Journal of Heredity 2014:105(1):28–38
doi:10.1093/jhered/est081
Advance Access Publication November 28, 2013

Out of the Alps: The Biogeography of a Disjunctly Distributed Mountain Butterfly, the Almond-Eyed Ringlet *Erebia alberganus* (Lepidoptera, Satyrinae)

Dirk Louy, Jan Christian Habel, Werner Ulrich, and Thomas Schmitt

From the Department of Biogeography, Trier University, Universitätsring 15, D-54286 Trier, Germany (Louy and Schmitt); Department of Ecology and Ecosystem Management, Technische Universität München, D-85350 Freising-Weihenstephan, Germany (Habel); and Nicolaus Copernicus University, Department of Animal Ecology, Pl-87100 Toruń, Poland (Ulrich).

Address correspondence to Dirk Louy at the address above, or e-mail: dirk.louy@gmail.com.

Abstract

Many studies on the biogeography of thermophilic and arctic–alpine species were performed during the past. Only little is known about species with intermediate characteristics. We analyzed the molecular biogeography of the butterfly *Erebia alberganus* (30 populations, representing 1106 individuals), sampled over the Alps, Apennines (Italy), and the Stara Planina (Bulgaria) using allozyme electrophoresis (17 loci). Genetic analyses revealed 3 major splits, with the strongest between the Stara Planina populations and all other populations, and a weaker split between the Alps and the Apennines. Individuals from the Apennines were genetically nested within the Alps group. The Alps cluster was segregated into 3 groups: the Southwestern, Western/Central, and Eastern Alps. The genetic diversities were highest for the Alps populations and significantly lower in the 2 isolates (Apennines, Stara Planina). The remarkable genetic split between Stara Planina and all other populations and the genetic distinctiveness of the former cluster might be interpreted as an ancient colonization event of this Balkan mountain range. The Apennines populations derive from a more recent expansion out of the Southwestern Alps. After surviving the Würm ice age most probably in the central Apennines, accompanied by genetic modification of some of these populations, northward expansion might have started from the western parts of the central Apennines reaching the northern Apennines during the early postglacial. The subtle genetic differentiation found among the Alps populations probably reflects 3 geographically disjunct Würm glacial centers located at the western slopes of the Southwestern Alps, at the southern slopes of the Central Alps, and in the Southeastern Alps.

Key words: *allozyme electrophoresis, climatic oscillations, colonization trajectories, disjunction, distinct refugia, nestedness analyses, range shifts*

Changes between long phases of cool climatic conditions and short periods of warm climatic conditions characterize the late Pleistocene (Quante 2010). These climatic shifts have caused large range changes of biota (Hewitt 2004). Depending on the biotic and abiotic requirements of species, these climatic oscillations caused divergent expansion and retraction dynamics. Warm-adapted species survived ice ages in refugia located at lower altitudes and latitudes but expanded to higher elevations and latitudes in the postglacial (de Lattin 1967; Hewitt 1996). Such refugia of thermophilic organisms were, for example, restricted to the southern European peninsulas (Iberia, Italy and the Balkans). The long-term isolation there has led to genetic changes, which still are detectable for many species, for example, over major parts of Europe (Hewitt 1999, 2004; Schmitt 2007).

In contrast to warm-adapted species, cold-adapted species often represent almost opposite patterns: They expanded their distribution range during cold stages and became restricted to mountains or Nordic refugia during interglacial phases (Holdhaus 1954; Schmitt 2009). Some of these species were widely distributed in the periglacial steppes between the northern ice shield and the glaciers of the southern mountains (Abbott et al. 2000; Muster and Berendonk 2006;

Schönswetter et al. 2006; Skrede et al. 2006; Mutanen et al. 2012).

These 2 biogeographically contrasting groups represent 2 extremes: 1) disjunct glacial refugia during the cold phases and postglacial northward expansion from these retreats versus 2) interconnected populations over large parts of the periglacial steppes of Central Europe during the cold phases, but current disjunction in arctic and alpine regions. However, many European species do not follow one of these two biogeographic patterns. Although species with boreo-montane distributions often show relatively similar patterns to arctic–alpine taxa (e.g., Despres et al. 2002; Habel et al. 2010; Michl et al. 2010), other cold-tolerant species show highly complex ice age distributions, often with numerous extra-Mediterranean retreats (Schmitt and Varga 2012). Furthermore, complex restrictions to the foothills of different European high mountain systems during ice ages are well known for species currently exhibiting an alpine-disjunct distribution, that is, those species now restricted to regions above timberline in high mountain systems (Schmitt 2009). The distributions of many of these species might have covered even smaller areas during cold stages than today (Stehlik et al. 2002; Schmitt et al. 2006). Although the biogeography of all these groups is already relatively well understood, little is known about species closely linked to mountain forest habitats and their glades (cf., Schmitt and Haubrich 2008).

One characteristic representative of this habitat is the butterfly *Erebia alberganus*. This species is restricted to the Alps, the Apennine Mountains (northern and central parts) in Italy, and the mountains of Stara Planina in Bulgaria and Korab in Macedonia (Kudrna et al. 2011). We sampled 1106 individuals at 30 sites from the Alps, Apennines, and Stara Planina, covering most of the species' entire current distribution range. We analyzed allozyme polymorphisms, a suitable marker in butterflies to study large-scale and long-term biogeographic patterns (cf., Schmitt 2007). Based on these data, we reconstruct the biogeographic history of *E. alberganus*. In particular, we want to reveal the evolution of the disjunctive distribution pattern of the species, especially by detecting its last ice age refugia and postglacial range modifications.

