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Allozyme polymorphisms in the small heath, *Coenonympha pamphilus*: recent ecological selection or old biogeographical signal?

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Allozyme electrophoresis is a common tool to analyse the genetic structure of populations with a focus on conservation and biogeography. An essential requirement for biogeographical analyses is the independence of the applied methods from ecological selection processes. We surveyed the genetic population structure of *Coenonympha pamphilus* as a model species to validate allozym electrophoresis as a method for such studies. We analysed 106 individuals of *C. pamphilus* of the Monte Baldo region (southern Alps, Italy) at different altitudes and 82 individuals from two populations north of the Alps (Germany). The F_{ST} among all populations was 9.7% but no significant differentiation was observed along the altitudinal gradient in the Monte Baldo region. Our results revealed no influence of the climate on the allozyme frequencies, thus excluding a major importance of recent selection processes for the observed differentiations. These results support the suitability of allozyme electrophoresis for biogeographical studies.

Introduction

In general, the question itself determines the selection of an appropriate method. In the case of analytical questions, the frame for interpreting the data obtained depends on the samples which are analysed and the method which is applied. Main parameters directly determining the selection of a method are its sensitivity, sensibility and selectivity. For biogeographical questions (cf. Hewitt 1996, 1999, 2000, 2001, 2004a, 2004b, Taberlet *et al*. 1998, Schmitt 2007), it is highly important to select methods, which clearly discriminate between long-term stochastic population processes and selective influences (e.g. local climatic and ecological conditions). The latter blur the analytical design of such a study. Allozyme analyses are frequently used in biogeographical studies, and the geographical patterns obtained are often interpreted biogeographically (e.g. Mensi *et al*. 1988, Porter & Geiger 1988, 1995, Porter & Shapiro 1989, Britten *et al*. 1994, 1995, Debinski 1994, Napolitano & Descimon 1994, Descimon 1995, Marchi *et al*. 1996, Jiggins & Davies 1998, Schmitt & Seitz 2001a, 2001b, 2002, 2004, Schmitt *et al*. 2002, 2003, 2005a, 2005b, 2006a, 2006b,

2007, Schmitt & Hewitt 2004a, 2004b, Schmitt & Krauss 2004, Habel *et al*. 2005, Schmitt & Müller 2007). However, this marker system was frequently critisized due to the selective pressure on enzyme loci (Eanes 1999). Thus many examples of active selection on allozyme loci are known including butterflies (Watt 1977, 1983, 1995, Eanes & Koehn 1978, Watt *et al*. 1983, 1996, 2003, Zalucki *et al*. 1987). Therefore, it is important to test whether these selective pressures on allozymes frequently affect the results for reasonable biogeographical interpretations.

A suitable test to answer this question has to be performed with a common species with restricted mobility of the individuals. Various populations in geographic proximity, but not in direct dispersal distance should be selected in a landscape representing rather different environmental conditions (e.g. climate) so that each population is subjected to a different environmental and thus evolutionary stress. This is best encountered in a mountain region because increasing altitudes are strongly correlated with changing ecological parameters (e.g. temperature, humidity, precipitation, insolation, duration of seasons). Additional samples should come from populations in distant regions reflecting climatically a part of the studied altitudinal transect.

We therefore selected a transect over an altitudinal gradient of 1000 m in the Monte Baldo region (eastern Italian Alps) and added one further sample from western and southern Germany. As a model species we selected the small heath, *Coenonympha pamphilus*. The species is highly adaptable to climatic conditions and widely distributed all over Europe. In comparison with other taxa of the genus *Coenonympha* it can be found in a very wide vertical range (Tolman & Lewingtion 1998). We analysed the genetic texture of six *C. pamphilus* populations by means of allozyme electrophoresis to answer the following questions:

1. Does a rather variable, common and widespread species like *C. pamphilus* show a high genetic diversity of its populations? If this is the case, we will be able to address the second question being central to this study.
2. Do different climatic regimes have selective influences on allozyme gene frequencies so that altitudinal gradients evolve, or do allozyme pattern simply reflect long-term stochastic processes?

