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Glacial survival and local adaptation in an alpine leaf beetle

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Abstract

The challenge in defining conservation units so that they represent evolutionary entities has been to combine both genetic properties and ecological significance. Here we make use of the complexity of the European Alps, with their genetic landscape shaped by geographical barriers and postglacial colonization, to examine the correlation between ecological and genetic divergence. Montane species, because of the fragmentation of their present habitat, constitute extreme cases in which to test if genetically distinct subgroups based on neutral markers are also ecologically differentiated and show local adaptation. In the leaf beetle *Oreina elongata*, populations show variation in host plant use and a patchy distribution throughout the Alps and Apennines. We demonstrate that despite very strong genetic isolation (F_{ST} = 0.381), variation in host plant use has led to differences in larval life-history traits between populations only as a secondary effect of host defence chemistry, and not through physiological adaptation to plant nutritional value. We also establish that populations that are more ecologically different in terms of larval performance are also more genetically divergent. In addition, morphological variation used to define subspecies appears to be mirrored in the population genetics of this species, resulting in almost perfect clustering based on microsatellite data. Finally, we argue from their strong genetic structure and congruent distribution that the subspecies of *O. elongata* were divided among the same glacial refugia within the Alps that have been proposed for alpine plants.

Keywords: Chrysomelidae, ice age refugia, life-history traits, microsatellites, *Oreina elongata*, phylogeography

Introduction

The rise of conservation issues in recent decades has challenged the concept of the taxon, as scientists have come to accept units below the species level as the focus of management efforts (Hey *et al*. 2003). Preserving the ability of a taxon to adapt requires some sophistication in the definition of the entities to be protected. Waples (1991) provided one of the most commonly used definitions. His evolutionary significant unit (ESU) is a population or group of closely connected populations that has two characteristics: (i) it is reproductively isolated from other such units, and (ii) it contributes substantially to the ecological or genetic diversity found within the species as a whole. This definition combines the two aspects of ecological significance and genetic differentiation. Their relative importance has been debated (Dimmick *et al*. 1999, 2001; Young 2001), but in practice most ESUs have been described on a genetic basis, often using neutral markers. Yet questions remain regarding the relevance of such a characterization of ESUs. It is not clear what being distinct at neutral markers really means for the evolution of populations, beyond suggesting a period of isolation, or reduced gene flow. This would allow local adaptation but only if there is also selection, and in fact if selection is strong enough, complete isolation is not required (in other words, isolation is neither necessary nor sufficient). Basing conservation effort on entities defined by variation at neutral genetic markers does not therefore necessarily imply preserving a taxon's ecological or behavioural diversity, nor its future potential. While future evolutionary challenges are impossible to predict, a correlation between variation in allele frequencies at neutral loci and ecological differences in populations would seem amenable to testing.

The Alps provide an ideal region in which to examine the interaction between isolation, genetic differentiation and local adaptation. They played a significant role during

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past glacial cycles as a barrier to dispersal and source of hybrid zones, and currently represent an extremely complex and fragmented landscape for mountain species. During the last glaciation, the advancing ice sheets drove most European species towards southern refugia. Their subsequent dispersal northwards shaped the genetic landscape of Europe. Typically, species found refuge in Iberia, Italy, the Balkans/Greece, and the Caspian/Caucasus region, and these sources then made varying contributions to the recolonization process (Hewitt 1996, 2000, 2004; Taberlet *et al*. 1998). For example, in species such as *Chorthippus parallelus* and *Alnus glutinosa*, the Alps and Pyrenees formed significant barriers and Balkan populations inherited most of northern Europe, while in others such as *Erinaceus europeus/concolor* and *Quercus* species, the contributions of the refugia were more equitable (Hewitt 1999).

While this is well documented for many lowland species, the glacial history of alpine species is much less certain. They too may have retreated to lower altitudes and latitudes, which for them would represent an increase in their range as the climate cooled. It is also possible that they survived within the mountain ranges in central or peripheral refugia where conditions were less harsh. Little data are available for animals (Schmitt *et al*. 2006), but studies are beginning to accumulate on alpine plants that show survival along the southern border of the Alps, at the northern edge, and even within the chain (Schönswetter *et al*. 2005). Survival within alpine refugia has important implications for the pattern of genetic variation we can expect within mountain species. Long-term survival and isolation would suggest the possibility of great genetic and adaptive differentiation between current populations, in contrast to the homogenization expected from dispersal from a limited number of refugia. Survival *in situ* would also provide an extended opportunity for co-evolution and local adaptation, unavailable to communities assembled from species from different refugia. Here we examine these ideas by testing for genetic and ecological differentiation among populations of an alpine beetle.

