

Distribution of genetic variance and isolation by distance in two leaf beetle species: *Oreina cacaliae* and *Oreina speciosissima*

STEFFI KNOLL* & MARTINE ROWELL-RAHIER

Laboratoire d'écologie animale et entomologie, Institut de Zoologie, Université de Neuchâtel, CH-2007 Neuchâtel, Switzerland

The distribution of genetic variance was investigated in two closely related *Oreina* leaf beetle species, *Oreina cacaliae* and *Oreina speciosissima*. Populations of these alpine beetles were sampled in mountainous areas of Western Europe, the total sampling area ranging from the Pyrenees to the Czech Republic. Allozyme electrophoresis of 21 (*O. cacaliae*) and 16 (*O. speciosissima*) loci revealed high genetic variability as expressed in a high percentage of polymorphic loci (only one monomorphic locus was found in each species) and high heterozygosities. No overall linkage disequilibrium was found in either species. Extensive heterozygote deficits were observed in several samples as reflected by high F_{IS} -values and high overall inbreeding coefficients (F_{IT}) of 0.349 (*O. cacaliae*) and 0.503 (*O. speciosissima*). The overall inbreeding coefficient was mainly attributable to within-population differentiation. The high heterozygote deficits are explained by a combination of inbreeding resulting in kinship groups and a sampling effect over several such kinship groups. No explanation of the observed patterns could be found in the host plant use or altitudinal location of the samples. For *O. cacaliae*, isolation by distance was found, but not for *O. speciosissima*. Gene flow estimates were in the range of $Nm = 0.8$ to 1.5.

Keywords: allozyme electrophoresis, Chrysomelidae, genetic variability, isolation by distance, *Oreina*, population structure.

Introduction

Historical, ecological and demographic processes are reflected in the genetic population structure of a species. Historically, vicariance events may lead to a biogeographical splitting of a species into two isolated and independently evolving lineages. Ecologically, intrinsic habitat discontinuities in space or time can lead to effective isolation at very small scales (Roderick, 1996 and references therein). For phytophagous insects, plant patches represent such habitat discontinuities in space that have been demonstrated as being responsible for population structuring (Guttmann *et al.*, 1989; Rank, 1992). If it changes to a new host plant species, a herbivore generally has to develop certain adaptations: to overcome plant defence mechanisms and to adapt to a different nutritional quality or to different natural enemies. Thus, different host plants (or, more generally, 'differential use of habitat') may also

cause divergence. Finally, demographic processes such as group size, dispersal and the mating system ultimately mould the genetic population structure of a species.

In the leaf beetle genus *Oreina* (Coleoptera, Chrysomelidae, Chrysomelinae) all of these factors have been proposed as being responsible for species divergence (Dobler *et al.*, 1996). Because they are mostly alpine species, these beetles must have undergone major habitat shifts during the last glaciation. Currently they live not only in geomorphologically and climatically very diverse environments, but within this habitat they also occur in locally subdivided groups on their host plant patches. Previous work has shown that there is considerable genetic variation within and between *Oreina* populations (Eggenberger & Rowell-Rahier, 1991; Rowell-Rahier, 1992). However, the limited number of populations in these studies could not provide an explanation for the observed patterns.

In this paper, the results are presented of a macrogeographical study on the distribution of

*Correspondence. E-mail: knoll@cefe.cnrs-mop.fr

genetic variability of two *Oreina* species, *Oreina cacaliae* and *O. speciosissima*. The first aim of the paper was to report and compare the genetic variability of these two sympatric sister species. Secondly, an explanation of the observed patterns was sought. Therefore, correlations were looked for between the observed genetic structure and ecological characteristics of the samples. Thereby the relative importance of different host plants, different geographical locations and different climatic regimes (as suggested from the large altitudinal range of the sites of these alpine beetles) were evaluated.

Materials and methods

Natural history of the beetles

The biogeographical range of *O. cacaliae* and *O. speciosissima* extends from north-eastern Spain, through southern France and throughout the Alps, and extends into the Karpats and the Czech and Slovakian mountains (Kühnelt, 1984). Both species are recorded between 500 m and 3000 m altitude. They occur sympatrically, feeding on the same host plant patches of perennial herbs (*Adenostyles* spp., *Senecio nemorensis-fuchsii* and *Petasites albus*), but *O. speciosissima* has a slightly broader ecological niche (concerning host plant spectrum, geographical range and type of chemical defence). Both species are ovoviviparous and have overlapping generations. Overwintered males and gravid females occur in early spring; larvae are laid from spring until early summer directly on the host plants. Larvae develop in four larval stages to adults either in the same year, or, if they are late, overwinter as L4 in the soil.

Oreina cacaliae and *O. speciosissima* have chemical defences (Hartmann *et al.*, 1997) and are aposematic. They show considerable colour polymorphism. Colour is still often used in the identification of subspecies or races (e.g. Kühnelt, 1984), although only the aedeagus is considered as a reliable character for species determination (Bourdonné & Doguet, 1991). The heritability of colour polymorphism, even though documented for other leaf beetles (Vasconcellos-Neto, 1988) is unknown for any species of the genus *Oreina*. Within populations, individuals of both species show only minor colour variations. Sympatric species of *Oreina* often show the same colour morph within one population (S. Dobler, pers. com.; pers. obs.). As the colours are thought to be warning signals to possible predators (birds), this could be mimicry and adaptation of individuals to the local dominating colour form and, consequently,

would be under strong selective control (Vasconcellos-Neto, 1988).

Sampling

Populations of the two *Oreina* species were sampled mainly in summer 1993, with some additional populations sampled during the summers of 1994 and 1995. The total sampling area ranged from the central Pyrenees to the Czech Republic (Table 1).