Material and Methods

Study Species

The Almond-eyed ringlet butterfly, *E. alberganus* (de Prunner, 1798), is widely distributed over the entire range of the Alps and 4 isolated exclaves located in the northern and central part of the Italian Apennines, the Bulgarian Stara Planina, and Korab Planina in Macedonia, where it is restricted to higher elevations (Kudrna et al. 2011). The species is split into several subspecies and forms, that is, the subspecies *E. alberganus phorcys* (Freyer, 1836) endemic to Bulgaria (Tshikolovets 2011) as well as the forms *E. alberganus* f. *caradjae* characteristic for higher elevations in the Eastern Alps and *E. alberganus* f. *tyrsus* common in the Western Alps (Tolman and Lewington 1997). The butterflies normally occur in high densities. They fly in one generation from mid-June to the beginning of August at flower-rich mountain forest glades and in open forests with sufficient undergrowth of flowers and grasses at intermediate altitudes (900–2200 m a.s.l. in the Alps, Sonderegger 2005; 800–2200 m a.s.l. in the Stara Planina; 1400–2000 m a.s.l. in the Apennines, own observations). The larvae feed on different species of grasses, for example, *Festuca* and *Anthoxanthum* species (Tolman and Lewington 1997; Sonderegger 2005).

Molecular Analyses

A total of 1106 individuals of *E. alberganus* were collected from 2004 to 2009 at 30 localities (with a mean of 37 individuals per site ranging from 37 to 40, but 4 populations only had 20 individuals) covering most of the entire distribution range of the species (including Alps, Apennines [Italy], and the Stara Planina [Bulgaria]; Figure 1; details on sampling locations in Table 1 and Supplementary Appendix I). The butterflies were netted in the field, frozen alive in liquid nitrogen, and stored under these conditions until further analyses.

Half of the abdomen of each imago was homogenized in Pgm buffer (Harris and Hopkinson 1978) by supersonic application followed by centrifugation at 8000 × *g* for 4 min. We ran cellulose acetate electrophoresis and stained the plates according to modified protocols given in Hebert and Beaton (1993). The following 17 loci were analyzed: MDH1, MDH2, GAPDH, MPI, FUM, PK, PEP_{LGG}, PGI, ME, PGM, G6PDH, AAT1, AAT2, 6PGDH, IDH1, IDH2, and GPDH. Details on running conditions are given in Supplementary Appendix II.

Statistics

Locus-specific allele frequencies and tests on Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were performed with the program ARLEQUIN 3.1 (Excoffier et al. 2005). Mean number of alleles and allelic richness (AR) were calculated in FSTAT 2.9.3.2 (Goudet 1995). AR was preferred over the mean number of alleles per locus as it corrects for differences in sample size. The percentage of polymorphic loci (P_{tot}) and the percentage of polymorphic loci with the most common allele not exceeding a proportion of 95% (P_{95}) were calculated with G-Stat (Siegismund 1993). Observed and expected heterozygosity were calculated in ARLEQUIN 3.1 (Excoffier et al. 2005). Differences in the population means of these parameters among groups were tested by Kruskal–Wallis analyses of variance (ANOVAs) using STATISTICA. Nonhierarchical and hierarchical analyses of molecular variance were calculated in ARLEQUIN 3.1 including 3 hierarchical levels: among regions (F_{CT}), among populations within regions (F_{SC}), and within populations.

A neighbor-joining tree was constructed with PHYLIP (Felsenstein 1993) based on Nei's (1972) distances. Bootstrap values were calculated based on 1000 replications. We chose the assignment method implemented in STRUCTURE 2.3.3 (Pritchard et al. 2000) using the admixture model to classify individuals based on multiple nuclear loci (cf., Hausdorf and Hennig 2010). To define the most probable number of populations (K) present in the data, we used the ad hoc criteria

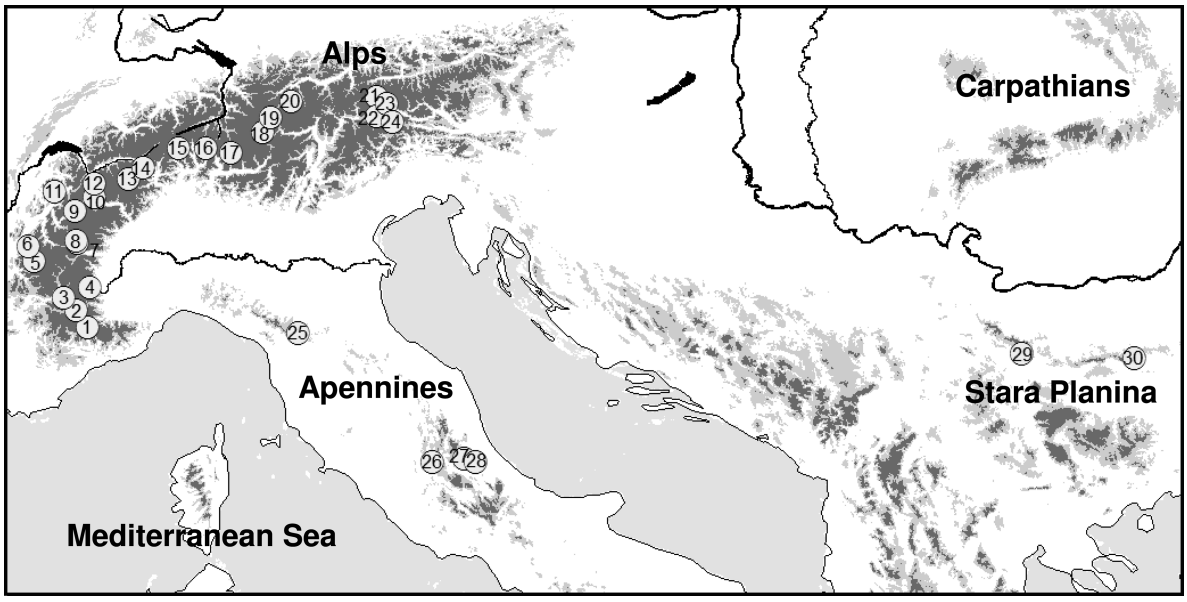

Figure 1. Locations of the sampling sites of *Erebia alberganus*. Numbers of sites coincide with all other figures and tables.

L(*K*) and Δ*K* proposed by Pritchard et al. (2000) and Evanno et al. (2005), ignoring high Δ*K* values for $K = 2$ as suggested by Hausdorf and Hennig (2010). Multiple analyses were run to explore whether results remain consistent. We performed 10 runs for each *K*, varying *K* from 1 to 15 with burn-in and simulation length of 150 000 and 500 000 runs, respectively.