The leaf-beetle *Oreina elongata* (Coleoptera: Chrysomelidae), although not endangered, provides an extreme case in which to test the congruence of ecological and genetic aspects in defining ESUs. *O. elongata* forms localized and probably strongly isolated populations at high altitudes in the Alps and Apennines. The beetles have a very limited altitudinal range (1500–2300 m above sea level) so the inhospitable peaks and valleys between sites are likely to represent real barriers. Furthermore, large distances separate populations and despite possessing wings, *O. elongata* has never been seen flying. Migration is therefore likely to be very low and we expect populations to have evolved genetic differentiation. We used microsatellite markers to survey genetic variation in 13 populations of *O. elongata* to answer our first question: (i) *Are mountain populations genetically distinct, suggesting that they are isolated?*

Subspecies have been described on the basis of morphological differentiation among groups of populations (including the form of the aedeagus and the cuticle microstructure, Daccordi & Ruffo 1976, 1986). These subspecies are separated geographically, with four in the Alps and three in the Apennines. Yet their existence has never been tested on a genetic level, leading to our second question: (ii) *Do the subspecies represent genetically distinct subgroups and is morphological differentiation correlated with genetic differentiation?*

Given the apparent isolation of these populations of *O. elongata*, we go on to test if they are also ecologically differentiated. For herbivorous insects, larval performance is particularly likely to adapt to differences in the host plants available to different populations, especially under the severe time constraints faced by alpine species. Throughout its geographical range, *O. elongata* varies in host plant use. Populations can be found that encounter only *Adenostyles* (*A. alliariae*, *A. glabra*, or *A. leucophylla*), only *Cirsium spinosissimum*, and occasionally those with access to both (Fig. 1). In mixed-host sites, beetles move repeatedly between species so would be expected to maintain adaptations to both genera (Gotthard *et al*. 2005). The two genera of host plants provide the insects with different forms of protection (Dobler *et al*. 1996). When feeding on *Adenostyles* species, the beetles sequester plant defence chemicals, pyrrolizidine alkaloids, and use them for their own defence. Conversely, beetles feeding on *Cirsium* synthesize cardenolides from sterol precursors to offset the absence of sequestrable defensive compounds in the host. Additionally, eggs and larvae of *O. elongata* on *C. spinosissimum* enjoy mechanical protection due to the numerous spines and hairs present on the host leaves (Ballabeni *et al*. 2001; Gotthard *et al*. 2005). The differences in host plant chemistry and the variation in host availability that forces specialization in these isolated populations is likely to result in local adaptation in larval performance (Mopper 1996). We compare larval growth on different hosts to test for local adaptation and use this data to answer the question: (iii) *Are genetically distinct populations also ecologically distinct?*

A history of recolonization will leave traces in the genetic structure of a species (Hewitt 1996, 2000, 2004). This structuring will be influenced by the sources and rates of colonization, and possibly by adaptation en route if this alters the probability of establishment in other sites. Although much has been written on postglacial recolonization in Europe, there is little known about the glacial refugia of montane insects. The refugia proposed for plants within the Alps (Schönswetter *et al*. 2005) may well have been shared by insects. The current distribution of *O. elongata* coincides with the locations of the refugia of alpine plants, and a test of genetic differentiation between these regions will allow us to answer a final question: (iv) *Did montane insects survive the last glaciation* in situ *in the Alps?*

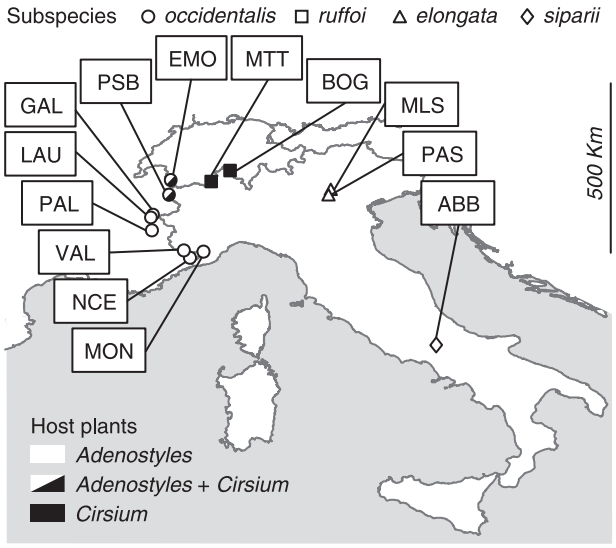

Fig. 1 The 13 populations of *Oreina elongata* sampled for the microsatellite analysis. Symbols vary between subspecies and shading indicates the host plants available in the field. Populations: ABB, Pizzone I ($n = 24$); BOG, Bosco Gurin CH ($n = 24$); EMO, Emosson dam CH ($n = 24$); GAL, Col du Galibier F ($n = 21$); LAU, Col du Lautaret F ($n = 24$); MLS, Giazza I ($n = 12$); MON, Monte Mongioie I ($n = 24$); MTT, Mattmark dam CH ($n = 24$); NCE, L'Authion F ($n = 24$); PAL, Lac Palluel F ($n = 21$); PAS, Monte Pasubio I ($n = 35$); PSB, Col du Petit Saint-Bernard F ($n = 24$); VAL, Terme di Valdieri I ($n = 24$).

