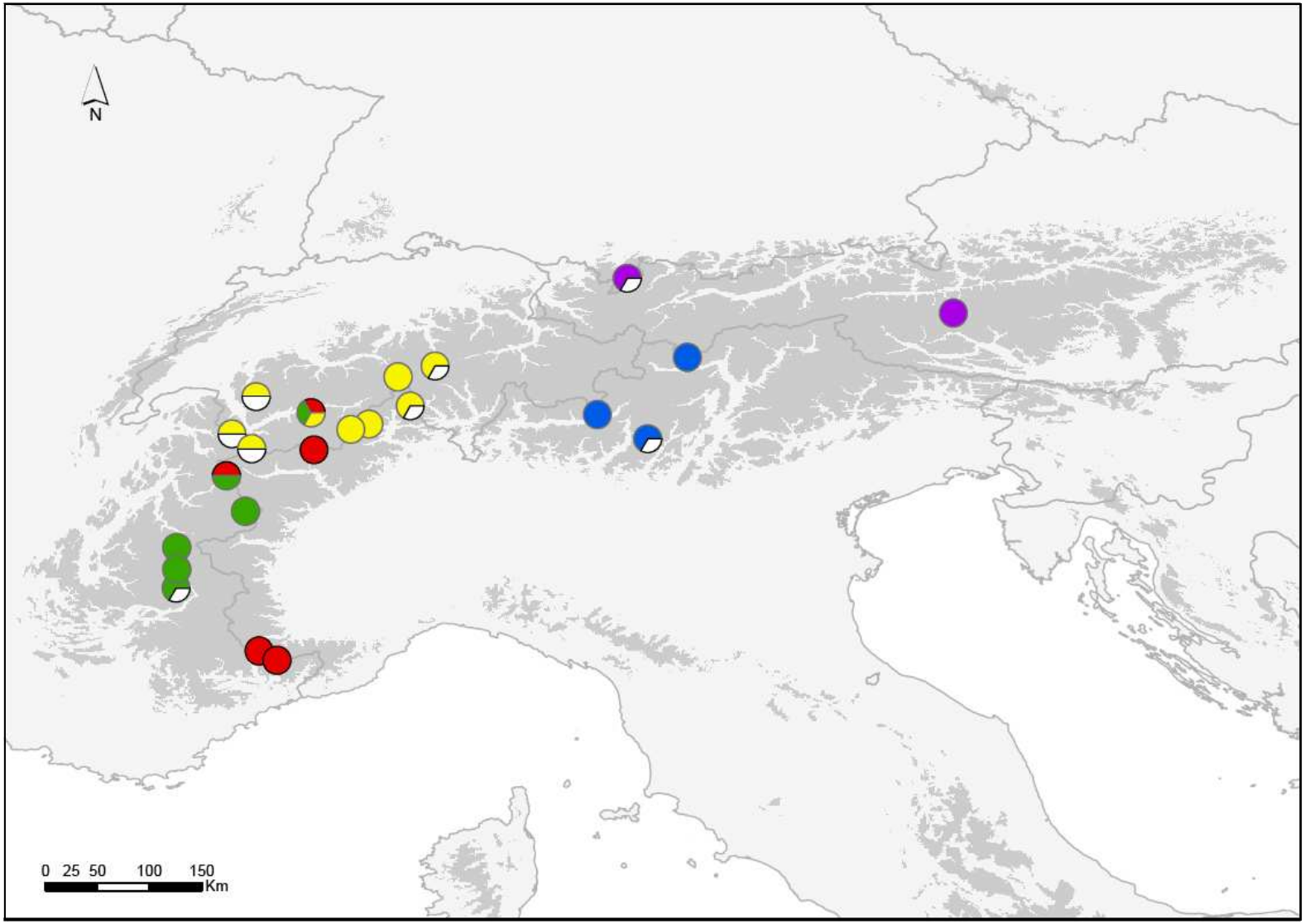

Figure S4

Chapter III

Phylogeography of two closely related species *Oreina cacaliae* / *Oreina speciosissima*: an example of sympatric wide spread montane insects

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Abstract

Climatic oscillations during the Quaternary influenced strongly the genetic structure of the contemporary fauna and flora in Europe. During cold periods many species were forced by the extension of ice sheets to migrate to southern refugia and recolonized northern Europe after retraction of the ice.

The patterns for many lowland species and a few high altitude species have been demonstrated during the last decade. Yet little is known for species covering a wide area.

Here we analyse the phylogeography of two closely related, sympatric alpine leaf beetles with distributions ranging from the Cantabrian Mountains in the west to the Carpathians in the east. Three mitochondrial regions and one nuclear region were sequenced for 120 *Oreina cacaliae* and 95 *O. speciosissima* from 87 populations across most of the distribution. The nuclear region ITS2 and morphological criteria separate the two species clearly, whereas the mtDNA phylogeny shows paraphyly for both species. Within the mtDNA phylogeny, 10 out of 12 analysed subspecies build monophyletic clades. Even though both species share the same habitat and occur in sympatry over the whole distribution their biogeographical groups are not congruent. Incongruence between the markers may be a result of hybridisation or sorting of ancestral polymorphism, obscuring the dates of population separation.

Introduction

The European fauna and flora was strongly influenced by the Quaternary glacial and interglacial cycles (Hewitt 2004). Oscillations between longer, dry and cold glacial periods and shorter, warm and more humid interglacial periods produced cycles of population contraction and expansion in many European organisms (Hewitt 1996; Williams *et al.* 1998). During glacial cycles, when the growing ice shelf and permafrost covered larger areas of northern Europe, many temperate species became extinct over much of their distribution, dispersed to new terrain or survived in southern refugia in the Iberian, Italian and Balkan Peninsulas (Hewitt 1996; 2000; Schmitt & Seitz 2001; Widmer & Lexer 2001; Hewitt 2004). During interglacial periods like the present, recolonization of northern Europe began from these refugia. For many species, mountain ranges such as the Alps or Pyrenees formed strict barriers and in regions where recolonizing lineages met, hybrid zones arose (Taberlet *et al.* 1998; Hewitt 2000). Thus lineages from separated refugia made different contribution to the genetic diversity of recolonized areas. Recent studies of arctic-alpine and high altitude species reveal a different scenario to that reconstructed for temperate species. In contrast to temperate and lowland species, arctic-alpine and high altitude species may have had much wider distributions during glacial periods and retreated to relatively small areas when the climate warmed. For the alpine flora, refugia within the Alps and at their southern, eastern and northern borders have been proposed on the basis of recent molecular studies (Stehlik *et al.* 2001; Stehlik *et al.* 2002; Schönswetter *et al.* 2005). For montane insects a very similar pattern has been described. For example, in *Erebia* butterflies refugia along the margins of glaciated high mountain ranges were postulated (Schmitt *et al.* 2006; Haubrich & Schmitt 2007; Schmitt & Haubrich 2008), for a widely distributed montane caddisfly Pauls *et al.* (2006) suggest persistence within several mountain ranges for some glacial cycles, and *O. elongata*, an alpine leaf beetle appears to have survived the Quaternary within or at the edge of the Alps and Apennines (Borer *et al.*).

Here we address the phylogeography of two closely related Alpine leaf beetles of the genus *Oreina* CHEVROLAT, 1837 (Coleoptera, Chrysomelidae). The genus *Oreina* contains about 28 species (Kippenberg 1994; 2008; and pers. comm.), which are found in isolated populations throughout the European mountains (from the Cantabrian Mountains in the west to the Carpathians in the east) with one species in western Siberia (Mikhailov 2008). Despite well developed and functional wings, they are rarely used (Kalberer *et al.* 2005). As a consequence, studies based on allozymes and microsatellite data suggest that even populations within small geographical distances have strong genetic differentiation and thus are highly isolated (Knoll & Rowell-Rahier 1998; Margraf *et al.* 2007). Within this genus two chemical defence strategies exist, which are correlated with host plant use. Species feeding on Apiaceae or Cardueae produce cardenolides *de novo* (Van Oycke *et al.* 1987; Eggenberger & Rowell-Rahier 1993b; 1993a), whereas species feeding on Senecioneae encounter pyrrolizidine alkaloid N-oxides (PAs) in several of their host plants. All species feeding on such plants are able to take up the alkaloids from their hosts and incorporate them into their defensive secretions (Pasteels & Rowell-Rahier 1991; Pasteels *et al.* 1995; Dobler *et al.* 1996).

We treat the two closely related species *Oreina cacaliae* SCHRANK, 1785 and *O. speciosissima* SCOPOLI, 1763, including seven of eight described subspecies for *O. cacaliae* and five of eight subspecies for *O. speciosissima* (Kippenberg 1994). Both species cover nearly the whole distribution with sympatric populations known from the Pyrenees to the Carpathians. The two species share many characteristics such as host plant use (oligophagous on several Senecioneae), habitat use (high-forbs) and lifecycle, but differ in their chemical defence and morphology. Whereas *O. cacaliae* is only able to sequester PAs, *O. speciosissima* can both sequester PAs and autogenously synthesize cardenolides. The external morphology of the cuticule as well as the aedeagus (male genitalia) differs and allows explicit determination of the two species.

In our study we examined the genetic structure of 87 populations (50 *O. cacaliae* and 37 *O. speciosissima*) of the two species across their whole distribution to answer the following questions:

1) Do the subspecies described on the basis of morphological criteria coincide with the clades defined by the phylogenetic analyses?
2) Do these two species share the same phylogeographical history?
3) Is the recent genetical structure a result of the last glaciation or is divergence within these species more ancient?

Materials and Methods

Sampling

Between 2004 and 2008, *Oreina cacaliae* and *Oreina speciosissima* were collected from 50 and 37 populations respectively (one to three beetles per site) covering almost the whole distribution (Table 1). Samples were preserved in pure ethanol and stored at -20°C. For the molecular analyses mainly males were chosen, in order to allow accurate species identification based on genitalia. Trees were rooted using two individuals of the closely related species, *Oreina virgulata* (Hsiao & Pasteels 1999).

DNA extraction, sequencing and alignment

Total genomic DNA was extracted from four legs of each individual using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Three regions of mtDNA and one nuclear region were amplified using universal insect primers: a fragment of *16S* ribosomal RNA [modLR-J-12887 (5'-CACCGGTTTGAACTCAGATC-3') with LR-N-13398 (Simon *et al.* 1994)]; cytochrome oxydase subunit I (*COI*) [C1-J-1751 with C1-N-2191 (Simon *et al.* 1994)]; cytochrome oxydase subunit II (*COII*) [modTL2-J-3037 with modC2-N-3661 (Mardulyn *et al.* 1997)]; and part of the nuclear region *ITS2* [ITS3 with ITS4 (Gomez-Zurita & Vogler 2003)]. Fragments

were amplified using a standard 30 µl PCR mix including: 3 µl of extracted DNA, 3 µl of 10X PCR buffer (Promega, Madison, USA), 3 µl of $MgCl_2$ solution (25 mM), 3 µl of dNTPs (1.5 mM), 0.5 µl of forward and reverse primer (Microsynth, Balgach, Switzerland), 0.3 µl of Taq DNA polymerase (Promega, Madison, USA), all made up to a final volume of 30 µl with purified MilliQ water. The PCR were run in a Biometra TGradient thermocycler (Biometra, Goettingen, Germany) using the following programs: for *16S* and *COI*, initial denaturation for 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 45°C, 1.5 min at 72°C), then final elongation of 8 min at 72°C; for *COII*, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 53°C, 2 min at 72°C), then final elongation of 8 min at 72°C; for *ITS2*, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 48°C, 1 min at 72°C), then final elongation of 8 min at 72°C. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. Sequencing by Macrogen Inc. (Seoul, South Korea) was carried out with both forward and reverse primers under BigDye TM terminator cycling conditions, purifying the products using ethanol precipitation and running them using an Automatic Sequencer 3730xl (Applied Biosystems, Foster City, USA).

Sequences (forward and reverse) were manually corrected using the software CHROMAS PRO 1.34 (Technelysium, Helensvale, Australia). The protein coding nucleotide sequences of *COI* and *COII* were checked for reading frame errors and termination codons in MEGA 4 (Tamura *et al.* 2007). Alignment was carried out using CLUSTALW Multiple Alignment (Thompson *et al.* 1994) within the software BIOEDIT 7.0.5.3, followed by minor manual correction.

Phylogenetic analyses

Individual and combined phylogenetic analyses were performed using the maximum parsimony (MP) and Bayesian Markov chain Monte Carlo (MCMC) criteria. Each locus and the combined data set (including the three mitochondrial markers) were analyzed using

parsimony ratchet (Nixon 1999) as implemented in PAUPrat (Sikes & Lewis 2001). Based on recommendations by Nixon (1999), ten independent searches were performed with 200 iterations and 15% of the parsimony informative characters perturbed. The shortest equally most parsimonious trees were combined to produce a strict consensus tree. Branch supports were calculated using the Bremer support (also known as decay index; Bremer, 1988) as implemented in TreeRot (Sorenson & Franzosa 2007). The Bremer support measures the number of extra steps in tree length required before a node collapses (Bremer 1988; Baker & DeSalle 1997). Bayesian MCMC was used to approximate the posterior probability distribution of the ITS2 marker, using the program MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003). Model selection for the data in the MCMC was carried out with MRMODELTEST2 v.2.3 (Nylander, 2004) based on the Akaike information criterion (Akaike, 1974). Two Metropolis-coupled Markov chains with incremental heating temperature of 0.1 were run for 20 x 10^6 generations and sampled every 1000 generations. The simulation was repeated six times, starting from random trees. Convergence of the MCMC was checked using the potential scale reduction factor proposed in MRBAYES 3.1.2 and the effective sample size criterion for each parameter as implemented in TRACER v.1.4 (Rambaut and Drummond, 2007). To yield a single phylogenetic hypothesis, the posterior distribution was summarized in a 50% majority rule consensus tree (the "halfcompat consensus tree" from MrBayes) after burn-in (for each analysis 5000 trees were discarded).

Divergence time estimation

To estimate phylogenetic relationships among individuals, partitioned Bayesian analysis was performed with BEAST 1.4.7 (Drummond & Rambaut 2007) and analyzed using TRACER v1.4 (Rambaut & Drummond 2007). The three mtDNA regions were represented as separate partitions in the analysis, with substitution models as estimated by MRMODELTEST (Nylander 2004) and four estimated alpha categories for the gamma term (Yang 1994). Three

independent runs of 30 x 10^6 generations were performed, sampling one tree every 1000 generations. A maximum clade credibility tree was generated after removing a burn-in of 10 x 10^6 generations of each run.

Due to the lack of fossils for *Oreina* beetles, direct calibration of the tree topologies was not possible. Instead, branch lengths and node ages were estimated by applying an arthropod mtDNA clock based on a review of published gene-specific mtDNA substitution rates in diverse arthropod taxa (Borer *et al.*), which suggests average pairwise divergence rates of 1.73% for COI, 1.38% for COII and 0.61% for 16S. We dated eight nodes using a relaxed clock with log-normal branch length distribution (Renner 2005; Drummond *et al.* 2006).

Results

For *O. cacaliae* and *O. speciosissima* 120 and 94 individuals, respectively, were sequenced and used for further analyses.

Phylogenetic reconstruction of the ITS2 data set

The alignment length of ITS2 was 635 bp with 12 potentially parsimony informative characters (hereafter PPIc) among 21 polymorphic characters. The best substitution model suggested by MRMODELTEST was JC69. Amplification failed in 18 cases (single individuals from 14 populations and two individuals from BIL and MAP).

Maximum parsimony and Bayesian inference methods produced highly congruent topologies with the same major nodes. We show only the MP topology with Bremer supports but add Bayesian posterior probabilities (hereafter bpp) for the well supported clades (Fig. 1). The ingroup, consisting of all individuals of *O. cacaliae* and *O. speciosissima*, is well supported with a Bremer support of eight and a bpp of 1.00. The ingroup splits into two groups, a polytomy containing all *O. speciosissima* individuals and a well supported clade (Bremer

support 1, bpp 0.96) containing all *O. cacaliae* individuals. Beside the clear separation of the two species, little structure is shown within each species.

Phylogenetic reconstruction of the mtDNA data sets

The total alignment length of the mtDNA was 1626 bp: 529 bp for *16S*, 462 bp for *COI* and 635 bp for *COII*. Among 158 variable characters, 120 were PPIc (representing 7.38% of the total number of characters), excluding the outgroup. The three mtDNA regions contribute as follows: *16S* (19 PPIc among 23 polymorphic sites), *COI* (41 PPIc among 51 polymorphic sites) and *COII* (60 PPIc among 84 polymorphic sites). The best substitution models suggested by MRMODELTEST were HKY+G for all three mtDNA regions. Amplification failed in 24 cases (for 16S, single individuals from six populations; for COI, single individuals from 5 populations and two individuals from RAI; for COII, single individuals from four populations, two individuals from MAP, BIL and three individuals from PDT). Over the three mtDNA regions, all individuals had at least two amplified regions.

In the maximum parsimony analysis *O. cacaliae and O. speciosissima* form a well defined ingroup supported with Bremer support of 69 (supporting Information Fig. S1). Individuals from both species are found in a basal polytomy, with *Oreina cacaliae* represented by individuals from the northern Apennines and *O. speciosissima* by individuals from the Carpathian Mountains. This is followed by a second polytomy containing both species (three populations of *O. cacaliae* from the Cantabrian and Pyrenees; *O. speciosissima* with populations from the Massif Central, Black Forest, Dolomites, southern Dinaric Alps and south-eastern Carpathians). The next divergence point is a polytomy with many clades. For *Oreina speciosissima* this includes two clades; one with individuals from the Ore Mountains and Bavarian Forest together with those from northern Croatia, Slovenia and the Alps (Bremer support = 4) and one with two individuals from VAL (with Bremer support = 1). Most of this polytomy is represented by clades of *Oreina cacaliae*. Besides many small clades

in the Alps three larger clades are defined (one containing specimens from the western Alps, Vosges, Black Forest and Bavarian Forest, one represented by specimens from Serbia, the Carpathians and Sudeten range, and the third includes specimens from the Massif Central and Montenegro).

Divergence time estimates

The Bayesian Inference analysis yielded a fully-resolved topology highly congruent with the MP analysis. For each parameter, convergence of the three independent runs was confirmed by the visual examination of their respective distributions in TRACER v1.4. The estimated sampling size (ESS) was >120 for all parameters. To obtain a half-compatible topology, nodes with supports < 0.5 were polytomized. The main seven nodes of interest are indicated on Figure 3. The basal node (node one) is strongly supported (bpp = 1), nodes two and three have lower supports (0.53 and 0.52 bpp), whereas nodes five, six and seven define clades with 0.71, 0.88 and 0.85 bpp, respectively, (Fig. 2). Nodes one two and three define three clades which are identical to the three polytomies in the MP approach. The dating analysis suggests that those three major clades were formed before the last glacial period (Table 2 and Fig. 3), with divergence spanning the last 2 million years. Here and in the discussion, we consider eight clades with bpp of 0.7-1.00 (Fig. 2). Clade I, including *O. cacaliae* from the northern Apennines and *O. speciosissima* from the Apuseni Mountains and Sudeten range, and clade II containing specimens from the southern Carpathians, form a polytomy that diverged 2.14 MYA (at node one). Clade III contains both species and diverged 1.26 MYA (node 2). Within clade III, *O. cacaliae* from the Cantabrians and Pyrenees split from *O. speciosissima* (containing specimens from the Massif Central, the Black Forest, the Dolomites, Montenegro, the southern Carpathians and one specimen from eastern Switzerland) about 0.73 MYA (node 4). Clade IV-VIII diverged 0.8 MYA (node 3) and form a polytomy. Clade IV contains *O. cacaliae* from the southern Alpes Maritimes. About 0.31 MYA (node 7) clade V containing

O. cacaliae, diverged into a clade with specimens from the Massif Central and a clade with specimens from Montenegro. *Oreina speciosissima* from the Alps, northern Croatia, Bavarian Forest and the Ore Mountains fall in clade VI. Clade VII comprises *O. cacaliae* from the Alps, the Vosges, the Black Forest, northern Croatia and the Bavarian Mountains. Approximately 0.38 MYA (node 6), this clade splits into one clade containing the individuals from the north-western populations and one broadpolytomy containing individuals from the eastern populations together with two *O. speciosissima* from the south eastern Alps. Clade VIII containing *O. cacaliae* diverged about 0.3MYA (node 5) into a clade containing specimens from the southern Carpathians, one with specimens from Serbia and the north-eastern Carpathians and a third clade comprising specimens from the High Tatras and Sudeten range.

Discussion

Do the subspecies described on the basis of morphological criteria coincide with the clades defined by the phylogenetic analyses?

The phylogenetic analysis based on the ITS2 region suggests a strict separation of *O. cacaliae* from *O. speciosissima* with a bpp of 0.96 and thus confirms the systematic classification based on morphological criteria. The two species can be unambiguously determined according to male genitalia and this separation is recovered in the analysis of the nuclear locus. In contrast the analysis of the three mtDNA regions using maximum parsimony and Bayesian inference suggests paraphyly of *O. cacaliae* and *O. speciosissima*, with members of both species falling in the clades that separate at node 1, 2, and 3. Much of the subspecies taxonomy however is recovered in the mtDNA analysis. In *Oreina cacaliae*, six (ssp. *senilis*, *barii*, *cacaliae* s. str., *bohemica*, *senecionis* and *albanica*) out of seven treated subspecies form well supported monophyletic clades. The subspecies *tussilaginis* is represented by two geographical groups which fall in separate clades, one in the Pyrenees and Cantabrian

Mountains (clade III) and the other in the Massif Central (clade V). In *Oreina speciosissima,* three (ssp. *convergens*, *juncorum* and *propria*) out of five treated subspecies form monophyletic clades whereas *ssp. speciosissima* occur in clade III and clade VI, and *ssp. fuscoaenea has* two groups in the polytomy of clade VI.

Do these two species share the same phylogeographical history?

Due to the low dispersal ability of *Oreina* beetles it is likely that the phylogeographical structure is strongly influenced by geographical isolation. In contrast to the situation in lowland species, warmer interglacial periods like the present may represent the most isolated periods, during which mountains act as refugia. During cold periods, even if they had to retreat from the highest altitudes a larger area of suitable habitat in lower altitudes would have become available.

Phylogeographical structure within Oreina cacaliae

There are distinct lineages in most of the major European mountain systems. The Alps form the centre of the species distribution, and are dominated by clade VII. This clade can be further separated into a north-western part in the Alps, the Bavarian Forest, the Black Forest and the Vosges Mountains and an eastern part in the Alps and northern Balkans. Eastern Europe is dominated by clade VIII, with some internal structure in the form of three clades. One in the Sudety Mountains and the High Tatras, one in the southern Carpathians, and a third in the Carpathians and Serbia. Populations from the Cantabrian and Pyrenees form the well defined clade III. Clade V has two disjunct pairs of populations, in the Massif Central and Montenegro. Finally, there are two clades represented by single populations, clade IV in the southern Alpes Maritimes and clade I in the Northern Apennines.

Phylogeographical structure within Oreina speciosissima

Clade VI covers the Alps, the northern Balkans, the Bavarian Forest and the Ore Mountains. In Eastern Europe clade I contain a population of the Sudety Mountains (GLA) and MAP in the Apuseni Mountains. The population BIL forms a well defined clade (clade II) in the southern Carpathians. Clade III covers a large area ranging from the southern Carpathians (SIN) to the Massif Central and Black Forest. Internal structure defines five clades (one in the southern Carpathians, one in Montenegro, two in the Dolomites and one in the Massif Central and Black Forest.

Incongruence between markers

The analysis of the nuclear ITS2 region and classical morphological criteria allow us to strictly separate the two species. This result is incongruent with the phylogeny based on the three mtDNA regions, which shows paraphyly in both species. The incongruence may be explained by two hypotheses: i) hybridisation and ii) ancestral polymorphism. i) Hybridisation could pull populations of the two species together within the analysis. There is one case that appears to show recent hybridisation in the eastern group of clade VII, where two individuals of *O. speciosissima* from VAL fall in a well supported group within a clade of *O. cacaliae*. Based on morphological criteria they are doubtlessly *O. speciosissima*. On the other hand, these two individuals share an identical sequence at COII with several individuals of *O. cacaliae*, a very similar COI genotype, and one identical and one very similar 16S genotype, respectively. This supports the hypothesis of a recent hybridisation event, within introgression of *O. cacaliae* into *O. speciosissima*. If hybridisation is the explanation of other cases of lineage mixing, it must be more ancient, because the sequences involved are not identical and the lineages are not currently sympatric. ii) On the other hand, it is possible that ancestral polymorphism could cause the observed paraphyly in the mtDNA analyses. If in an ancestral population a locus is polymorphic, this can be retained through species divergence,

but with lineage sorting the gene genealogy may then not reflect the population separation (Degnan & Rosenberg 2009).

Despite these caveats, it seems we can still reveal the evolutionary history of these species. Terminal clades largely consist of single species, recover many of the morphologically-defined subspecies, and seem to reveal biogeographical regions that may represent refugial populations. Lineage sorting appears to have occurred within these glacial regions, so that we can recover their evolutionary history, but not interpret their dating as the times of refugial isolation.

Is the contemporary genetical structure a result of the last glaciation or is divergence within species more ancient?

The molecular clock hypothesis, even if providing only a approximate dating method, show that divergence within *O. cacaliae* and *O. speciosissima* started long before (2 MYA) the last glacial maximum [18 KYA (COHMAP 1988)]. Thus divergence of clades was influenced by several glacial and interglacial cycles. Divergence time within these two species can be considered as recent compared with the divergence times suggested for *Oreina elongata* (Borer *et al.*) but older than divergence suggested for *Oreina gloriosa* (Borer et al. , thesis chapter 3) where the regions show divergence in mtDNA and AFLP analyses but not yet in external morphological criteria.