Nestedness Analyses

We assessed the degree of nestedness with the NODF (nestedness by overlap and decreasing fill; Almeida-Neto et al. 2008) metric that measures the degree of ordered decline in allele incidence along a predefined environmental or diversity gradient (Ulrich et al. 2009; Habel et al. 2013). We tested whether hypothesized West to East and North to South colonization trajectories are in accordance with a stepwise decrease in allele diversity. Idiosyncrasy analysis (Ulrich et al. 2009) assessed the deviation of each site from a perfect nested subset pattern. We used the nestedness contribution (the difference in the degree of nestedness with and without a focal site; Saavedra et al. 2011) to infer the role of each site in the decline of allele diversity. We calculated the respective contributions for the 14 gene loci with more than 3 alleles. Although raw scores depend on matrix fill and allele numbers, we normalized them by calculating the respective standardized effect sizes (SES = μ/σ; μ: mean, σ: standard deviation [SD]) where expected means and SDs came from an equiprobable reshuffling of occurrences within the allele × site matrix. Low SES values imply higher degrees of unexpected occurrences of alleles and therefore point to possible introgression.

Furthermore, we assessed patterns of allele co-occurrence using the common C-score metric (Stone and Roberts 1992; Ulrich and Gotelli 2013), a normalized count of the total number of {{1,0},{0,1}} submatrices within the presence–absence matrix. High values in C-score point to a segregated pattern of allele co-occurrence within populations. To assess spatial turnover of alleles, we sorted rows and columns according to the first axis of correspondence analysis (seriation) and linked the order of sites and total numbers of alleles per site to the latitude and longitude of the study sites (Ulrich and Gotelli 2013). We quantified the spatial turnover of alleles by the squared coefficient of correlation r^2 between the row and column numbers of the ordinated matrix (Ulrich and Gotelli 2013).

To test for statistical significance, we used a permutation approach (Gotelli and Ulrich 2013) and compared observed metrics with the distribution of metrics obtained from 200 randomized matrices. Although there is no a priori reason constraining potential incidences of alleles across the sample sites apart from selection pressure and bottleneck effects, we used the equiprobable null model *ee* that controls only for the total number of alleles across all sample sites but does not fix total numbers of incidences for certain alleles among sites or allele richness within sites (Gotelli 2000). All calculations were done with the software applications NODF (Almeida-Neto and Ulrich 2011) and Turnover (Ulrich and Gotelli 2013).

Results

Genetic Diversity

No linkage disequilibrium and no significant deviations from HWE were observed for any pair of loci. Tests were performed using the conventional Bonferroni correction as well as the false discovery rate controlling procedure (described in Benjamini and Hochberg 1995; Benjamini et al. 2001), both with identical results. Therefore, further analyses were performed using standard algorithms in population genetics.

Table 1 Genetic diversities of all *Erebia alberganus* populations analyzed

Region	Site	*A*	*AR*	*APc*	*APp*	H_e	H_o	P_{tot}	P_{95}
Alps	I-Colle della Lombarda-1	2.18	1.91	20.00	5.71	11.6	11.2	58.8	47.1
	F-Col de Larche-2	(1.65)	1.65	10.34	0	12.1	13.2	52.9	41.2
	F-Vars-3	1.77	1.64	6.67	0	11.8	10.9	64.7	47.1
	I-Serre Uberto-4	1.82	1.65	12.90	0	12.0	12.7	58.8	35.3
	F-Les Deux Alpes-5	2.00	1.80	11.76	2.94	9.2	9.9	58.8	41.2
	F-le Rivier d'Allemond-6	(1.77)	1.76	6.67	0	11.4	11.2	52.9	41.2
	F-Petit Cenis-7	(1.59)	1.59	3.70	0	7.1	5.6	41.2	29.4
	F-Lanslebourg-8	1.41	1.36	4.17	0	4.8	4.9	29.4	17.6
	F-La Rosière near Seez-9	1.65	1.57	3.57	0	14.5	15.1	35.3	35.3
	I-St. Rhemy, Aosta valley-10	1.88	1.66	6.25	3.13	11.0	10.0	52.9	29.4
	F-Col des Aravis-11	1.65	1.58	3.57	0	11.4	11.3	47.1	29.4
	CH-Rive Haut-12	1.71	1.60	0	0	10.3	9.6	52.9	23.5
	CH-Täschalp-13	(1.53)	1.53	11.54	3.85	9.6	10.3	41.2	29.4
	CH-Simplon Alpien-14	1.88	1.65	0	0	6.5	6.6	52.9	17.6
	CH-Passo Campolungo-15	1.77	1.56	6.67	0	7.5	7.1	58.8	23.5
	CH-San Bernadino-16	1.88	1.70	9.38	3.31	12.2	11.0	58.8	29.4
	CH-Casaccia-17	1.65	1.53	10.71	3.57	7.5	7.2	41.2	23.5
	CH-Tscherv-18	1.71	1.58	10.31	0	9.9	9.9	58.8	41.2
	CH-Vnà-19	1.88	1.80	12.50	6.25	13.4	10.2	52.9	35.3
	A-Verpeil near Feichten-20	1.77	1.64	13.79	0	11.8	11.6	58.8	35.3
	I-Rein in Taufers-21	1.88	1.71	9.38	0	13.2	13.2	58.8	41.2
	A-Kalkstein-22	1.53	1.47	11.54	0	10.9	11.3	41.2	23.5
	A-Sajatmähder, Virgental-23	1.77	1.68	16.67	3.33	15.0	13.9	64.7	52.9
	A-Connyalm-24	1.71	1.64	10.34	0	14.9	12.5	64.7	47.1
Mean (±SD)		**1.78** (±0.17)	**1.64** (±0.11)	**8.85** (±4.91)	**1.34** (±2.05)	**10.82** (±2.67)	**10.43** (±2.59)	**52.4** (±9.63)	**34.1** (±9.83)
Italy	I-Cutigliano-25	1.47	1.44	0	0	13.8	12.1	35.3	35.3
	I-Terminillo-26	1.94	1.75	3.03	3.03	12.4	11.0	58.8	47.1
	I-Prati di Tivo-27	1.18	1.18	0	0	3.4	2.9	17.6	17.6
	I-Monte Termoggia-28	1.53	1.50	0	0	10.3	8.9	47.1	35.3
Mean (±SD)		**1.53** (±0.31)	**1.47** (±0.23)	**0.76** (±1.52)	**0.76** (±1.52)	**9.98** (±4.61)	**8.73** (±4.10)	**39.7** (±17.58)	**33.8** (±12.16)
Stara Planina	BG-Monastirishte-29	1.35	1.27	0	0	3.8	3.7	35.3	17.6
	BG-Triglav-30	1.53	1.38	3.85	3.85	4.4	4.0	47.1	17.6
Mean (±SD)		**1.44** (±0.13)	**1.33** (±0.08)	**1.93** (±2.72)	**1.93** (±2.72)	**4.10** (±0.42)	**3.85** (±0.21)	**41.2** (±8.34)	**17.6** (±0.0)
Total mean (±SD)		**1.71** (±0.22)	**1.59** (±0.16)	**7.31** (±5.43)	**1.30** (±1.97)	**10.26** (±3.28)	**9.77** (±3.16)	**50.0** (±11.53)	**32.9** (±10.46)