One sample always consisted of beetles randomly taken from only one host plant patch, a patch being defined by continuous plant cover. Adult beetles were picked by hand from their host plants, brought alive to the laboratory and stored in liquid nitrogen. Samples were always more than 5 km apart. Because host plant patches can be very large (up to 1 km²) and beetles very abundant (densities from 0.8 to 3 beetles m⁻², S. Knoll & M. Rowell-Rahier, unpubl. obs.), sometimes only a fraction of the patch was sampled (the sampled area rarely exceeded 100 m²). Earlier studies (Rowell-Rahier, 1992) have documented high F_{IS} -values for both species and explained them by a Wahlund effect. Therefore the supposedly smallest possible 'random mating unit' (beetles at the same host plant patch) was sampled to avoid sampling over more than one neighbourhood. All patches sampled were clearly dominated by one host plant species (and were classified as such), even when more than one host plant species was present in the area. Each patch was located on 1 : 25000 topographical maps and this was recorded, together with host plant species and colour of the beetles as patch characteristics (Table 1).

Allozyme electrophoresis

Thoracic muscles were homogenized in 0.1 M Tris-EDTA buffer with 2-Mercaptoethanol, pH 7.0. The following 16 enzyme systems were scored in standard horizontal starch gel electrophoresis (12% Sigma starch; for methods see Hillis & Moritz, 1990): ACOH (aconitate hydratase EC 4.2.1.3), DDH (NADH-diaphorase EC 1.8.1.4), MDHP (malate dehydrogenase EC 1.1.1.40) and GPI (glucose-6-phosphate isomerase EC 5.3.1.9) on TCA (0.001 M Tris-citrate buffer, pH 6.7); AAT (aspartate aminotransferase EC 2.6.1.1, two loci), PEP (LA) (peptidase EC 3.4.-.-, two loci), ARK (arginine kinase EC 2.7.3.3, three loci), IDH (isocitrate dehydrogenase EC 1.1.1.42, two loci) and EST (esterase, nonspecific) on TCB (0.05 M Tris-citrate buffer, pH 8.7); and FUMH (fumarate hydratase EC

4.2.1.2), FDH (formaldehyde dehydrogenase EC 1.2.1.1), GAPDH (glyceraldehyde-3-phosphate dehydrogenase EC 1.2.1.12), G3PDH (glycerol-3-phosphate dehydrogenase EC 1.1.1.8), SOD (superoxide dismutase EC 1.15.1.1, two loci), TPI (triose-phosphate isomerase EC 5.3.1.1) and AO (aldehyde oxidase EC 1.2.3.1, two loci) on EBT (0.2 M Tris-borate buffer, pH 8.6). For *O. cacaliae* 21 loci could be consistently scored, for *O. speciosissima* 16 loci (Table 2). Banding patterns of all reported

enzyme systems followed the ones reported in the literature (Hillis & Moritz, 1990).

Statistical analyses

Calculations of allele frequencies and tests for linkage disequilibrium and Hardy–Weinberg equilibrium were performed using GENEPOP version 2.0 (Raymond & Rousset, 1995). Associations of genotypes between loci were tested for each population

Table 1 Locations and environmental and ecological parameters of the sampling sites: colour-type as body colour/stripe colour

	Mountain ridge	Altitude (m)	Latitude	Longitude	Host plant	Colour-type
<i>Oreina cacaliae</i>						
Adelboden	Alps, CH	1500	46°28.10'	7°32.10'	<i>Adenostyles</i>	Blue-green/–
Albula	Alps, CH	1820	46°38.30'	9°49.20'	<i>Senecio</i>	Blue/–
Appenzell	Alps, CH	1300	47°16.00'	9°27.48'	<i>Adenostyles</i>	Blue/–
Cascade	Vosges, F	950	48°05.00'	7°05.00'	<i>Adenostyles</i>	Green/blue
Col Aubisque	Pyrenees, F	1100	42°57.00'	1°40.00'	<i>Adenostyles</i>	Green/blue
Ferret	Alps, CH	1614	45°55.20'	7°05.50'	<i>Adenostyles</i>	Blue-green/–
Hirschbach	Alps, D	1400	47°50.00'	11°40.00'	<i>Adenostyles</i>	Green/blue
Hohwald	Vosges, F	650	48°25.00'	7°20.00'	<i>Adenostyles</i>	Green/blue
Höllental	Alps, D	1100	47°25.00'	11°05.00'	<i>Petasites</i>	Green/blue
Kandersteg	Alps, CH	1490	46°28.15'	7°39.20'	<i>Adenostyles</i>	Blue-green/–
Lieserwasen	Vosges, F	850	48°57.00'	6°50.00'	<i>Senecio</i>	Green/blue
Madonna	Alps, I	1825	46°13.00'	10°49.00'	<i>Adenostyles</i>	Blue/–
Morgins	Alps, CH	1400	46°13.55'	6°47.30'	<i>Adenostyles</i>	Green/blue
Nova Pec	Karkonosze, Cz	850	48°45.00'	13°55.00'	<i>Senecio</i>	Green/blue
Ochsenalp	Alps, CH	1920	46°48.45'	9°38.55'	<i>Adenostyles</i>	Blue/–
Safien Thalkirch	Alps, CH	1700	46°37.50'	9°16.35'	<i>Adenostyles</i>	Blue/–
Schneekoppe	Karkonosze, Cz	1200	50°35.00'	15°50.00'	<i>Senecio</i>	Green/–
Tschiertschen	Alps, CH	1860	46°47.55'	9°37.00'	<i>Adenostyles</i>	Blue/–
Vals	Alps, CH	1600	46°36.00'	9°09.10'	<i>Adenostyles</i>	Blue/–
Vrin	Alps, CH	1450	46°40.10'	9°05.40'	<i>Adenostyles</i>	Blue/–
Zastler	Black Forest, D	1170	47°53.30'	8°00.20'	<i>Petasites</i>	Green/blue
<i>Oreina speciosissima</i>						
Altwater	Praded, Cz	700	50°10.00'	17°20.00'	<i>Petasites</i>	Green/blue
Appenzell	Alps, CH	1300	47°16.00'	9°27.48'	<i>Adenostyles</i>	Green/blue
Boubin	Bohemian forest, Cz	990	49°56.00'	13°50.00'	<i>Senecio</i>	Green/red
Hirschbach	Alps, D	1400	47°50.00'	11°40.00'	<i>Petasites</i>	Green/blue
Höllental	Alps, D	1100	47°25.00'	11°05.00'	<i>Petasites</i>	Green/blue
Hörnli	Alps, CH	2300	46°45.50'	9°36.50'	<i>Cirsium</i>	Black/–
Kiental	Alps, CH	1350	46°38.00'	7°45.00'	<i>Adenostyles</i>	Green/blue
Kralov	Praded, Cz	700	50°10.00'	17°20.00'	<i>Senecio</i>	Green/blue
La Lecherette	Alps, CH	1150	46°27.00'	7°06.55'	<i>Petasites</i>	Green/blue
Morgins	Alps, CH	1400	46°13.55'	6°47.30'	<i>Adenostyles</i>	Green/blue
Nova Pec	Karkonosze, Cz	850	48°45.00'	13°55.00'	<i>Senecio</i>	Green/red
Safien-Rainmatte	Alps, CH	1416	46°42.20'	9°19.30'	<i>Petasites</i>	Green/blue
Tschiertschen	Alps, CH	1730	46°48.05'	9°36.50'	<i>Adenostyles</i>	Green/blue
Vrin	Alps, CH	1450	46°40.10'	9°05.40'	<i>Adenostyles</i>	Green/blue
Zamecek	Bohemian forest, Cz	750	50°60.00'	13°60.00'	<i>Senecio</i>	Green/red
Zastler	Black Forest, D	1170	47°53.30'	8°00.20'	<i>Petasites</i>	Green/blue