Drusus discolor, a caddisfly sharing its geographical distribution with *O. cacaliae* and *O. speciosissima* as well as the suggested divergence time (2 MY) (Pauls et al. 2006), shows slightly more diverged but congruent biogeographic groups as defined for *O. cacaliae.* The separation within the Alps into a north-western region and an eastern region (*O. cacaliae*) has already been shown for several alpine plants (Schönswetter *et al.* 2005) as well as for other terrestrial and aquatic insect species (Pauls *et al.* 2006; Haubrich & Schmitt 2007; Schmitt & Haubrich 2008). The biogeographical groups of another caddisfly (*Rhyacophila aquitanica)*

are highly congruent with clade III of *O. speciosissima (Balint et al. 2008)*. The presence of largely congruent biogeographical patterns in several high altitude species occupying similar ecological niches (the species compared above to *Oreina* are all dependent on humid habitats) argues for a change in thinking from single species scenarios to multi-species phylogeography.

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Table 1. Sampled populations of a) *Oreina cacaliae* and b) *O. speciosissima* with their geographical coordinates, subspecies and number of treated individuals. Shaded rows indicate sympatric populations.

a)

Population	Site	Altitude (m a. s. l.)	Geographical coordinates	Subspecies	Sample Size
ARL	Arlbergpass (A)	1354	47°08 'N, 10°12 'E	*cacaliae*	3
BAD	Badgastein (A)	1963	47°06 'N, 13°07 'E	*cacaliae*	2
GAM	Gamsblick (A)	1317	47°11 'N, 12°28 'E	*cacaliae*	1
JOH	Johnsbach (A)	1587	47°32 'N, 14°33 'E	*cacaliae*	3
KOR	Koralpe (A)	1564	46°48 'N, 14°56 'E	*cacaliae*	2
LOS	Loser (A)	1598	47°39 'N, 13°47 'E	*cacaliae*	3
VAL	Valentinsalm (A)	1457	46°37 'N, 12°54 'E	*cacaliae*	3
VSJ	Velebit Sjeverni (HR)	1375	45°25 'N, 14°37 'E	*cacaliae*	4
DOM	Domasov (CZ)	832	50°06 'N, 17°15 'E	*senecionis*	2
KST	Karlova Studanka (CZ)	1252	50°04 'N, 17°14 'E	*senecionis*	1
VUP	Velka Upa (CZ)	1061	50°40 'N, 15°43 'E	*senecionis*	2
AUB	Aubisque (F)	1459	42°58 'N, 00°18'W	*tussilaginis*	2
BAL	Ballon d'Alsace (F)	882	47°48 'N, 06°50 'E	*cacaliae*	2
CDC	Col de la Charbonnière (F)	1149	48°26 'N, 07°19 'E	*cacaliae*	3
CDS	Col de la Schlucht (F)	1199	48°04 'N, 07°02 'E	*cacaliae*	2
FEN	Col de Fenestre (F)	2479	44°06 'N, 07°21 'E	*senilis*	3
GAL	Col du Galibier (F)	1999	45°05 'N, 06°26 'E	*cacaliae*	2
LDB	Lac du Ballon (F)	1011	47°54 'N, 07°05 'E	*cacaliae*	1
MDR	Mont Dore (F)	1428	45°33 'N, 02°47 'E	*tussilaginis*	3
PMA	Puy Mary (F)	1550	45°06 'N, 02°40 'E	*tussilaginis*	2
BUH	Bühlertal (D)	865	48°39 'N, 08°13 'E	*cacaliae*	2
FIN	Finsterau (D)	1103	48°56 'N, 13°33 'E	*bohemica*	3
WAL	Waldkirch (D)	956	48°04 'N, 08°00 'E	*cacaliae*	2
KAR	Karersee (I)	1570	46°24 'N, 11°34 'E	*cacaliae*	2
LAS	Lago Santo (I)	1273	44°24 'N, 10°00 'E	*barii*	3
MPI	Monte Pizzoc (I)	1305	46°02 'N, 12°23 'E	*cacaliae*	1
PDT	Passo del Tonale (I)	1784	46°15 'N, 10°36 'E	*cacaliae*	2
PDU	Passo Duran (I)	1477	46°18 'N, 12°05 'E	*cacaliae*	2
PPF	Passo Pian delle Fugazze (I)	1296	45°44 'N, 11°09 'E	*cacaliae*	2
PRO	Passo Rolle (I)	1969	46°17 'N, 11°46 'E	*cacaliae*	2
KOL	Kolasin (MNE)	1451	42°49 'N, 19°37 'E	*albanica*	3
ZAB	Zabljak (MNE)	1377	43°09 'N, 19°03 'E	*albanica*	3
KOS	Koscielisko (PL)	995	49°16 'N, 19°51 'E	*senecionis*	1
ZAK	Zakopane (PL)	1006	49°16 'N, 20°00 'E	*senecionis*	2
PET	Petrosani (RO)	1088	45°24 'N, 23°31 'E	*senecionis*	3
SIB	Sibau (RO)	1087	47°35 'N, 24°55 'E	*senecionis*	2
SIN	Sinaia (RO)	1362	45°21 'N, 25°31 'E	*senecionis*	3
KOP	Kopaonik (SRB)	1700	43°29 'N, 20°46 'E	*cacaliae*	3
MGO	Mali Golik (SLO)	1212	45°59 'N, 13°50 'E	*cacaliae*	2

POV	Podvolovljek (SLO)	1173	46°16 'N, 14°42 'E	*cacaliae*	3
PRE	Predmeja (SLO)	1142	45°55 'N, 13°50 'E	*cacaliae*	2
VRS	Vrsic Pass (SLO)	1387	46°25 'N, 13°44 'E	*cacaliae*	2
EUR	Posada de Valéon (S)	1294	43°07 'N, 04°52'W	*tussilaginis*	3
RAI	Rio Aiguamoix (S)	1636	42°39 'N, 00°55 'E	*tussilaginis*	3
CDM	Col-des-Mosses (CH)	1716	46°23 'N, 07°07 'E	*cacaliae*	3
GER	Gerschnialp (CH)	1228	46°48 'N, 08°22 'E	*cacaliae*	5
KAN	Kandersteg (CH)	1314	46°28 'N, 07°39 'E	*cacaliae*	3
LAF	La Fouly (CH)	1571	45°56 'N, 07°05 'E	*cacaliae*	3
MET	Mettmen (CH)	1183	46°57 'N, 09°05 'E	*cacaliae*	1
TSC	Tschiertschen (CH)	1315	46°48 'N, 09°36 'E	*cacaliae*	3

A, Austria; CH, Switzerland; CZ, Czech Republic; D, Germany; F, France; I, Italy; MNE, Montenegro; PL, Poland; RO, Romania; S, Spain; SRB, Serbia; SLO, Slovenia;

b)

Population	**Site**	**Altitude (m a. s. l.)**	**Geographical coordinates**	**Subspecies**	**Sample Size**
ARL	Arlbergpass (A)	1354	47°08 'N, 10°12 'E	*speciosissima*	1
BAD	Badgastein (A)	1963	47°06 'N, 13°07 'E	*speciosissima*	3
GAM	Gamsblick (A)	1317	47°11 'N, 12°28 'E	*speciosissima*	3
JOH	Johnsbach (A)	1587	47°32 'N, 14°33 'E	*speciosissima*	3
LOS	Loser (A)	1598	47°39 'N, 13°47 'E	*speciosissima*	2
LUN	Lunz am See (A)	865	47°50 'N, 15°02 'E	*speciosissima*	3
VAL	Valentinsalm (A)	1457	46°37 'N, 12°54 'E	*speciosissima*	3
RIS	Risnjak (HR)	1303	45°25 'N, 14°37 'E	*speciosissima*	3
MDR	Mont Dore (F)	1428	45°33 'N, 02°47 'E	*convergens*	3
ALT	Altenberg (D)	771	50°45 'N, 13°42 'E	*speciosissima*	3
FEB	Feldberg (D)	1189	47°51 'N, 08°00 'E	*speciosissima*	3
FIN	Finsterau (D)	1103	48°56 'N, 13°33 'E	*speciosissima*	3
OBE	Oberstdorf (D)	1216	47°24 'N, 10°18 'E	*speciosissima*	3
KAR	Karersee (I)	1570	46°24 'N, 11°34 'E	*speciosissima*	3
MPI	Monte Pizzoc (I)	1305	46°02 'N, 12°23 'E	*speciosissima*	2
PDT	Passo del Tonale (I)	1784	46°15 'N, 10°36 'E	*speciosissima*	3
PDU	Passo Duran (I)	1477	46°18 'N, 12°05 'E	*speciosissima*	3
PPF	Passo Pian delle Fugazze (I)	1296	45°44 'N, 11°09 'E	*speciosissima*	2
KOL	Kolasin (MNE)	1451	42°49 'N, 19°37 'E	*ssp. propria*	2
GLA	Snieznik (PL)	1000	50°12 'N, 16°52 'E	*fuscoaenea*	1
BIL	Lacul Bâlea (RO)	2100	45°36 'N, 24°36 'E	*juncorum*	2
MAP	Muntii Apuseni (RO)	1031	46°27 'N, 22°50 'E	*juncorum*	2
PET	Petrosani (RO)	1088	45°24 'N, 23°31 'E	*juncorum*	1
SIN	Sinaia (RO)	1362	45°21 'N, 25°31 'E	*juncorum*	1
MGO	Mali Goliak (SLO)	1212	45°59 'N, 13°50 'E	*speciosissima*	4
OKR	Okreslju (SLO)	1394	46°22 'N, 14°35 'E	*speciosissima*	3
POD	Podnanos (SLO)	1145	45°48 'N, 14°03 'E	*speciosissima*	1
PRE	Predmeja (SLO)	1142	45°55 'N, 13°50 'E	*speciosissima*	2
VRS	Vrsic Pass (SLO)	1387	46°25 'N, 13°44 'E	*speciosissima*	2

BRU	Brülisau (CH)	1093	47°16 'N, 09°27 'E	*speciosissima*	3
CDM	Col-des-Mosses (CH)	1716	46°23 'N, 07°07 'E	*speciosissima*	3
GER	Gerschnialp (CH)	1228	46°48 'N, 08°22 'E	*speciosissima*	2
KAN	Kandersteg (CH)	1314	46°28 'N, 07°39 'E	*speciosissima*	3
LAF	La Fouly (CH)	1571	45°56 'N, 07°05 'E	*speciosissima*	3
MET	Mettmen (CH)	1183	46°57 'N, 09°05 'E	*speciosissima*	2
TSC	Tschiertschen (CH)	1315	46°48 'N, 09°36 'E	*speciosissima*	5
ZIN	Zinal (CH)	1679	46°08 'N, 07°37 'E	*speciosissima*	3

A, Austria; CH, Switzerland; CZ, Czech Republic; D, Germany; F, France; I, Italy; MNE, Montenegro; PL, Poland; RO, Romania; S, Spain; SRB, Serbia; SLO, Slovenia;

Table 2. Ages (in millions of years) of several nodes in the *O. cacaliae / O. speciosissima* phylogeography, based on a log-normal relaxed clock model.

	Lognormal	
Node	Mean	95% C.I.
1	2.14	1.40-3.10
2	1.26	0.87-1.72
3	0.8	0.56-1.12
4	0.73	0.46-1.07
5	0.3	0.17-0.47
6	0.38	0.23-0.56
7	0.31	0.13-0.55

Figure legends

Figure 1. Strict consensus tree of the MP analysis of the ITS2 region. Node supports are given by Bremer supports and bpp (italics). Species are indicated by label colour: blue = *O. cacaliae* and red = *O. speciosissima*.

Figure 2. Half-compatible topology of the BEAST analysis of the three mtDNA regions. Species are indicated by label colour: blue = *O. cacaliae* and red = *O. speciosissima*. Node supports are given by Bayesian posterior probabilities. Dates for the numbered nodes are given in Table 2 and discussed in the text. Branches illustrated with \\ are reduced in length by 4,5 MY.

Figure 3. Geographical distribution of the sampled populations of a) *Oreina cacaliae* and b) *O. speciosissima*. The eight clades defined in the BEAST analysis are represented by colour: dark brown = clade I, dark red = clade II, purple = clade III, pink = clade IV, dark blue = clade V, light blue = clade VI, green = clade VII, yellow = clade VIII. Sympatric populations are indicated by shading of the population label. The dashed line indicates the split into two claeds within clade VII. Topographic shading shows altitudes above 1000m. Colours

Supporting Information

Figure 1S. Strict consensus tree of the MP analysis of the three mtDNA regions. Node supports are given by Bremer supports (decay index) ≥ 1. Species are indicated by label colour: blue = *O. cacaliae* and red = *O. speciosissima*.

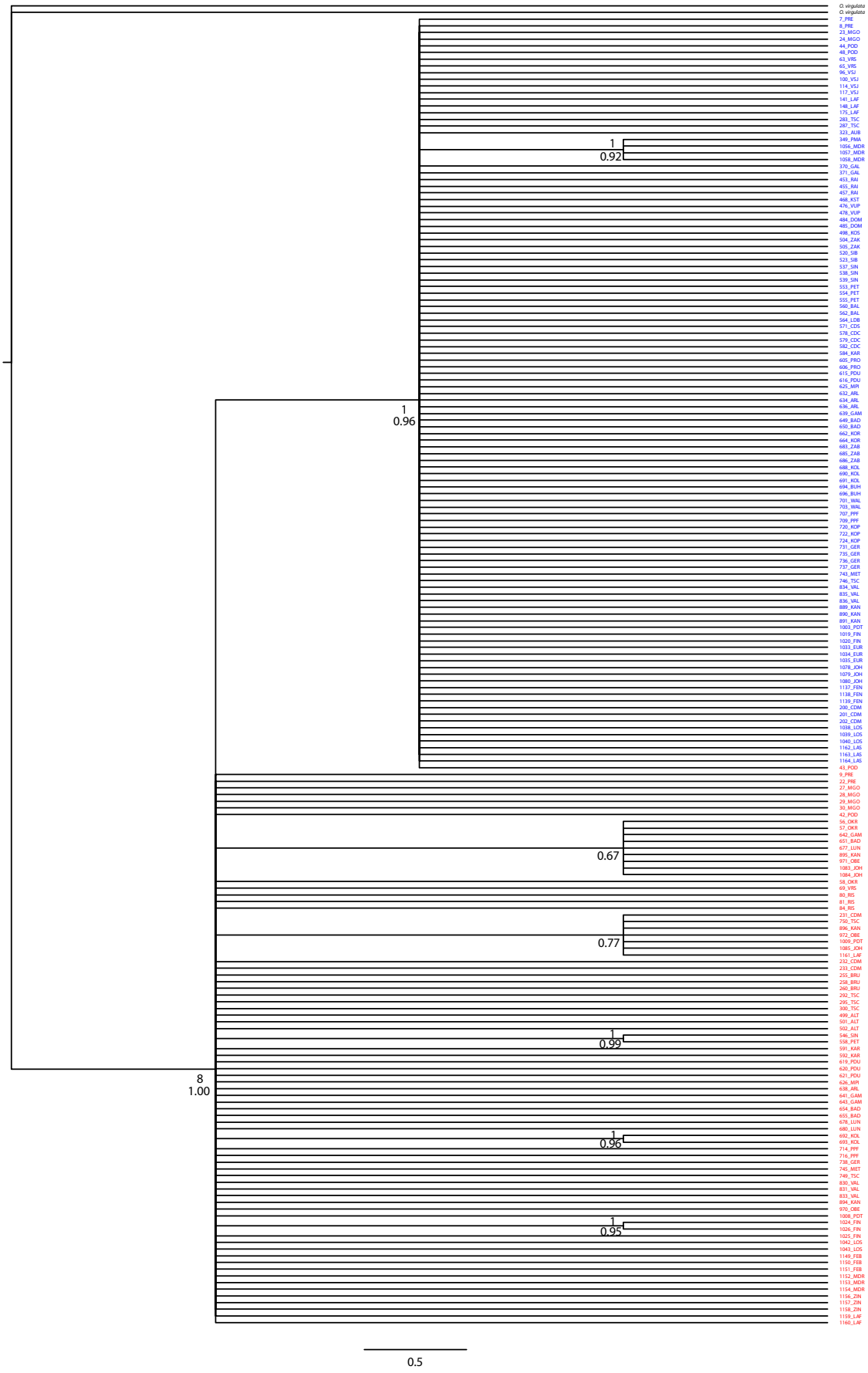

O. virgulata
O. virgulata
7_PRE
8_PRE
23_MGO
24_MGO
44_POD
48_POD
63_VRS
65_VRS
96_VSJ
100_VSJ
114_VSJ
117_VSJ
141_LAF
148_LAF
175_LAF
283_TSC
287_TSC
323_AUB
349_PMA
1056_MDR
1057_MDR
1058_MDR
370_GAL
371_GAL
453_RAI
455_RAI
457_RAI
468_KST
476_VUP
478_VUP
484_DOM
485_DOM
498_KOS
504_ZAK
505_ZAK
520_SIB
523_SIB
537_SIN
538_SIN
539_SIN
553_PET
554_PET
555_PET
560_BAL
562_BAL
564_LOB
571_CDS
578_CDC
579_CDC
582_CDC
584_KAR
605_PRO
606_PRO
615_PDU
616_PDU
625_MPI
632_ARL
634_ARL
636_ARL
639_GAM
649_BAD
650_BAD
662_KOR
664_KOR
683_ZAB
685_ZAB
686_ZAB
688_KOL
690_KOL
691_KOL
694_BUH
696_BUH
701_WAL
703_WAL
707_PPF
709_PPF
720_KOP
722_KOP
724_KOP
731_GER
735_GER
736_GER
737_GER
743_MET
746_TSC
834_VAL
835_VAL
836_VAL
889_KAN
890_KAN
891_KAN
1003_PDT
1019_FIN
1020_FIN
1033_EUR
1034_EUR
1035_EUR
1078_JOH
1079_JOH
1080_JOH
1137_FEN
1138_FEN
1139_FEN
200_CDM
201_CDM
202_CDM
1038_LOS
1039_LOS
1040_LOS
1162_LAS
1163_LAS
1164_LAS
43_POD
9_PRE
22_PRE
27_MGO
28_MGO
29_MGO
30_MGO
42_POD
56_OKR
57_OKR
642_GAM
651_BAD
677_LUN
895_KAN
971_OBE
1083_JOH
1084_JOH
58_OKR
69_VRS
80_RIS
81_RIS
84_RIS
231_CDM
750_TSC
896_KAN
972_OBE
1009_PDT
1085_JOH
1161_LAF
232_CDM
233_CDM
255_BRU
258_BRU
260_BRU
292_TSC
295_TSC
300_TSC
499_ALT
501_ALT
502_ALT
546_SIN
558_PET
591_KAR
592_KAR
619_PDU
620_PDU
621_PDU
626_MPI
638_ARL
641_GAM
643_GAM
654_BAD
655_BAD
678_LUN
680_LUN
692_KOL
693_KOL
714_PPF
716_PPF
738_GER
745_MET
749_TSC
830_VAL
831_VAL
833_VAL
894_KAN
970_OBE
1006_PDT
1024_FIN
1026_FIN
1025_FIN
1042_LOS
1043_LOS
1149_FEB
1150_FEB
1151_FEB
1152_MDR
1153_MDR
1154_MDR
1156_ZIN
1157_ZIN
1158_ZIN
1159_LAF
1160_LAF
1
0.92
1
0.96
0.67
0.77
1
0.99
8
1.00
1
0.96
1
0.95
0.5

Figure 1

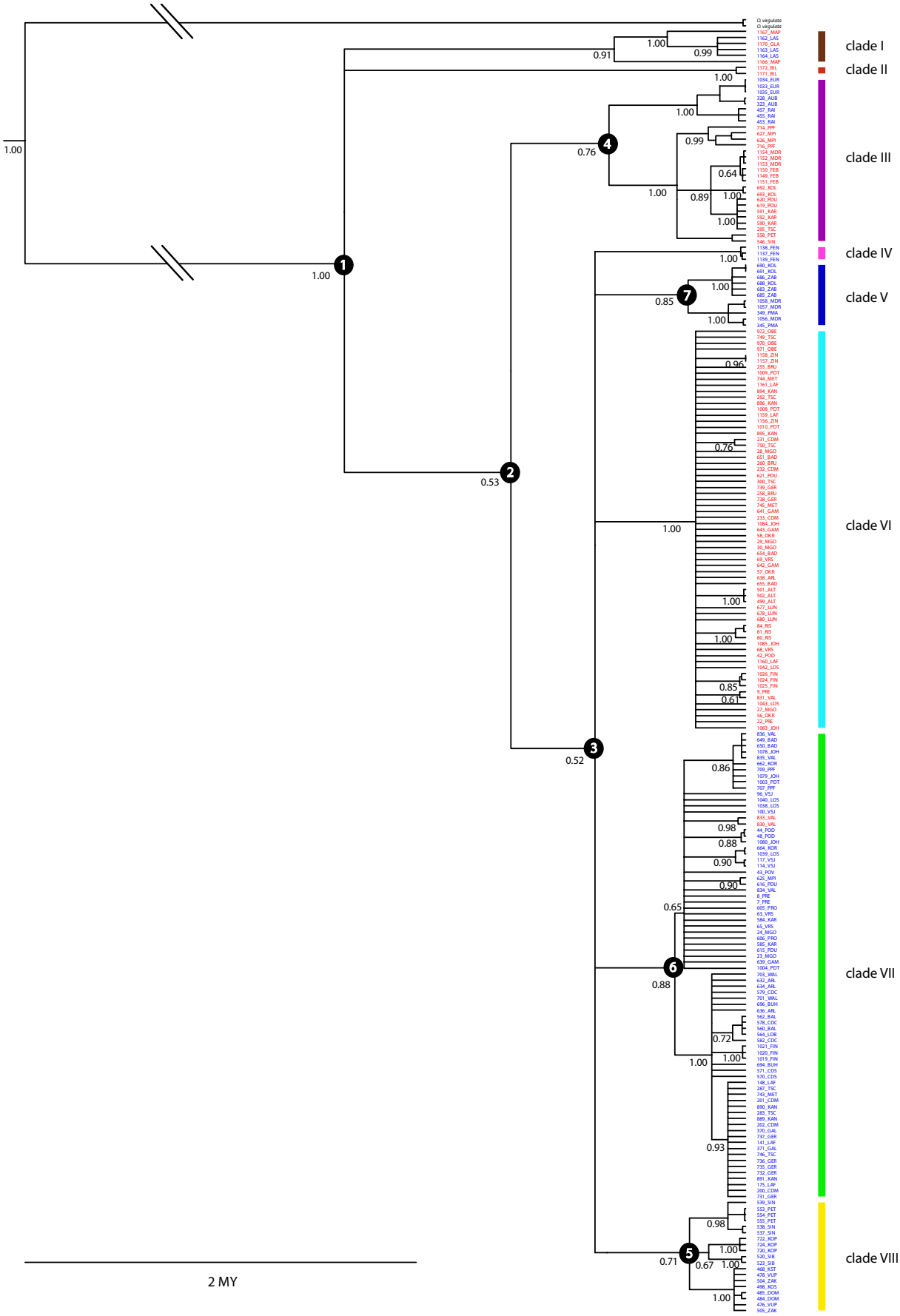
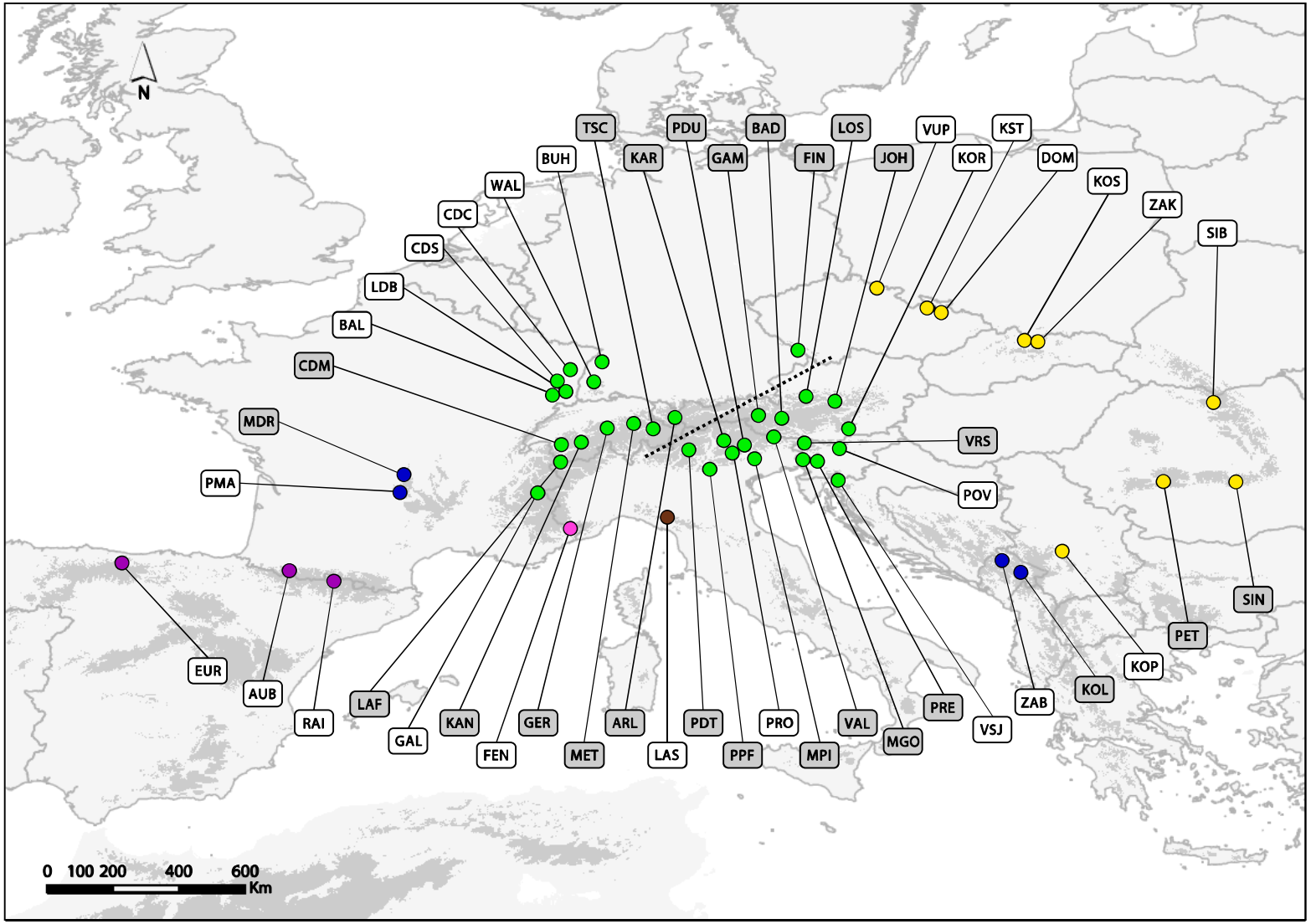

clade I
clade II
clade III
clade IV
clade V
clade VI
clade VII
clade VIII
2 MY

Figure 2


N
TSC
PDU
BAD
LOS
VUP
KST
BUH
KAR
GAM
FIN
JOH
KOR
DOM
WAL
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ZAK
CDC
SIB
CDS
LDB
BAL
CDM
MDR
VRS
PMA
POV
SIN
PET
KOP
EUR
KOL
AUB
ZAB
RAI
LAF
PRE
KAN
GER
ARL
PDT
PRO
VAL
VSJ
GAL
MGO
FEN
MET
LAS
PPF
MPI
0 100 200 400 600
Km