The following parameters are given: mean number of alleles (*A*) (values in parenthesis were excluded for calculating mean values due to small sample sizes), AR, fraction of private alleles endemic to one mountain system (*APc*) and restricted to one population (*APp*), expected heterozygosity (H_e), observed heterozygosity (H_o), and total percentage of polymorphic loci (P_{tot}) and of loci with the most common allele not exceeding 95% (P_{95}). Site names including running numbers coincide with all other figures and tables. AR was calculated based on the lowest number of individuals (20 individuals). Except for *A* and AR, all values are given in percentage. Bold values are means.

The genetic diversities varied significantly among the 30 populations analyzed. Highest genetic diversities were found in populations from the Alps (means: A 1.75, AR 1.64, H_e 10.8%, H_o 10.4%, P_{tot} 52.4%, and P_{95} 34.1%), intermediate values for the Apennines (means: A 1.53, AR 1.47, H_e 9.9%, H_o 8.7%, P_{tot} 39.7%, and P_{95} 33.8%), and lowest for populations from Stara Planina (means: A 1.44, AR 1.33, H_e 4.1%, H_o 3.9%, P_{tot} 41.2%, and P_{95} 17.6%). Population-based Kruskal–Wallis ANOVAs obtained a significant difference among regions for the parameter AR; the parameters A, H_e, and H_o were marginally significant ($0.05 < P < 0.1$). The frequency of private alleles (i.e., restricted to a single mountain region) was highest for the Alps (mean 8.85%; 23 endemic alleles), intermediate for the populations from Stara Planina (mean 1.93%; 1 endemic allele), and lowest for the populations from the Apennines (mean 0.76% alleles; 1 endemic allele). This difference was significant (Kruskal–Wallis ANOVA: $P = 0.006$). However, the mean frequency of alleles endemic to a single population was highest for Stara Planina (mean 1.93%), intermediate for the Alps (1.34%), and lowest for the Apennines (0.76%). All parameters of genetic diversities are given for all populations in Table 1. Population-specific allele frequencies are given in Supplementary Appendix III.

Genetic Differentiation

The neighbor-joining dendrogram assigned the populations into 3 main clusters: Alps, Apennines, and Stara Planina. The group built by the 2 Stara Planina populations is strongly supported by the highest bootstrap values obtained for any node of the neighbor-joining phenogram (95%). The support for the entire branch containing the 4 Apennines populations is moderate (60%), but 71% is reached for the 2 most differentiated populations from Terminillo and the northern Apennines. The populations from the Alps form 3 subtle subclusters with moderate bootstrap support: Southwestern Alps west of the Alps' main chain (population 1, 3, 5, 6, 7, 8), Western and Central Alps (2, 4, 9–20, 22), and Eastern Alps (21, 23, 24) (see Figure 2).

The Bayesian structure analyses for the detection of the most probable K yielded 2 maxima: $K = 3$ (39.3) and $K = 5$ (18.9) (see Supplementary Appendix IV). For $K = 3$, STRUCTURE detected the same main clusters as the neighbor-joining dendrogram: Alps, Apennines, and Stara Planina. However, 1) the Eastern Alps populations (21–24, East and South Tyrol; note that population 22 [East Tyrol] in this analysis is part of the Eastern Alps group in contrast to cluster analysis) showed remarkable similarity with the Stara Planina populations and 2) the populations from the Western and Central Alps were composed of a complex mix out of the 3 detected gene pools, in some populations showing striking similarity with the Apennines populations. For $K = 5$, we yielded similar genetic assignments, but the groups Eastern Alps, Apennines, and Stara Planina represented clearly independent gene pools each, without major overlap with the still much more diverse Western and Central Alps group (Figure 3).

The overall genetic differentiation among all populations was high and explained about 17.5% of the total variance ($F_{ST} = 0.175$, $P < 0.001$), whereas the inbreeding coefficient within populations was low, albeit significant ($F_{IS} = 0.040$, $P < 0.001$), maybe due to some moderate Wahlund effect. The above delimited genetic groups showed differing levels of genetic differentiation among populations. Thus, the Alps populations showed relatively high genetic differentiation among each other ($F_{ST} = 0.1384$, $P < 0.0001$). Although the Southwestern Alps subgroup was relatively homogeneous, the 2 other subgroups showed much higher among population variation (Table 2A). Also, the 4 Apennines populations showed relatively high among populations differentiation ($F_{ST} = 0.1199$, $P < 0.0001$), whereas the 2 populations from Stara Planina showed no significant genetic differentiation.