separately. To test for Hardy–Weinberg equilibrium, results of the probability test are presented, which corresponds to the exact test for Hardy–Weinberg equilibrium. Where there are more than four alleles at a locus, instead of the complete enumeration a Markov chain method was used to estimate, in 100 batches and 1000 iterations per batch, the P -values and standard errors (Raymond & Rousset, 1995). For all tests a sequential Bonferroni correction for multiple comparisons was applied.

As measures of genetic variability the number of alleles per locus, percentage of loci polymorphic and the unbiased heterozygosity estimate as well as observed heterozygosity were calculated, using the program BIOSYS-1 (Swofford & Selander, 1981).

Unbiased estimates of F -statistics were calculated with FSTAT (Goudet, 1995) according to the formulae given in Weir & Cockerham (1984). Standard deviations for single-locus estimates were calculated by jackknifing over populations, and standard errors for means by jackknifing over loci.

A possible relationship was tested between, as dependent variables, the observed genotype and one morphological trait (colour) and, as possible

Table 2 Numbers of populations of *Oreina* fixed (frequency of the most common allele $p = 1$) or polymorphic (frequency of the most common allele $p < 0.95$; 95% criterion) for different loci

Locus	<i>O. speciosissima</i>		<i>O. cacaliae</i>	
	$p = 1$	$p < 0.95$	$p = 1$	$p < 0.95$
<i>Sod2</i>	12	4	—	—
<i>Aat</i>	2	14	1	19
<i>Acoh</i>	7	9	4	15
<i>Ao1</i>	1	15	1	18
<i>Ark2</i>	14	1	18	0
<i>Ddh</i>	9	5	2	18
<i>Fdh</i>	7	7	0	19
<i>Fumh</i>	6	10	11	5
<i>Gapdh</i>	0	15	0	21
<i>Gpi</i>	2	14	13	6
<i>Idh1</i>	10	3	18	1
<i>Idh2</i>	14	0	19	1
<i>Pep(La)1</i>	1	14	7	13
<i>Pep(La)2</i>	5	10	1	20
<i>Sod1</i>	14	1	2	15
<i>Tpi</i>	1	14	4	15
<i>Ao2</i>	—	—	1	20
<i>Ark1</i>	—	—	11	10
<i>Ark3</i>	—	—	10	7
<i>Est</i>	—	—	1	20
<i>G3pdh</i>	—	—	1	20
<i>Mdhp</i>	—	—	0	20

explanatory factors, host plant association and geographical and altitudinal isolation (nonparametric Mantel tests, e.g. Manly, 1985). The following five distance matrices were used: the genetic distance matrix (Rogers's modified genetic distance); a 'colour-type' matrix; the geographical distance matrix; the altitudinal distance matrix; and a 'host plant' matrix. For the 'colour-type' matrix, the observed colours were coded in distinct classes and the distance between samples with beetles of the same colour was coded as 0 and that between samples with beetles of different colours as 1. In the same way, the 'host plant' matrix coded the distance between patches dominated by the same plant species with 0, and that between patches dominated by different plant species with 1. Latitude and longitude of the sampling sites and altitude were recorded from 1 : 25000 topographical maps and the distance matrices were calculated from these. For each patch these characteristics are given in Table 1. Mantel tests were performed with the program R (Legendre & Vaudor, 1991). Based on 10000 permutations, r -values and the significance of correlations are reported.

To account for correlations between the explanatory matrices (geographical distance, altitude and host plant), partial Mantel tests were conducted following the method of Smouse *et al.* (1986) whenever appropriate.

Isolation by distance was further tested as described by Slatkin (1993). N_m -values as measures of gene flow were calculated by the private alleles method adjusted for sample sizes (Barton & Slatkin, 1986) and compared to those calculated from F_{ST} -values.