Figure 3a

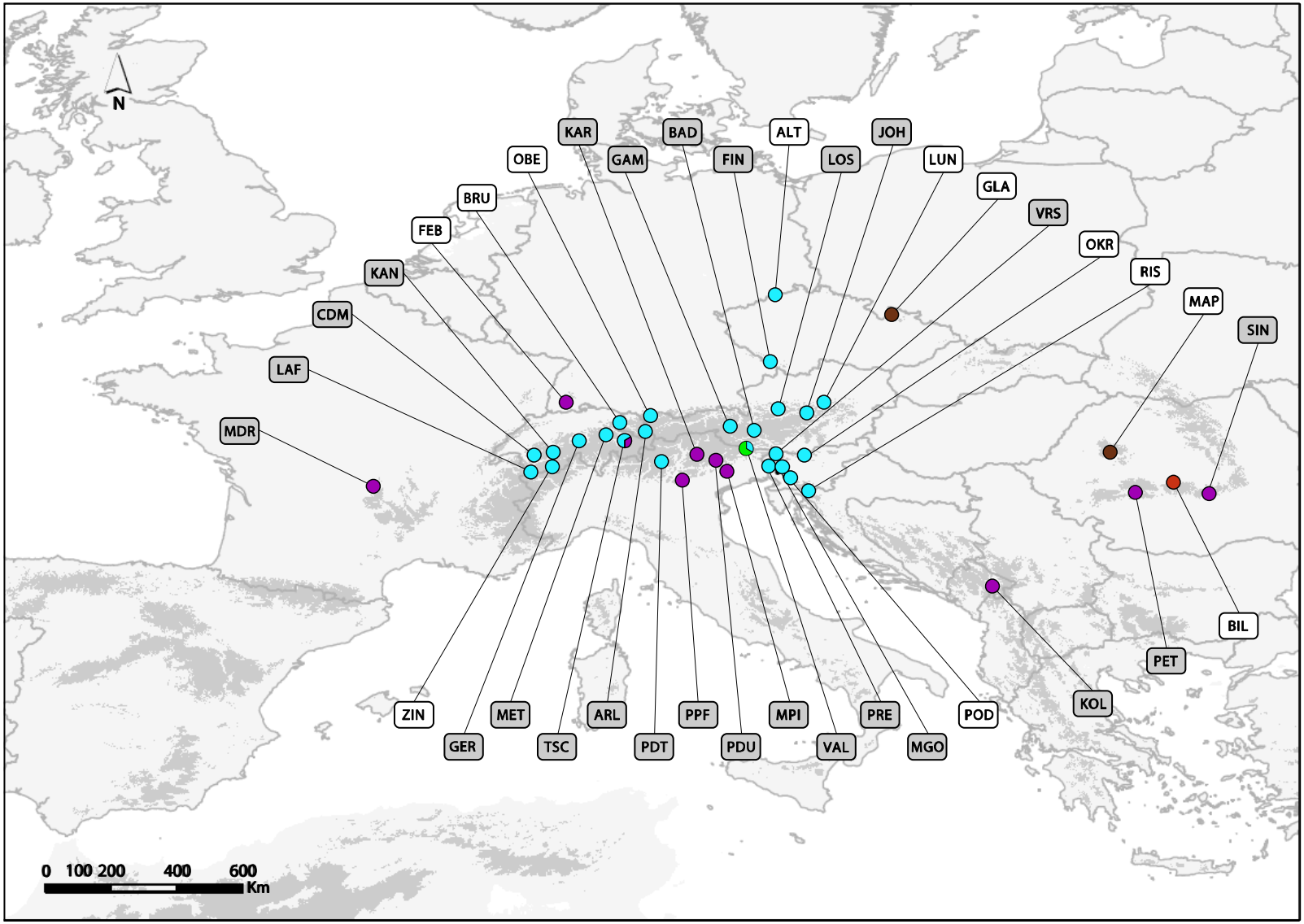
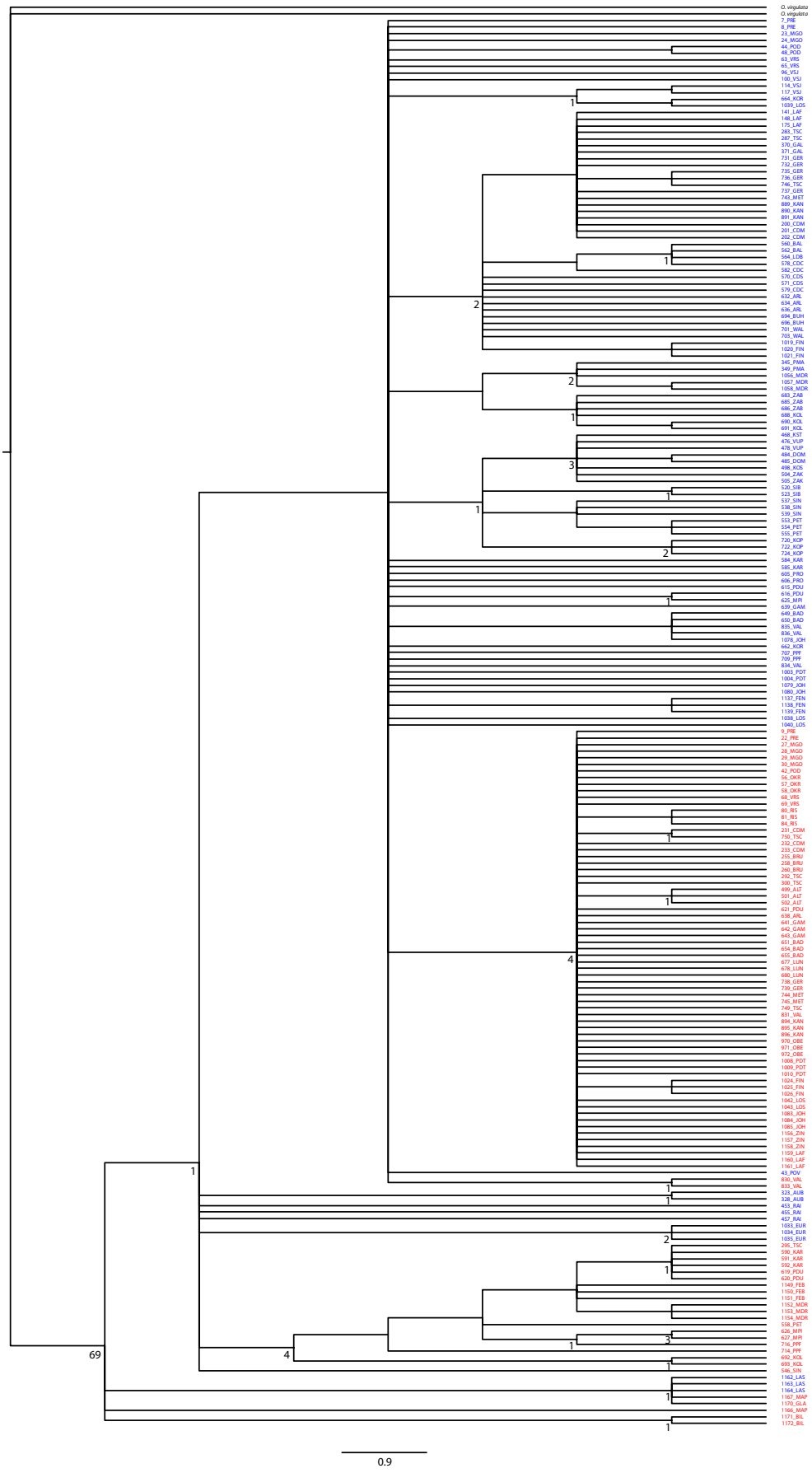

N
KAR
BAD
ALT
JOH
OBE
GAM
FIN
LOS
LUN
BRU
GLA
FEB
VRS
OKR
KAN
RIS
MAP
CDM
SIN
LAF
MDR
BIL
PET
KOL
ZIN
MET
ARL
PPF
MPI
PRE
POD
GER
TSC
PDT
PDU
VAL
MGO
0 100 200 400 600
Km

Figure 3b


O. virgulata
O. virgulata
7_PRE
8_PRE
23_MGO
24_MGO
44_POD
48_POD
63_VRS
65_VRS
96_VSJ
100_VSJ
114_VSJ
117_VSJ
664_KOR
1039_LOS
141_LAF
148_LAF
175_LAF
285_TSC
287_TSC
370_GAL
371_GAL
731_GER
732_GER
735_GER
736_GER
746_TSC
737_GER
743_MET
889_KAN
890_KAN
891_KAN
200_CDM
201_CDM
202_CDM
560_BAL
562_BAL
564_LDB
578_CDC
582_CDC
570_CDS
571_CDS
579_CDC
632_ARL
634_ARL
636_ARL
694_BUH
696_BUH
701_WAL
703_WAL
1019_FIN
1020_FIN
1021_FIN
345_PMA
349_PMA
1056_MDR
1057_MDR
1058_MDR
683_ZAB
685_ZAB
686_ZAB
688_KOL
690_KOL
691_KOL
468_KST
476_VUP
478_VUP
484_DOM
485_DOM
498_KOS
504_ZAK
505_ZAK
520_SIB
523_SIB
537_SIN
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553_PET
554_PET
555_PET
720_KOP
722_KOP
724_KOP
584_KAR
585_KAR
605_PRO
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615_PDU
616_PDU
625_MPI
639_GAM
649_BAD
650_BAD
835_VAL
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1078_JOH
662_KOR
707_PPF
709_PPF
834_VAL
1003_PDT
1004_PDT
1079_JOH
1080_JOH
1137_FEN
1138_FEN
1139_FEN
1038_LOS
1040_LOS
9_PRE
22_PRE
27_MGO
28_MGO
29_MGO
30_MGO
42_POD
56_OKR
57_OKR
58_OKR
68_VRS
69_VRS
80_RIS
81_RIS
84_RIS
231_CDM
750_TSC
232_CDM
233_CDM
255_BRU
258_BRU
260_BRU
292_TSC
300_TSC
499_ALT
501_ALT
502_ALT
621_PDU
638_ARL
641_GAM
642_GAM
643_GAM
651_BAD
654_BAD
655_BAD
677_LUN
678_LUN
680_LUN
738_GER
739_GER
744_MET
745_MET
749_TSC
831_VAL
894_KAN
895_KAN
896_KAN
970_OBE
971_OBE
972_OBE
1008_PDT
1009_PDT
1010_PDT
1024_FIN
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1026_FIN
1042_LOS
1043_LOS
1083_JOH
1084_JOH
1085_JOH
1156_ZIN
1157_ZIN
1158_ZIN
1159_LAF
1160_LAF
1161_LAF
43_POV
830_VAL
833_VAL
323_AUB
328_AUB
453_RAI
455_RAI
457_RAI
1033_EUR
1034_EUR
1035_EUR
295_TSC
590_KAR
591_KAR
592_KAR
619_PDU
620_PDU
1149_FEB
1150_FEB
1151_FEB
1152_MDR
1153_MDR
1154_MDR
558_PET
626_MPI
627_MPI
716_PPF
714_PPF
692_KOL
693_KOL
546_SIN
1162_LAS
1163_LAS
1164_LAS
1167_MAP
1170_GLA
1166_MAP
1171_BIL
1172_BIL
0.9

Figure S1

Chapter IV

Ecological speciation within the alpine leaf beetle *Oreina speciosissima* (Coleoptera, Chrysomelidae)

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Abstract

Interest in the origin of species and finding explanations for the enormous diversity of species that can be observed in the world around us have absorbed evolutionary biologist for over 150 years. Species not only differ greatly among each other but often show a remarkable phenotypic and genotypic variation within their own taxon. Within species variation is the basis of species formation. Here we describe a possible case of a group currently undergoing speciation- the alpine leaf beetle *Oreina speciosissima s. str*, and *Oreina speciosissima troglodytes* (Coleoptera, Chrysomelidae). Based on morphological criteria the subspecies *O. s. s.str*. and *O. s. troglodyte*s as well as intermediate forms were described. The phylogeny of three mtDNA regions (16S, CO1 and COII) showed very little structure and was insufficient to elucidate divergence and to compare the classification based on morphological criteria with molecular data. AFLP genotyping, however, showed clear structure which was highly congruent with the classification of those individuals that fell clearly into morphological categories. Those that were intermediate in morphology were present at the base of both subspecies clades and therefore suggest that the divergence between the two subspecies is not yet complete. We believe that this is ecological speciation, driven by the presence of different ecological niches in certain habitats and explore possible mechanisms that could cause the observed divergence between these two subspecies.

Introduction

Over the last 150 years, numerous species concepts have been postulated by evolutionary biologists. Species concepts can be defined in order to help us classify organisms in a systematic manner, in order to help us understand the occurrence of distinct entities in nature, or for some other goals (Coyne and Orr 2004). Van Valen (1976) defined the ecological species concept as follows: "A species is a lineage (or a closely related set of lineages), which occupies an adaptive zone minimally different from that of any other lineage in its range and which evolves separately from all lineages outside its range". The driving force behind ecological speciation is divergent natural selection between environments or in other words; reproductive isolation of populations or sub-populations by means of adaptation to different environments or niches. (Schluter 2000, 2001; Via 2001; Schluter 2009). Ecological selection is a consequence of individual based interactions with the environment (Rundle and Nosil 2005).

The hypothesis of ecological speciation predicts that divergent selection between ecological niches is the driving force of reproductive incompatibility. Ecologically divergent pairs of populations will show higher levels of reproductive incompatibility than more similar population pairs. Another prediction is that traits under selection often affect reproductive compatibility (Nosil *et al.* 2009).

The fauna and flora of Northern Europe was strongly affected by climatic oscillations during the Quaternary. Multiple glacial and interglacial cycles strongly influenced the genetic structure of most temperate taxa. During glacial cycles many lowland species were forced to retreat to southern refugia in the Iberian, Italian and Balkan peninsulas (Hewitt 1996, 2000; Widmer and Lexer 2001; Hewitt 2004). For many species, mountain ranges such as the Alps were barriers to their dispersal northwards during interglacial periods (Taberlet *et al.* 1998;

Hewitt 2000). As a consequence, lineages from these separated refugia contributed differently to the genetic diversity of northern regions. In contrast to lowland species, recent molecular studies regarding high altitude species propose refugia within the Alps as well as at their southern, eastern and northern borders (Borer *et al.* ; Stehlik *et al.* 2002; Schönswetter *et al.* 2005; Pauls *et al.* 2006; Schmitt *et al.* 2006) . Even if high altitude species persisted during cold periods *in situ* in the Alps, climate oscillations may have influenced the altitudinal distribution of populations between glacial and interglacial periods.

Here we present the phylogeography of two subspecies of an alpine leaf beetle, *Oreina speciosissima s.str.* and *O.s. troglodytes. Oreina* beetles occur in isolated populations throughout the European mountain ranges. Typically beetles are found in moist, shaded or semi-shaded areas, often in the vicinity of mountain streams at altitudes between 800 and 2800m above sea level. *Oreina s.s.str.* is widely distributed nearly across the whole range of the genus *Oreina,* whereas *O. s. troglodytes* is restricted to the Swiss and neighboring Italian Alps. In the Swiss Alps both species occur in the same general area but are separated by altitude (Kippenberg 1994). *Oreina s. s.str.* is found at lower altitudes (up to 1700m), in typical high-forb habitats, feeding on a variety of plants from the family of Asteraceae (e.g. *Adenostyles, Senecio and Petasites). Oreina s. s.str.* -like most other *Oreina* species- can be found throughout the day on or in the vicinity of its host plants. On the other hand, *O.s.troglodytes* occurs in stone runs with very scarce and patchy vegetation above 1900m. During daytime beetles are found hiding in crevices and under loose rocks close to its main host plant *Doronicum clusii.* Besides the differences in host plant use and the separation by altitude, the two described subspecies differ in the shape of male genitalia (aedeagus) and coloration. *Oreina s. s.srt* are brightly coloured in metallic green or blue whereas the colouration in *O. s. troglodytes* is generally darker and matte (Kippenberg 1994).

In the present study we analyzed the genetic structure of thirteen populations of *O. speciosissima s.str.* and *O. speciosissima troglodytes* across the Swiss Alps. Sequencing of three mtDNA and one nuclear marker as well as AFLP genotyping was used to answer following questions:

1. Do molecular data support the same grouping based on differences in morphology and altitude?
2. Is the observed divergence between the two subspecies recent?
3. Do differences in altitude alone explain the observed differences?
4. Are there ecological factors associated with the divergence of the two subspecies?

Materials and Methods

Sampling

During the 2004 and 2008 seasons, *Oreina speciosissima s.srt. and O. speciosissima troglodytes* were collected from 13 populations(Fig. 1 and Table 1). Samples were preserved in pure ethanol and stored at -20°C. For all populations, three individuals were chosen for the phylogeographic analysis, using only males to be sure of accurate identification based on genitalia.

DNA extraction, sequencing and alignment

Total genomic DNA was extracted from four legs of each individual using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Three regions of mtDNA and one nuclear region were amplified using universal insect primers: a fragment of 16S ribosomal RNA [modLR-J-12887 (5'-CACCGGTTTGAACTCAGATC-3') with LR-N-13398 (Simon *et al.* 1994)]; cytochrome oxydase subunit I (COI) [C1-J-1751 with C1-N-2191 (Simon *et al.* 1994)]; cytochrome oxydase subunit II (COII) [modTL2-J-3037 with modC2-N-3661 (Mardulyn *et al.* 1997)]; and

part of the nuclear region ITS2 [ITS3 with ITS4 (Gomez-Zurita & Vogler 2003)]. Fragments were amplified using a standard 30 µl PCR mix including: 3 µl of extracted DNA, 3 µl of 10X PCR buffer (Promega, Madison, USA), 3 µl of $MgCl_2$ solution (25 mM), 3 µl of dNTPs (1.5 mM), 0.5 µl of forward and reverse primer (Microsynth, Balgach, Switzerland), 0.3 µl of Taq DNA polymerase (Promega, Madison, USA), all made up to a final volume of 30 µl with purified MilliQ water. The PCR were run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) using the following programs: for 16S and COI, initial denaturation for 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 45°C, 1.5 min at 72°C), then final elongation of 8 min at 72°C; for COII, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 53°C, 2 min at 72°C), then final elongation of 8 min at 72°C; for ITS2, initial denaturation of 1.5 min at 93°C, 36 cycles (35 s at 93°C, 1 min at 48°C, 1 min at 72°C), then final elongation of 8 min at 72°C. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's specifications. Sequencing by Macrogen Inc. (Seoul, South Korea) was carried out with both forward and reverse primers under BigDye TM terminator cycling conditions, purifying the products using ethanol precipitation and running them using an Automatic Sequencer 3730xl (Applied Biosystems, Foster City, USA).

Sequences (forward and reverse) were manually corrected using the software CHROMAS PRO 1.34 (Technelysium, Helensvale, Australia). The protein coding nucleotide sequences of COI and COII were checked for reading frame errors and termination codons in MEGA 4 (Tamura *et al.* 2007). Alignment was carried out using CLUSTALW Multiple Alignment (Thompson *et al.* 1994) within the software BIOEDIT 7.0.5.3, followed by minor manual correction.

Phylogenetic analyses of the mtDNA data sets

Individual and combined phylogenetic analyses were performed using the maximum parsimony (MP) and Bayesian Markov chain Monte Carlo (MCMC) criteria. Each partition and the combined mtDNA data set were analyzed using parsimony ratchet (Nixon 1999) as implemented in PAUPrat (Sikes and Lewis 2001). Ten independent searches were performed with 200 iterations and 15% of the parsimony informative characters perturbed (Nixon 1999). The shortest most parsimonious trees were combined to produce a strict consensus tree. Branch supports were calculated using the Bremer support (also known as decay index; Bremer, 1988) as implemented in TreeRot (Sorenson and Franzosa 2007). The Bremer support measures the number of extra steps in tree length required before a node collapses (Bremer 1988; Baker and DeSalle 1997). Bayesian MCMC was used to approximate the posterior probability distribution of AFLP, each mitochondrial marker and the combined mtDNA data set, using the program MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). Parameters for the AFLP data set were set as follows: "datatype = restriction" and "coding = noabsencesites".

Model selection for the mtDNA data partitions in the MCMC was carried out with MRMODELTEST2 v.2.3 (Nylander 2004) based on the Akaike information criterion (Akaike 1973). Two Metropolis-coupled Markov chains with incremental heating temperature of 0.1 were run for 30 million generations and sampled every 1000th generation. The simulation was repeated six times, starting from random trees. Convergence of the MCMC was checked using the potential scale reduction factor proposed in MRBAYES 3.1.2 and the effective sample size criterion for each parameter as implemented in TRACER v.1.4 (Rambaut and Drummond 2007). To yield a single hypothesis of the phylogeny, the posterior distribution was summarized in a 50% majority rule consensus tree (the "halfcompat consensus tree" from MrBayes) after burn-in (for each analysis 10000 trees were discarded). With the combined

mtDNA data set, each locus was allowed to have partition-specific model parameters (Ronquist and Huelsenbeck 2003; Nylander 2004).

Congruence among mtDNA markers

Before combining mtDNA markers and applying a total evidence approach (Kluge 1989), we investigated topological congruence among markers. Pairwise incongruence length difference (ILD; (Farris *et al.* 1994)) tests were performed following the snowball procedure (Planet and Sarkar 2005) as implemented in the program mILD (Planet and Sarkar 2005). This procedure fits with the total evidence approach (Kluge 1989; Lecointre and Deleporte 2005).

Trees were rooted using two individuals of the closely related species, *Oreina virgulata* (Hsiao & Pasteels 1999).

AFLP

The restriction of extracted DNA was performed using a standard 30 µl mix including: 7.5 µl of extracted DNA, 3 µl of NEbuffer 2, 0.3 µl of 100 x BSA (10 mg/ml), 0.06 µl of MseI), 0.6 µl of EcoRI, all made up to a final volume of 30 µl with purified MilliQ water (all reagents by New England BioLabs, Ipswich, Massachusetts). The restriction was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) for two hours at 37°C followed by inactivation of the restriction enzymes at 65°C for 20 min.

To perform ligation of the adapters, 20 µl of the restriction mix was added to a standard 20 µl mix, including: 4 µl of 10 x T4 ligase buffer (Promega, Madison, USA), 1.44 µl of EcoRI adapter (5'-AATTGGTACGCAGTCTAC-3' and 5'-CTCGTAGACTGCGTACC-3'), 1.44 µl of MseI adapter (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3'), 0.2 µl of T4 ligase (3 U/µl, Promega, Madison, USA), all made up to a final volume of 20 µl with purified MilliQ water. The ligation was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) for two hours at 37°C.

The amplification process was started with a preamplification, in which only one selective base pair was added to the primer, followed by a selective amplification with two additional base pairs. This two-step procedure avoids the occurrence of nonspecific bands and reduces background noise (Vos *et al.* 1995). For the preamplification, 2 µl of the ligation mix was added to 28 µl of a PCR mix consisting of: 6 µl of 5 x GoTaq flexi buffer (Promega, Madison, USA), 2.4 µl of $MgCl_2$ (25mM), 0.75 µl of dNTPs (10mM), 0.75 µl of EcoRI primer + A (10 µM, 5'-GACTGCGTACCAATTCA-3'), 0.75 µl of MseI primer + A (10 µM, 5'-GATGAGTCCTGAGTAAA-3'), 0.15 µl of Taq DNA polymerase (Promega, Madison, USA) and mad up to a final volume of 28 µl with purified MilliQ water. The preamplification was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) using the following program: initial denaturation for 2 min at 94°C, 29 cycles (45 s at 94°C, 45 s at 56°C, 1 min at 72°C), then final elongation of 10 min at 72°C.