Materials and methods

Ecological differentiation

Study populations. Beetles were collected during the last week of June 2002 from five populations: Mattmark dam (MTT) and Bosco Gurin (BOG) in the Swiss Alps, where the beetles are found on *Cirsium spinosissimum*; Col du Lautaret (LAU) in the Vanoise region of France and L'Authion (NCE) around Nice in France, where the beetles live on *Adenostyles glabra* exclusively; and Col du Petit Saint-Bernard (PSB) on the border between France and Italy, where they feed on both *C. spinosissimum* and *Adenostyles alliariae* in mixed patches (Verdon *et al*. 2007).

Experimental design. All experiments were conducted at the Col du Petit Saint-Bernard. Field-collected females were kept individually and their eggs placed according to family in Petri dishes with a moistened chalk bottom covered with a filter paper to maintain humidity.

Seven families of 12 larvae from each population were then used to compare larval performance on different host plants. Larvae from each family were assigned to one of three diets (*A. alliariae*, *A. glabra* and *C. spinosissimum*), weighed, and reared in individual Petri dishes like those described above where they were provided with a cutting of their treatment host plant. Every third day, each larva was weighed, its food renewed and the chalk moistened. When they reached a weight of 30 mg, larvae were transferred to plastic boxes containing a cutting of their treatment host plant and soil in which to pupate and overwinter. On 23 September 2002, larvae were dug out and weighed to obtain their prepupal weight.

Statistical analysis. After ln transformation of the weight data, regression lines were fitted to the individual growth trajectories. The slope of each line estimates the ln of the exponential growth factor and was used as a measure of growth rate (Margraf *et al*. 2003).

The effects of diet, origin (host plant in the source population), population, family and their interactions on growth rate and prepupal weight were tested with mixed-model analyses of variance (ANOVAS). Diet, origin, population and their interactions were considered as fixed factors whereas family and the family–diet interaction were considered random effects. Family was nested within population, which was in turn nested within origin. Analyses were carried out using the JMP statistical package with type III sums of squares (SAS 1989).

Heritability values and genetic correlations between performance on pairs of hosts are not presented here, because for both performance traits, there was no significant genetic variation found among families in the ANOVAS (Table 1), and family terms were not significant in one-way ANOVAS carried out separately for each population/diet combination.

Genetic differentiation

Study populations. During the summer 2001, beetles were collected from 13 populations of *Oreina elongata* throughout the Alps and Apennines, including the five used in the ecological experiment, and representing four of the seven subspecies and several combinations of host plants (see Fig. 1 for ecological details and sample sizes). They were starved overnight to avoid contamination by plant material contained in the gut, preserved in pure ethanol and stored at –80 °C as quickly as possible.

Molecular methods. DNA was extracted using the PUREGENE kit (Gentra Systems) from the head, thorax and legs only, to ensure that no plant material contained in the gut could contaminate the samples.

In total, 305 individuals were scored for six autosomal microsatellite loci, running each individual twice on separate gels. For more information on the loci and the laboratory protocol, refer to Margraf *et al*. (2005).

Statistical analysis. For some loci, null alleles were fixed in certain populations (individuals repeatedly failed to amplify,

Table 1 ANOVAs for the two performance parameters: larval growth rate and prepupal weight. 'Origin' is a term coding for the host plant in the source population

	SS	MS	DF	*F*	*P*
Response: growth rate					
Origin	0.07418	0.03709	2	170.931	< 0.001
Population [origin]	0.00266	0.00133	2	6.438	0.008
Family [population, origin]	0.00544	0.00021	26	0.811	0.716
Diet	0.01458	0.00729	2	29.232	< 0.001
Origin * diet	0.00474	0.00118	4	5.007	< 0.001
Population * diet [origin]	0.00113	0.00028	4	1.060	0.390
Family * diet [population, origin]	0.01348	0.00026	52	1.167	0.228
Response: prepupal weight					
Origin	2003.26	1001.63	2	47.093	< 0.001
Population [origin]	6.70	3.35	2	0.095	0.910
Family [population, origin]	759.07	30.36	25	1.633	0.068
Diet	87.19	43.59	2	2.443	0.095
Origin * diet	144.12	36.03	4	2.253	0.066
Population * diet [origin]	96.44	24.11	4	1.202	0.327
Family * diet [population, origin]	936.81	18.74	50	1.352	0.079

SS, sum of squares; MS, mean square; DF, degrees of freedom; F, Fratio.

while giving polymerase chain reaction products for other loci). MICROCHECKER (Van Oosterhout *et al*. 2004) was used to produce a corrected data set by adjusting allele frequencies individually for each locus and population to take into account the frequency of null alleles estimated with Brookfield's ESTIMATOR 2 (Brookfield 1996). This software identifies the presence of null alleles by a characteristic excess of homozygotes that is evenly distributed across homozygote classes, but is not found at all loci (because that would suggest inbreeding). It assumes a single null allele per locus. All estimates of population differentiation were carried out using both the real data and the corrected data set and the values were usually very similar, but unless specified the results presented here were calculated using the corrected data set. At the same time, MICROCHECKER was used to test for another source of errors in microsatellite genotyping, large allele dropout, which was found to be absent.