Hierarchical variance analyses supported the genetic assignment into 3 genetic clusters (Alps, Apennines, and Stara Planina) with a high F_{CT} value ($F_{CT} = 0.1100$,

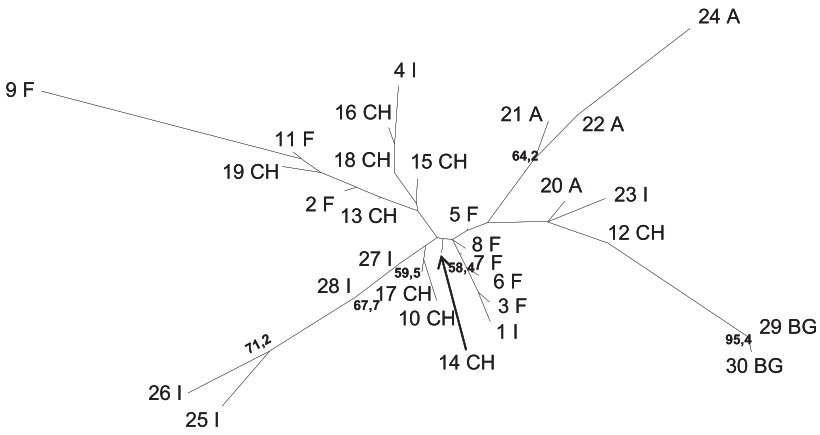

Figure 2. Neighbor-joining phenogram based on Nei's (1972) genetic distances with bootstrap values (>50) (derived from 1000 replicates) of 30 populations of *Erebia alberganus*. Numbers of localities coincide with all figures, tables, and Supplementary Appendix I. Country abbreviations are given with the population numbers.

$P < 0.0001$). The strongest genetic split was detected between Apennines (population 25–28) and Stara Planina (population 29–30) ($F_{CT} = 0.3087$, $P < 0.0001$). The differentiation between Alps and Apennines was much less ($F_{CT} = 0.0715$, $P < 0.0001$), but populations from the Alps and from Stara Planina showed a strong differentiation between these 2 groups ($F_{CT} = 0.1475$, $P < 0.0001$). The genetic differentiation among the 3 subgroups within the Alps ($F_{CT} = 0.0620$, $P < 0.0001$; following the results of the neighbor-joining dendrogram and results derived from STRUCTURE analyses) was almost as high as the differentiation between Alps and Apennines. Furthermore, the 4 Apennines populations are well distinguished into 2 groups (i.e., 25 and 26 vs. 27 and 28) with their differentiation in the same order of magnitude ($F_{CT} = 0.0710$, $P < 0.0001$) than the one between Alps and all Apennines populations (see Table 2).

Spatial Distribution of Alleles—Nestedness Analyses

Our nestedness analysis yielded a strong signal of decline in AR along a west–east gradient in the Alps (Table 3). All SES scores were positive, and 7 were statistically significant

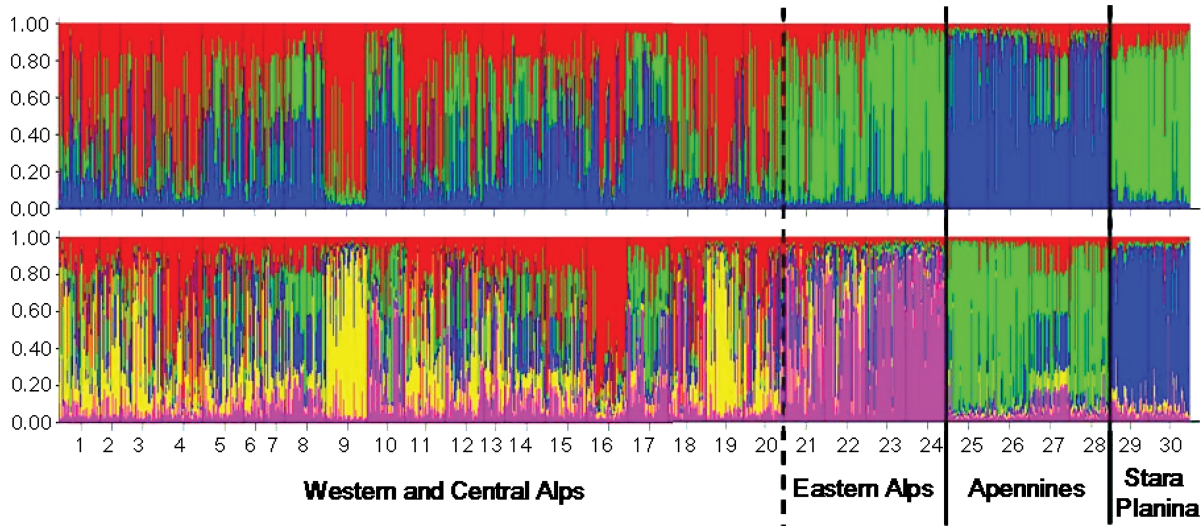

Figure 3. Results of the Bayesian structure analyses of *Erebia alberganus* populations calculated with STRUCTURE, for $K = 3$ (upper plot) and $K = 5$ (lower plot), supported by highest ΔK values (see Supplementary Appendix IV). Population numbers coincide with all other figures and tables. Population clusters and subclusters are identified at the bottom.

Table 2 Nonhierarchical (A) and hierarchical (B) analyses of molecular variance to test for significance and the degree of genetic differentiation among *Erebia alberganus* populations

Populations/regions	Among populations (F_{ST})	Among individuals within populations (F_{IS})	Within individuals
(A) Nonhierarchical molecular variance analyses			
All populations	0.1837 (0.1749***)	0.0403 (0.0465***)	0.8260
Alps	0.1472 (0.1384***)	0.0303 (0.0330**)	0.8861
Alps (Southwest)	0.0442 (0.0528***)	0.0248 (0.0313)	0.7675
Alps (West/Central)	0.1328 (0.1262***)	0.0263 (0.0286)	0.8936
Alps (East)	0.0985 (0.0817***)	0.0572 (0.0517)	1.0504
Apennines	0.1148 (0.1199***)	0.1018 (0.1209***)	0.7406
Central Apennines	0.0949 (0.1145***)	0.0888 (0.1209**)	0.6458
Stara Planina	−0.0019 (−0.0055)	0.0254 (0.0725)	0.3247
(B) Hierarchical molecular variance analyses			
Group(s)	Among groups (F_{CT})	Among populations within groups (F_{SC})	Within individuals
Alps vs. Apennines vs. Stara Planina	0.1241 (0.1100***)	0.1378 (0.1372***)	0.8259
Alps vs. Stara Planina	0.1749 (0.1475***)	0.1410 (0.1394***)	0.8404
Stara Planina vs. Apennines	0.3436 (0.3087***)	0.0870 (0.1131***)	0.6455
Apennines vs. Alps	0.0807 (0.0715***)	0.1432 (0.1366***)	0.8635
Population 25, 26 vs. 27, 28	0.0696 (0.0710***)	0.0684 (0.0751***)	0.7406
Alps (3 groups)	0.0678 (0.0620***)	0.1098 (0.1070***)	0.8861

Variance values (top line) with the respective F statistics (in parentheses). Groupings were created according to the results of STRUCTURE analyses.
$^{**}P < 0.01$, $^{***}P < 0.001$.