For the selective amplification, 3 µl of the 20 times diluted preamplification mix was added to 17 µl of a PCR mix including: 6 µl of 5 x GoTaq flexi buffer (Promega, Madison, USA), 2.4 µl of $MgCl_2$ (25mM), 0.75 µl of dNTPs (10mM), 1.2 µl of EcoRI primer + ACA (10 µM, 5'-GACTGCGTACCAATTCACA-3'), 1.2 µl of MseI primer + AXX (10 µM, 5'-GATGAGTCCTGAGTAAAXX-3'), 0.15 µl of Taq DNA polymerase (Promega, Madison, USA) and made up to a final volume of 17 µl with purified MilliQ water. We used the following MseI primer + AXX (AGC, ACG and AAC) each with 5-FAM fluorescently labelled EcoRI primer + ACA. The selective amplification was run in a Biometra TGradient thermocycler (Biometra, Göttingen, Germany) using the following program: initial denaturation for 2 min at 94°C, 14 cycles (30 s at 94°C, 30 s at 65°C, 1 min at 72°C) in which the 65°C annealing temperature was decreased by 0.7°C each cycle, followed by 24 cycles (30 s at 94°C, 30 s at 56°C, 1 min at 72°C), completed by a final elongation of 5 min at 72°C.

AFLP analysis

PCR products were analysed using the GeneScan technology with a capillary sequencer (ABI 3730XL, Applied Biosystems, Foster City, CA; the service was provided by Macrogen Inc. Seoul, South Korea). Resulting electropherograms were analysed with PeakScanner (ABI, peak detection parameters: default parameters with the addition of a light peak smoothing) in order to detect and calculate the size of AFLP bands. The scoring was performed using an automated scoring R CRAN package, RawGeno (Arrigo *et al.* 2009). The library was settled as follows: scoring range = 100 – 250 bp for the first two pairs of primers and 100-280 for *E*coRI-ACA/MseI-AAC, minimum intensity = 50 rfu, minimum bin width = 0, maximum bin width = 1 bp and closely sized bins (5%) were removed. Finally, the matrices of the three scored primer pairs were concatenated into one binary matrix where individuals and bands were stored as lines and columns, respectively. Ten individuals were amplified twice in order to determine the overall reproducibility rate.

Phylogenetic analyses of the AFLP data set

Phylogenetic analyses of the AFLP data were performed using the maximum parsimony (MP) and Bayesian Markov chain Monte Carlo (MCMC) criteria. Maximum parsimony analysis (including Bremer support analysis) was performed as described for the mtDNA. Parameters for the Bayesian MCMC analysis in MRBAYES were set as followed: "datatype = restriction" and "coding = noabsencesites". Four metropolis-coupled Markov chains with incremental heating temperature of 0.1 were run for 5 million generations and sampled every 1000th generation. The simulation was repeated six times, starting from random trees. Convergence of the MCMC was checked using the potential scale reduction factor proposed in MRBAYES 3.1.2 and the effective sample size criterion for each parameter as implemented in TRACER v.1.4 (Rambaut and Drummond 2007). To yield a single hypothesis of the

phylogeny, the posterior distribution was summarized in a 50% majority rule consensus tree (the “halfcompat consensus tree” from MrBayes) after burn-in (for each analysis 1500 trees were discarded).

Results

Phylogenetic reconstruction of mtDNA data sets

From 39 sequenced (AFLP and mtDNA) individuals, 37 mtDNA and 28 AFLP samples resulted in satisfactory amplification. In order to obtain the equal numbers and identities of samples, we discarded some mtDNA sample data, with one exception (NUF) where one less mtDNA sequence was obtained (Table 1).

The ITS2 region showed no variation in our sequenced individuals and was therefore excluded from further analyses. However the three mtDNA regions were polymorphic. Total alignment length of the three mtDNA fragments was 1632 bp : 529 bp for 16S, 470 for COI and 633 bp for COII. Excluding the outgroup, 30 characters were potentially parsimony informative (hereafter PPIc) among 37 variable characters. The three mtDNA regions contribute as follows: *16S* (3 PPIc among 5 polymorphic sites), *COI* (13 PPIc among 16 polymorphic sites) and *COII* (14 PPIc among 16 polymorphic sites). The best substitution models suggested by MRMODELTEST were: HKY for *16S* and HKY+G for *COI* and *COII*. Amplification failed in two cases of population TAN, one individual for COI and one for COII.

The mILD analysis showed no incongruence among the three mtDNA markers (*COI* and *COII*, *P* value = 1.00; *COI* and *16S*, *P* value = 1.00; *COII* and *16S*, *P* value = 1.00). Consequently we applied the total evidence approach and combined the three mtDNA regions into a single matrix for the following analyses.

Maximum parsimony and Bayesian inference methods produced highly congruent topologies with the same major nodes. We show only the MP topology with Bremer supports and Bayesian posterior probabilities (hereafter bpp) (Fig. 2). The ingroup is well supported with a Bremer support of 42 and a bpp of 1.00. The ingroup splits into two groups, a well supported clade (Bremer support = 10 and bpp = 1.00) containing all individuals from GRA and a polytomy (Bremer support = 5 and bpp = 1.00) containing all other individuals. Apart from a clade containing all individuals of GSB and one with two individuals from CDM and one from TSC, there is no structure within the polytomy.

AFLP

The AFLP analysis produced a total of 530 bands (171, 166 and 173 for EcoRI-ACA/MseI-AGC, EcoRI-ACA/MseI-ACG and *E*coRI-ACA/MseI-AAC, respectively) with an average of 254 bands per individual. Among 510 variable characters, 458 were PPIc. When comparing band patterns in replicates, we computed an overall reproducibility rate of 96.1%.

Phylogenetic reconstruction of AFLP data

Maximum parsimony and Bayesian inference methods produced highly congruent topologies with the same major nodes. Here we show only the MP topology with Bremer supports and Bayesian posterior probabilities (Fig. 3). Due to a lack of an outgroup, we applied a midpoint root. At the base there is a separation into two well supported clades (clade I and clade II), each with a bpp of 0.94. Within clade I we define two well supported clades with a bpp of 0.96 and 0.91 respectively (clade Ia and clade Ib). Within clade II we define three clades (clade IIa, clade IIb and clade IIc, each with some internal structure) with bpp values of 1.00, 0.79 and 0.98 respectively. Clade I contains eight individuals with strict *O. s. troglodytes* morphology (all of clade Ib with the exception of UMB) and 5 individuals with an intermediate morphology (all of clade Ia and UMB from clade Ib). Furthermore all specimens

in clade I originate from the same habitat type (stone run). Clade II contains nine specimens with a strict *O. s. s.str.* morphology (clade IIc) and six individuals with an intermediate morphology (clade IIa and IIb), whereas all individuals from clade II originate from high-forb habitats.

Discussion

Do molecular data support the same classification based on differences in morphology and altitude?

The phylogeography based on mtDNA analyses shows very little structure. The obtained patterns support neither geographical distribution nor systematic grouping of the beetles. AFLP genotyping on the other hand, which considers the whole genome, clearly shows two clades with some structure within each of them. This structure does not appear to have a geographical cause, but rather a systematical grouping congruent with the classification based on morphological characters. Both clades contain individuals with a strict morphology (clade I *O. s. troglodytes* and clade II *O. s. s.str*.) and at the base of each clade are some individuals with an intermediate morphology. The fact that we obtain very little structure based on mtDNA markers which are known to be variable (Simon *et al.* 1994) might be explained by the history of our system where divergence started recently and there was little time for mutations to occur. Additionaly, the strength of AFLP genotyping lies in the consideration of the whole genome, while sequencing analysis is much more narrow in its consideration of single gene regions.

Is the observed divergence between the two subspecies recent?

Based on our analyses we are not able to date the divergence of the two defined clades. With regard to the altitudinal distribution we can assume that *Oreina* beetles were not able to

survive cold periods at high altitudes which were covered by ice. Recent studies of alpine plants and insects have postulated that survival in refugia at the edges of the Alps occurs, and we believe this may be a realistic history of these two populations (Borer *et al.* ; Schönswetter *et al.* 2005; Pauls *et al.* 2006; Schmitt *et al.* 2006; Borer, thesis chapter III). Thus we can imagine that divergence between the two subspecies may have begun relatively recently, after the last glacial maximum 18'000 YA. Further, the absence of any geographical structure within the mitochondrial phylogeny could be a sign of parallel evolution of ecotypes and subspecies divergence within the Swiss Alps.

Do different altitudes explain the observed differences?

Focusing on individuals showing strict morphology, the altitude groups both subspecies into well separated clades. When we include all analyzed individuals, none of the morphological characters previously described or altitude can explain the topology of the AFLP phylogeny. Whereas the habitat type alone defines the two major clades (clade I and clade II). In both clades individuals with intermediate morphology share the habitat type with individuals having strict morphology which highlights the importance of habitat.

Are there ecological factors causing the divergence of the two subspecies?

With regard to the fact that we are able to define the two clades by the habitat type, we assume that several ecological factors are involved in the mechanism of reproductive isolation responsible for the morphological and behavioral differences we observed between *O. s. s.str*. and *O. s. troglodytes*.

Pre-zygotic reproductive isolation can be due to divergent host plant preferences and cause partial reproductive isolation between herbivorous insect populations that mate on the plant they feed (Tavormina 1982; Katakura *et al.* 1989; Craig *et al.* 1993; Feder *et al.* 1994; Funk 1998; Via 1999; Linn *et al.* 2003). *Oreina* beetles have been often encountered by the authors

in the process of mating, so the above mentioned mechanism for reproductive isolation seems to be very likely. This is further supported with the fact that microsatellite and allozyme data suggest that *Oreina* beetles have very limited dispersal abilities (Knoll and Rowell-Rahier 1998; Margraf et al. 2007).

A less obvious but possible mechanism of reproductive isolation between the study groups might be temporal isolation. Since *O. s. troglodytes* beetles are generally are found at higher altitudes than *O. s. s.str*, the growing season of their host plant (and subsequently of the beetles themselves) starts later and ends sooner than that of their lowland conspecifics. On a daily scale *Oreina ssp.* inhabiting stone runs seem to be nocturnal where *Oreina ssp.* living in high-forbs can be found on their host plant round the clock. *Oreina* beetles mate continuously throughout the season and have no clearly defined mating period. Different daily rhythms, generation times or differences in season length will therefore only pose temporal reproductive barriers between the two studied groups at the beginning and end of the growing season.

Both habitat and temporal isolation seem to have evolved as by-products of adaptation to different environments. High altitude plants start their growing season later than low altitude ones for the simple reason that mountain tops are still covered with snow until late spring. The change from diurnal rhythm into a nocturnal one is likely to correlate with the increase of exposure to UV-radiation within the stone run areas which are the home of *O. s. troglodytes*. However habitat and temporal isolation may have been favored by selection because of the fact that traits which enhance fitness in e.g. the low land environment decreased it in the high altitude environment or vice versa. Alternatively habitat and temporal isolation could also have been favored by selection as a result of resource competition between populations of conspecifics or closely related species or in order to decrease in likelihood of heterospecific matings (Rundle and Nosil 2005).

Rundle & Nosil, 2005 argue that 'immigrant inviability' is an important and underestimated form of pre-zygotic isolation that can occur when migrants from one environment suffer a reduced survival in the other, being poorly adapted to their non-native habitat. The reduced gene flow that stems from this phenomenon is a direct result of decreased encounter rate between heterospecifics due to the reduced migrant survival rate. Since populations of *O. s. troglodytes* and *O. s s.str.* are sometimes separated by relatively short distances, and may perhaps not be completely allopatric, this form of pre-zygotic isolation is worth noting.

Another form of pre-zygotic isolation can arise when individuals from different populations do not recognize or fail to attract each other. Proof for an ecological basis for this type of isolation is difficult to find because of the interaction between signal traits in one sex and preferences in the other (Rundle and Nosil 2005). Differences between populations in both signals and preferences generally evolve as a by-product of mate choice evolution which in turn is a product of natural selection, sexual selection and genetic drift (Coyne and Orr 2004). Differences in traits between *Oreina speciosissima troglodytes* and *Oreina speciosissima s.str.* that can be the product of ecologically based sexual selection are the reduction in body size at higher altitudes and differences in elytrum texture resulting in less 'shiny' beetles at higher altitudes. Although mate-choice experiments (Matthias Borer, thesis chapter 5) with *Oreina gloriosa* did not reveal preferences in tests with similar and differently colored males and females (and these individuals do not show differences in elytrum texture), this feature might still play a decisive role in mate recognition and choice of *Oreina speciosissima s.str.* and *O. s. troglodytes.*

Following a top-down approach we identified several phenotypic traits that are likely under divergent selection. Two of these traits (elytrum color and aedeagus morphology) are liable to be associated with reproductive isolation as well.

Conclusions

Evolutionary biology still has to deal with a lot of uncertainties that surround speciation. As is true for most studies that consider ecological speciation, we merely considered mechanisms of reproductive isolation that are likely to stem from ecologically-based divergent natural selection. The relative contribution of divergent selection compared to non-adaptive processes such as genetic drift, founder events and population bottlenecks is still largely unclear (Rundle and Nosil 2005). Our study shows quite a clear structure within the *O. speciosissima s. str.* and *O. s. troglodytes* group based on habitat types. We discussed several possible mechanisms which could drive the divergence between the two subspecies, however, our study does not provide clear answers to these questions. Further, more detailed studies within *Oreina ssp*. could possibly answer some of the questions surrounding the likeliness of divergent, locally adapted groups stemming intermediate groups.

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Table 1. Sampled populations of *O. speciosissima* with their geographical coordinates, subspecies, habitat type, number of treated individuals and year of collection.

Code	Population	Altitude (m a.s.l.)	Geographical coordinates	Subspecies	Habitat	Sample size		Year
						sequencing	AFLP	
KAN	Kandersteg	1314m	46°28'21"N, 07°39'23"E	*speciosissima*	hf	2	2	2004
TSC	Tschiertschen	1325m	46°48'55"N, 09°36'31"E	*speciosissima*	hf	1	1	2004
CDM	Col des Mosses	1716m	46°23'26"N, 07°07'30"E	*speciosissima*	hf	3	3	2005
TAN	Lac Taney	1389m	46°20'38"N, 06°50'01"E	*speciosissima*	hf	3	3	2008
GRA	Le Grammont	1974m	46°21'15"N, 06°49'04"E	*troglodytes**	sr	3	3	2008
NUF	Nufenenpass	2172m	46°28'41"N, 08°22'36"E	*troglodytes**	hf	2	3	2008
GSB	Grosser St. Bernhard	2410m	45°52'04"N, 07°10'27"E	*troglodytes**	hf	3	3	2008
BET	Bettmerhorn	2628m	46°24'44"N, 08°04'33"E	*troglodytes*	sr	1	1	2008
UMB	Umbrailpass	2647m	46°32'53"N, 10°25'43"E	*troglodytes**	sr	2	2	2008
BER	Berninapass	2315m	46°24'37"N, 10°01'36"E	*troglodytes*	sr	1	1	2008
ALB	Albulapass	2324m	46°34'46"N, 09°50'15"E	*troglodytes*	sr	2	2	2008
MUM	Muottas Muragl	2735m	46°30'27"N, 09°56'29"E	*troglodytes*	sr	2	2	2008
JUL	Julierpass	2373M	46°28'02"N, 09°43'35"E	*troglodytes*	sr	2	2	2008

*, morphology variable; hf, high-forb; sr, stone run.

Figure legends

Figure 1. Distribution of the 13 sampled populations of *O. speciosissima s. str*. and *O. speciosissima troglodytes***.**

Figure 2. Strict consensus tree of the MP analysis of the AFLP data. Subspecies are indicated by label colour: blue = *O. speciosissima s.str.,* red = *O. speciosissima troglodytes* and gray = intermediate forms. Node supports are given by Bremer supports (decay index) ≥ 1 and Bayesian posterior probabilities (italic).

Figure 3. Half-compatible topology of the MRBAYES analysis of the AFLP data. Subspecies are indicated by label colour: blue = *O. speciosissima s.str.,* red = *O. speciosissima troglodytes* and gray = intermediate forms. Node supports are given by Bremer supports ≥ 1 and Bayesian posterior probabilities (italic). Habitat types are indicated by the coloured bars: green = high-forb and grey = stone run. Defined clades are discussed in the text.

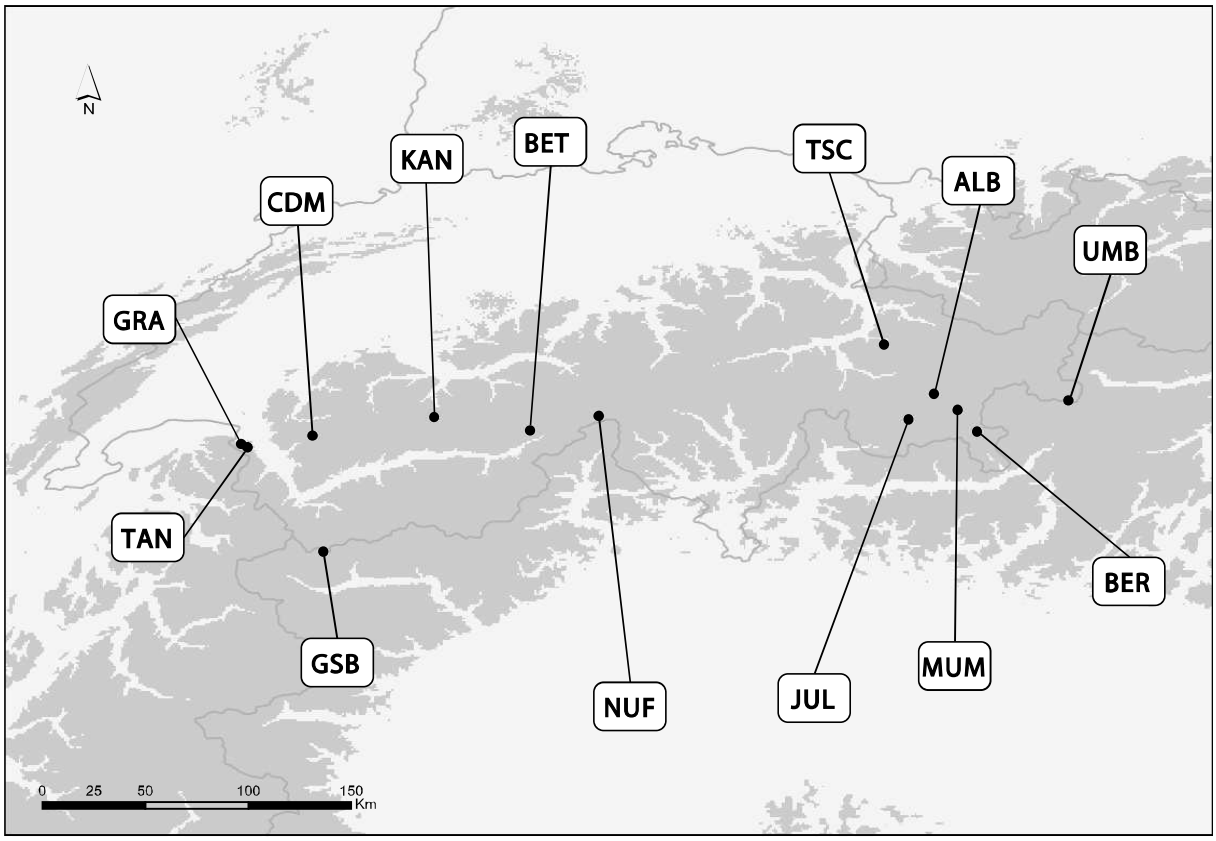
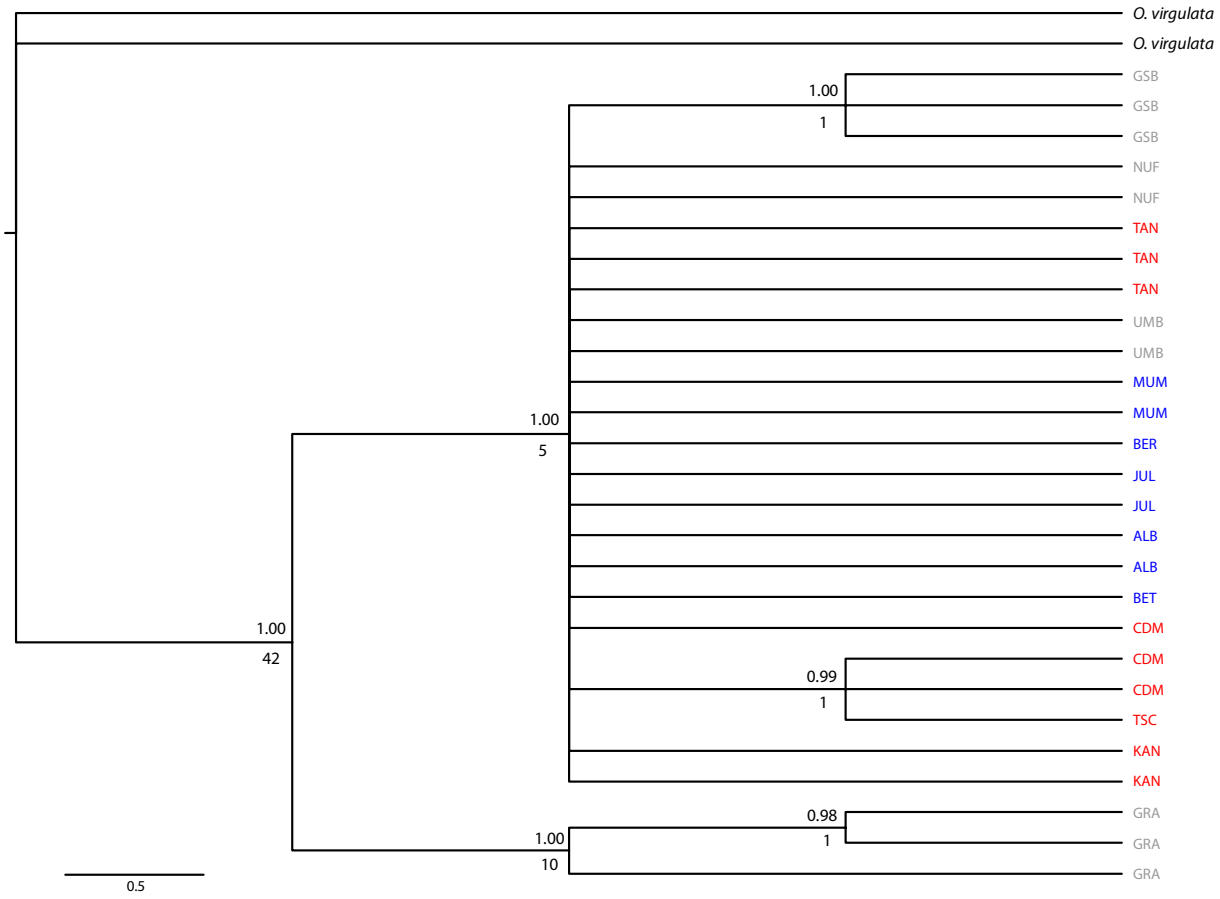

N
KAN
BET
TSC
CDM
ALB
UMB
GRA
TAN
BER
GSB
MUM
NUF
JUL
0 25 50 100 150
Km

Figure 1


O. virgulata
O. virgulata
GSB
GSB
GSB
NUF
NUF
TAN
TAN
TAN
UMB
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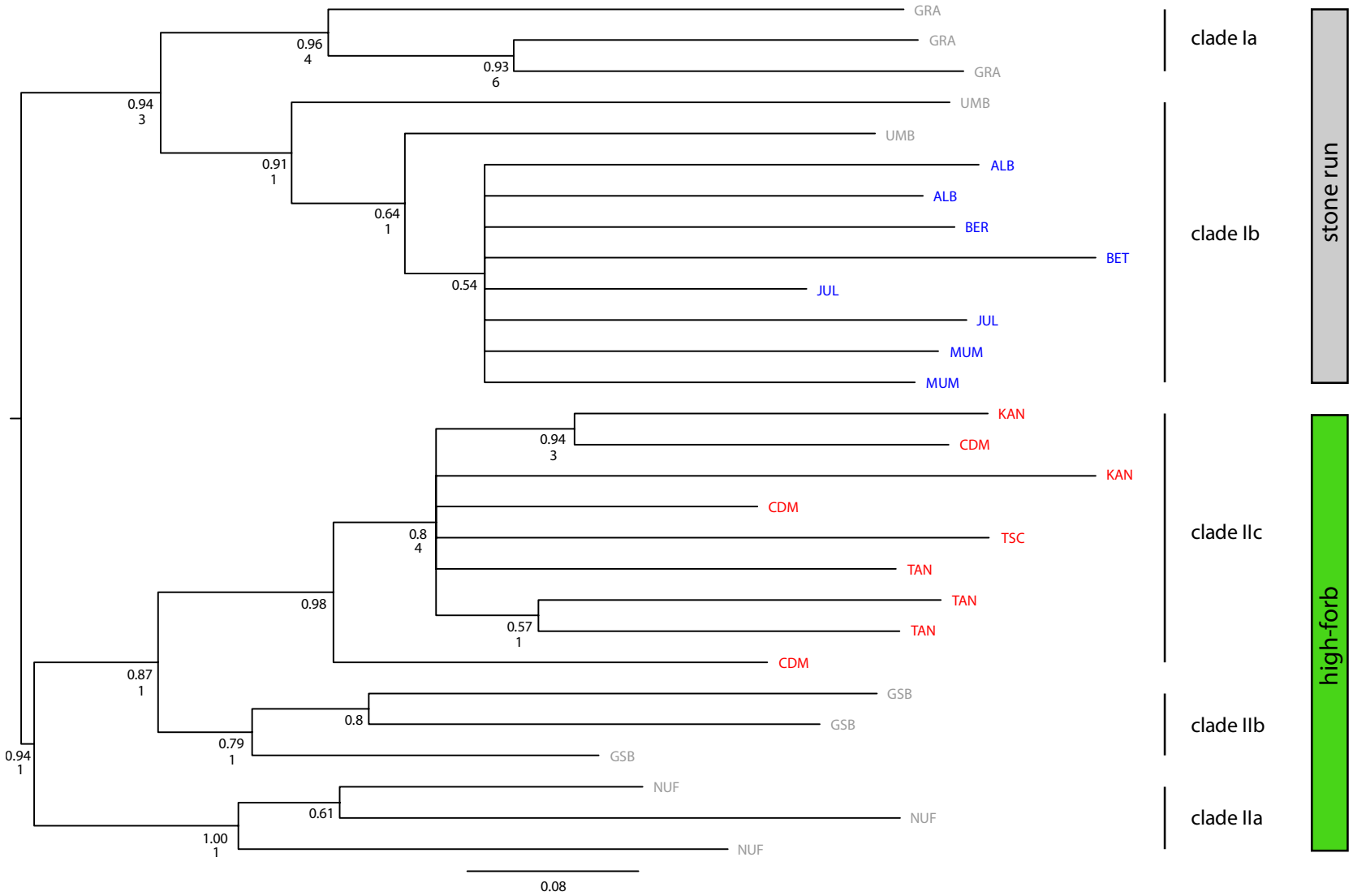
BER
JUL
JUL
ALB
ALB
BET
CDM
CDM
CDM
TSC
KAN
KAN
GRA
GRA
GRA
1.00
1
1.00
5
1.00
42
0.99
1
0.98
1
1.00
10
0.5

Figure 2


GRA
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KAN
CDM
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CDM
TSC
TAN
TAN
TAN
CDM
GSB
GSB
GSB
NUF
NUF
NUF
0.96
4
0.93
6
0.94
3
0.91
1
0.64
1
0.54
0.94
3
0.8
4
0.98
0.57
1
0.87
1
0.8
0.79
1
0.94
1
0.61
1.00
1
0.08
clade Ia
clade Ib
clade IIc
clade IIb
clade IIa
stone run
high-forb

Figure 3

Chapter V

The influence of colour on mate choice in a colour-polymorphic alpine leaf beetle

Matthias Borer, Alessandro Stähli and Russell E. Naisbit

Abstract

The principal element in sexually reproducing organisms is mate choice where the mating preferences strongly select for the best individuals. Attention has mainly been directed towards the handicap principal, where the costs maintain the honesty of signals, and thus create directional sexual selection. However, in populations containing polymorphic individuals over a long time, such directional sexual is unlikely, because this would tend to drive a polymorphic population towards a monomorphism over time.