Linkage disequilibrium between loci was tested using ARLEQUIN (Schneider *et al*. 2000). Departure from Hardy–Weinberg equilibrium (HWE) was assessed using exact tests given by GENEPOP (Raymond & Rousset 1995), as was genetic differentiation among populations. The program uses the procedure of Weir & Cockerham (1984) to give multilocus estimates of Wright's *F* statistics, F_{IT}, F_{IS} and F_{ST} (Wright 1969), and also calculates R_{ST} (Slatkin 1995). The package was used to estimate isolation by distance [for both F_{ST} against distance and $F_{ST}/(1 - F_{ST})$ against ln(distance)], and the number of effective migrants (Nm) using the private alleles method (Slatkin 1985) to compare with that obtained by transforming F_{ST} (Wright 1969). The program DIPLINTA.F (Barker 2005) was used to test the significance of *F* statistics based on the genotype frequencies in the corrected data set, using *t*-tests based on standard errors estimated by jackknifing across populations.

The genetic distinctness of subspecies was first assessed using Mantel tests. Nei's standard genetic distances between populations were estimated using the R 4 package (Casgrain & Legendre 1997). To assess their influence on genetic differentiation in *O. elongata*, population affiliation to subspecies and geographical distances between populations were used to calculate similarity matrices that were then transformed into distance matrices (d = 1–s). We performed Mantel tests (999 permutations) using the R 4 package to assess the correlation coefficient between pairwise population genetic distances and the two distance matrices. Second, a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) was used to estimate the partitioning of genetic variance among subspecies, among populations within subspecies and within populations using GENALEX (Peakall & Smouse 2006). Finally, the grouping of populations was examined by cluster analysis. MICROSATELLITE ANALYSER (Dieringer & Schlotterer 2003) was used to bootstrap (999 permutations) the calculation of genetic distance between each pair of populations. The 999 distance matrices were then used as input files for the NEIGHBOR and CONSENSE procedures in PHYLIP (Felsenstein 2005) to produce a UPGMA tree with bootstrapped nodes. Very similar topologies were recovered when using *D*s (Nei 1978), *D*a (Nei *et al*. 1983), *D*c (Cavalli-Sforza & Edwards 1967) and with UPGMA and neighbour-joining methods. We present only *D*s, which is thought to most accurately represent branch lengths (Takezaki & Nei 1996).

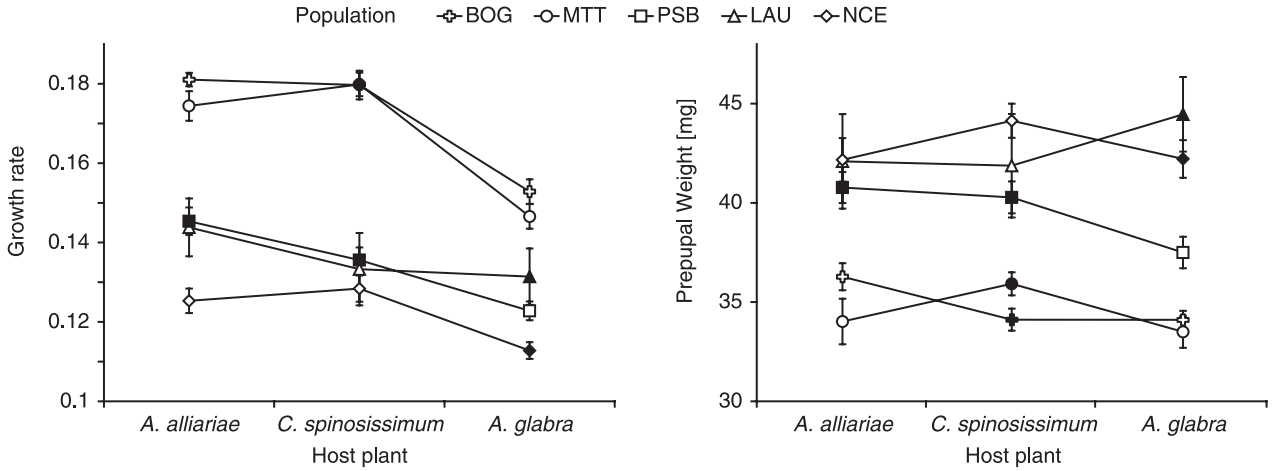

Fig. 2 Interaction plots of the two performance parameters, larval growth rate (see methods) and prepupal weight, showing means and standard errors on each host plant, with the natural hosts shaded.

To test if ecological resemblance is correlated with genetic similarity, a matrix of larval performance was created using mean growth rate and mean prepupal weight on each of the three host plants for each of the five populations treated in the ecological analysis (a matrix of five populations × six performance variables). This data was standardized and turned into a Euclidean distance matrix, which was then compared to the Nei's genetic distance matrix for those same five populations using a Mantel test (999 permutations). In addition, populations were clustered according to genetic and performance distances in two separate trees using the UPGMA method.