Results

Allele frequencies

These are available from the authors on request. For each species only one locus was monomorphic (95% criterion) in all samples (*Ark2* in *O. cacaliae* and *Idh2* in *O. speciosissima*). All loci except *Gapdh* for both species and *Fdh* and *Mdhp* for *O. cacaliae* were also fixed in at least one sample, some samples being fixed for different alleles (Table 2). A mean of 6.1 alleles per locus was found in *O. cacaliae* and of 5.9 alleles per locus in *O. speciosissima*; when excluding rare alleles ($p < 0.05$) these numbers decreased to 2.2 and 2.1, respectively.

No latitudinal, longitudinal or altitudinal cline was found for any of the alleles with an overall frequency of $p > 0.10$.

Linkage disequilibrium

1 *Oreina speciosissima*. After applying sequential Bonferroni procedures only one combination of loci out of 158 possible tests gave a significant *P*-value at the 5% level, *Fumh/Aat* in Safien-Rainmatte.

2 *Oreina cacaliae*. Out of 1987 possible tests, six showed a significant deviation from random distribution of genotypes after applying sequential Bonferroni corrections: in Ferret *Ddh/Tpi*; in Kandersteg *Acoh/Ark2*; in Zastler *Ddh/Tpi*; and in Lieserwasen *G3pdh/Gapdh*, *Acoh/Pep(La)2* and *G3pdh/Mdhp*.

Hardy–Weinberg equilibrium

1 *Oreina speciosissima*. With the exact test, 24.6% (31 out of 126 possible tests) were significantly different from Hardy–Weinberg expectations after applying Bonferroni correction. Overall, for 76.9% of the polymorphic loci genotypic distribution deviated from Hardy–Weinberg equilibrium. Not surprisingly, the over all loci, over all populations test showed highly significant deviation from Hardy–Weinberg equilibrium ($\chi^2_{176} = \infty$, $P < 0.001$).

2 *Oreina cacaliae*. With the exact test, 13.6% (39 out of 287) possible tests were significantly different from Hardy–Weinberg expectations after applying Bonferroni correction. Overall, for 52.4% of the polymorphic loci genotypic distribution deviated from Hardy–Weinberg equilibrium. Again, the over all loci, over all populations test showed highly significant deviation from Hardy–Weinberg equilibrium ($\chi^2_{432} = \infty$, $P < 0.001$).

Genetic variability measures

The number of alleles per locus found in single samples was the only measure of genetic variability dependent on sample size and was therefore not considered. Percentage of polymorphic loci for *O. cacaliae* ranged from 33.3% (Nova Pec) to 80.9% (Adelboden and Ferret), and for *O. speciosissima* from 31.3% (Zamecek) to 81.3% (Safien-Rainmatte). Unbiased heterozygosity values for *O. cacaliae* ranged from 0.138 (Nova Pec) to 0.365 (Ochsenalp), and for *O. speciosissima* from 0.140 (Appenzell) to 0.370 (Safien-Rainmatte) (Table 3).

As for single alleles, no significant correlation of any measure of genetic variability with latitude, longitude or altitude could be found.

F-statistics

1 *Oreina speciosissima*. A wide range of F_{IS} -values was observed in the different samples, ranging from

–0.072 to +0.637 (Table 3). The overall inbreeding coefficient was high, with a value of F_{IT} of 0.503 ± 0.056 . This was mostly attributable to the within-population component ($F_{IS} = 0.350 \pm 0.042$), although there was also considerable among-population differentiation ($F_{ST} = 0.236 \pm 0.043$).

Table 3 Variability measures, F_{IS} -values and date of sampling (H.unb., unbiased estimate of heterozygosity; H.dc, observed heterozygosity; % loci, % loci polymorphic (95% criterion); F_{IS} , mean F_{IS} -value; date of sampling: (1) before larviposition; (2) after larviposition but before emergence of the new generation adults; (3) after the emergence of the new generation adults

	H.unb.	H.dc	% loci	F_{IS}	Date
<i>Oreina cacaliae</i>					
Adelboden	0.337	0.257	80.9	0.270	2
Albula	0.331	0.224	66.7	0.368	2
Appenzell	0.305	0.274	76.2	0.102	2
Cascade	0.252	0.169	71.4	0.301	1
Col Aubisque	0.222	0.155	57.1	0.284	2
Ferret	0.358	0.283	80.9	0.164	1
Hirschbach	0.286	0.203	76.2	0.348	1
Hohwald	0.299	0.203	66.7	0.276	1
Höllental	0.256	0.178	57.1	0.328	1
Kandersteg	0.339	0.244	66.7	0.282	1
Lieserwasen	0.278	0.215	57.1	0.222	1
Madonna	0.197	0.124	52.4	0.377	3
Morgins	0.345	0.369	71.4	–0.104	1
Nova Pec	0.138	0.103	33.3	0.391	2
Ochsenalp	0.365	0.300	71.4	0.186	2
Safien Thalkirch	0.291	0.221	66.7	0.265	3
Schneekoppe	0.234	0.175	47.6	0.241	2
Tschiertschen	0.243	0.198	57.1	0.206	2
Vals	0.298	0.285	61.9	0.094	3
Vrin	0.328	0.265	61.9	0.195	3
Zastler	0.272	0.246	61.9	0.111	1
<i>Oreina speciosissima</i>					
Altwater	0.180	0.128	50.0	0.277	2
Appenzell	0.140	0.084	37.5	0.478	2
Boubin	0.194	0.174	62.5	0.049	2
Hirschbach	0.216	0.085	68.8	0.637	1
Höllental	0.175	0.097	43.8	0.415	1
Hörnli	0.268	0.197	56.3	0.301	3
Kiental	0.303	0.149	62.5	0.517	1
Kralov	0.267	0.215	62.5	0.280	2
La Lecherette	0.236	0.261	50.0	–0.072	1
Morgins	0.208	0.145	56.3	0.305	1
Nova Pec	0.164	0.159	37.5	0.042	2
Safien-Rainmatte	0.370	0.197	81.3	0.466	3
Tschiertschen	0.342	0.183	62.5	0.487	2
Vrin	0.161	0.142	50.0	0.078	3
Zamecek	0.181	0.213	31.3	–0.060	1
Zastler	0.257	0.163	56.3	0.393	1

2 *Oreina cacaliae*. Again, there was a great range of F_{IS} -values, ranging from -0.104 to $+0.391$ (Table 3). The overall inbreeding coefficient was somewhat lower than in *O. speciosissima*, ($F_{IT} = 0.349 \pm 0.024$) and, again, mostly attributable to the within-population component ($F_{IS} = 0.229 \pm 0.029$), although there was also considerable differentiation between populations ($F_{ST} = 0.155 \pm 0.028$).