Here we test if colour is involved in mate choice within a polymorphic alpine leaf beetle. *Oreina gloriosa* occurs in green and blue morphs, with monomorphic as well as polymorphic populations known. We test for male and female mate choice in individuals from mixed and pure populations. There was no significant assortative mating in any of the populations.

Introduction

Mate choice is the principal element of sexual selection in many species and significantly affects an individual's fitness. Evolutionary biologists have directed much attention towards the handicap principle (Zahavi 1975) where the cost maintains the honesty of signals such as the peacock's enormous and colourful train as a display of the state of health, toxicity (Pasteels *et al.* 1990; Bowers 1992; Andersson & Iwasa 1996), or "good genes" (Andersson 1994). In this case sexual selection is directional because only males in the best physical condition are able to pay these costs (Cotton *et al.* 2004) and hence are more likely to mate with females than less conspicuous rivals. In populations with different colour morphs over a long time, as our study organisms seem to have, we do not expect such directional selection. The colour polymorphism within a population survive if there is completely random mating, since this will not change allele frequencies. Mate choice for the same colour (assortative mating) on the other hand, would tend to lead a population to become monomorphic over time.

Beetles of the genus *Oreina* (Coleoptera: Chrysomelidae) are found in isolated populations throughout the European Mountains (Daccordi & Ruffo 1976; Kippenberg 1994; Margraf *et al.* 2007). Most of them are brightly coloured in metallic blue, green and red.

Within the genus two different chemical defence strategies exist, which are correlated with host plant use. Species feeding on Apiaceae or Cardueae produce cardenolides *de novo* (Van Oycke *et al.* 1987; Eggenberger & Rowell-Rahier 1993b; 1993a), whereas species feeding on Senecioneae encounter pyrrolizidine alkaloid N-oxides (PAs) in several of their host plants. All species feeding on such plants are able to take up the alkaloids from their hosts and incorporate them into their defensive secretions (Pasteels & Rowell-Rahier 1991; Pasteels *et al.* 1995; Dobler *et al.* 1996).

Cardenolides are produced in small volumes but at high concentrations (Rowell-Rahier *et al.* 1995) and stored exclusively in the pronotal and elytral glands of adults (Pasteels & Rowell-Rahier 1991). *Oreina* larvae store the defence compounds in the whole body (Pasteels *et al.* 1988; Ehmke *et al.* 1991; Rowell-Rahier *et al.* 1991).

Oreina gloriosa (FABRICIUS, 1781), a species with an alpine distribution only, occurs in blue and green morphs with a blue longitudinal stripe on each elytra and feeds only on the umbelliferous plant *Peucedanum ostruthium* (Apiaceae). Their host lack PAs, so *O. gloriosa* have only cardenolides for their chemical defense. Populations of *O. gloriosa* can contain mostly green morphs or blue morphs however there exists mixed populations comprising both colour morphs. In this species, body size and toxicity are two parameters that influence sexual selection. Large males with high concentration or high amount of cardenolides have higher fitness compared with smaller males. On the other hand, large females with a high toxin concentration mated sooner and heavier females mated more often (Labeyrie *et al.* 2003). With the present knowledge about sexual selection in *O. gloriosa* there seems to be reciprocal male and female choice for well defended and large individuals. Labeyrie *et al.* (2003) suggest that sexual selection in *O. gloriosa* may contribute to the maintenance of defensive traits in this leaf beetle. However, toxin secretion has only been observed when the beetles were disturbed or attacked by predators but never during copulation. Due to the fact that the defence glands are under neural regulation (Schooneveld *et al.* 1991) it might be possible that small amounts of toxins are released in a sexual context and act as pheromones (Trigo & Brown 1990; Attygalle *et al.* 1991; Dussourd *et al.* 1991; Eggenberger & Rowell-Rahier 1993b; Amano *et al.* 1999). Untill now there is no evidence for this hypothesis, but there may also be other parameters, for example colour, influencing mate choice in this leaf beetle.

The expression of colour in *O. gloriosa* is probably genetically determined and thus the choice of a mate partner influences the inheritance of colour and moreover the colour pattern

of a population. It is also likely that *Oreina* beetles are able to see colours, especially green blue and UV, as has been demonstrated for *Leptinotarsa decemlineata* (Coleoptera; Chrysomelidae) (Doring & Skorupski 2007), so they should be able to distinguish the two morphs.

But how can we explain the occurrence of monomorphic populations and mixed populations? Assortative mating is one potential explanation. To test if colour plays a role in mate choice in *Oreina gloriosa*, we carried out experiments to answer three main questions:

- Is colour involved in mate choice in *Oreina gloriosa*?
- Do beetles from monomorphic populations prefer their own colour?
- If there is choice, which sex chooses?

If one or more of these questions can be answered with yes, mate choice may influence the level of polymorphism of populations.

Material and Methods

During the seasons 2005, 2006 and 2007, beetles were collected from three populations in the Swiss Alps. Green morphs were taken at Saas Grund (SAG; altitude: 1639m; N 46°06’15”/ E 07°56’57”), a strongly green-dominated population. The blue morphs we collected from La Fouly (LAF; altitude: 1500m; N45°56’10”/ E 07°05’36”) which is a blue dominated population. Col-des-Mosses (CDM; altitude: 1716m; N 46°23’26”/ E 07°07’30”), one of the rare mixed populations we know, was chosen to collect beetles from both morphs. In our system we investigate a monophagous beetle from three very similar habitats with little altitudinal variation and small geographical distances between populations (max. 70 km) within the same mountain range. That way we reduce a few potential ecological and environmental parameters (e.g. host plant effect, altitude effect etc.) that could have an important influence on our beetles and thus on the experiments.

In the field collected beetles were separated by site and colour. Thereafter in the laboratory the sex determination took place. The beetles of each group were kept in individual plastic boxes (19 x 9 x 8.5 cm) with moist household paper on the base and fresh leaves of their host plant *Peucedanum ostruthium.* All boxes were kept in an incubator (UMS Cooled Incubator) with a diurnal temperature of 17°C and 13°C during the night (regulated with a WEST 4400 Setpoint Programmer). Temperature changes occured gradually over 2 hours and the light cycle spanned 12 hours (regulated with a MaxiRex CD1). The eight boxes containing our experimental groups were checked twice a day during the first three days to detect potential mistakes in sex determination.

The fourth day after beetle collection we started with the mate choice experiment. Round plastic boxes with a diameter of 10cm and a height of 5cm were used as mating arenas. They were equipped with moist household paper on the base and a small piece of *P. ostruthium* was put in the centre of the arena.

The following experiments were carried out

Experiments were carried out in quartets containing a blue male & female and a green male & female, placed in a mating arena. We used three different quartet combinations. In combination A, a blue male and female from the mixed population (CDM) were placed together with a green individual of both sexes from the mixed population (CDM). Combination B included a blue and green male from the mixed population (CDM) together with one green female (SAG) and one blue female (LAF). For the third combination, C, a female of each colour from the mixed population (CDM) were placed with a male from the green population (SAG) and a male from the blue population (LAF) (Fig.1a). As we could only collect a limited number of beetles per site, in particular green females from the mixed population (CDM), we reused the individuals in new quartet combinations (Fig.1.b). Hence

all beetles were tested twice, but always in two different types of quartet. To avoid a size advantage (Labeyrie *et al.* 2003) we were careful to choose beetles of a very similar size.

The quartets were left until the first couple mated. After four and a half hours the experiment was stopped even if there was no mating. We considered a mating as successful only if the aedeagus was connected to the female genitalia.

If there is choice based on colour we expect to see assortative mating in at least some combinations. If there is a preference in monomorphic populations, we would expect assortative mating in B/E if this is due to female choice and C/F, if due to male choice. The data were analysed using G-tests. There were insufficient replicates to carry out a fulltest of association between male and female colour, so we test counts of assortative versus disassortative matings for a departure from equality.

Results

G -test: Goodness of fit

There were no significant departures from random mating in any of the three combinations [combination A/D, Table 1.a); df = 1, G = 3.18, q = 1.02, G_{adj} = 3.13; **p = 0.0769**], [combination B/E, Table 1.b); df = 1, G = 0, G_{adj} = 0; **p = 1**], [combination C/F, Table 1.c); df = 1, G = 2.04, q = 1.03, G_{adj} = 1.98; **p = 0.159**].

Furthermore, we analysed the combined data for all three groups together (total assortative matings and total disassortative matings) and again there was no departure from random mating [Table 1.d); df = 1, G_{pooled} = 2.86; **p = 0.091**].

The summary of assortative and disassortative matings (Table 2) highlights the fact that in the combination A/D and C/F the ratio of assortative and disassortative matings is two to one, or very close to it. To test if there is a difference between the three different combinations (A/D,

B/E and C/F), we carried out a G-test of heterogeneity. This showed significant heterogeneity (df = 2, G_h = 25.27; **p < 0.001)**.

Discussion

There was no significant assortative mating in any of the three experiments suggesting that *Oreina gloriosa* shows random mating with respect to colour. Hence we are tempted to say that in *O. gloriosa*, colour is not involved in mate choice.

We expected disassortative mating in combination A/D because random mating might enable the survival of polymorphism in this population. Table 2 highlights the fact that assortative matings are nearly twice as frequent as disassortative ones. Despite the non-significant result it shows a trend towards a preference for mating partners of the same colour. Such preferences would allow an unstable equilibrium at the point where morph frequencies are equal, if the fitness of the two morphs are equal. As soon as one colour morph becomes more common, sexual selection would act against the rare morph and could turn a mixed population into a monomorphic one.

A preference for the other colour morph (disassortative mating) could potentially maintain polymorphism by creating negative frequency dependent selection. However, there is no hint of this in our data.

To test whether there is male or female choice we designed the combinations B/E and C/F. Assortative mating in B/E would indicate female choice by colour in monomorphic populations and assortative mating in C/F male choice. It is clear that there is no assortative mating in combination B/E. On the other hand, combination C/F shows a tendency for assortative matings with a ratio of two to one compared with disassortative matings. Conspicuously there was no mating between green males and green females within the third combination.

The significant difference between the three mating pair combinations showed by the G-test of heterogeneity might indicate the presence of choice and variation between populations that is not detectable with our current data set. Future work with more replicates and repeated populations of each type could therefore reveal a role for colour in the mate choice of these leaf beetles.

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Table 1.a-d. The observed numbers of matings in each type of experiment. a) both males and females are from the mixed population; b) the two males are from the mixed population whereas the females are from blue-dominated and green-dominated populations; c) both females are from the mixed population and the two males are from a blue and green-dominated population. The total numbers of all observed matings within the three combinations are shown in d).

a)

A/D		**Female**		
		blue	green	Total
Male	blue	13	2	15
	green	9	8	17
	Total	22	10	**32**

b)

B/E		**Female**		
		blue	green	Total
Male	blue	16	12	28
	green	8	4	12
	Total	24	16	**40**

c)

C/F		**Female**		
		blue	green	Total
Male	blue	12	2	14
	green	4	0	4
	Total	16	2	**18**

d)

Total		**Female**		
		blue	green	Total
Male	blue	41	16	57
	green	21	12	33
	Total	62	28	**90**

Table 2. Summary of the number of assortative matings and disassortative matings observed in the three combinations A/D, B/E and C/F.

	Assortative mating	**Disassortative mating**	Total
A/D	21	11	**32**
B/E	20	20	**40**
C/F	12	6	**18**
Total	**53**	**37**	90

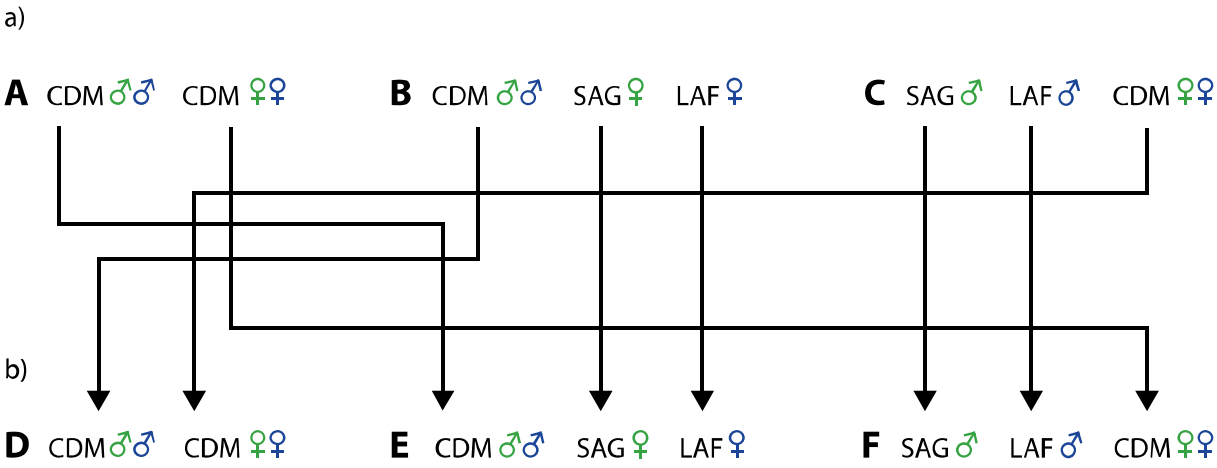

Figure 1. Three different quartets of four *O. gloriosa* in the mate choice experiment. The origin, colour and sex are indicated. a) shows the initial quartets whereas b) illustrates the pairing for the new quartets. The way individuals were swapped for the new quartets, is shown by arrows.

Chapter VI

Positive frequency-dependent selection on warning colour in alpine beetles

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Abstract

Müller's theory of warning colour and mimicry, despite forming a textbook example of frequency-dependent selection, has rarely been demonstrated in the wild. This may in part be due to the difficulty of measuring selection on mobile prey species. Here we demonstrate that this selection acts in alpine leaf beetle communities, by using tethered beetles exposed to natural predators. Individuals that match the locally predominant colour morph have log-odds of week-long survival increased by a factor of 1.67 over those that do not match. Such selection, in concert with variation in community composition, could generate geographic variation in warning colour. However, in the face of this purifying selection, the within-population polymorphism seen in several *Oreina* species remains paradoxical.

Introduction

Müller's theory of warning colour and mimicry is based on the argument that in two unpalatable species that share a habitat, if they are dissimilar predators must eat a certain number of each in order to learn that they are distasteful, whereas if they are identical, members of both species benefit by sharing the cost of education (Müller 1879; Mallet & Joron 1999; Ruxton *et al.* 2004). This generates selection for resemblance between unpalatable species within a habitat (Müllerian mimicry) as well as positive frequency-dependent selection (purifying selection) within a species, because in both cases common forms benefit from protection and rare or novel variants suffer increased predation. The principle is widely used as a textbook example of frequency-dependent selection and has seen renewed interest in the literature, with suggestions that mimetic shifts may influence both speciation and community structure (Jiggins *et al.* 2001; Elias *et al.* 2008). Despite this, the survival value has rarely been demonstrated with natural prey and predators in the wild, apart from examples in neotropical *Heliconus* butterflies (Benson 1972; Mallet & Barton 1989; Kapan 2001). This is probably in large part due to the difficulty of measuring selection in mobile species, which complicates the practical and statistical techniques needed to estimate survival from recapture probabilities.

Here we demonstrate positive frequency-dependent selection in the alpine leaf beetle, *Oreina gloriosa*, using tethered individuals in natural populations. Beetles of the genus *Oreina* are found in isolated populations throughout the mountains of Europe (Kippenberg 1994; Margraf *et al.* 2007). They possess two chemical defence strategies correlated with their host plant use. Species feeding on Apiaceae or Cynareae (Asteraceae) synthesise cardenolides, whereas those feeding on Senecioneae (Asteraceae) are able to sequester host-derived pyrrolizidine alkaloids (Dobler *et al.* 1996). This chemical defence is accompanied by what appears to be warning colouration, in bright metallic blues and greens, often in combination with blue or red stripes.

Likely predators include many that hunt visually, in particular birds such as the snow finch *Montifringilla nivalis*, as well as predators that are less reliant on vision, like shrews and insects.

These beetles are relatively sedentary and feed during the day exposed on the upper leaves of their host plants, so provide an ideal system in which to test frequency dependent selection by predators in the wild. The use of leashes allows natural movement by the beetles but certain detection of predation, thereby avoiding the need to simultaneously model the probabilities of resighting, dispersal, and predation events. Our focus is on *Oreina gloriosa*, a species that is chemically defended by cardenolides and is monophagous on *Peucedanum ostruthium*. It occurs throughout the Alps and has two colour morphs: green and blue, each with blue stripes. We use this variation to compare the survival of blue and green individuals from mixed populations when exposed to predation in sites dominated by green or blue morphs.

Materials and Methods

Experiments were carried out in communities close to the tree line (altitudes of 1592-2182m above sea level) along the side valleys of the Rhone valley in southwestern Switzerland. In these sites the beetles are found in forest clearings and open habitats in patches of a high forb plant community that is often dominated by *Oreina* host plants, including *Peucedanum ostruthium*, *Adenostyles alliariae*, *Chaerophylum villarsii* and *Heracleum sphondylium*.

Tethered experimental beetles were placed in natural *Oreina* communities during the alpine summers between July 2005 and September 2008. In each replicate, 10 green and 10 blue *Oreina gloriosa* individuals (always all collected from the same mixed population, either La Fouly or Col des Mosses) were attached to randomly chosen *Peucedanum* plants throughout a host-plant patch. The plants were marked with plastic tags and the beetles attached using leashes made of 0.4m lengths of fine transparent plastic thread (Perlon, 10mm diameter), tied

at one end between the prothorax and elytra and at the other end to an upper node of the host plant. At the same time, the beetles naturally present were censused to determine the predominant colour at the site, taking the entire *Oreina* community into account (from one to six common species, including *O. gloriosa*, *O. speciosa*, and *O. cacaliae*). The sites were then visited one week later to record the survival of each tethered beetle.

The data were analysed using logistic regression in R (Team 2009), in a model for survival probability with terms for site (to take into account the paired nature of the design) and beetle colour (coded as "local" or "foreign"). To test for an overall survival advantage of one morph over the other, the analysis was repeated with beetle colour coded as "green" or "blue", and to exclude the possibility of consistent differences between the two types of site, the analysis was repeated a third time with the sites simply coded as "green-dominated" or "blue-dominated". The data were well approximated by a binomial distribution (with dispersion factor of 1.19, compared to a value of 1 for an ideal binomial distribution) so there was no need to use quasibinomial estimation.

Results

The experiment was carried out at twenty sites, half blue-dominated and half green-dominated. There was significant variation between sites in their overall level of predation (Table 1), with survival values of between 43% and 95%. Beetle colour also had a significant effect on survival probability. Matching the locally predominant colour increased the log-odds of week-long survival by a factor of 1.67 (with 95% confidence interval of 1.01 to 2.77). This advantage was similar for blue beetles in blue-dominated sites (1.91) and green beetles in green-dominated sites (1.42). There were no consistent differences between the two types of site in their overall levels of predation (deviance = 0.613, df = 1, p = 0.434), nor were there

overall differences in survival between green and blue beetles (deviance = 0.540, df = 1, p = 0.462).

Discussion

There was a strong effect of colouration on survival, with a significant benefit to matching the locally predominant colouration. Visually hunting predators therefore appear to be an important factor in survival, and they generalise across beetle species in their avoidance learning of colouration. The effect of colour is likely to include contributions from both positive frequency dependent selection on colour within *O. gloriosa,* and from Müllerian mimicry across the entire *Oreina* community.

There was considerable variation between sites in the overall levels of predation, but this is not unexpected and would be influenced by many factors, including the weather during the replicate and the local predator community composition. There was also variation in the relative predation on local and foreign colours. This might be a result of differences in the relative frequency of blue and green morphs leading to variation in the strength of frequency-dependent selection, as well as differences in the contribution of visual and non-visual predators.

This mode of predation would have opposite effects on the expected levels of geographic and within-population polymorphism. It forms a mechanism by which geographic variation in community composition could bias the process of convergence and generate within-species geographic variation in colouration. *Oreina* communities are particularly susceptible to vary geographically due to variation in the host plants available and as a result of stochastic effects of their limited dispersal ability, the isolated nature of their habitats and the fact that the entire region was subjected to repeated extinction and recolonisation during Quaternary glacial cycles (Margraf *et al.* 2007;Borer, thesis chapter I-III). This local adaptation in response to

the community could therefore be responsible for the observation that several species show great variation in colouration across their distribution (Knoll & Rowell-Rahier 1998; Margraf *et al.* 2007). In contrast, such predation should eliminate within-population polymorphism because it generates strong purifying selection. The remarkable variation seen in many *Oreina* species is therefore paradoxical (Joron & Mallet 1998; Mallet & Joron 1999). Future work will be devoted to examining what other factors, such as sexual selection or dispersal, may contribute to this diversity.

Acknowledgements

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Table 1. Logistic regression analysis of the week-long survival of *Oreina gloriosa* individuals depending on their colour (coded as "local" or "foreign" depending on whether or not they matched the locally predominant colour).

Source	DF	Deviance	Residual DF	Residual Deviance	P (Chi)
null			39	72.807	
site	19	43.498	20	29.309	0.001
beetle colour	1	4.057	19	25.252	0.044

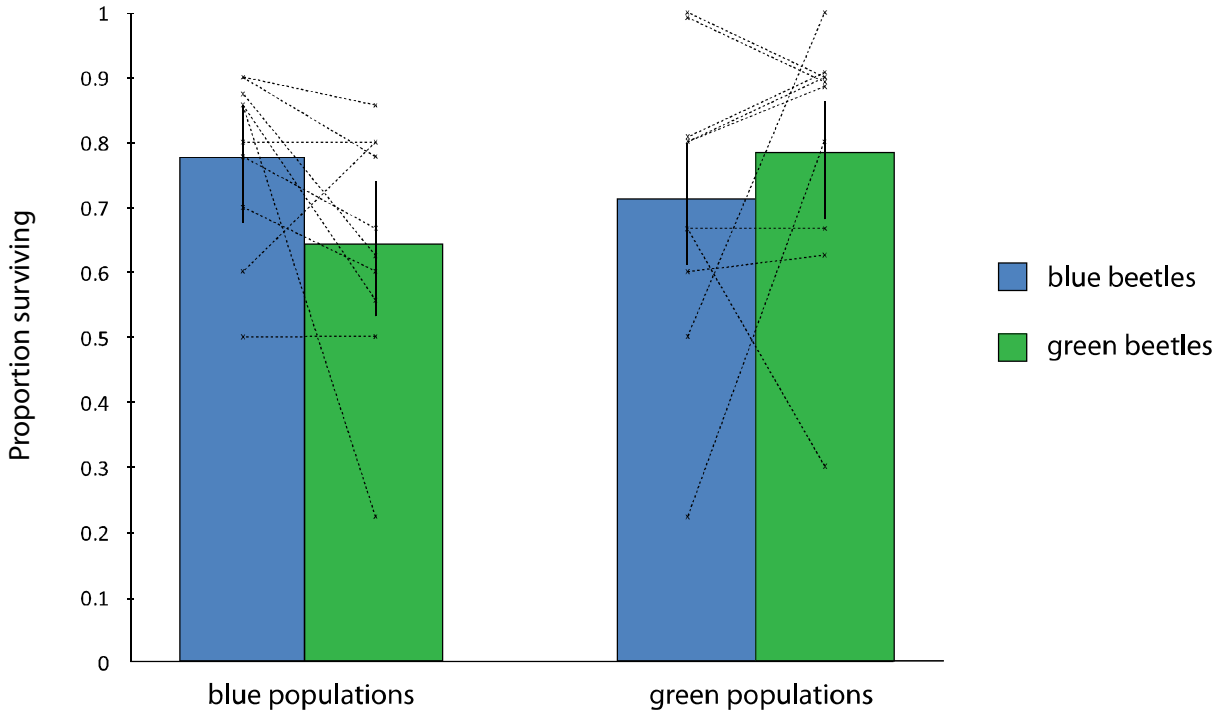

Figure 1. Week-long survival estimates for blue and green beetles at blue- and green-dominated sites. Error bars show exact binomial (Clopper-Pearson) 95% confidence intervals for survival probability.