Table 3 Nestedness and co-occurrence analysis of 14 loci of *Erebia alberganus* with at least 3 alleles for Alps and Stara Planina and for the Alps site 1 and the Italian sites

Locus	6PGDH	G6PDH	GOT1	GOT2	GPDH	IDH1	IDH2	MDH1	MDH2	MPI	PEP_{LGG}	PGI	PGM	PK
Number of alleles	5	3	5	6	3	4	6	5	3	3	3	7	5	3
Alps only														
Matrix sorted according to longitude and allele incidences														
NODF	**4.05**	**5.79**	0.09	**4.64**	**2.94**	**2.9**	0.32	0.28	1.45	0.26	0.25	**2.78**	**2.71**	1.15
Matrix sorted according to the first correspondence axis														
r^2	**−4.89**	**−2.02**	**−2.06**	**−3.35**	**−2.27**	**−4.73**	**−4.51**	**−2.46**	**−2.10**	**−4.36**	**−3.16**	**−5.89**	**−5.64**	**−2.48**
C-score	**−6.971**	**−3.574**	**−6.25**	**−7.221**	**−3.727**	**−3.851**	**−7.131**	**−6.342**	**−2.624**	**−3.906**	**−3.425**	**−7.535**	**−7.253**	**−3.267**
Italian sites														
Matrix sorted according presumed colonization trajectory and allele incidences														
NODF	**2.99**	1.37	0.45	**2.96**	−0.98	0.06	−0.02	−1.34	**2.91**	**2.00**	**2.31**	1.50	1.03	1.71
Matrix sorted according to the first correspondence axis														
r^2	**−2.13**	−1.28	−1.48	−1.25	−1.38	−0.59	**−3.68**	**−4.10**	−0.64	−1.85	−1.44	**−4.43**	**−3.99**	−1.37
C-score	**−2.28**	−1.21	**−2.18**	**−2.08**	−1.46	−0.83	**−2.71**	**−2.26**	−1.50	−1.28	−1.55	**−3.29**	**−2.31**	−1.64

In the case of nestedness analysis, the allele site matrices were sorted according to the total number of allele occurrences and to the presumed colonization trajectory from west to east (Alps only, sites 1 to 24) and north to south (Italian sites only: sites 1, 25–28). For the co-occurrence analysis, alleles and sites were sorted according to the first axis of correspondence analysis to maximize allele segregation. Given are SES scores (*ee* null model). Significant scores ($P < 0.05$) are given in bold.

at the 1% error level. For the north–south gradient (Italian sites), 5 out of 14 SES values were significant in spite of the low power of the test due to the small number of sites. In line with the nested pattern of allele occurrences, we did not find an indication of a vicariant pattern of allele occurrence. The first correspondence axis used for the turnover analysis of Alps and Apennines was not significant in any case (all $P > 0.1$) if correlated with longitude and latitude, respectively (not shown), corroborating the lack of allele turnover among Alps and Apennines. The co-occurrence and turnover analysis (Table 3) for both colonization trajectories pointed to strong patterns of allele aggregation as expected for a nested pattern and consequently for an ordered loss of alleles along the colonization trajectories. The idiosyncrasy analysis of the Alps populations pointed particularly to the most western site 1 as contributing least to the nested pattern and thus having the comparably highest number of unexpected occurrences or absences of alleles (Figure 4). High degrees of idiosyncrasy also characterized the Western Alps population 4, the Central Alps population 17, and the Eastern Alps population 22.

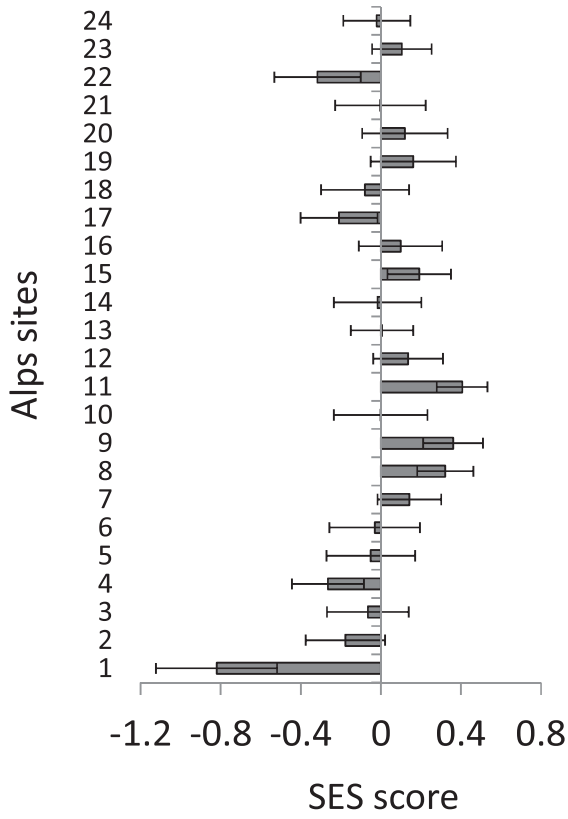

Figure 4. Average levels of normalized (SES transformed) nestedness contribution and the respective standard errors of the 24 Alps populations of *Erebia alberganus* calculated from the 14 gene loci with at least 3 alleles contained in Table 2.