For both species, loci varied in their contributions to these values, some showing F_{ST} -values not different from zero (Table 4). The high values for

Idh1 in *O. cacaliae* are caused by the single sample from the Pyrenees; removing this sample resulted in F_{IT} , F_{IS} and F_{ST} for *Idh1* not different from zero. *Idh1* is highly polymorphic in the Pyrenean sample (six alleles) and monomorphic (95% criterion) in all other samples of *O. cacaliae*.

Mantel tests

1 *Oreina speciosissima*. In the pairwise Mantel tests of the five distance matrices (genetic distance,

Table 4 F -statistics for individual loci in two *Oreina* species; standard errors were calculated by jackknifing over populations

	F_{IS}	F_{ST}	F_{IT}
<i>Oreina cacaliae</i>			
<i>Aat</i>	0.134 ± 0.100	0.355 ± 0.104	0.443 ± 0.046
<i>Acoh</i>	0.246 ± 0.093	0.428 ± 0.134	0.572 ± 0.169
<i>Ao1</i>	0.198 ± 0.021	0.034 ± 0.076	0.225 ± 0.073
<i>Ao2</i>	0.300 ± 0.017	0.063 ± 0.032	0.344 ± 0.035
<i>Ark2</i>	0.519 ± 0.082	0.183 ± 0.114	0.608 ± 0.130
<i>Ark3</i>	0.867 ± 0.013	-0.020 ± 0.268	0.863 ± 0.264
<i>Ark4</i>	0.255 ± 0.019	0.053 ± 0.127	0.293 ± 0.141
<i>Ddh</i>	0.100 ± 0.072	0.127 ± 0.079	0.215 ± 0.052
<i>Est</i>	0.184 ± 0.039	0.149 ± 0.064	0.306 ± 0.056
<i>Fdh</i>	0.171 ± 0.049	0.130 ± 0.044	0.278 ± 0.036
<i>Fumh</i>	0.246 ± 0.011	0.030 ± 0.174	0.268 ± 0.178
<i>G3pdh</i>	0.279 ± 0.019	0.071 ± 0.064	0.330 ± 0.075
<i>Gapdh</i>	0.298 ± 0.053	0.167 ± 0.058	0.416 ± 0.045
<i>Gpi</i>	0.308 ± 0.016	0.043 ± 0.155	0.337 ± 0.163
<i>Idh1</i>	0.313 ± 0.648	1.386 ± 0.685	1.475 ± 0.130
<i>Idh2</i>	0.173 ± 0.232	0.453 ± 0.304	0.587 ± 0.096
<i>Mdhp</i>	0.181 ± 0.022	0.058 ± 0.046	0.228 ± 0.046
<i>Pep(La)1</i>	0.294 ± 0.083	0.175 ± 0.059	0.418 ± 0.046
<i>Pep(La)2</i>	0.342 ± 0.060	0.124 ± 0.057	0.422 ± 0.064
<i>Sod2</i>	0.238 ± 0.054	0.158 ± 0.076	0.357 ± 0.088
<i>Tpi</i>	0.203 ± 0.051	0.117 ± 0.070	0.297 ± 0.059
<i>Oreina speciosissima</i>			
<i>Aat</i>	0.397 ± 0.083	0.256 ± 0.077	0.548 ± 0.108
<i>Acoh</i>	0.270 ± 0.013	-0.005 ± 0.127	0.266 ± 0.127
<i>Ao1</i>	0.307 ± 0.031	0.133 ± 0.097	0.399 ± 0.112
<i>Ark3</i>	-0.034 ± 0.037	0.026 ± 0.006	-0.009 ± 0.039
<i>Ddh</i>	0.672 ± 0.119	0.182 ± 0.242	0.760 ± 0.199
<i>Fdh</i>	0.602 ± 0.143	0.665 ± 0.098	0.875 ± 0.138
<i>Fumh</i>	0.720 ± 0.125	0.528 ± 0.057	0.859 ± 0.144
<i>Gapdh</i>	0.336 ± 0.056	0.215 ± 0.063	0.478 ± 0.078
<i>Gpi</i>	0.031 ± 0.027	0.091 ± 0.092	0.118 ± 0.107
<i>Idh1</i>	0.425 ± 0.017	0.136 ± 0.283	0.503 ± 0.321
<i>Idh2</i>	0.011 ± 0.009	-0.014 ± 0.002	-0.003 ± 0.009
<i>Pep(La)1</i>	0.352 ± 0.050	0.114 ± 0.087	0.424 ± 0.101
<i>Pep(La)2</i>	0.275 ± 0.029	0.091 ± 0.100	0.341 ± 0.103
<i>Sod1</i>	0.768 ± 0.045	0.062 ± 0.505	0.812 ± 0.481
<i>Sod2</i>	0.591 ± 0.046	0.273 ± 0.052	0.700 ± 0.081
<i>Tpi</i>	0.366 ± 0.091	0.307 ± 0.063	0.557 ± 0.095

Table 5 Results of the pairwise Mantel tests; significant results ($P < 0.05$) are in bold type. For details of the different distance matrices tested see text