Finally, to test the hypothesis of alpine glacial refugia, the AMOVA and Mantel tests were repeated after excluding the Apennine population, ABB, to check for significant genetic differentiation between the groups of populations separated in putative glacial refugia.

Results

Ecological differentiation

Growth rate differed significantly according to the plant available in the source population (origin term in Table 1). Overall, beetles from populations that encounter only *Cirsium* (MTT and BOG) grew faster than those from the three other populations (Fig. 2). Diet also had a significant effect and there was a significant interaction between diet and host of origin. There was significant variation between populations within the origin groups, but neither family nor its interaction with diet were statistically significant.

Origin was the only factor that had a significant effect on prepupal weight (Table 1). Despite their higher growth rate, larvae from MTT and BOG reached a lower weight before pupation (Fig. 2).

Table 2 *F* statistics for *Oreina elongata*. Standard deviations (in parentheses) were estimated using DIPLINTA.F by jackknifing over populations and were used to calculate significance with *t*-tests

Locus	F [F_{IT}]	Θ [F_{ST}]	f [F_{IS}]
CAA3	0.227 (0.081)***	0.219 (0.074)***	0.010 (0.054)
CAA5	0.318 (0.056)***	0.272 (0.040)***	0.063 (0.044)***
CAB1	0.702 (0.129)***	0.530 (0.116)***	0.354 (0.211)***
CAB6	0.682 (0.097)***	0.580 (0.089)***	0.232 (0.112)***
CAO7	0.390 (0.085)***	0.368 (0.061)***	0.036 (0.115)
TGG6	0.578 (0.132)***	0.479 (0.252)***	0.250 (0.206)***
All	0.449 (0.092)***	0.381 (0.069)***	0.106 (0.055)**

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Genetic differentiation

All six microsatellite loci were variable, ranging in polymorphism between three and 15 alleles. Three out of the six loci (CAB1, CAB6, CAO7) did not amplify in the individuals from BOG, MTT and PAS, CAO7 did not amplify in ABB individuals and CAB1 in MLS. This suggests changes in the annealing site of the primers, resulting in null alleles. MICROCHECKER (Van Oosterhout *et al*. 2004) gave estimates of null alleles frequencies of 2.3% to 45.7% (overall null allele frequencies: CAA3 26.1%; CAA5 19.8%; CAB1 45.7%; CAB6 32.0%; CAO7 43.1% and TGG6 2.3%). Exact tests for departure from Hardy–Weinberg equilibrium on the original data indicated a significant deficit of heterozygotes for four out of six loci (CAA3, CAA5, CAB1 and CAB6) and overall through the multilocus test. Hierarchical analysis confirmed the deficit of heterozygotes within populations (F_{IS} = 0.396). Correcting the data set for null alleles decreased the overall value of F_{IS} to 0.106 (Table 2). However, the exact tests for departure from

Table 3 Estimates of population differentiation (F_{ST} and R_{ST}) per locus and overall, for the original data and the data set corrected for the presence of null alleles. Values of R_{ST} in parentheses were calculated after excluding the complex locus, CAB1

	F_{ST}	R_{ST}
Original data set		
CAA3	0.288	0.544
CAA5	0.360	0.681
CAB1	0.424	0.446
CAB6	0.558	0.692
CAO7	0.241	0.211
TGG6	0.427	0.369
All	0.367	0.561 (0.570)
Corrected data set		
CAA3	0.219	0.435
CAA5	0.272	0.614
CAB1	0.530	0.484
CAB6	0.580	0.768
CAO7	0.368	0.050
TGG6	0.479	0.369
All	0.381	0.498 (0.499)

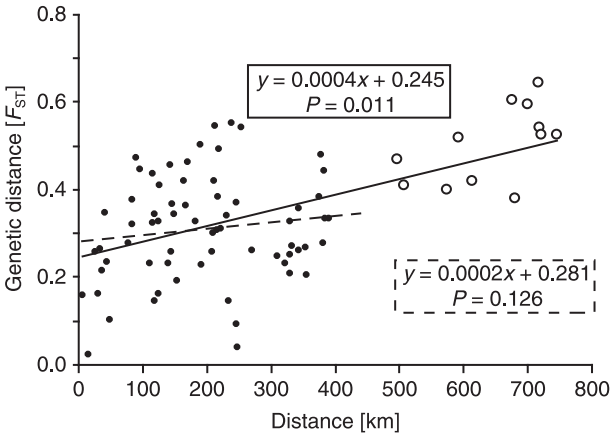

Fig. 3 Plot of genetic distance against geographical distance. *P* values were calculated using Spearman's rank correlation coefficients in GENEPOP. The solid line includes all populations and the dashed line all populations but the most distant one, ABB (for which the values are shown as open circles).

HWE remained significant, which suggests that in addition to the presence of null alleles, there is also significant inbreeding. We found no linkage disequilibrium between the loci and no difference between males and females in F_{ST} or F_{IS} values.