Conclusion

In contrast to lowland species, *Oreina* beetles are likely to have had wider distributions during glacial periods, and their current distribution in isolated habitats in the European mountains represents interglacial survival in refugia. The phylogeographic patterns of the four studied species are not perfectly congruent. Nevertheless, within the Alps, where all species occur, we can define some lines of separation that are present in more than one species. In the western Alps the separation between *O. elongata ruffoi* and *O. e. occidentalis* coincide with that between clade two and three in *O. gloriosa*. In the south of the central Alps *O. elongata elongata* shares a very similar distribution to the lineage of *O. speciosissima s. str*. of clade III in the Dolomites. The differences in phylogeographic patterns, in particular the fact that *O. cacaliae* and *O. speciosissima* have widely distributed lineages across much of the Alps, might largely be due to different dispersal abilities. The addition of work on further host plant species would open the way to a community phylogeography of the European mountain system.

Appendix I

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Cladistics
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The European Alps as a crossroads of genetic lineages in a sessile mountain leaf-beetle species complex

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1. Title Page

Title: The European Alps as a crossroads of genetic lineages in a sessile mountain leaf-beetle species complex

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Short running title: Phylogeography of a European mountain leaf-beetle species complex.

2. Abstract

Although many alpine insects have similar distributions, they might demonstrate different dispersal abilities reflected in different phylogeographical histories. In the last decade, large scale phylogeographies have mostly focused on species presenting substantial dispersal abilities and only few studies have emphasized on non-motile organisms. In order to fill this gap, the present study aims at describing phylogeographic patterns of three sister species of sessile alpine leaf beetles: *Oreina speciosa*, *O. ganglbaueri* and *O. alpestris* (Coleoptera, Chrysomelidae). Based on more than 700 sequences from three mitochondrial and one nuclear genes, we depict the phylogenetic relationships between individuals from 63 sites sampled across the main European mountain massifs. Our results demonstrate the paraphyletic status of *O. speciosa* and *O. alpestris*, but address the existence of several well-segregated lineages with relatively narrow distributions, such as the Pyrenean *O. ganglbaueri*. As one could expect in the case of sessile species, most of these narrow-endemic lineages are likely to have diverged for a long time, sufficient to allow the development of effective reproductive barriers. However, our results also highlight the existence of admixed populations, notably in the Alpine massif, which was covered by ice until recently. This result addresses the ability of sessile species to re-colonize previously glaciated areas in a relatively short span of time and demonstrates the importance off the Alps as a crossroads of genetic lineages in alpine insects.

3. Table of contents

Introduction

Long before the application of molecular techniques based on DNA, the current distribution areas of many European species were already supposed to be the result of Pleistocene climatic oscillations that begun about 2Mya (Coope 1994, Hewitt 2000, Schmitt 2007). During glacial periods, species went extinct over large parts of their range, dispersed to new locations or survived in refugia. When the climate warmed again, they recolonized the previously ice-covered areas. In the last decade, sequence-based phylogeographies revealed that the biogeographic histories of temperate and arctic-alpine species were likely to be different (e.g., Hewitt 2001; Schmitt et al., 2005). Temperate species survived during cold periods in southern refugia in Iberia, Italy, the Balkans and Caucasus region, repopulating northern Europe at the end of the Last Glacial Maximum and creating hybrid zones in the areas where the expanding genomes met (Hewitt 2000). In contrast, alpine species or those with an arctic-alpine disjunctive distribution must have been widespread during glacial maxima and probably covered cold steppes in most parts of Europe, whereas they were restricted to remote environments during interglacial stages like that we are currently experiencing (Hewitt 2004). As a consequence, some species with formerly large distributions continue to persist only as relict species in relatively small areas.

Large scale phylogeographies of the European fauna have been investigated in several groups, such as vertebrates (e.g. Taberlet *et al.*, 1998; Böhme et al, 2007; Salomone et al, 2007; Sotiropoulos et al, 2007) and insects (e.g., crickets [Hewitt 2001], butterflies [Schmitt et al, 2006; Haudrich and Schmitt, 2007; Habel et al, 2008; Espeland et al, 2007], hoverflies [Milankov et al, 2008], mayflies [Williams et al, 2006], and beetles [Gomez-Zurita and Vogler, 2003; Cardoso and Vogler, 2005; Cardoso et al, 2009]). Unexpectedly, high levels of differentiation among populations and regions were found in several of these studies, and their authors had to deal with intricate scenarios of extinction and recolonization through the Quaternary to explain the current

spatial genetic structure of populations. As a consequence, many species were considered as species complexes encompassing high levels of genetic, ecological and morphological variation. Interestingly, nearly all published phylogeographic studies among European taxa involved organisms with substantial migratory abilities, foreseeing rather clearly their recent postglacial re-colonization patterns. However, one might ask whether or not patterns of recolonization should be so clear in sessile species, which by definition are highly limited in their dispersal abilities. A rational expectation in such organisms is a marked pattern of isolation by distance, with disjunctive areas of the species' distribution being highly isolated genetically from each other.

Here, we aim to tackle this problem by presenting the phylogeography of three sister species of sessile Alpine leaf beetles within the genus *Oreina* Chevrolat, 1837 (Coleoptera, Chrysomelidae). This genus includes 28 species (Kippenberg 1994, 2008 and pers. comm.) found throughout the mountain regions of Europe, with some incursions into the lowlands, and western Siberia. Most are oligophagous on either Apiaceae or Asteraceae (Jolivet et al., 1986). *Oreina* species are generally considered to be non-flying despite possessing completely developed red-coloured wings, and the recorded cases of flight in a few species are usually rare (Kalberer et al. 2004). As a consequence, studies based on allozyme and microsatellite data suggest that populations are highly isolated (Knoll and Rowell-Rahier 1998, Margraf *et al*. 2007). Here, we treat the three sister taxa *Oreina speciosa*, *O. ganglbaueri* and *O. alpestris*. These species share many characteristics, such as their general external morphology, highly variable coloration, life cycle, habitat, host plants (all are oligophagous on several species of Apiaceae), and autogenous cardenolide production (Dobler *et al.* 1996, Triponez *et al.* 2007) and they have been differentiated mainly on the basis of detailed features of male genitalia. Even though *O. alpestris* has a much wider distribution over the European mountains (from the Pyrenees to the

Carpathians) than *O. speciosa* (Alps, Jura, Massif Central and northern Balkans) and *O. ganglbaueri* (restricted to the Pyrenean-Cantabric range), the taxa are often found in sympatry in the Alps and Pyrenees. In this study, our objectives are as follows:

1) to determine whether morphological characteristics defining these taxa coincide with major gene pools and if this can be reconciled with the classical view of species definition.
2) to examine the main phylogeographic patterns of these taxa at a Europe-wide scale to determine to what extent their limited dispersal abilities have affected their spatial genetic structure.

Finally, we will place our findings in the wider framework of the spatial genetic structure and dispersal patterns of sessile organisms as a result of their response to Pleistocene climate oscillations.

Material and Methods

Oreina sampling

Sites within the European Alpine System were visited during the summers of 2005, 2006 and 2007. In total, the three sister species were collected in 63 sites (see Table 1), comprising mostly male specimens, with a total of 211 collected specimens. All sampled insects were determined, mostly on the basis of the males' genitalia shape and with the help of some external morphological features for females, following Kippenberg (1994). Outgroups were sampled among four closely-related species according to Hsiao and Pasteels (1999), namely *O. bifrons*, *O. cacaliae*, *O. gloriosa* and *O. liturata*.

DNA extraction, PCR amplification and Cycle sequencing

Total genomic DNA was extracted from an average of three individuals per population, using the DNeasy® Tissue Kit (Qiagen, Hilden, Germany). Extraction was performed using 4–6

legs of each individual. One nuclear region and three mtDNA regions were amplified using the following primers: partial internal transcribed spacer (*ITS2*) region (ITS3 and ITS4 from Gomez-Zurita and Vogler, 2003), *16s* ribosomal RNA (LR-N-13398 and LR-J-12883 from Simon et al. 1994), partial cytochrome oxidase I (*COI*) (C1-J-1751 and C1-N-2191 from Simon et al. 1994) and partial cytochrome oxidase II (*COII*) (modTL2-J-3037 and modC2-N-3661 from Mardulyn et al. 1997). Amplification was carried out in a standard 30 µl PCR reaction including: 3 µl of 10X PCR buffer (Promega, Madison, WI, USA), 3 µl of a $MgCl_2$ solution (25 mM), 3 µl of dNTPs (1.5 mM), 0.5 µl of forward and reverse primers (10 mM), 0.3 µl of Taq DNA polymerase (Promega, Madison, WI, USA), 3 µl of extracted DNA, all made up to 30 µl with purified MilliQ water. The PCR reactions were run in a TGradient thermocycler (Biometra, Goettingen, Germany) with the following program: initial denaturation at 93°C for 1 min 30 s; 35 cycles comprising denaturation steps at 93°C for 1 min 30 s, annealing steps at 45°C (*16s rRNA*, *COI*) or at 53°C (*COII*, *ITS2*) for 1 min, extension steps at 72°C for 2 min; and final extension at 72°C for 8 min. The PCR product purification and sequencing was carried out by Macrogen (Seoul, South Korea). Sequencing was performed with both forward and reverse primers under BigDyeTM terminator cycling conditions, purifying the reacted products by using ethanol precipitation, and running them using an Automatic Sequencer 3730XL (Applied Biosystem, Foster City, USA).

Sequence alignment

Sequences (forward and reverse) were manually corrected and assembled using the software CHROMAS PRO 1.34 (Technelysium, Helensvale, Australia). Alignments of *ITS2* and *16s rRNA* were carried out using CLUSTALW Multiple Alignment (Thompson *et al.* 1997) within the software BIOEDIT 7.0.5.3, followed by minor manual correction. For *COI* and *COII*, alignment was trivial as all sequenced fragments were of the same size. For each defined partition, the best-

fit substitution model was selected using MrAIC.pl 1.4.3 (Nylander 2004) based on the Akaike information criterion (AIC; Akaike 1974). The three mtDNA partitions were shown to be congruent using the mILD test (Planet and Sarkar 2005) and a supermatrix comprising *16s rRNA*, *COI* and *COII* was built. In contrast, the mILD analysis revealed that *ITS2* was incongruent with the mtDNA regions and it was treated separately.

Phylogenetic reconstructions

To estimate phylogenetic relationships among individuals, both the mtDNA supermatrix and the *ITS2* alignment were analyzed using parsimony ratchet (Nixon, 1999) as implemented in PAUPrat (Sikes and Lewis, 2001). Based on recommendations by Nixon (1999), ten independent searches were performed with 200 iterations and 15% of the parsimony informative characters perturbed using PAUP* version 4.0b10 (Swofford, 2003). The shortest equally most parsimonious trees were combined to produce a majority-rule consensus tree. Node support was determined by computing Bremer support values as implemented in TREEROT.V3 (Sorenson and Franzosa 2007) and using PAUP* version 4.0b10 (Swofford, 2003).

Bayesian analyses (Nylander et al. 2004) were also performed separately for the *ITS2* region and the mtDNA supermatrix (treating the three mtDNA regions as separate partitions), using MRBAYES version 3.1 (Huelsenbeck and Ronquist 2001), with substitution models as estimated by MRAIC (Nylander et al. 2004) and four estimated alpha categories for the gamma term (Yang 1994). Four simultaneous Monte Carlo Markov Chains were run for 10^8 generations in two independent runs, saving a tree every 1000 generations. Convergence of the MCMC runs was tested by computing the Potential Scale Reduction Factor (Gelman and Rubin 1992) criterion as implemented in MRBAYES, and by determining the Effective Sample Size using TRACER 1.4.1 (Rambaut and Drummond 2008). Accordingly, the burn-in period was set to $3*10^7$ generations until stationarity in the likelihood value was established among the runs, so that 30000 sample

points were discarded. The last 70000 trees were used to calculate the half-compatible topology (*i.e.*, majority-rule) and the Bayesian posterior probability (BPP) at each node. Bremer support values were also determined on the half-compatible topology, as implemented in TREEROT.V3 and using PAUP* version 4.0b10.

Spatial Genetic Structure

Based on the topologies and node supports obtained both for mtDNA and *ITS* analyses, supported clades were defined and then displayed on geographical maps using ARCGIS 9.1 (ESRI, Redlands, CA, USA), by representing each population as a pie-chart showing the number of samples from each clade.

Results

Morphological determination

By using the classical determination key for *Oreina* species from Freude et al (1976) that distinguishes *O. speciosa*, *O. ganglbaueri* and five subtypes for *O. alpestris* (defined along a morphological continuum), we could only distinguish four discrete genitalia types, representing the genitalia shapes of *O. speciosa*, *O. ganglbaueri* and two well-defined variations of *O. alpestris*. We will refer to these two *O. alpestris* entities as "*alpestris* α" and "*alpestris* β" (Fig. 1). The geographical distribution of the specimens we collected for each of these four morphotypes is represented in Fig. 1. Whereas *O. ganglbaueri* was strictly restricted to the Pyrenees, *O. speciosa* and *O. alpestris* showed a wider distribution area. Interestingly, the genitalia type "*alpestris* β" has an intermediate morphology between "*alpestris* α" and *O. speciosa*. Except in two populations, one in Abruzzi and one in the northern Carpathians, types "*alpestris* α" and "*alpestris* β" were never found together. Moreover, type "*alpestris* β" appears to be restricted to the edges of the distribution and was never found in the Alps. In contrast, all *O.*

alpestris populations sympatric with *O. speciosa* (in other words, Alpine populations) always showed an "*alpestris* α" morphology.

Sequence polymorphism

Amplification of mtDNA regions was successful for 185 specimens, whereas *ITS2* was successfully amplified for 184 specimens (with 158 specimens amplified both for mtDNA and *ITS2* regions). The genetic analysis yielded a total of 1442 bp for the three mtDNA regions: 513 bp for *16s rRNA* (20 parsimony-informative sites among 35 polymorphic sites), 345 bp for partial *COI* (54 parsimony-informative sites among 68 polymorphic sites), and 584 bp for partial *COII* (63 parsimony-informative sites among 85 polymorphic sites). The total length of the nuclear *ITS2* region was 611 bp (43 parsimony-informative sites among 73 polymorphic sites). All sequences were deposited in GenBank (accession numbers xxxxxx to yyyyyy). The best-fit substitution models for each of the four regions were as follows: Hasegawa–Kishino–Yano (HKY) model with gamma parameter for *ITS2* and *16s rRNA*; General Time Reversible (GTR) model with gamma parameter and a proportion of invariable sites for *COI* and *COII*.

MtDNA phylogenetic analyses

The MP analysis yielded a well-resolved mtDNA majority-rule consensus tree, based on 125 equally parsimonious trees (650 steps; CI=0.55; RI=0.89). *Oreina speciosa*, *O. ganglbaueri* and *O. alpestris* clustered together with a Bremer support of 2 and supports ranged from 0 to 14 for clades within the ingroup (see Fig. 2). However, among the three species, only *O. ganglbaueri* was monophyletic, with *O. alpestris* and *O. speciosa* being mutually paraphyletic. The half-compatible consensus tree obtained through Bayesian inference analyses largely confirmed the topology obtained by MP (see supplementary file #1). Overall, BPP values were relatively high, confirming the monophyly of the clade comprising the three ingroup taxa with very high support (BPP=0.98), as well as the monophyly of *O. ganglbaueri* (BPP=1). Bremer

indexes on the Bayesian topology supported the ingroup with a value of 3, and ranged between 0 and 14 within the ingroup, even if basal clusters were slightly less well supported than in the MP analysis. When comparing the broad-scale topologies produced by MP and Bayesian Inference, only two inconsistencies were apparent: *Oreina ganglbaueri* diverged one step earlier in the MP analysis than in the Bayesian Inference analysis, and a small group comprising five *O. speciosa* individuals from northern Italy (Dolomites and Piemonte regions) fell in two different clades depending on the reconstruction method. Although the two methods yielded highly similar results, we discuss our results based on the MP topology due to its higher level of Bremer support in basal nodes. However, in order to define entities that were supported by both methods, we consider only clades supported by Bremer indexes ≥ 1 and BPP ≥ 0.8. Globally, the tree consists of seven major clades, referred to as M1 to M7 (see Fig. 2). Interestingly, the spatial genetic structure of these clades was revealed to be highly consistent with geographic patterns (see Fig. 3 and supplementary file #2, in which the distribution of each clade is considered independently and shown in parallel with the topology of one of the most parsimonious trees):

- The most basal clade, M1, is composed of *O. alpestris* individuals from the southern Balkans (Serbia and Montenegro) together with one population from the eastern Alps in Austria;
- The next clade, M2, groups all *O. ganglbaueri*.
- The following four clades, M3 to M6, are composed of both *O. speciosa* and *O. alpestris* specimens:
    - M3 is composed of beetles from several places in the Alps without clear geographical clustering, plus two populations from western Dolomites and Engadina, strictly restricted to this cluster;

- M4 groups together specimens from southern, central and eastern Alps, as well as two populations from western Dolomites and Adamello, strictly restricted to this cluster;
- M5 comprises beetles from the northern part of the occidental and oriental Alps, as well as from the Jura, the Black Forest and the Carpathians;
- M6, which shows a relatively high level of genetic diversity, is mostly composed of specimens from southern and occidental mountains; several well-supported subclades, showing a well-defined geographical structure restricted to a given massif, can be further delimited as follows: M6a comprises beetles from the French Massif Central, M6b groups all specimens from the Central Apennines, M6c is composed of beetles from the Pyrenean-Cantabric range, M6d corresponds to specimens from the southern Alps and the Ligurian Apennines, and M6e comprises beetles from the western Alps and the Jura;

- Finally, M7 is purely composed of *O. speciosa* specimens, all of them ranging across the Alpine Arc; samples within this clade show little substructure, with the exception of a group (indicated by a "§" on topologies) composed of five individuals from the Dolomites and Piemont.

ITS phylogenetic analyses

The MP analysis yielded an *ITS* majority-rule consensus tree based on 2009 equally parsimonious trees (78 steps; CI=0.85; RI=0.93). The resulting topology was, however, much

less well-resolved than the mtDNA topology for the ingroup taxa and most samples were embedded in polytomies. Bremer supports ranged from 1 to 2 for clades within the ingroup, and *O. speciosa*, *O. ganglbaueri* and *O. alpestris* clustered together with a Bremer support of 3 (see Fig. 4). Again, only *O. ganglbaueri* was monophyletic among the three species, with *O. alpestris* and *O. speciosa* being mutually paraphyletic. The half-compatible consensus tree obtained through Bayesian inference analyses confirmed the topology obtained by MP (data not shown), although the splitting of major clades was not identical. BPP values were much lower than in the mtDNA analysis, even if the monophyly of the clade comprising the three taxa was confirmed with a high support (BPP=1.00), as well as the monophyly of *O. ganglbaueri* (BPP=0.98). Bremer indexes on the Bayesian topology also supported the ingroup with a value of 3, and ranged between 1 and 2 within the ingroup. In order to be as consistent as possible with the mtDNA analyses, only clades supported by Bremer indexes ≥ 2 and BPP ≥ 0.95 will be discussed in the light of the MP topology (*NB.* threshold levels were increased compared to the mtDNA analyses in order to remove biases related to the much lower level of polymorphism within the *ITS* dataset; *i.e.*, the *ITS* tree was more than eight times shorter than the mtDNA tree). Overall, only two clusters, referred to as N1 and N2, were well defined, both being nested in a wide polytomy (see Fig. 4 and Fig. 5):

- N1 groups all *O. ganglbaueri* specimens;
- N2 is composed of *O. alpestris* specimens from the Alps, the Black Forest, the Balkans and the Carpathians (but none of the *O. alpestris* from the Appenines and the Pyrenees).

The other specimens (*i.e.*, all *O. speciosa* and the *O. alpestris* from the Appenines and the Pyrenean-Cantabric range) are loosely included into one single polytomy (referred to as N0).

Comparison between morphological and phylogenetic data

In order to discuss the nature of inconsistencies among morphological, mtDNA and *ITS* data, we provide a contingency table (Table 2) containing information from unambiguously determined specimens (excluding females and specimens with missing genetic information).

- Only the group composed of specimens with an *O. ganglbaueri* morphology (5 specimens) showed an absolute congruence with between mtDNA and *ITS* patterns (respectively clustered into M2 and N2).
- Within the *ITS* cluster N1, more than 80% of the samples demonstrated an "alpestris α" morphology and conversely, more than 80% of the specimens characterized by an "alpestris α" morphology were found within the N1 cluster; among them, about one third of those specimens exclusively composed the most basal mtDNA clade, M1 (distributed in the Balkanic region as well as in South-eastern Austria), whereas the other two thirds were distributed within the more widespread mtDNA clades M3, M4 and M5 (which also contain specimens with an *O. speciosa* morphology). The remaining specimens in the N1 cluster showed an "alpestris β" morphology.
- The other "alpestris β", together with a few samples with an "alpestris α" morphology, and 100% of the *O. speciosa* samples were not defined through the *ITS* analysis. Among the *O. speciosa* specimens, 60% form the mtDNA clade M7, spread across the Alps but with a low level of within-clade genetic differentiation (with the exception of the group formed by five individuals from the Piemonte and the Dolomites that might show long-branch attraction, and that clustered into M5 in the Bayesian Inference analysis). The other 40% were distributed across mtDNA clades M3, M4, M5 and particularly M6. The

remaining specimens with an “alpestris α” morphology clustered in clades M3 and M6, whereas most beetles with an “alpestris β” morphology clustered into clade M6. The M6 clade is therefore the only mtDNA clade composed of beetles with three different morphologies. It is also the clade encompassing the greatest genetic and geographic distance, and several of its sub-clades demonstrate a consistent geographic distribution (see above).

Discussion

Partial incongruence between morphology, ITS *and mtDNA phylogenies*

Based on the simple criterion of the shape of male genitalia (and on a few external criteria for females), the species circumscription of each taxon studied here seemed quite evident for classical coleopterologists, with the exception of *O. alpestris* (formerly known as *O. variabilis*, see Kippenberg 1994, Dobler *et al.* 1996) which showed some local variation and which was split into five to eight subspecies, depending on the authors. However, based on our phylogenetic approach, only *O. ganglbaueri* can be objectively considered as a segregated entity, both from the morphological and genetic point of view. It can be seen as a micro-endemic restricted to the central valleys of the Pyrenean range, which has remained isolated for a sufficient length of time for the development of barriers to hybridization that impede introgression of *O. ganglbaueri* with other specimens from *O. alpestris*, even in sympatry (as in population SP1 for instance); the male genitalia of *O. ganglbaueri* are by far the largest in size (among all *Oreina* species studied here), a feature that could act as a physical pre-zygotic barrier. Interestingly, the *O. ganglbaueri* clade (M2) splits very early in the mtDNA phylogeny, attesting to the ancient history of this lineage in this mountain range. The consistency between morphology and phylogeny is also strong in the most basal clade of the mtDNA phylogeny (M1), strictly composed of *O. alpestris* specimens

from the Balkans and Eastern Austria, with an "alpestris α" morphology and belonging to the N1 nuclear clade. This argues in favor of a very ancient history of this lineage as well, and similar to the case of *O. ganglbaueri*, it may have survived long enough in allopatry in the Balkanic region to have developed reproductive barriers despite its current sympatry with *O. speciosa* in Austria (as in population VAL for instance). Globally, this well-circumscribed entity contrasts with the high level of morphological and phylogenetic variation found in other *O. alpestris* samples from across the European mountain ranges. This heterogeneity could be explained by the following two antithetic hypotheses:

- first, the sympatric status of *O. alpestris* with *O. speciosa* (in the Alps) and *O. ganglbaueri* (in the Pyrenean range) might have created an opportunity for selection due to outbreeding depression to act to reduce hybridization and accelerate the development of pre-zygotic barriers. It is well known that genitalia are among the fastest evolving characters in insects, and that selection on these structures may even increase speciation rates (Polihronakis, 2009). As a consequence, it might have led to independent evolution of similar homoplasic features of the male genitalia in disparate populations that were then grouped by taxonomists;
- second, the wide range of morphological and phylogenetic variation could simply be the result of several independent events of introgression with other lineages.