	Genotype		Colour-type		Distance (km)		Altitude (m)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
<i>Oreina cacaliae</i>								
Genotype								
Colour-type	-0.012	0.452						
Distance (km)	0.511	0.004	0.064	0.180				
Altitude (m)	-0.023	0.458	0.430	0.001	0.182	0.089		
Host plant	0.071	0.336	-0.014	0.505	0.214	0.103	0.029	0.267
<i>Oreina speciosissima</i>								
Genotype								
Colour-type	0.403	0.002						
Distance (km)	0.233	0.048	0.681	0.002				
Altitude (m)	0.277	0.042	0.431	0.002	0.378	0.007		
Host plant	0.114	0.071	0.489	0.001	0.361	0.002	0.268	0.004

colour, host plant, geographical distance and altitude) all combinations were significantly correlated, except for the relationship between genotype and host plant (Table 5). After removing the interaction of altitude and geographical distance, geographical distance was still positively correlated with colour-type ($r = 0.62$, $P < 0.002$), but not with genetic distance ($r = 0.14$, $P = 0.142$), and altitude was not significantly correlated with either genotype ($r = 0.21$, $P = 0.131$) or colour-type ($r = 0.26$, $P = 0.082$). Geographical distance was also positively related to colour-type after removing host plant effects ($r = 0.62$, $P = 0.001$), so it can be concluded that there is a geographical component determining colour-type. However, there was still a positive host plant component on colour-type, even when geographical effects were removed ($r = 0.36$, $P = 0.003$).

2 *Oreina cacaliae*. In the pairwise Mantel test, colour-type was positively correlated with altitude and allozyme genotype was positively correlated with geographical distance. No other combination showed positive correlations (Table 5).

Isolation by distance and gene flow

Slatkin's method (Slatkin, 1993) indicated isolation by distance for *O. cacaliae* [$\log(Nm) = 1.01 - 0.41 \log(\text{km})$, $r^2 = 0.32$; significant relationship ($P < 0.001$, Mantel test, 1000 permutations)], but not for *O. speciosissima* [$\log(Nm) = -0.39 + 0.09 \log(\text{km})$, $r^2 = 0.03$; nonsignificant relationship ($P > 0.1$, Mantel test, 1000 permutations)]. This is analogous to the result obtained from the Mantel test, where a posi-

tive correlation between geographical distance and genetic distance could only be observed for *O. cacaliae*. The sample from the Pyrenees seems to be more greatly differentiated than could be explained by distance alone, because removal of this sample flattened the slope of the regression [$\log(Nm) = 0.91 - 0.36 \log(\text{km})$; $r^2 = 0.23$], whereas removal of the other most distant sample from the Czech Republic did not [$\log(Nm) = 1.06 - 0.43 \log(\text{km})$, $r^2 = 0.32$].

Nm -values, calculated as a measure of gene flow from the frequencies of private alleles, were very similar for both species (1.33 for *O. speciosissima* and 1.51 for *O. cacaliae*). Nm -values calculated from F_{ST} were both smaller, 0.81 for *O. speciosissima* and 1.36 for *O. cacaliae*.

Discussion

Distribution of genetic variance

Oreina cacaliae and *O. speciosissima* displayed a high genetic variability. Measures of genetic variability were in the same range for both species, not supporting the hypothesis of greater genetic variability in the species with broader ecological niche. In comparison with studies of other chrysomelid species, the percentage of polymorphic loci is very high; the heterozygosity values, however, are similar to those reported in Rowell-Rahier (1992) and for other Chrysomelidae (references in Knoll *et al.*, 1996).

Considerable genetic variation was found, both within and among populations. Moreover, the within-population component was larger in both species than the amount of genetic variation

attributable to differentiation among populations (F_{IS} -values greater than F_{ST} -values). With a high amount of within-population variance, a significant amount of between-population differentiation is expected (Wright, 1978). The high F_{IS} -values reported here correspond to those reported in an earlier study (Rowell-Rahier, 1992). On the other hand, the difference in F_{ST} -values between the two species suggested in the previous study could not be confirmed. The much lower F_{ST} -value (0.051) for *O. speciosissima* formerly reported was probably an artefact, because only three samples were studied and because of the wide range of single between-population F_{ST} -values in the present study.

The high F_{IS} -values in this study result from substantial heterozygote deficits in almost all populations. This is not an effect of one or two single loci, nor do all loci show a similar amount of heterozygote deficit, as would be the result of inbreeding or a Wahlund effect. A homogeneous heterozygote deficit over nine polymorphic loci was, for instance, observed in the cave-dwelling beetles of the genus *Speonomus* (Crouau-Roy, 1988) and was explained by inbreeding.

Three possible explanations are generally invoked to explain heterozygote deficits: the presence of null alleles, inbreeding or a Wahlund effect. The possibility of null alleles can be excluded because no missing genotypes (homozygous null alleles) were found in the gels.

Bilton (1992), in a study on the dytiscid beetle *Hydroporus glabriusculus*, found a similar pattern of high overall genetic variance, mainly caused by high F_{IS} -values. He attributed this to the sampling procedure conducted over several different aggregations. The sampling procedure in the study reported here was explicitly designed to avoid Wahlund effects, sampling only small and continuous host patches, within which the beetles were spaced evenly. Although the beetles do not disperse much (S. Knoll & M. Rowell-Rahier, unpubl. obs.), a substructuring of independent groups within these patches seems unlikely.

Inbreeding should result in a homogeneous effect at all loci, which was not found here. We explain the pattern found by a combination of inbreeding resulting in kinship groups and a sampling effect over several different closely related groups.