Material and methods

Species

The small heath, *Coenonympha pamphilus* (Lepidoptera, Nymphalidae, Satyrinae), is a species widely distributed from northern Africa to the Far East and parts of western Mongolia. Its northern border reaches 69°N in Fennoscandia. In the south, it is distributed on most of the Mediterranean islands (Higgins & Riley 1993, Tolman & Lewington 1998, Kudrna 2002). The larvae feed on Poaceae as *Festuca ovina*, *F. rubra*, *Poa annua*, *Anthoxanthum odoratum*, *Cynosurus cristatus*, *Dactylis glomerata*, and *Nardus stricta*. Sometimes eggs of *C. pamphilus* can be found on various other species (Tolman & Lewington 1998).

Coenonympha pamphilus is plurivoltine with an adaptable alternation of generations. Larvae from each generation are able to hibernate until the following spring (Higgins & Riley 1993). The flight season of the butterflies is quite long. The imagos appear throughout spring, summer and autumn from February (northern Africa and Spain; end of May in northern Europe) until November. Open grassy places and patches with short vegetation are preferred (Settele *et al*. 2005). Their very variable habitats have been found from sea level up to the lower alpine level (Tolman & Lewington (1998): 1950 m a.s.l., Higgins and Riley (1993): 1800 m a.s.l.). Especially the number of individuals and the appearance of the first generation varies strongly with altitude and geographical locality of the habitat (Tolman & Lewington 1998).

Collection sites

Four populations of *C. pamphilus* were sampled on the slopes of the Monte Baldo, (Italian Alps). Two additional samples from central Europe were added (Römersköpfchen and Leimberg, Germany) (Fig. 1).

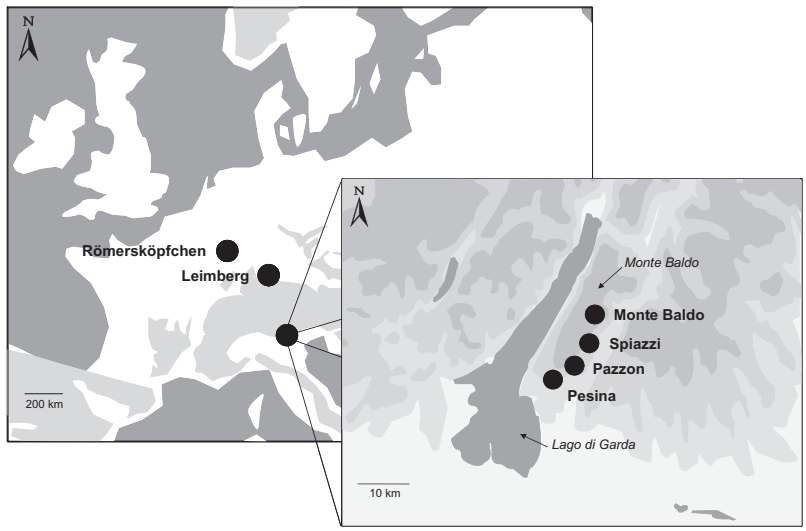

Fig. 1. Locations of sample sites. Four populations of *Coenonympha pamphilus* were sampled from the slopes of Monte Baldo (Italy). Two samples from Germany were added to the analyses.

The areas in the vicinity of the sample sites are characterized by agriculture with meadows and pastures. None of the habitats in the Monte Baldo area is separated by ecological or geographical barriers along the transect. The site near Pesina (250 m a.s.l.) was used as an olive grove with sub-Mediterranean climate. Pazzon (450 m a.s.l.) is located on the coline altitudinal level, and the sample was collected from an intensively cultivated meadow. The intensity of agricultural land-use decreased with increasing altitude, and thus the third site near Spiazzi (900 m a.s.l.) was an extensively used mountain pasture. The highest site on the Monte Baldo (1200 m a.s.l.) was a subalpine meadow. The German populations were sampled from semi-natural calcareous grasslands.

Sampling and electrophoresis

One hundred and six individuals of *C. pamphilus* from the Monte Baldo region were taken in early September 2004. Additional 82 individuals from two German populations were analysed. The sample sizes ranged from 15 to 41 individuals with a mean of 31 (± 10.4 SD) individuals per population (Table 1). After netting in the fields the imagos of *C. pamphilus* were immediately frozen in liquid nitrogen and stored under these conditions until further analyses in the laboratory.