The overall F_{ST} value revealed very high genetic differentiation among populations ($F_{ST} = 0.381$), and the estimates were reasonably consistent and significant across all loci (Table 2). R_{ST}, the measure of differentiation designed especially to accommodate the high polymorphism of microsatellites (Slatkin 1995), gave a higher value of 0.498 (Table 3). While five of the loci are simple repeats (four dinucleotides and one trinucleotide), CAB1 has a more complicated motif (an interrupted dinucleotide) (Margraf *et al*. 2005). This locus is less likely to meet the assumptions of the measure, but calculating R_{ST} after excluding it gave an almost identical value ($R_{ST} = 0.499$). The estimated number of migrants was reasonably consistent between the private allele method (Nm = 0.251) and Wright's method (Nm = 0.406).

A comparison of the results of calculations based on the original data and that produced by MICROCHECKER shows that despite the addition of a high frequency of null alleles, this correction produced very little change in the values for F_{ST} and R_{ST} (Table 3).

Pairwise estimates of F_{ST} between populations ranged from 0.022 to 0.644 over geographical distances of between 6.5 and 746 km. The isolation-by-distance (IBD) test performed by GENEPOP (for F_{ST} against distance: slope = 0.0004, intercept = 0.2446, $P = 0.011$, for $F_{ST}/(1 - F_{ST})$ against ln(distance): slope = 0.2045, intercept = –0.4956, $P = 0.009$) confirmed the Mantel correlation between pairwise genetic distance and population geographical distance ($r = 0.692$, $P < 0.001$). They show a very shallow but significant IBD relationship among the sampled populations (solid line in Fig. 3). However, if the most distant of populations, ABB, is removed from the analysis the relationship disappears (slope = 0.0002, intercept = 0.2810, $P = 0.126$, dashed line in Fig. 3).

The division of populations into four subspecies was strongly significant with a Mantel *r* of 0.546 ($P = 0.001$). This is confirmed by the AMOVA (Table 4) and can be seen in the UPGMA diagram based on Nei's *D*s (Fig. 4), where

Table 4 The analysis of molecular variance (AMOVA) among subspecies. Values in parentheses indicate results when excluding the locus CAB1

Source of variation	SS	MS	DF	%	Statistics	*P*
Among subspecies	80 013	26 671	3	62 (47)	$R_{RT} = 0.619$ (0.473)	0.001 (0.001)
Among populations/subspecies	13 237	1 471	9	9 (13)	$R_{SR} = 0.228$ (0.245)	0.001 (0.001)
Individual/within populations	60 877	102	597	29 (40)	$R_{ST} = 0.706$ (0.602)	0.001 (0.001)
Total	154 127	28 244	609			

SS, sum of squares; MS, mean square; DF, degrees of freedom; %, percentage of variation explained.

Table 5 The analysis of molecular variance (AMOVA) among groups of populations from supposed refugia. Values in parentheses indicate results when excluding the locus CAB1

Source of variation	SS	MS	DF	%	Statistics	*P*
Among refugia	75 098	37 549	2	65 (50)	R_{RT} = 0.649 (0.501)	0.001 (0.001)
Among populations/refugia	13 237	1 471	9	8 (12)	R_{SR} = 0.219 (0.234)	0.001 (0.001)
Individual/within populations	58 861	107	550	27 (38)	R_{ST} = 0.726 (0.618)	0.001 (0.001)
Total	147 196	39 127	561			

SS, sum of squares; MS, mean square; DF, degrees of freedom; %, percentage of variation explained.

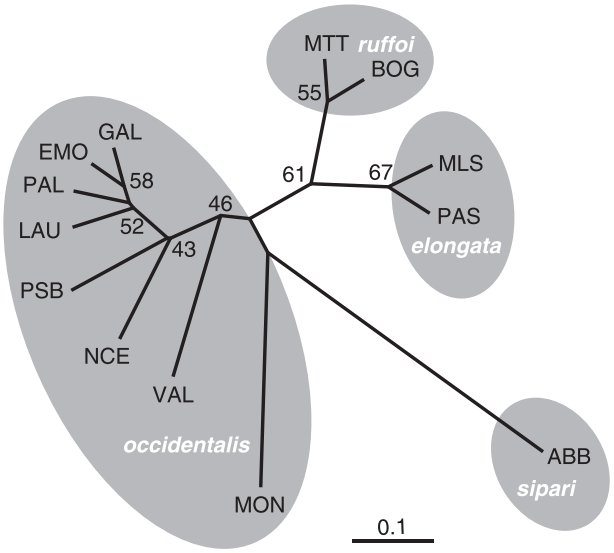

Fig. 4 UPGMA diagram of the 13 populations of *Oreina elongata* based on Nei's standard genetic distances (*D*s). Values at the nodes of the branches indicate bootstraps from 999 iterations, showing only those above 40%. Shaded areas group populations by subspecies.

only MON falls outside of a perfect clustering according to subspecies.

Mantel tests showed a high correlation between the distance based on mean larval performance of the five populations and their genetic distances (Mantel $r = 0.681$) although it was not statistically significant ($P = 0.106$). Nevertheless, the topologies of the two trees are perfectly congruent (Fig. 5).