Discussion

Allozymes, Still a Useful Tool for Biogeographic Analyses in Butterflies

Although the easy accessibility of different DNA analytical techniques has largely advanced biogeography (Avise 2000), the previously often used allozyme polymorphisms are still a useful tool in some species groups (Schmitt 2007). One of these species groups are butterflies, which often show highly polymorphic allozyme loci (e.g., Johannesen et al. 1996; Schmitt et al. 2005). However, allozymes are under selective pressure (Eanes 1999), and several examples (also for butterflies) are known demonstrating the influence of selective pressures on allele frequencies (e.g., Watt et al. 1996, 2003; Karl et al. 2008, 2009). However, other studies on butterflies showed that neither altitudinal gradients (Besold et al.

Table 4 Genetic diversities of different European Satyrid butterflies obtained from allozyme analyses

Species	*A*	H_e	H_o	P_{tot}	P_{95}	Reference
E. alberganus	1.7 (±0.2)	10.3 (±3.3)	9.8 (±3.2)	50.0 (±11.5)	32.9 (±10.5)	This article
E. medusa	2.0 (±0.3)	15.6 (±3.4)	14.5 (±3.5)	55.6 (±14.5)	41.4 (±9.2)	Schmitt (2007)
E. epiphron	2.1 (±0.3)	15.4 (±2.4)	—	69.7 (±16.1)	43.2 (±7.2)	Schmitt et al. (2006)
E. euryale	2.3 (±0.5)	15.6 (±2.9)	15.2 (±3.0)	68.5 (±14.7)	41.2 (±12.6)	Schmitt and Haubrich (2008)
E. melampus	1.6 (±0.3)	8.9 (±1.6)	9.7 (±4.7)	42.8 (±20.5)	25.0 (±6.0)	Haubrich and Schmitt (2007)
E. sudetica	1.7	6.4	6.7	55.6	22.2	Haubrich and Schmitt (2007)
C. arcania	2.0 (±0.5)	16.6 (±5.8)	14.9 (±5.0)	52.3 (±19.7)	41.5 (±16.6)	Besold et al. (2008a)
C. pamphilus	2.9 (±0.2)	25.3 (±1.6)	23.3 (±1.4)	82.4 (±4.2)	63.9 (±5.8)	Besold et al. (2008b)
C. darwiniana	2.6	17.5 (±4.2)	16.1 (±3.5)	63.9 (±4.0)	38.9 (±15.7)	Schmitt and Besold (2010)
C. gardetta	2.2	13.7 (±0.3)	14.3 (±0.8)	61.2 (±7.8)	41.7 (±3.9)	Schmitt and Besold (2010)
C. macromma	2.4 (±0.2)	23.1 (±4.9)	21.8 (±1.3)	61.2 (±7.8)	50.0 (±15.7)	Schmitt and Besold (2010)
M. jurtina	2.4 (±0.4)	17.2 (±4.2)	11.4 (±1.7)	70.8 (±9.5)	43.7 (±12.5)	Habel et al. (2009a)

The following parameters are given: mean number of alleles (*A*), expected heterozygosity (H_e), observed heterozygosity (H_o), and total percentage of polymorphic loci (P_{tot}) and of loci with the most common allele not exceeding 95% (P_{95}). *E*, *Erebia*; *C*, *Coenonympha*; *M*, *Maniola*.

2008a) nor the sampling time along a generation (Kühne and Schmitt 2010) had significant influences on the allele frequencies of all loci analyzed in these studies. Furthermore, studies on butterflies applying allozymes in combination with mitochondrial DNA (e.g., Hammouti et al. 2010; Vila et al. 2011) or microsatellites (e.g., Habel et al. 2011b) always revealed similar biogeographic patterns. Therefore, allozyme studies have always to be seen in the light of possible influences of selection, but this, in general, also applies to all other genetic markers. As the resolution of allozymes is particularly good in butterflies, this marker is still useful for biogeographic analysis and interpretation in this species group.

Low Genetic Diversity, but Moderately High Genetic Differentiation

The genetic diversity of *E. alberganus* populations is low (cf., Nève 2009; Table 4), also compared with most other *Erebia* species, as *E. medusa*, *E. epiphron*, *E. momos*, or *E. euryale* (Schmitt et al. 2006; Haubrich and Schmitt 2007; Schmitt 2007; Schmitt and Haubrich 2008). However, similarly low values of genetic diversity were found for *E. melampus* sensu strictu and *E. sudetica* (Haubrich and Schmitt 2007). The low genetic diversity of *E. alberganus* is further emphasized if compared with other Satyrinae butterflies, such as the widely distributed species *Coenonympha arcania*, *C. pamphilus*, *C. glycerion*, *Melanargia galathea*, or *Maniola jurtina* (Besold et al. 2008a, 2008b; Habel et al. 2009a; Habel et al. 2011a; Louy et al. 2013). Even Satyrinae butterflies with restricted occurrences, such as the local Alpine endemics *C. darwiniana* and *C. macromma* or the Southeastern European species *C. rhodopensis* show considerably higher genetic diversity despite of their small geographic ranges (Schmitt and Besold 2010; Louy et al. 2013).

We argue that the low genetic diversity of *E. alberganus* is the consequence of a demographic contraction to a small geographic region, most likely during some glacial cold stage (see below). This relationship between low genetic diversity and small and isolated populations is a well-known phenomenon (Habel and Schmitt 2012), for example, supported by relict populations of the Red Apollo *Parnassius apollo* (Descimon 1995; Habel et al. 2009b), or other rare, localized species in Europe and North America (e.g., Debinski 1994; Britten et al. 1995; Gadeberg and Boomsma 1997).

Comparing the genetic differentiation among populations and among population groups reveals a moderately high differentiation (cf., Nève 2009). This level of differentiation is in the order of magnitude of other mountain butterfly species for which range fragmentations are assumed for about 2 full glacial–interglacial cycles (e.g., Schmitt et al. 2006; Schmitt and Haubrich 2008).