The whole abdomen of each imago was homogenized in a Pgm-buffer solution (Harris & Hopkinson 1978) with ultrasound and was centrifuged 5 min at 17 000 g. We used cellulose

Table 1. Number of individuals of *Coenonympha pamphilus* sampled (*N*), sample location, country (International Car Codes (ICC)), date of capture, elevation, longitude and latitude (Gauss-Krüger-Coordinates) of all sample sites.

Location	Country (ICC)	*N*	Date of capture	Elevation (m a.s.l.)	Longitude	Latitude
Pesina	I	33	11.IX.2004	250	10°46´E	45°37´N
Pazzon	I	23	10.IX.2004	450	10°49´E	45°38´N
Spiazzi	I	35	10.IX.2004	900	10°50´E	45°40´N
Monte Baldo	I	15	9.IX.2004	1200	10°51´E	45°42´N
Leimberg-Gruibingen, Göppingen	D	41	30.V.2004	700	9°39´E	48°36´N
Römersköpfchen	D	41	18.V.2004	300	6°55´E	50°00´N

acetate plates for allozyme electrophoresis of the abdominal tissues. For running conditions, we applied those used by Schmitt *et al*. (2006b) for the satyrine butterfly *Erebia epiphron*, but the locus MPI was not scorable and PK had two different loci (Table 2). Thus, a total of 18 allozyme loci was analysed.

Statistical analyses

Alleles were labelled according to their relative mobility during electrophoresis, starting with '1' for the slowest. Allele frequencies and parameters of genetic diversity (i.e. mean number of alleles per locus *A*, expected heterozygosity H_e, observed heterozygosity H_o, total percentage of polymorphic loci P_{tot} and percentage of polymorophic loci with the most common allele not exceeding 95% P_{95}) were computed using G-STAT (Siegismund 1993). The same software was used to perform the Mantel test.

Hierarchical *F*-statistics were estimated to describe genetic differentiation of groups of populations. Locus by locus AMOVAs, hierarchical genetic variance analysis, and the Hardy-Weinberg equilibrium and linkage disequilibrium were calculated with ARLEQUIN 3.01 (Excoffier *et al*. 2005). The χ^2-test (corrected for small sample sizes according to Levene) was used to test the significance of deviations from Hardy-Weinberg equilibrium. Sequential Bonferroni corrections were performed as described in Rice (1989).

We calculated genetic distances (Nei 1972) and constructed phenograms, using the Neighbor-joining algorithm (Saitou & Nei 1987) with PHYLIP (Felsenstein 1993). Bootstraps based on 1000 iterations were calculated with the same software.

Kruskal-Wallis ANOVA, Mann-Whitney *U*-test, Friedman test, Pearson correlation analysis and linear regression analysis were carried out with SPSS 10.

In addition to population-based analyses we applied two different individual based statistical tools. The Neighbor-joining analysis was used in combination with the genetic similarity index at the level of single individuals (Nei & Li 1979, Lynch 1990), and Markov chain Monte Carlo simulations (MCMC) implemented in STRUCTURE (Prichard *et al*. 2000) represent a model based approach. For these calculations we selected the admixture model and predefined four separate hypothetical populations ($k = 4$) relating to the four Monte Baldo sample sites to test the hypothesis of an inhomogenous genetic population structure along the altitudinal cline.

Distance matrices based on the genetic similarity index (Nei & Li 1979, Lynch 1990) between individuals, group related descriptive statistics and

Table 2. Electrophoresis conditions for the different enzymes analysed for *Coenonympha pamphilus*. TC: Tris-citrate pH = 8.2 (Richardson *et al*. 1986), TG: Tris-glycine pH = 8.5 (Hebert & Beaton 1993), TM: Tris maleic acid pH = 7.0 (adjusted from TM pH = 7.8 (Richardson *et al*. 1986)). All buffers were run at 200 V.