The genetic distinctness of populations from different possible refugia in the Alps was verified by the significant Mantel correlation ($r = 0.467$, $P = 0.009$), and the AMOVA (Table 5).

Discussion

Are mountain populations genetically distinct, suggesting that they are isolated?

The populations of *Oreina elongata* showed a very high degree of genetic differentiation, with F_{ST} of 0.381. This corresponds to an estimate of around two migrants every five generations (Nm = 0.406), consistent with the prediction that due to the absence of flight in this species and to the difficulty in crossing unsuitable habitats in valleys, these high-altitude populations are very isolated. In fact, migration is likely to be absent. According to a mark–recapture study at the Col du Petit Saint-Bernard (PSB), the maximum dispersal distance observed was 80 m over 2 years (D. Conconi, unpublished results). Moreover, a recent genetic study at the same site using RAPD (random amplified polymorphic DNA) analyses has shown genetic differentiation between patches of *O. elongata* separated by only 1 km (Hirter 2003). The system is therefore unlikely to have reached an equilibrium between migration and drift, and the very high value of F_{ST} is probably the result of zero migration and the gradual decay of genetic similarity since population founding, rather than constant very low migration (Whitlock & McCauley 1999).

This interpretation is supported by the very weak or nonexistent IBD relationship, in which F_{ST} only increases

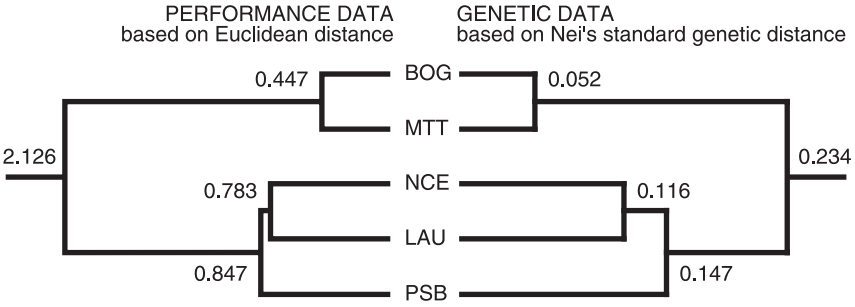

Fig. 5 UPGMA diagrams based on Euclidean distance in the mean larval performance data and on Nei's standard genetic distances between the five tested populations. Values at the nodes of the branches indicate distances at which the two groups join.

by 0.04 per hundred kilometres and all pairwise values of F_{ST} are consistently high (from 0.022 to 0.644 over distances of 6.5–746 km). As expected for a 'sedentary species', gene flow across the entire spatial scale is so weak that genetic differentiation barely correlates with geographical distance (Peterson & Denno 1998). Any similarity that exists between populations is likely to be a remnant of the original colonization process.

Correction of the allele frequencies for the presence of null alleles using MICROCHECKER had very little effect on the per-locus and overall values of F_{ST}. This is despite relatively high estimates of null allele frequencies, and suggests that the presence of null alleles in a data set will not necessarily greatly bias estimates of genetic differentiation between populations.

There are surprisingly few other studies of genetic differentiation in high-altitude species within the Alps. A brief survey reveals an eclectic mix of organisms, which together suggest that, as would be expected, the Alps do indeed introduce genetic structure into species distributions. For instance, strong genetic differentiation between populations within the range is seen in the caddisfly *Drusus discolor* (Pauls *et al*. 2006), the Norway spruce *Picea abies* (Gugerli *et al*. 2001), the globeflower *Trollius europaeus* (Despres *et al*. 2002), and *Eryngium alpinum* (Gaudeul *et al*. 2000). In more mobile species, F_{ST} is lower but still significant, for instance in the larch budmoth *Zeiraphera diniana* (Emelianov *et al*. 2004) and the burnet moth *Zygaena exulans* (Schmitt & Hewitt 2004). Mountains elsewhere have a similar effect, dividing high-altitude species as well as forming barriers between populations of lowland species (Godt *et al*. 1996; Knowles 2001; Crespi *et al*. 2003; Ruiz-Garcia 2003; Tremetsberger *et al*. 2003; DeChaine & Martin 2005; Ge *et al*. 2005).

Do the subspecies represent genetically distinct subgroups?

The UPGMA diagram based on genetic distances clusters the populations almost perfectly according to the four subspecies, indicating that they form distinct genetic subgroups. Only MON falls outside of its subspecies and is grouped in the analysis marginally more closely with ABB, but this could be the result of long-branch attraction. According to the Mantel test and AMOVA, subspecies described on the basis of morphological characters appear to be supported by genetic data. This result suggests that morphological differentiation as measured by subspecies description (Daccordi & Ruffo 1976, 1986) is correlated with genetic differentiation at microsatellite loci.

Are genetically distinct populations also ecologically distinct?