The level of differentiation among mountain sibling species, in general, is considerably higher than among the genetic groups of *E. alberganus* (e.g., Haubrich and Schmitt 2007; Schmitt and Besold 2010). Also, ecologically similar species, but with lower dispersal capacity as the mountain ground beetle *Carabus irregularis*, show much deeper splits among their genetic groups (e.g., about 2 My between Alps and Carpathians) than *E. alberganus* (Homburg et al. 2013). On the other hand, most species with typical Mediterranean expansion centers and a supposed divergence time among lineages lasting only the last ice age (e.g., Schmitt et al. 2005; Habel et al. 2009a, 2011a) show more shallow genetic differentiations among groups. For these reasons, we assume that the divergence time reflected in the allozyme patterns of *E. alberganus* does not surpass the Riss glaciation (i.e., 2 full glacial–interglacial cycles). However, the overall differentiation is too high to be the product of very young (i.e., postglacial) processes.

Range Dynamics of *E. alberganus* over Time

The Alps, and here particularly the Southwestern Alps, represent the genetic hotspot and, most likely, evolutionary center of *E. alberganus*. This region harbors the highest population genetic diversity (reflected by genetic diversity indices), and also the forma *tyrsus* is restricted to this area. This might indicate persistence of the species in the region of the Southwestern Alps with only altitudinal shifts, at least over the last 2 glacial–interglacial cycles. As all other extant *E. alberganus* populations are genetically nested within these populations, they are most likely to have derived from expansions out of this

region. In general, the importance of the Southwestern Alps for survival and evolution of alpine and arctic–alpine species is well known (reviewed in Schönswetter et al. 2005; Varga and Schmitt 2008; Schmitt 2009; Taberlet et al. 2012). In contrast to *E. alberganus*, Taberlet et al. (2012) demonstrated in a meta-analysis on plants that the Southwestern Alps, although particularly rich in endemic species, do not represent a center of genetic diversity and genetic endemism; they therefore concluded that many species surviving here did so in small refuge areas resulting in genetic impoverishment; they also assumed that these refuge populations did not largely expand over the Alps during the postglacial range expansion.

Within the Alps' populations, all our analyses supported a distinct Southeastern Alps group (with the only ambiguity of the South Tyrol population 21). However, this group was genetically nested within the Western and Central Alps populations. Furthermore, the transition between these 2 groups apparently is abrupt, and the forma *caradjae* is restricted to the Southeastern Alps. The most likely scenario for such a pattern is expansion from one center with subsequent fragmentation and survival in 2 refuges. As the distribution of *E. alberganus* currently is continuous all over the Southern Alps (and presumably was so during the entire postglacial period), this eastward expansion most likely occurred during the last interglacial (i.e., the Eemian or Riss–Würm interglacial). Subsequently, *E. alberganus* might have become isolated in a southeastern Würm ice age refugium, reexpanding over major parts of the Southeastern Alps in the postglacial. As in the case of the assumed Southwestern Alps refugium, the existing literature gives strong evidences for the importance of the Southeastern Alps and adjoining regions for the survival of mountain taxa (reviewed in Schönswetter et al. 2005; Schmitt 2009; Taberlet et al. 2012) but also for many species with typical continental distributions (reviewed in Schmitt and Varga 2012).

Among the Western and Central Alps' populations, all provenances from the French Alps west of the Alpine Main Chain show strong genetic cohesiveness and form, albeit with weak support, a subgroup with little genetic differentiation among the individual populations. However, this genetic split through the Southwestern Alps is not as deep as the split through the eastern Central Alps. Consequently, the age of separation should be more recent. As the East–West split in the Alps most likely is of early Würm glacial age, the Southwestern Alps split might just go back to the late Würm maybe separating 2 regional refugia on both sides. Similar splits in this region are also known for the alpine species *P. phoebus* (Descimon 1995) and *E. epiphron* (Besold J, Brandt S, Schmitt T, unpublished data).

The genetic diversity of *E. alberganus* populations differed strongly among the 3 mountain regions, with minimum values in Stara Planina (Bulgaria). The strong genetic differentiation of Stara Planina from all other populations, but the strong nestedness of these Bulgarian populations in the populations from the Alps, support an earlier split between these 2 geographic groups than among the Alps populations (i.e., pre-Würm). Most probably, this vicariance event was followed by long-term isolation of populations in the eastern Balkans, well justifying the subspecific status of this group as *E. alberganus phorcys*. Two processes might be relevant in shaping the low genetic diversity in the Stara Planina cluster: 1) loss of genetic information in the wake of the eastward colonization process (cf., Hewitt 1996; Habel et al. 2013) and 2) continuous geographic isolation enhancing the effects of environmental stochasticity and population fluctuation within these small populations (cf., Channell and Lomolino 2001; Melbourne and Hastings 2008).

The Apennines populations were genetically nested within the Western and Central Alps populations and showed a considerably weaker genetic differentiation (in particular Gran Sasso) from the Alps than Stara Planina. Therefore, the colonization of the Apennines is likely to have happened more recently. Two time slots might be possible for this expansion along the Apennines chain: 1) the early Würm ice age with survival in the Apennines and 2) the early postglacial with rapid genetic modification over the last few thousands of years. However, 2 aspects support the first scenario. 1) The differentiation between the Central Apennines regions Gran Sasso and Terminillo was relatively strong. A differentiation velocity as necessary for a postglacial age of this expansion is little likely if compared with the assumed average differentiation rate in *E. alberganus* and the currently high population densities of the species in central Italy (Schmitt T, personal observations). 2) The northern Apennines showed a strong genetic cohesiveness with the Terminillo population, but both are considerably more differentiated from the Alps populations than Gran Sasso. This is the opposite pattern to the one expected under a recent expansion scenario. Therefore, in our eyes, this pattern is best explained by 1) an early Würm expansion to the Central Apennines, 2) Würm survival in this region with differentiation among the different mountain blocks of the Central Apennines, and 3) early postglacial northward readvance of the Terminillo group along the Apennines chain, thus representing the source for the genetically closely related Northern Apennines populations.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Funding

Konrad Adenauer Foundation (to D.L.); Forschungsfonds of Trier University (to T.S. and D.L.).

Acknowledgments

Sampling permits were provided where necessary. We thank Dennis Rödder (Bonn, Germany) for creating Figure 1. We thank 2 anonymous referees for improving earlier versions of this manuscript.

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Received March 27, 2013; First decision May 2, 2013; Accepted October 15, 2013

Corresponding Editor: Adalgisa Caccone