Enzyme system (abbrev.)	EC no.	Number of loci	Buffer	Homogenate applications	Run time (min)
Mdh	1.1.1.37	2	TC	2	40
Me	1.1.1.40	1	TG	3	30
Idh	1.1.1.42	2	TM	3	50
6pgdh	1.1.1.44	1	TM	3	50
G6pdh	1.1.1.49	1	TM	2	40
Gpdh	1.1.1.8	1	TM	4	45
Gapdh	1.2.1.12	1	TC	3	45
Aat	2.6.1.1	2	TM	3	40
Pk	2.7.1.40	2	TC	2	30
Apk	2.7.3.3	1	TG	1	30
Fum	4.2.1.2	1	TC	3	45
Pgi	5.3.1.9	1	TG	1	30
Pep_{LGG}	5.4.11/13	1	TC	3	30
Pgm	5.4.2.2	1	TG	2	30

transformations of 4-d data into 3-d data sets were computed with our own software. Algorithms therein were thoroughly tested in advance.

Results

Seventeen out of 18 scored loci were polymorphic. *Gapdh* was the only monomorphic locus for all samples analysed. The number of distinguished alleles (in total 69 alleles) ranged from two to six per locus with a mean of 4.0 (± 1.2 SD). Allele frequencies of all polymorphic loci are shown in the Appendix.

Based on the allele frequencies, we calculated several population genetic parameters. The mean number of alleles per locus (*A*) ranged from 2.6 to 3.2, with a mean of 2.9 (± 0.2 SD). The percentage of polymorphic loci with the most common allele not exceeding 95% (P_{95}) ranged from 57% to 72% with a mean of 64% (± 6% SD); for the total percentage of polymorphic loci (P_{tot}) we received values from 78% to 89%, mean 82% (± 4% SD). The mean expected heterozygosity (H_e) was 25.3% (± 1.6% SD), ranging from 23.8% to 26.8%, and for the mean of the observed heterozygosity (H_o), we observed 23.3% (± 1.4% SD) varying from 21.6% to 25.2%. Details for all populations are given in Table 3. No significant differences of the genetic diversities of all parameters were detected between the Monto Baldo region and Germany (all *U*-tests: $p > 0.06$).

The great majority of the loci did not deviate from the Hardy-Weinberg equilibrium. After the Bonferroni correction, only one case of significant deviation was detected (*G6pdh*: population 'Monte Baldo'). No significant genetic linkage disequilibrium was observed. All loci showed banding patterns consistent with known quaternary structures (Richardson *et al*. 1986). Therefore, further analyses could be performed using standard population genetic approaches.

The total genetic variance of all six populations was quite high (2.498) with 0.2412 (F_{ST}: 9.7%, $p < 0.0001$) genetic variance among populations and 0.1914 (F_{IS}: 8.4%, $p < 0.0001$) genetic variance among individuals within populations. Genetic distances (Nei 1972) among these samples ranged from 0.004 to 0.073 with a mean of 0.040 (± 0.030 SD). Unbiased Neighbor-joining phenograms based on these genetic distances (Fig. 2) distinguished two major entities: *C. pamphilus* from north of the Alps (Germany) and samples from the Monte Baldo region (Italy). This differentiation was clearly supported by bootstrap values. The genetic distance (Nei 1972) between the two northern samples was 0.011, and the range was 0.004 to 0.015 with a mean of 0.009 (± 0.004 SD) in the Monte Baldo region.

The alleles 3 of *Mdh1* and 2 of *G6pdh* were limited to the Monte Baldo region whereas the alleles 5 of *Pgi* and 4 of *Pgm* were restricted to the German samples. This differentiation was mirrored by hierarchical variance analyses (vari-

Table 3. Parameters of genetic diversity of all studied populations of *Coenonympha pamphilus*. Abbreviations: H_e: expected percentage of heterozygosity; H_o: oberserved percentage of heterozygosity; P_{95}: percentage of polymorphic loci with the most common allele not exceeding 95%; P_{tot}: total percentage of polymorphic loci; *A*: mean number of alleles per locus; *N*: number of individuals (calc.).