It is, however, not possible to favor one or the other hypothesis based on our dataset, since genome-wide markers and experimental crosses would be required to resolve the question. Nonetheless, our analyses clearly highlight the paraphyletic status of *O. alpestris*. In addition to the specimens found in the Balkans (M1), several *O. alpestris* with an "alpestris α" morphology

come from the southern and central Alps (M3-M4) and northern Alps plus Carpathians (M5). The other *O. alpestris* specimens (from the Apennines and the Pyrenean-Cantabric range), which cluster into clade M6 together with *O. speciosa* specimens, mostly have an "alpestris β" morphology. Therefore, their genitalic structures (more similar to those from *O. speciosa*) as well as their phylogenetic position (very close to *O. speciosa*) suggest that they are intermediate between the two nominal species. Regarding specimens with a strict *O. speciosa* morphology, their undefined position in the *ITS* phylogeny, as well as the later branching of the clades in which they fall (together with specimens showing other morphologies) argues for an incomplete development of barriers to reproduction with other lineages. Nonetheless, the existence of one major mtDNA clade, M7, strictly composed of central European *O. speciosa* could represent evidence for a long history of this specific lineage in the Alps. Although it is not possible to unequivocally interpret the history of the different lineages and morphotypes addressed in this study, our results globally suggest that we are dealing with a species complex rather than with three discrete species. Reproductive barriers seem to be incomplete, and despite their low dispersal ability, *Oreina* lineages within these taxa tend to intermix where they meet within the Alps. Only lineages that have been isolated for a long time in remote mountain ranges (i.e., *O. ganglbaueri* in the Pyrenees and the M1 clade of *O. alpestris* in the Balkans) seem to have developed barriers to hybridization that maintain their status as non-introgressed entities.

Taxonomic considerations

Among the different lineages identified by the mtDNA analysis, some do match perfectly a series of subtaxa described on the base of morphological features and/or distribution patterns (see Kippenberg 1994, 2008). For instance, the mtDNA clades M6a, M6b and M6c correspond respectively to the microendemics *O. speciosa* ssp. *lugdunensis* (Wse.) from French Massif Central, *O. alpestris* ssp. *marsicana* (Luig.) from Appenines and *O. alpestris* ssp. *nigrina* (Suffr.)

from Pyrenees. Other entities only partly match described subtaxa, or at least do show a larger distribution than previously thought. This is notably the case of M6d, which corresponds to *O. speciosa* ssp. *pretiosa* (Suffr.) from Swiss Jura (but expanding into Massif Central and the Swiss western Alps as addressed by our results) or M1, which might correspond to *O. alpestris* ssp. *balcanica* (Wse.) described from Macedonia and Bulgaria (ranging from Serbia and Montenegro to southern Austria as shown here). Similarly, a partial match is found with clade M5, which could be an assemblage of three described subspecies of *O. alpestris*: ssp. *variabilis* (Wse.) (from the Alps and Black Forest), ssp. *banatica* (Wse.) (from central Carpathians and Transsylvania) and ssp. *alpestris* s. str. (Schumm.) (from northern Carpathians and Sudets). Finally, at the species level, there is a perfect match between mtDNA clade M2, nuclear clade N1, and *O. ganglbaueri*. Some congruence therefore exists between phylogenetic clades and microendemic taxa, although the species status of *O. alpestris* and *O. speciosa* is definitively much more complex than previously thought with these two taxa being reciprocally paraphyletic both in the mtDNA and nuclear topologies. To our opinion, a good starting point to clarify systematics in this group would be to merge *O. alpestris*, *O. speciosa* and *O. ganglbaueri* into one single species, until the careful revision of the genus would allow defining species according to the main phylogenetic clades discussed here.

Phylogeography of the species complex

Setting aside the morphological description of species, some noticeable patterns from the clades obtained with the mtDNA topology can be informative about the phylogeography of this species complex. First of all, the European regions classically considered as species refugia during Pleistocene climatic oscillations can be identified here. The two most basal mtDNA lineages comprise respectively beetles from the Pyrenees and from the Balkanic range (in yellow and black in Fig. 2, Fig. 3 and supplementary file #2), two areas known for having played the role

of Pleistocene refugia. The M5 clade includes pure populations in the Carpathians and the Black Forest (in violet on the corresponding figures), which may be related to putative Northern refugia (Stewart and Lister, 2001).

When considering the Alps, the picture gets more intricate. Some pure populations of the M3 and M4 lineages (eg, western Dolomites [IT2, IT3], Adamello [IT5], Swiss Inn valley [SUS]) could represent persistent refugia, with range expansion leaving just a few representatives at the eastern and western edges of the Alps. The M6 clade is also marginal within the Alps, but has pure populations in the Pyrenees, Massif Central, Jura and Apennines. The source of the M7 clade is less clear, since it shows an even distribution throughout the alpine arc. In addition, numerous populations include individuals from different mtDNA clades. For instance, some northern Alpine populations harboured individuals with mtDNA originating in the Carpathians; other eastern Alpine populations included a Pyrenean haplotype. Interestingly, the case of the Jura is similar to that of the Alps regarding the level of phylogenetic variation found in populations, with influences from the Alps and Massif Central. As a mark of their status as a crossroads in Europe, the Alps and the Jura are the European mountain ranges that show the highest level of admixture by distantly-related mtDNA haplotypes.

Conclusion: the Alps as a crossroads in sessile alpine organisms

The alpine European Fauna and Flora encompass thousands of plant and animal species. Among them many taxa show limited dispersal abilities. This is for instance the case of numerous soil invertebrates, as well as sessile phytophagous organisms. Compared to plants or flying animals, many more generations would be needed by these organisms to recolonize previously glaciated areas and to fill the gaps between disjunctive portions of their contemporaneous distribution. Since the effectiveness of reproductive barriers is directly correlated with the extent to which drift and selection have induced divergence of populations, sessile organisms are more prone than

highly motile species to develop partial or complete barriers to crossing between lineages. This was notably the case in the leaf beetle species complex studied here, in which some lineages were not divergent enough to impede admixture, whereas other had sufficient time to develop effective reproductive barriers. Understanding how such complexes (in which the level of intercrossing ability varies among regions and lineages) behave under outbreeding depression conditions and with local adaptation represents the next step to understand their evolutionary history, in the Alpine natural laboratory.

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6. Tables

Table 1. Geographical details of the sampled populations. The names of the collected morphological species are also indicated.

Code	Mountain massif	Country	Detailed location	Altitude	Latitude Longitude	Species collected
ABZ1	Appenines	Italy	Vado di Sole	1650	N 42°23'51.70" E 013°47'19.40"	O. alpestris
ABZ2	Appenines	Italy	Sarnano1	1333	N 42°58'57.40" E 013°15'00.20"	O. alpestris
ABZ5	Appenines	Italy	Sarnano2	1431	N 43°01'27.90" E 013°13'25.20"	O. alpestris
AI1	Alps	Switzerland	Brulisau	1600	N 47°17'04.08" E 009°29'05.90"	O. alpestris / O. speciosa
AUT1	Alps	Austria	Arlbergpass	1354	N 47°08'03.80" E 010°12'20.90"	O. speciosa
AUT2	Alps	Austria	Mittersill (Gamsblick)	1317	N 47°11'25.20" E 012°28'37.50"	O. speciosa
AUT3	Alps	Austria	Badgastein (Stubnerkugel)	1876	N 47°06'35.40" E 013°07'36.20"	O. alpestris
AUT5	Alps	Austria	Koralpe	1564	N 46°48'30.50" E 014°56'34.10"	O. speciosa
BE1	Alps	Switzerland	Kandersteg	1314	N 46°28'21.10" E 007°39'23.00"	O. alpestris / O. speciosa
CZ4	Carpathians	Czech Republic	Karlova Studanka	1267	N 50°04'13.30" E 017°14'42.30"	O. alpestris
DOL2	Alps	Italy	Passo Rolle	1969	N 46°17'46.00" E 011°46'56.00"	O. speciosa
DOL3	Alps	Italy	Passo Duran	1477	N 46°18'45.20" E 012°05'29.20"	O. speciosa
EUR	Cantabrics	Spain	Pico de Europa	1295	N 43° 07'40.44" W004°52'38.82"	O. alpestris
FR1	Jura	France	Cret de la Neige	1715	N 46°15'04.96" E 005°55'28.54"	O. speciosa
FR3	Pyrenees	France	Vernet-les-Bains	1737	N 42°30'00.20" E 002°24'30.40"	O. alpestris
FR4	Pyrenees	France	Col de Port	1334	N 42°53'39.10" E 001°26'50.40"	O. alpestris
FR5	Pyrenees	France	Col de Peyresourde	1548	N 42°47'50.00" E 000°27'10.60"	O. ganglbaueri
FR8	Massif Central	France	Puy Mary	1550	N 45°06'40.90" E 002°40'51.80"	O. speciosa
FR9	Massif Central	France	Puy de Dome	1292	N 45°46'07.70" E 002°57'33.70"	O. speciosa
FR11	Alps	France	Col du Galibier	1999	N 45°05'07.40" E 006°26'19.10"	O. speciosa
FR13	Alps	France	Ailefroide	1700	N 44°53'41.50" E 006°26'44.70"	O. speciosa
FR14	Alps	France	Abries	1886	N 44°48'49.10" E 006°58'28.30"	O. speciosa
FR16	Alps	France	Saint-Martin Vesubie	1795	N 44°06'37.10" E 007°18'41.10"	O. speciosa
GL1	Alps	Switzerland	Schwanden	1500	N 46°57'28.30" E 009°05'55.90"	O. speciosa
GR1	Alps	Switzerland	Tschiertschen	1400	N 46°48'42.00" E 009°36'40.00"	O. alpestris / O. speciosa
GRH	Alps	Austria	Grosser Hengst	1615	N 47°27'6.15" E 014°25'50.86"	O. speciosa
HR2	Dinarics	Croatia	Risnjak	1402	N 45°25'39.50'' E 014°37'19.40''	O. speciosa
HR4	Dinarics	Croatia	Sjeverni Velebit	1422	N 44°48'28.00'' E 014°58'14.70''	O. speciosa
IT1	Alps	Italy	Col du Petit St-Bernard	1996	N 45°42'12.00" E 006°52'29.40"	O. speciosa
IT2	Alps	Italy	Passo Pian delle Fugazze	1296	N 45°44'47.30" E 011°09'32.30"	O. alpestris

Table 1. continued

Code	Mountain massif	Country	Detailed location	Altitude	Latitude Longitude	Species collected
IT3	Alps	Italy	Monte Baldo	1247	N 45°47'03.60" E 010°52'14.00"	O. speciosa
IT4	Alps	Italy	Val Daone	1319	N 46°01'19.80" E 010°30'47.60"	O. speciosa
IT5	Alps	Italy	Passo del Tonale	1784	N 46°15'53.40" E 010°36'46.80"	O. speciosa
IT7	Appenines	Italy	Passo del Penice	1141	N 44°47'26.20" E 009°18'12.70"	O. alpestris
IT8	Alps	Italy	Terme di Valdieri	1419	N 44°12'10.30" E 007°16'13.60"	O. speciosa
IT9	Alps	Italy	Crissolo (Piano del Re)	1342	N 44°41'59.00" E 007°09'11.20"	O. speciosa
IT11	Alps	Italy	Breuil-Cervinia	2149	N 45°55'40.10" E 007°37'53.30"	O. speciosa
IT12	Alps	Italy	Macugnaga	1343	N 45°57'58.50" E 007°56'14.00"	O. speciosa
JU1	Jura	Switzerland	Undervelier	550	N 47°17'57.06" E 007°13'24.32"	O. speciosa
LOT	Alps	Switzerland	Lotschental	1888	N 46°26'21.67" E 007°52'23.46"	O. speciosa
MON2	Dinarics	Montenegro	Savnik (Slatina)	1363	N 42°59'55.50" E 019°09'58.00"	O. alpestris
NE1	Jura	Switzerland	Motiers	760	N 46°54'09.63" E 006°36'59.38"	O. speciosa
PL2	Carpathians	Poland	Zakopane	1016	N 49°16'31.80" E 019°51'12.10"	O. alpestris
RO1	Carpathians	Romania	Monti Rodnei	1111	N 47°35'54.90" E 024°55'21.20"	O. alpestris
RO2	Carpathians	Romania	Sinaia	1386	N 45°21'26.50" E 025°31'05.70"	O. alpestris
SER1	Dinarics	Serbia	Kopaonik	1700	N 43°20'32.40" E 020°46'01.90"	O. alpestris
SLO2	Alps	Slovenia	Predmeja	1142	N 45°55'56.70'' E 013°50'31.20''	O. speciosa
SLO8	Alps	Slovenia	Logarska Dolina	1394	N 46°22'09.50'' E 014°35'04.70''	O. speciosa
SLO9	Alps	Slovenia	Dom na komni	1261	N 46°16'58.10'' E 013°47'13.40''	O. speciosa
SLO10	Alps	Slovenia	Vrsic pass	1387	N 46°25'29.00'' E 013°44'34.60'	O. speciosa
SO1	Jura	Switzerland	Weissenstein	1274	N 47°15'03.40" E 007°29'07.70"	O. speciosa
SP1	Pyrenees	Spain	Salardu	1636	N 42°39'57.90" E 000°55'06.70"	O. alpestris / O. ganglbaueri
SUR	Alps	Switzerland	Sur	1515	N 46°31'16.40" E 009°37'27.00"	O. speciosa
SUS	Alps	Switzerland	Susch	1486	N 46°44'50.13" E 010° 4'28.92"	O. speciosa
SW3	Black Forest	Germany	Zastler	1068	N 47°54'13.40" E 007°58'58.00"	O. alpestris
VAL	Alps	Austria	Valentinshalm	1540	N 46°36'29.48" E 012°57'18.89"	O. alpestris / O. speciosa
VD1	Alps	Switzerland	Col des Mosses	1843	N 46°23'13.93" E 007°07'48.60"	O. alpestris / O. speciosa
VS2	Alps	Switzerland	Sanetsch	1680	N 46°18'26.66" E 007°20'07.46"	O. speciosa
VS4	Alps	Switzerland	Saas Almagell	1620	N 46° 06'15.76" E 007°56'57.93"	O. speciosa
VS5	Alps	Switzerland	Les Haudères	1436	N 46°04'52.00" E 007°30'18.10"	O. speciosa
VS6	Alps	Switzerland	Emosson	1944	N 46°03'55.30" E 006°55'43.90"	O. speciosa
VS7	Alps	Switzerland	Chandolin	2000	N 46°14'41.85" E 007°36'10.01"	O. speciosa
VS8	Alps	Switzerland	La Fouly	1571	N 45°56'10.20" E 007°05'36.10"	O. speciosa

Table 2. Contingency table showing relations and number of specimens between morphological categories, mtDNA and nuclear phylogenetic clades.

		mtDNA clustering							
***ITS* clustering**	**genitalic morphology**	M1	M2	M3	M4	M5	M6	M7	**Total**
N0	alpestris α			1			5		**6**
	alpestris β						15		**15**
	speciosa			9	11	2	19	61	**102**
Total N0				10	11	2	39	61	**123**
N1	ganglbaueri		5						**5**
Total N1			5						**5**
N2	alpestris α	8		1	6	10			**25**
	alpestris β					5			**5**
Total N2		8		1	6	15			**30**
Total		**8**	**5**	**11**	**17**	**17**	**39**	**61**	**158**

7. Illustrations

Figure 1. Drawings of the four genitalia morphotypes examined in this study with their respective spatial distributions. A. type *ganglbaueri*; B. type *speciosa*; C. type *alpestris* β; D. type *alpestris* α.

Figure 2. Majority-rule consensus tree of the MP analysis for the three mtDNA regions *COI*, *COII* and *16s rRNA* in a total evidence approach. Label names comprise the population code (see Table 1), the number of the specimen (a, b, c, d, e, f, g), followed by the genitalic morphology (ALPα, ALPβ, GAN, SPE) for males specimens. In female specimens (highlighted with a "*"), the global body shape and morphology allowed us to identify the three species, coded by ALP, GAN and SPE (although we could not give detailed determinations regarding ALPα, ALPβ). Bremer support values are indicated on each node. Main clades are defined with codes ranging from M1 to M7. The group illustrated with a "§" switched position between the MP and the Bayesian inference analyses (see supplementary material #1).

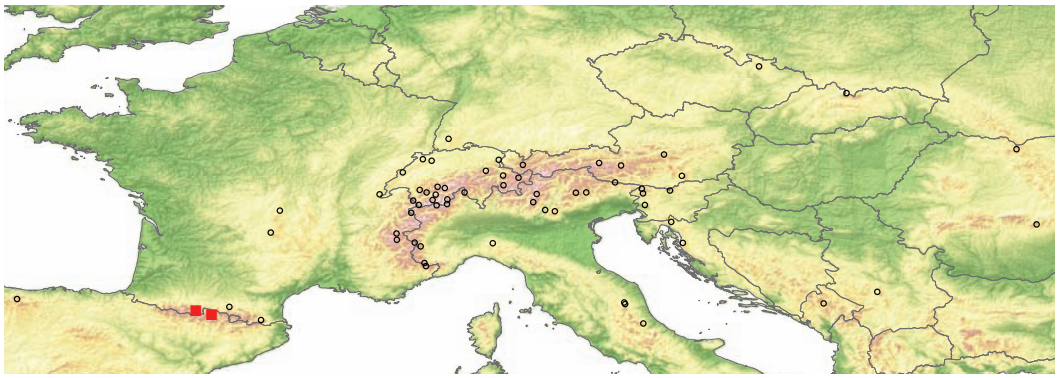
Figure 3. Geographical distribution of each mtDNA clade identified in the MP analysis. Colors refer to clades identified in Figure 2 and codes correspond to populations detailed in Table 1.

Figure 4. Majority-rule consensus tree (equal branch-length cladogram) of the MP analysis for the nuclear region *ITS2*. Label names are as in caption of Figure 2. Bremer support values are

indicated on each node. The two main clades are defined with codes N1 and N2. Specimens that are not comprised in a well-supported clade (N1 or N2) are considered as belonging to group N0.

Figure 5. Geographical distribution of each nuclear clade identified in the Maximum Parsimony analysis. Colors refer to N1 and N2 clades, as identified in Figure 4. Samples in white correspond to the loosely defined N0 group.

A.



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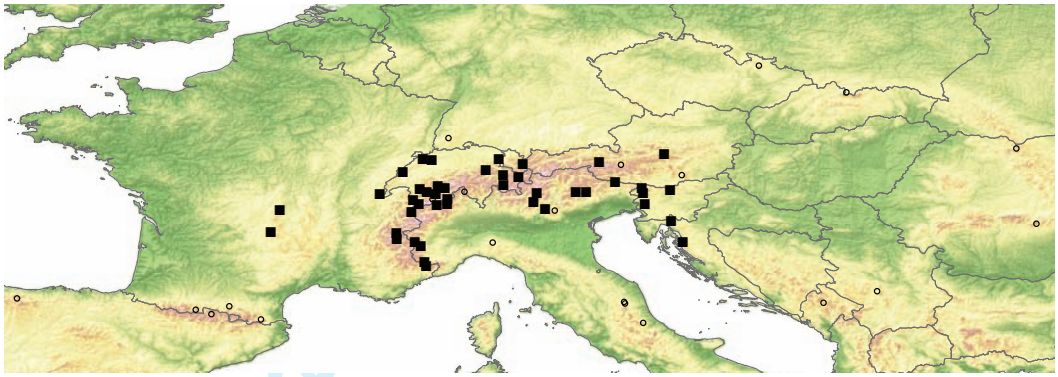
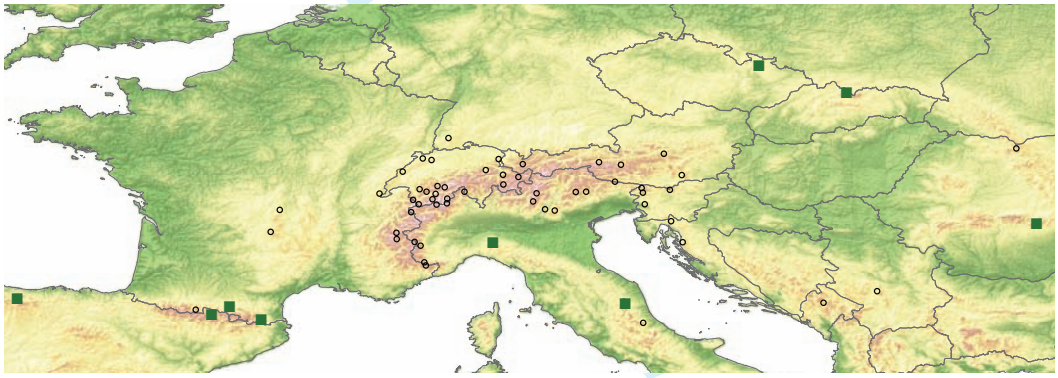


C.



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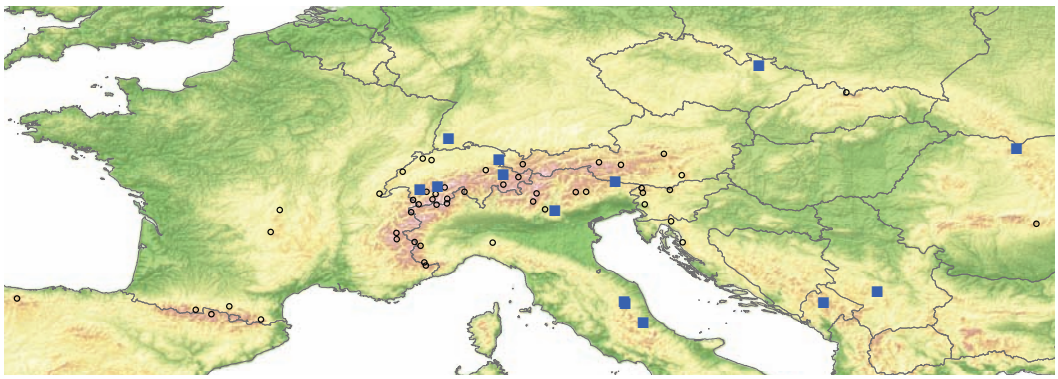

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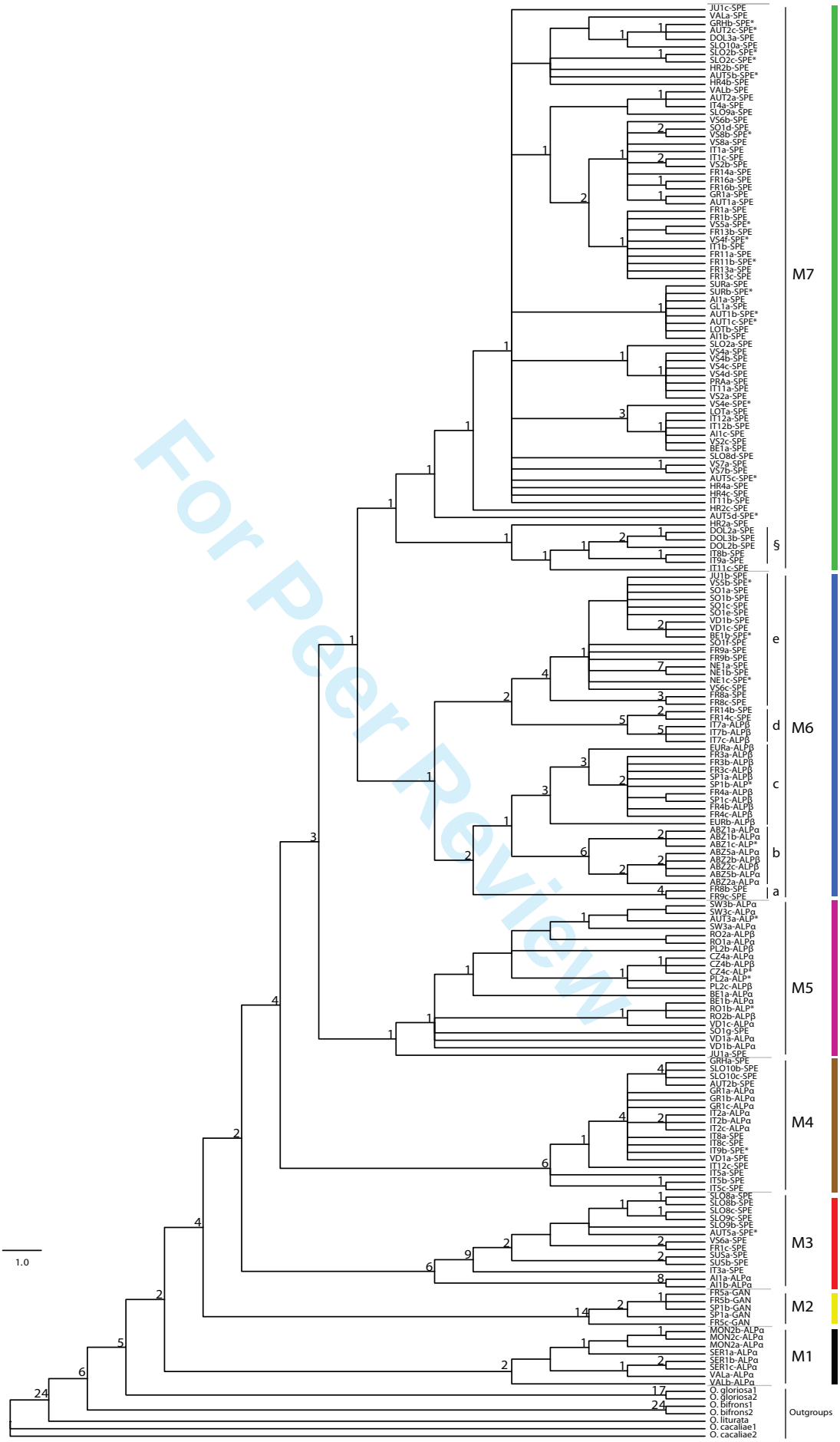