Very low or nonexistent migration between populations would allow their unimpeded divergence by both genetic drift and local adaptation. The populations of *O. elongata* differed in life-history traits depending on the plant available at the site of their origin and this response varied between diets (i.e. for growth rate, the origin, diet and origin × diet terms were significant in the ANOVA). However, we found no strong evidence of local adaptation in the analysis: in general, populations did not grow faster on their natural host(s), either in comparison with other populations grown on that host, or with their own performance on alternative hosts. In particular, all populations showed a reduced growth rate on *Adenostyles glabra*, suggesting that this plant constitutes a low-quality host. This is perhaps related to the presence of additional toxins, sesquiterpenes, in the leaves of *A. glabra* (Hägele & Rowell-Rahier 1999; then called *Adenostyles alpina*). Nevertheless, the smallest difference between larval growth rate on *A. glabra* and on the two other hosts was found for NCE and LAU, the two populations found naturally on *A. glabra*.

The *Dreina elongata ruffoi* populations (MTT and BOG) showed a higher larval growth rate and a lower prepupal weight than the *O. e. occidentalis* populations across all diets. This suggests a different life-history strategy, which might be related to differences in the availability of defensive compounds at these sites. MTT and BOG populations live on *Cirsium spinosissimum* exclusively and cannot take advantage of the pyrrolizidine alkaloids (PAs) produced by *Adenostyles* species. Instead, they rely on the synthesis of cardenolides, which have been shown to be less effective than PAs in protecting the beetles from predators, at least from naïve birds (Rowell-Rahier *et al*. 1995). Beetles that have a shorter developmental time and therefore a reduced period of exposure to predators might have been selected in these PA-free populations. This result confirms the independent evolution of the subspecies and reveals a secondary adaptation to the host plant present in the field based on defence chemistry.

Local adaptation can be demonstrated when each deme shows improved fitness in its own habitat relative to foreign demes brought into that habitat (Kawecki & Ebert 2004). An earlier study had concluded that there was evidence for slight local adaptation in two populations of *O. elongata*, but did not actually include their natural hosts (Ballabeni *et al*. 2003). Here, we do not see a clear pattern of local adaptation in these populations due to the two masking effects of (i) the poor quality of *A. glabra*, and (ii) the accelerated life history strategy of the *O. e. ruffoi* group. However, the populations performed better on their natural host than could be expected on the basis of the quality of the host. This constitutes a weak criterion of local adaptation since some populations consistently outperform others on all hosts. Yet it indicates that even though not sufficient to detect the final result, selection might still be acting in an ongoing process of local adaptation in these populations. Among other traits, oviposition choice shows no evidence

Fig. 6 Map of Italy and neighbouring countries showing the congruence between the distribution of the subspecies of *Oreina elongata* (shaded areas and names, modified from Daccordi & Ruffo 1976) and the glacial refugia proposed for alpine plants (dashed outlines and numbers, modified from Schönswetter *et al*. 2005).

of local adaptation to host availability (these same populations all prefer *C. spinosissimum*, Verdon *et al*. 2007).

For this species, ESUs defined at the level of subspecies would fall along the major lines of separation in terms of genetic distinctness. This would also capture the subdivision according to ecological differentiation as this is correlated with genetic distinctness. Ecological data may therefore not be necessary to define groups that encompass the ecological diversity within a species. Furthermore, shortcuts to avoid the need for complicated ecological tests may be difficult to apply: although the simplest feature to measure, host availability, was a good predictor of overall ecological differentiation within the five population data set, the detailed pattern of larval performance was not predictable from host plant use alone. This is because ecological differentiation seems to have been driven indirectly by local adaptation to plant defence chemistry, rather than by direct physiological adaptation to plant nutritional quality.

Did montane insects survive the last glaciation in situ *in the Alps?*

The very strong differentiation between populations suggests a long period of isolation. This is unlikely to be a product of drift following homogenization by postglacial recolonization. Populations of *O. elongata*, therefore, probably survived the last glaciation *in situ* within the Alps. There is a very close correspondence between the ranges of the subspecies of *O. elongata* and the glacial refugia described for alpine plants (Fig. 6). The distributions of the four alpine subspecies (*O. e. occidentalis*, *O. e. ruffoi*, *O. e. elongata* and *O. e. styriaca*, Daccordi & Ruffo 1976) coincide with the four most commonly described refugia for plants (refugia I–IV in Schönswetter *et al*. 2005). This strongly suggests that the beetles too survived the glacial periods in populations at the southwestern, southern and eastern borders of the Alps. This is further supported by the lack of similarity between the Italian ABB population and any of those in the Alps, which would refute the idea that *O. elongata* took refuge that far south during the glaciation. For alpine species, the ice ages may therefore have led to movement southwards within the mountain chain, but not the large scale migration seen in lowland species.

In summary, as predicted by the lack of flight in *O. elongata* and the barriers formed by the mountains, we found very strong genetic differentiation between populations. Yet, despite this isolation, the differences in host plant availability in the field have not given rise to the expected pattern of local adaptation of larval performance. The ice-age history of this species appears to be one of long-term isolation and subspecies formation, with adaptation of their life-history traits to the defence chemistry of the host plants.

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