Location	H_e (%)	H_o (%)	P_{tot} (%)	P_{95} (%)	*A*	*N*
Pesina	23.8	23.8	83.3	55.6	3.11	32.5
Pazzon	26.5	25.2	88.9	66.7	2.83	22.6
Spiazzi	22.9	22.7	83.3	61.1	3.00	34.8
Monte Baldo	26.8	24.1	77.8	66.7	2.67	14.9
Italy (mean ± SD)	25.0 ± 1.9	24.0 ± 1.0	83.3 ± 4.5	62.5 ± 5.3	2.90 ± 0.19	26.2 ± 9.2
Leimberg	26.2	22.1	77.8	61.1	2.61	40.6
Römersköpfchen	25.6	21.6	83.3	72.2	3.17	40.2
Germany (mean ± SD)	25.9 ± 0.4	21.9 ± 0.4	80.6 ± 3.9	66.7 ± 7.8	2.89 ± 0.39	40.4 ± 0.3
Total (mean ± SD)	25.3 ± 1.6	23.3 ± 1.4	82.4 ± 4.2	63.9 ± 5.8	2.90 ± 0.23	30.9 ± 10.2

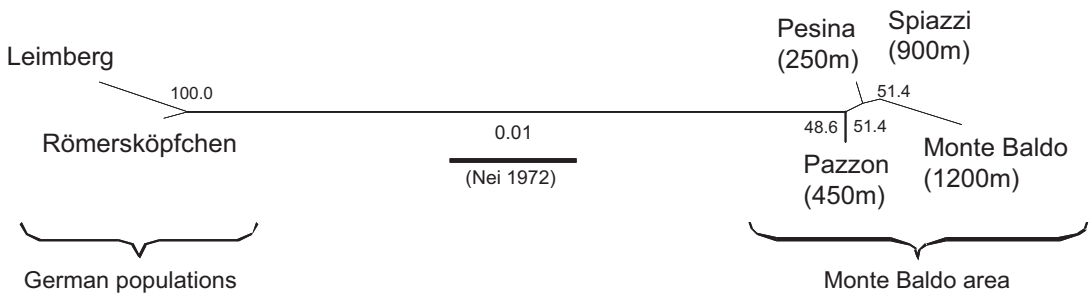

Fig. 2. Population based Neighbor-joining phenogram of all samples of *Coenonympha pamphilus* based on genetic distances according to Nei (1972). Bootstrap values are given at the nodes.