Similar arguments have been used for the tree-hopper *Enchenopa binotata* (Guttmann *et al.*, 1989). In this species, high differentiation among samples of nymphs on different branches of their host trees and high F_{IS} -values within these branches were explained by sampling over the offspring of only a

few females. This argument implies that later in the season (before mating) there is dispersal and thus a mixing of these sibling groups. No smaller F_{IS} -values were found in the populations sampled just before larviposition, the time when most dispersal and mixing should have taken place. F_{IS} -values were not related to the date of sampling, so results from the present study do not represent a structuring which is not significant for the population.

The extent of inbreeding (mating with relatives) and whether this occurs by chance (because of limited dispersal) or by assortative mating can only be speculated about. There is no information about mate choice or parentship in *Oreina* species. In the field frequent mating can be observed up to the time of larviposition and it is known from laboratory studies that these frequent matings do not result in more larvae (S. Dobler, pers. comm.). Also, males of the previous year's generation were often observed mating with newly hatched, still soft females. Overlapping generations, in combination with low vagility, should increase the possibility and magnitude of inbreeding.

In conclusion, it is suggested that *Oreina* 'host plant patch' populations do not mate at random and probably constitute a mix of more or less closely related kin groups.

Causes of population structuring

A correlation of population variability with environmental parameters is an indication of local adaptation and selection (e.g. Manly, 1985). For three explanatory factors, host plant, geographical distance and altitude, tests were made for an influence on the population structure. For both species there are no host plant effects at the macrogeographical scale investigated here. This is in agreement with the results of a phylogenetic study of the genus, which documented low host fidelity and flexibility in host affiliations for the genus (Dobler *et al.*, 1996). Auto-genous defence, in combination with aposematism, is expected to promote independence from the host plant (Dobler *et al.*, 1996). Because *O. cacaliae* has given up the possibility of autogenous defence, relying exclusively on sequestration of host secondary compounds, a closer association to its host as reflected in its smaller host plant spectrum would have been expected. At a microgeographical scale Kreslavsky *et al.* (1976) reported host races for *O. cacaliae* from a morphometric study based on length of elytrae. However, a morphometric study of 12 characters conducted on a subset of the populations presented here, ranked length of elytrae as not

very informative for investigating differentiation between *O. cacaliae* or *O. speciosissima* populations. The most informative characters were those measured on the aedeagus and the length of tarsi (Gallusser, 1996).

For *O. cacaliae*, the observed population structure seems to result from isolation by distance, which, in turn, cannot be explained by selection and adaptation in geographically different areas, as indicated by the absence of any clinal pattern. Rather, the pattern of isolation by distance seems to be imposed by limited gene flow and geographical distance in an otherwise homogeneous species as originally proposed by Wright (1978). In a recent review of population structure of phytophagous insects, Peterson (1996) shows that isolation by distance is a general feature of the population structure of sedentary species on a macrogeographical scale.

The colour of *O. cacaliae* showed a strong correlation with altitude: at higher altitudes, beetles were dark blue. Melanism at high altitude is a common feature of many insect species and is commonly explained by providing better ultraviolet protection.

Colour-types are often used in chrysomelids to identify 'races' or 'subspecies' (e.g. Kühnelt, 1984 for *Oreina*), but so far no genetic differentiation can be shown for colour forms (Verdyck *et al.*, 1996). Colour-type was used in the present study as a morphological character and no correlation with geographical distance was found. By contrast, in a detailed morphometric study of *O. cacaliae* (Gallusser, 1996), identical patterns were found for the morphological variation as for allozymic variation (namely 'isolation by distance' for both data sets). However, the study revealed that there is no correlation between the morphological data set and the allozyme data set, although data were obtained from the same individuals. Geographical distance is influencing both of them independently.

For *O. speciosissima* none of the tested environmental factors (geographical distance, altitude and host plant) was correlated with genotype. However, colour-type was correlated with geographical distance and host plant. This might reflect a flaw in the sampling regime employed in this study. The populations from the Czech Republic all belong to a specific colour-type (green with red stripes, found nowhere else), and the population from Hörnli is the only black one and the only one on *Cirsium spinosissimum* (Table 1). *Oreina speciosissima* showed no indication of isolation by distance and low *N_m*-values around one, which could be interpreted as indicating a species not at equilibrium with virtually no ongoing gene flow (Slatkin, 1993).

However, the sampling for *O. speciosissima* populations was not as intensive as for *O. cacaliae*, and it might not have the power to detect isolation by distance (Slatkin & Maddison, 1990; Slatkin, 1993). This seems more likely, as there is no explanation for why *O. cacaliae* should have reached an equilibrium state, whereas *O. speciosissima* has not.

Conclusion

Both species show comparable amounts of genetic variation and considerable population structuring. In *O. cacaliae* the observed structure can be explained by the isolation-by-distance model—limited gene flow in a sedentary species over larger distances—whereas for *O. speciosissima* no such explanation could be found. For both species, no host plant effect can be detected. Obviously, demographic processes play an important role in determining the distribution of genetic variation in *Oreina* species and merits further investigation.

Acknowledgements

We would like to thank B. Benrey, S. Dobler, J. Goudet, C. Liepert, J.M. Pasteels and T. Turlings for helpful comments on an earlier draft of the manuscript. C. Knoll and G. Schwarzbözl helped with sampling in the Pyrenees and H. Kippenberg kindly indicated *Oreina* sites. This work was supported by the Swiss National Science Foundation (grant no. 31-33669.92).