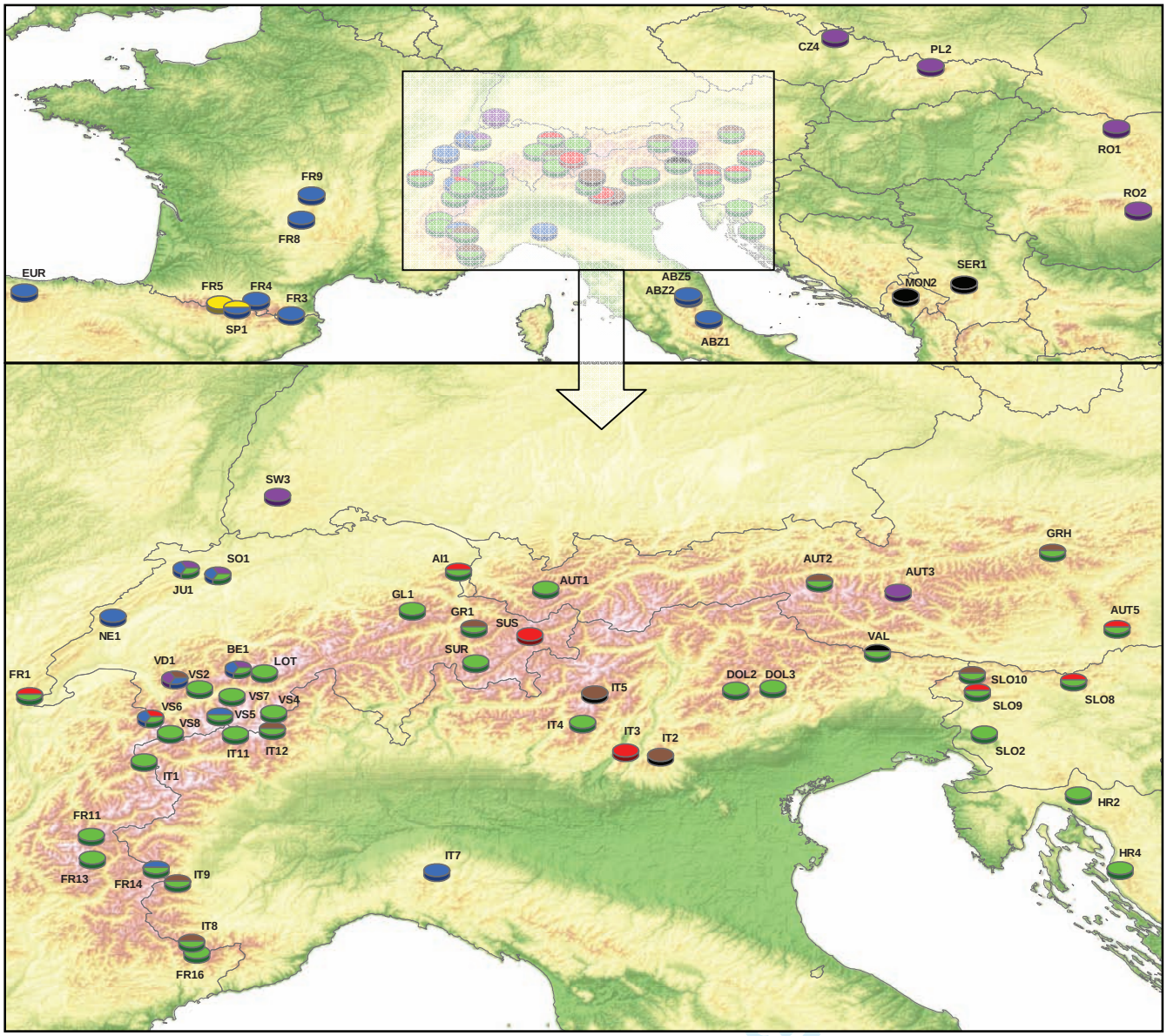
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RO1
RO2
FR9
FR8
EUR
FR5
FR4
FR3
SP1
ABZ5
ABZ2
ABZ1
SER1
MON2
SW3
GRH
SO1
JU1
AI1
AUT1
AUT2
AUT3
AUT5
GL1
GR1
SUS
SUR
NE1
VAL
FR1
VD1
BE1
LOT
VS2
VS7
VS4
VS6
VS8
VS5
IT11
IT12
IT1
IT5
IT4
IT3
IT2
DOL2
DOL3
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SLO9
SLO8
SLO2
HR2
HR4
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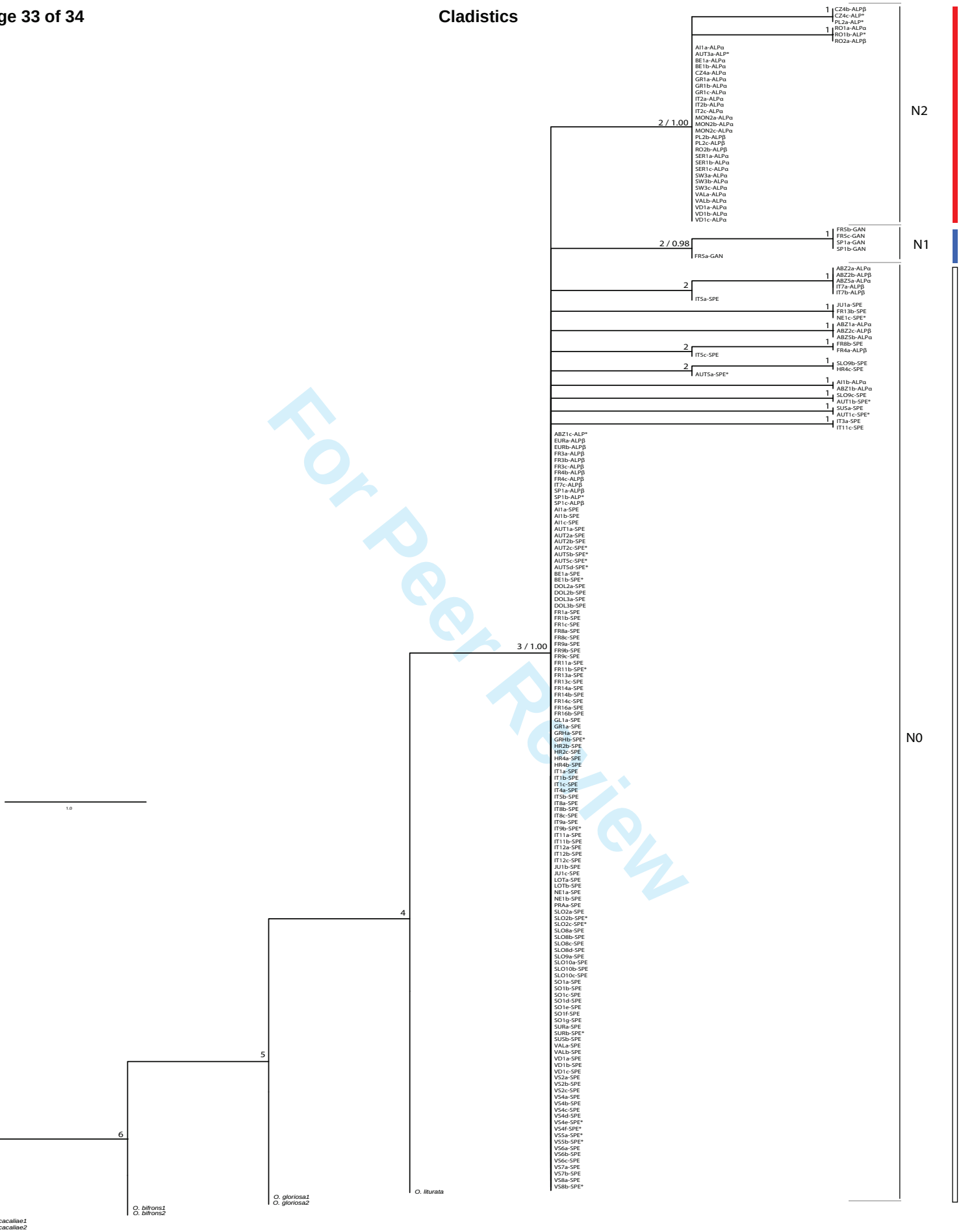
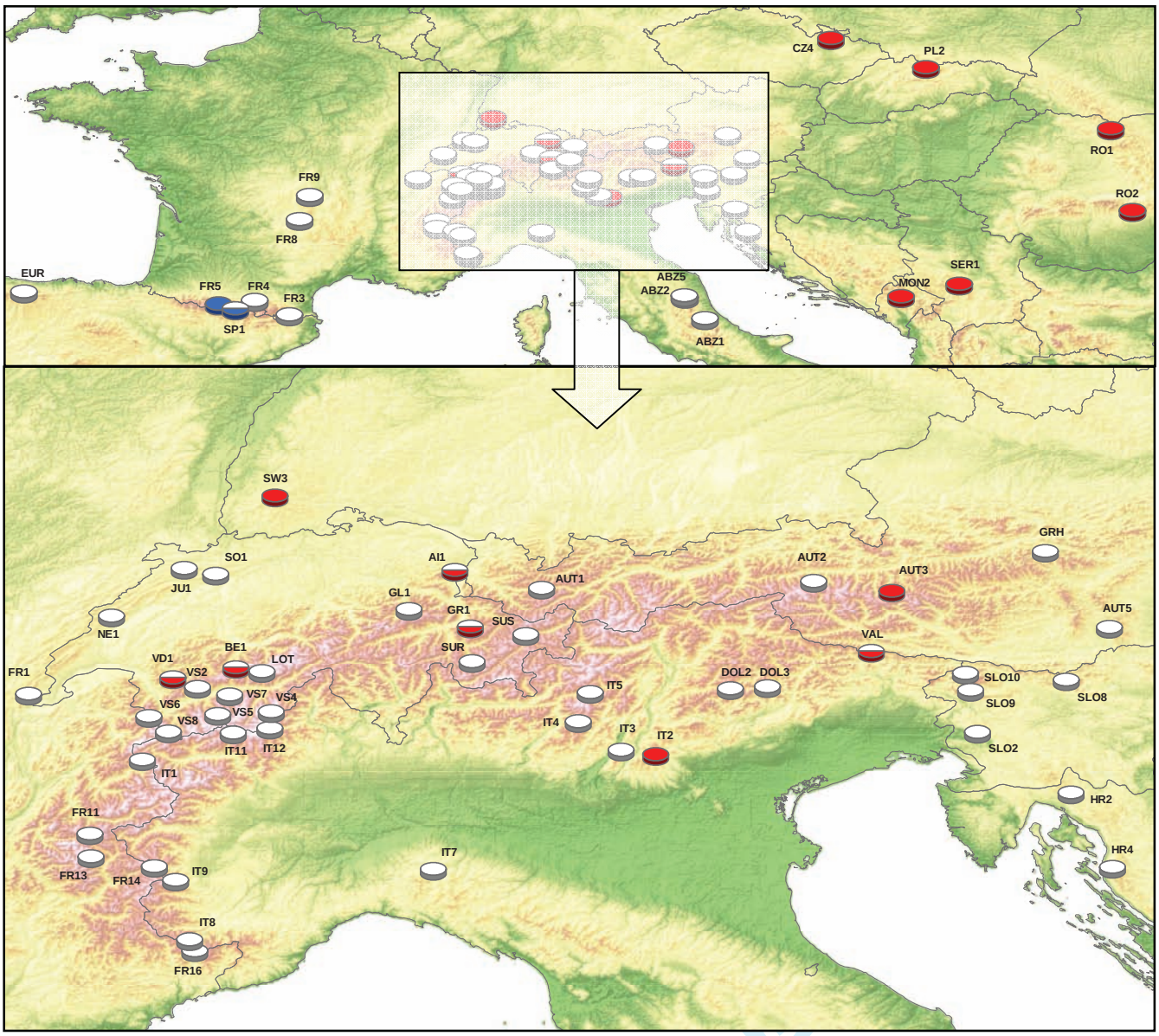
FR13
FR14
IT9
IT7
IT8
FR16

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PL2
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RO2
FR9
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EUR
FR5
FR4
FR3
SP1
ABZ5
ABZ2
ABZ1
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MON2
SW3
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AI1
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GR1
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SUR
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VS8
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IT5
IT4
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IT2
DOL2
DOL3
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HR2
HR4
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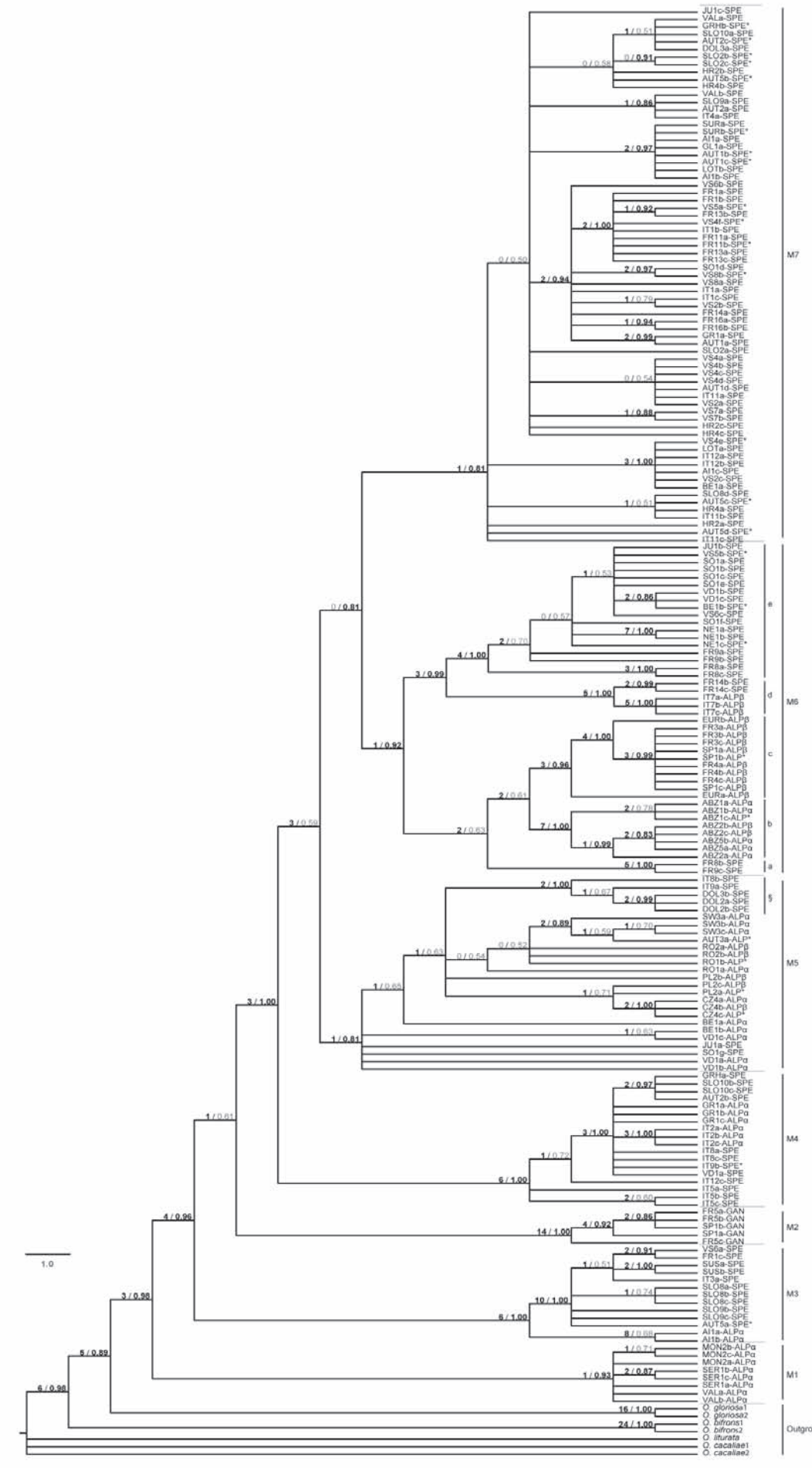
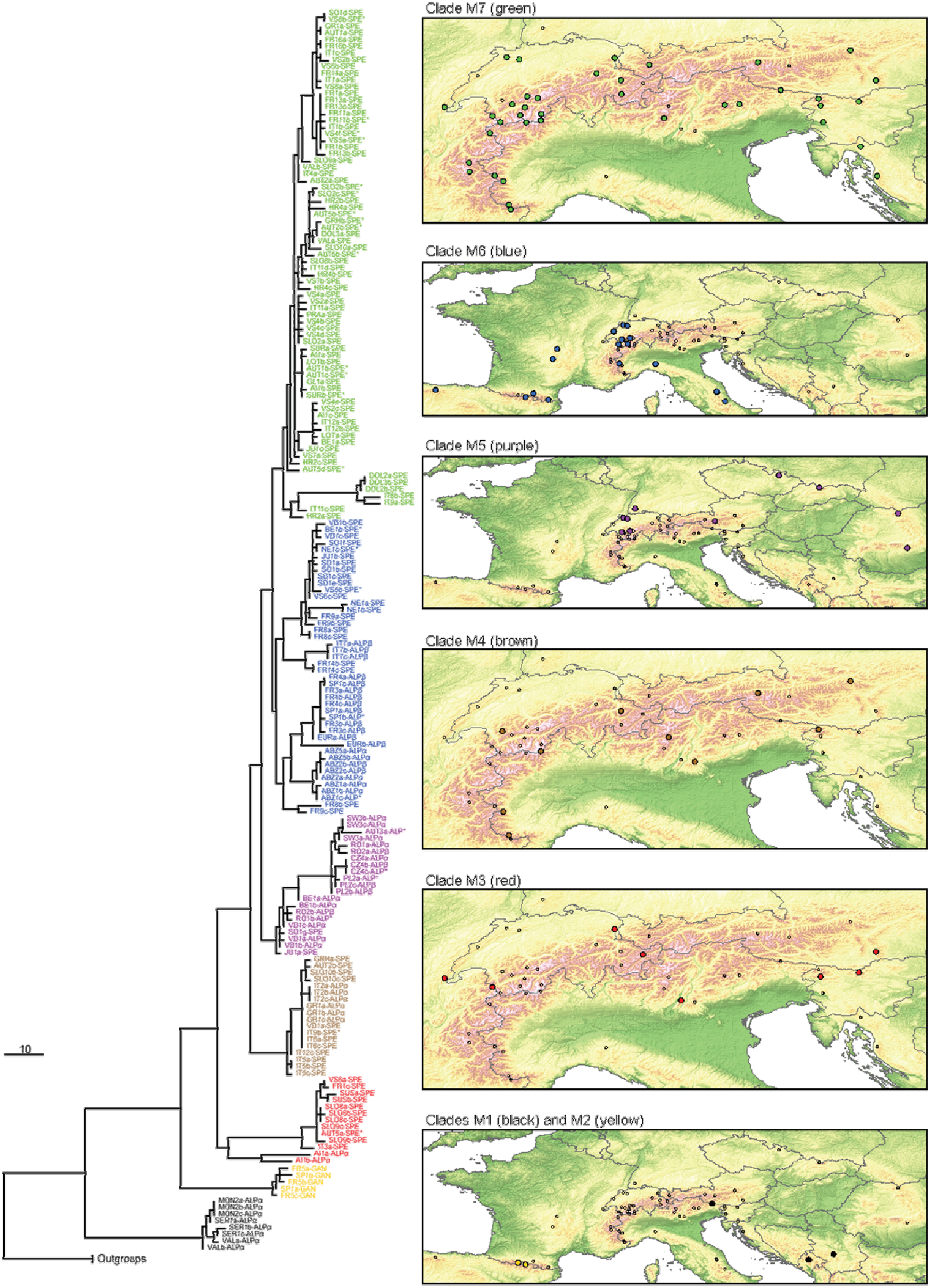


Supplementary file #1: Half-compatible topology of the MrBayes analysis, with the corresponding Bremer support values and BPP on each node. Major clades from the MP analysis are shown to the right of the topology. The group indicated by § switched position between the MP and Bayesian analyses (see Fig.2).



Supplementary file #2: One of the 125 equally parsimonious trees, highlighting mtDNA clades M1 to M7, with their corresponding spatial distribution on the maps.

Appendix II

MATTHIAS BORER

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CURRICULUM VITAE

PERSONAL DATA

Date of Birth	18th January 1978
Birthplace	Schlieren (ZH), Switzerland
Citizen of	Kleinlützel (SO)
Nationality	Swiss
Personal Status	Single

EDUCATION

2004 – 2009

PhD Thesis in Evolutionary Entomology.
University of Neuchâtel, Switzerland.

Subject of PhD Thesis: 'Phylogeography and biodiversity in an alpine leaf beetle genus'.
Supervisors: Prof. Dr. Martine Rahier and PD Dr. Russell E. Naisbit.

1999 – 2004

Master of Science in Biology including specialisation in Community Ecology.
University of Bern, Switzerland.

Subject of Master Thesis: 'Spatial distribution of *Apion onopordi* infestation on creeping thistle *Cirsium arvense*'.
Supervisors: Prof. Dr. Wolfgang Nentwig and PD Dr. Sven Bacher.

1990 – 1999	Gymnasium at Kantonsschule Solothurn. Qualification: Matura Type E. Solothurn, Switzerland.
1987 – 1990	Primary School. Derendingen, Switzerland.
1985 – 1987	Primary School. Dornach, Switzerland.

WORK EXPERIENCE

2005 – 2009	Substitute teacher at secondary schools and gymnasium.
2004 – 2009	Assistant responsible for the practical work: Introduction to entomology for the second year biology students. Theoretical courses, excursions and determination.
01/2007 – 02/2007	Entomological expert in a field study about plant-pollinator mutualism in Uruguay. Direct behavioural observations in the field with wild specimens under natural weather conditions. Supervisors: Prof. Dr. Redouan Bshary and Anneken Brandenburg.
01/2006	Entomological expedition in W-Malaysia. Collection of tropical insects focused on praying mantids.
04/2004 & 02/2005	Entomological expedition in Cameroon. Collection of tropical insects focused on praying mantids.
2002 – 2003	Assistant at the department of Community Ecology at the Zoological Institute, University of Bern, Switzerland.
2002	Faunistic ecological course (entomological inventory) in the natural reserve ‘Pfynwald’, Switzerland. Supervisor: Prof. Dr. Jürg Zettel.

COMPLEMENTARY EXPERIENCE

2000 – Present	'Insect-Workshops' at different schools (primary, lower-grade and secondary schools).
12/2008	Presentation for the Entomological Society of Bern at the Museum of Natural History in Bern. Title: 'Die Gattung *Oreina* – banale Blattkäfer oder eine phylogenetische Wunderkiste?'.
03/2008	Presentation for the Entomological Society of Neuchâtel at the Museum of Natural History in Neuchâtel. Title: 'Les Mantes – des insects fascinants'.
09/2006	Workshop 'Conflicts of Interest in Mutualistic Interactions'. University of Neuchâtel, Switzerland.
05/2006	Workshop 'NCCR Plant Survival'. University of Neuchâtel, Switzerland. Presentation: 'A Europe-wide phylogeography of *Oreina* leaf beetles and their host plants'.
2004	Presentation for the Entomological Society of Bern at the Museum of Natural History in Bern. Title: 'Mantodea – faszinierende Lauerjäger'.

LANGUAGES

German	mother tongue
French	fluent in spoken and written
English	fluent in spoken and written

CONFERENCES & SYMPOSIA

06 – 08 February 2008	Biology 08. Lausanne, Switzerland. Poster Presentation: 'The Phylogeography of an Alpine Leaf Beetle'.
30/01 – 01/02 (2008)	NCCR Plant Survival International Conference: Plant Species Concepts and Evolution. Neuchâtel, Switzerland. Poster Presentation: 'The Phylogeography of an Alpine Leaf Beetle'.

26 – 28 Octobre 2007	50. Deutsche Koleopterologentagung. Weinstadt- Beutelsbach, Germany.
16 – 17 February 2006	Biology 06. Geneva, Switzerland.
08 – 10 September 2004	IIIème Cycle Romand en Sciences Biologiques: Host Recognition by Parasites and Parasitoids. University of Neuchâtel, Switzerland.

PUBLICATIONS

Naisbit R.E., Borer M., van Noort T. and Rahier M. 'Positive frequency-dependent selection on warning colour in alpine beetles'. (in prep.).

Borer M., van Noort T., Buerki S., Arrigo N., Rahier M., Alvarez N and Kippenberg H. 'Ecological speciation within the alpine leaf beetle *Oreina speciosissima* (Coleoptera, Chrysomelidae)'. (in prep.).

Borer M., Triponez Y., Alvarez N., Rahier M. and Naisbit R.E. 'Phylogeography of two closely related species *Oreina cacaliae / Oreina speciosissima*: an example of sympatric wide spread montane insects'.

Borer M., Buerki S., Arrigo N, Naisbit R.E., Rahier M. and Alvarez N. 'Comparative phylogeography in an Alpine antagonism: the monophagous leaf beetle *Oreina gloriosa* (Coleoptera; Chrysomelidae) and its host plant *Peucedanum ostruthium*'. (in prep.)

Borer M., Alvarez N., Margraf N., Rahier M. and Naisbit R.E. (2009). 'The phylogeography of an alpine leaf beetle: divergence within *Oreina elongata* spans the Quaternary'. *Evolution* (submitted).

Haenni J. P. & Borer M. 2007. 'First Report of a Nemestrinidae (Diptera) parasitoid of the Mantodea'. *Studia dipterologica* **14**/1, 61-65.

Moravie M. A., Borer M. & Bacher S. 2006. 'Neighbourhood of host plants influences oviposition decisions of a stem-boring weevil'. *Basic and Applied Ecology*, vol. **7**/6, 545-554.

EXTRACURRICULAR ACTIVITIES

Entomology	Breeding of tropical insects since 1986 (Mantodea, Phasmatodea and Coleoptera). Specialisation in biology, life history and systematics of Mantodea. Member of the IGM ('Interest Group Mantodea').

	Member of the Entomological Society of Bern.
	Member of the Entomological Society of Neuchâtel.
	Member of the Swiss Systematic Society.
Sports	Track and Field (Swiss elite in decathlon until 2004), Ice-Hockey and hiking.

REFERENCES

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