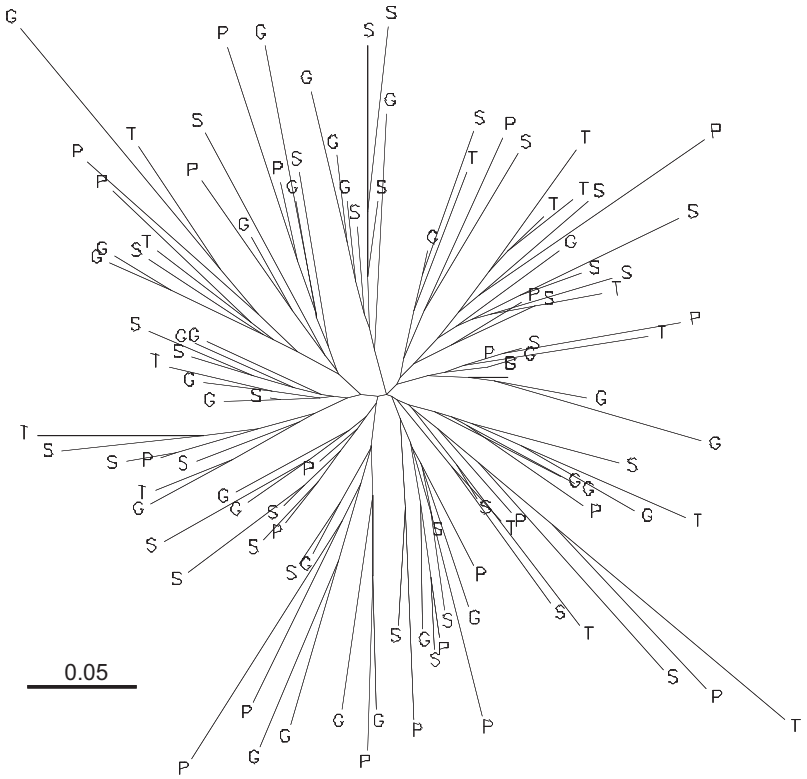

Fig. 3. Individual based Neighbor-joining diagram of four sample locations of *Coenonympha pamphilus* from the Monto Baldo region. We used the genetic similarity index (Nei & Li 1979) to compute the distance matrix. Every single branch represents one individual data set. Letters at the branch tips abbreviate the corresponding locations (G: Pesina (250 m a.s.l.), P: Pazzon (450 m a.s.l.), S: Spiazzi (900 m a.s.l.), T: Monte Baldo (1200 m a.s.l.)).

ance among groups: 0.3714, F_{CT} = 14.03%, p < 0.0001). The variance among populations within groups was considerably lower (0.0186, F_{SC}: 0.81%, p = 0.0068). Variance analysis including all four Monte Baldo populations showed no significant differentiation among populations (variance among populations: 0.0104, F_{ST}: 0.5%, p = 0.95). The variance within populations was considerably higher (among individuals: 0.0602, F_{IS}: 2.7%, p = 0.1104; within individuals: 2.1424). The differentiation among the German samples was also not significant (F_{ST}: 1.6%, p = 0.0772).

Two individual based clustering methods were applied including all 106 individuals taken at the four Monte Baldo sampling sites to test for possible sub-structures in this region. A Neighbor-joining phenogram based on the genetic similariy index (Nei & Li 1979) among individuals showed no groupings (Fig. 3). Thus, no genetic differentiation going alongside with the altitudinal gradient and different climatic conditions of the sample sites was observed. This estimation was confirmed by the Markov chain Monte Carlo simulation with four predefined clusters

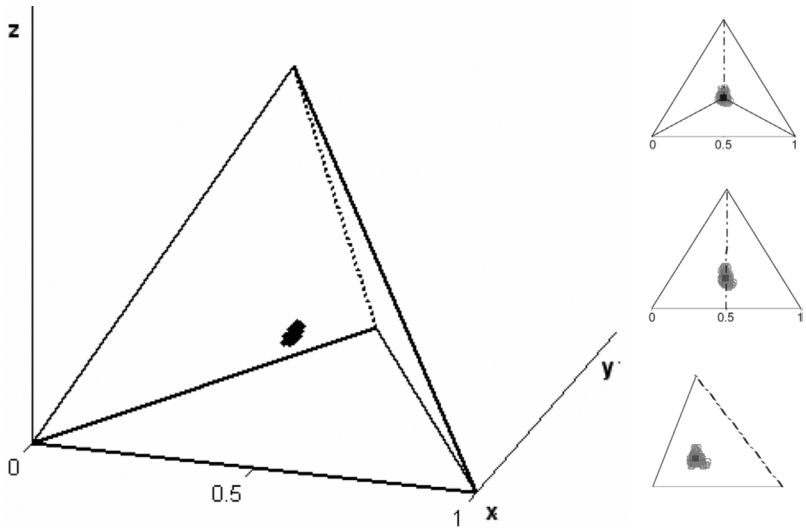

Fig. 4. Individual oriented THE-diagram (tetrahedron) based on the Markov chain–Monte Carlo (MCMC) simulations. Every single individual data set from four *Coenonympha pamphilus* sample stations from the Monte Baldo region (106 individuals) is represented by a single dot within the THE-coordinates. A texture of four clusters was tested, representing four altitude levels (250 m, 450 m, 900 m, 1200 m) corresponding to the four corners of the tetrahedron. Four views (three-dimensional (on the left-hand side), top, front and side view (on the right-hand side, top down)) are shown, the backside edge of each tetrahedron is displayed as a dotted line.

($k = 4$) corresponding to the four altitude levels. All individuals of the four samples clustered together in the centre of the tetrahedron (Fig. 4). The standard deviations of relative probabilities of all individuals to be classified to one of four clusters ranged from 0.007 to 0.012 ($N = 106$). A Friedman rank test for homogenity of probabilities of individuals was applied and revealed no significant differentiation among the populations of the Monte Baldo region ($\chi^2 = 2.448$, $p = 0.49$).