References

- BARTON, N. H. AND SLATKIN, M. 1986. A quasi-equilibrium theory of the distribution of rare alleles in a subdivided population. *Heredity*, **56**, 409–415.
- BILTON, D. T. 1992. Genetic population structure of the postglacial relict diving beetle *Hydroporus glabriusculus* Aubé (Coleoptera: Dytiscidae). *Heredity*, **69**, 503–511.
- BOURDONNÉ, J.-C. AND DOGUET, S. 1991. Données sur la biosystème de *Chrysolina* L.S. *Ann. Soc. Ent. Fr.*, **27**, 29–64.
- CROUAU-ROY, B. 1988. Genetic structure of cave-dwelling beetles populations: significant deficiencies of heterozygotes. *Heredity*, **60**, 321–327.
- DOBLER, S., MARDULYN, P., PASTEELS, J. M. AND ROWELL-RAHIER, M. 1996. Host-plant switches and the evolution of chemical defense and life history in the leaf beetle genus *Oreina*. *Evolution*, **50**, 2373–2386.
- EGGENBERGER, F. AND ROWELL-RAHIER, M. 1991. Chemical defence and genetic variation. Interpopulational study of *Oreina gloriosa* (Coleoptera: Chrysomelidae). *Naturwissenschaften*, **78**, 317–320.

- GALLUSSER, S. 1996. *Etude des differences morphometrique entre populations d'Oreina cacaliae, speciosissima et elongata*. Travail de diplôme, Institut de Zoologie, Université de Neuchâtel, Neuchâtel, Switzerland.
- GOUDET, J. 1995. FSTAT v. 1.2. A computer program to calculate *F*-statistics. *J. Hered.*, **86**, 485–486.
- GUTTMANN, S. I., WILSON, T. AND WEIGT, L. A. 1989. Microgeographic genetic variation in the *Enchenopa binotata* complex (Homoptera: Membracidae). *Ann. Entomol. Soc. Am.*, **82**, 225–231.
- HARTMANN, T., WITTE, L., EHMKE, A., THEURING, C., ROWELL-RAHIER, M. AND PASTEELS, M.J. 1997. Selective sequestration and metabolism of plant derived alkaloids by chrysomelid leaf beetles. *Phytochemistry*, **45**, 489–497.
- HILLIS, D. M. AND MORITZ, C. 1990. *Molecular Systematics*. Sinauer Associates, Sunderland, MA.
- KNOLL, S., ROWELL-RAHIER, M., MARDULYN, P. AND PASTEELS, J. M. 1996. Spatial genetic structure of leaf beetle species with special emphasis on alpine populations. In: Jolivet, P. H. A. and Cox, M. L. (eds) *Chrysomelidae Biology*, vol. 1, *The Classification, Phylogeny and Genetics*, pp. 379–388. SPB Academic Publishing bv, Amsterdam.
- KRESLAVSKY, A. G., SOLOMATIN, V. M., MIKHEEV, A. V. AND GRITZENKO, V. V. 1976. Intrapopulation ecological differentiation in a leaf beetle *Chrysochloa cacaliae*. *Zool. Zh.*, **55**, 1163–1171.
- KÜHNELT, W. M. 1984. Monographie der Blattkäfergattung *Chrysochloa* (Coleoptera, Chrysomelidae). *Sitzungsber. Österr. Akad. Wiss. Math.-naturw. Kl., Abt. I*, **193**(6–10) 171–287.
- LEGENDRE, P. AND VAUDOR, A. 1991. Le Progiciel R. *Analyse Multidimensionnelle, Analyse Spatiale*. Departement de sciences biologiques, Université de Montréal, Canada.
- MANLY, B. F. J. 1985. *The Statistics of Natural Selection on Animal Populations*. Chapman and Hall, London.
- PETERSON, M. A. 1996. Long-distance gene flow in the sedentary butterfly, *Euphilotes enoptes* (Lepidoptera: Lycaenidae). *Evolution*, **50**, 1990–1999.
- RANK, N. E. 1992. A hierarchical analysis of genetic differentiation in a montane leaf beetle *Chrysomela aenei-collis* (Coleoptera: Chrysomelidae). *Evolution*, **46**, 1097–1111.
- RAYMOND, M. AND ROUSSET, F. 1995. GENEPOP (version 1.2): A population genetics software for exact tests and ecumenicism. *J. Hered.*, **86**, 248–249.
- RODERICK, G. K. 1996. Geographic structure of insect populations: gene flow, phylogeography, and their uses. *Ann. Rev. Ent.*, **41**, 325–352.
- ROWELL-RAHIER, M. 1992. Genetic structure of leaf beetle populations: microgeographic and sexual differentiation in *Oreina cacaliae* and *O. speciosissima*. *Entomologia exp. appl.*, **65**, 247–257.
- SLATKIN, M. 1993. Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, **47**, 264–279.
- SLATKIN, M. AND MADDISON, W. P. 1990. Detecting isolation by distance using phylogenies of genes. *Genetics*, **126**, 249–260.
- SMOUSE, P. E., LONG, J. C. AND SOKAL, R. R. 1986. Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Syst. Zool.*, **35**, 627–632.
- SWOFFORD, D. L. AND SELANDER, R. B. 1981. BIOSYS-1: A Fortran program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *J. Hered.*, **72**, 281–283.
- VASCONCELLOS-NETO, J. 1988. Genetics of *Chelymorphism cribraria*, Cassidinae: colour patterns and their ecological meanings. In: Jolivet, P., Petitpierre, E. and Hsiao, T. H. (eds) *Biology of the Chrysomelidae*, pp. 217–232. Kluwer Academic Publishers, Dordrecht.
- VERDYCK, P., DE WOLF, H., BACKELJAU, T. AND HULSELMANS, J. 1996. A genetic study of two colour forms of *Phyllotreta cruciferae* (Chrysomelidae: Alticinae). In: Jolivet, P. H. A. and Cox, M. L. (eds) *Chrysomelidae Biology*, vol. 1, *The Classification, Phylogeny and Genetics*, pp. 389–397. SPB Academic Publishing bv, Amsterdam.
- WEIR, B. S. AND COCKERHAM, C. C. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution*, **43**, 1349–1368.
- WRIGHT, S. 1978. *Evolution and the Genetics of Populations*, vol. 4, *Variability Within and Among Natural Populations*. University of Chicago Press, Chicago, IL.