To test for isolation by distance we applied Mantel tests and correlation analyses. The calculations were based on all Monte Baldo samples. As genetic parameters we considered genetic distances (Nei 1972), as geographical parameters we included geographical distances based on Gauss-Krüger coordinates as well as vertical distances based on altitudinal levels (Table 1). Neither of these tests showed significant correlations (geographical distances: $r^2 = 0.258$, Mantel test: $p = 0.112$; altitudinal distances: $r^2 = 0.218$, Mantel test: $p = 0.09$). The percentages of polymorphic loci and the observed and expected heterozygosity as well as the number of alleles did not significantly correlate with the elevation in the Monte Baldo region (Pearson correlations: all $p > 0.25$).

Discussion

The Neighbor-joining phenogram (Fig. 2) clearly unraveled a genetic structure consisting of two major entities. The populations north of the Alps (Germany) are strictly seperated from those populations south of the Alps (Monte Baldo). The genetic differentiation between both groups showed a mean genetic distance (Nei 1972) of 0.066 and F_{CT} of 14.0%. Genetic differentiations in this order of magnitude are frequently observed among groups of populations of butterflies, for which a long-term isolation during Pleistocene (often Würm or Riss glacial vicariance) is frequently assumed as a reason for these differences (Schmitt & Seitz 2001a, 2001b, Habel *et al*. 2005, Schmitt *et al*. 2005a, 2005b, 2006b, 2007, Dennis & Schmitt 2008). However, more samples would be necessary for a more comprehensive analysis.

The differentiation within both groups lack relevance and revealed no remarkable genetic

structures (North genetic distance: 0.011, F_{ST} = 1.6%; South genetic distance: 0.009, F_{ST} = 0.5%). We further applied various individual and population based statistical methods to unravel eventual genetic substructures of the Monte Baldo populations, which may indicate correlations between the location of the population sampled and the allozyme gene frequencies. The tests for isolation by distance (vertical and horizontal), correlation analyses, Neighbor-joining tree in combination with the individual similarity index (Fig. 3) showed no sub-structuring among the Monte Baldo populations. The results of the MCMC simulation in combination with the THE graph (Fig. 4) clearly emphasize the homogeneity of the allele frequencies of the Monte Baldo populations despite the strong vertical climatic change from warm-temperate to mountain and subalpine levels. Although the distribution of *C. pamphilus* in the Monte Baldo region is fragmented by forested areas (own observations) and the dispersal capacity of the species is relatively limited (Bink 1992), no significant differentiation was observed.

However, an influence of recent environmental conditions on the allelic structure of some few loci (especially *Pgm* and *Pgi*) has been demonstrated for some lepidopteran species, but the overall effect on the global genetic differentiation among populations was relatively weak in the majority of cases (e.g. Eanes & Koehn 1978, Zalucki *et al*. 1987, 1993, Gu 1991, Goulson 1993). The only case of strong intraspecific differentiation assumed to result from recent environment-driven evolution was given for *Colias* butterflies in North America (Watt *et al*. 2003), but may even be interpreted as two sympatric cryptic species with micro-allopatry.

On the contrary, the geographically distant *C. pamphilus* populations in Germany showed significantly different allele frequencies from the Monte Baldo. This strongly supports that the rather different climatic conditions along this altitudinal gradient are not influencing the observed allelic structure of the populations, especially as the climatic conditions of these German populations can be found within the climatic gradient along our altitudinal transect in northern Italy. Therefore, these results support the assumption that the observed remarkable genetic structures in *C. pamphilus* are not the result of recent evolutionary and environmental processes, but reflect ancient biogeographical patterns as e.g. glacial vicariance effects.

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Appendix. Allele frequencies of all polymorphic loci of all six samples of *Coenonympha pamphilus*.

		Römersköpfchen Germany	Leimberg Germany	Pesina Italy	Pazzon Italy	Spiazzi Italy	Monte Baldo Italy
Idh1	1	0	0.025	0.078	0.095	0.071	0.033
	2	0.938	0.875	0.797	0.738	0.829	0.867
	3	0.063	0.100	0.109	0.143	0.100	0.100
	4	0	0	0.016	0.024	0	0
Idh2	1	0.025	0	0	0	0	0
	2	0.013	0	0.121	0	0.057	0
	3	0.938	0.902	0.667	0.705	0.686	0.667
	4	0	0	0	0	0	0.033
	5	0.025	0.098	0.212	0.295	0.243	0.300
	6	0	0	0	0	0.014	0
Mdh1	1	0	0.061	0.078	0.065	0.014	0.100
	2	0.410	0.293	0.422	0.304	0.486	0.433
	3	0	0	0.031	0.043	0.086	0.133
	4	0.551	0.598	0.328	0.457	0.286	0.2
	5	0.038	0.049	0.141	0.130	0.129	0.133
Mdh2	1	0	0.012	0.015	0	0	0
	2	0.939	0.963	0.970	0.913	0.929	0.900
	3	0	0	0	0.022	0.029	0.033
	4	0.061	0.024	0.015	0.043	0.029	0.067
	5	0	0	0	0.022	0.014	0
6Pgdh	1	0	0	0.015	0	0	0
	2	0	0	0	0	0.014	0.067
	3	0.900	0.962	0.879	0.864	0.929	0.867
	4	0.038	0.038	0.091	0.091	0.057	0.067
	5	0.063	0	0.015	0.045	0	0
G6Pdh	1	0.012	0	0	0	0.014	0
	2	0	0	0.061	0.022	0.086	0.133
	3	0.976	0.939	0.894	0.935	0.814	0.667
	4	0.012	0.061	0.045	0.043	0.086	0.200
Pgi	1	0	0	0	0	0.014	0
	2	0.012	0	0.152	0.087	0.100	0.100
	3	0.207	0.293	0.576	0.543	0.514	0.467
	4	0.744	0.610	0.197	0.283	0.286	0.367
	5	0.024	0.073	0	0	0	0
	6	0.012	0.012	0.061	0.087	0.086	0.067
	7	0	0.012	0.015	0	0	0
Pgm	1	0.012	0	0	0	0	0
	2	0.049	0	0	0	0	0
	3	0.390	0.207	0.015	0.109	0.071	0.033
	4	0.012	0.012	0	0	0	0
	5	0.476	0.707	0.788	0.783	0.786	0.833
	6	0.061	0.073	0.182	0.109	0.114	0.100
	7	0	0	0.015	0	0.029	0.033
Pep	1	0.038	0	0.016	0.130	0.030	0.033
	2	0.887	0.866	0.984	0.717	0.924	0.900
	3	0.063	0.134	0	0.152	0.045	0.067
	4	0.013	0	0	0	0	0
Aat1	1	0.013	0	0	0	0.014	0
	2	0.788	0.659	0.985	1	0.957	0.933
	3	0.200	0.305	0	0	0.029	0.067
	4	0	0.037	0.015	0	0	0
Aat2	1	0	0	0	0.022	0	0
	2	0.025	0.012	0.030	0	0.029	0
	3	0.138	0.220	0.333	0.391	0.386	0.567

continued

Appendix. Continued.

		Römersköpfchen Germany	Leimberg Germany	Pesina Italy	Pazzon Italy	Spiazzi Italy	Monte Baldo Italy
	4	0.463	0.378	0.561	0.522	0.571	0.367
	5	0.338	0.390	0.061	0.065	0.014	0.067
	6	0	0	0.015	0	0	0
	7	0.013	0	0	0	0	0
	8	0.013	0	0	0	0	0
	9	0.013	0	0	0	0	0
Gpdh	1	0	0	0.030	0.022	0	0
	2	0.939	1	0.924	0.935	1	1
	3	0.012	0	0	0	0	0
	4	0.037	0	0.045	0.043	0	0
	5	0.012	0	0	0	0	0
Fum	1	0.615	0.461	1	0.957	0.971	1
	2	0.385	0.513	0	0.043	0.029	0
	3	0	0.026	0	0	0	0
Me	1	0.025	0	0.016	0.043	0.014	0.033
	2	0	0.012	0	0	0	0
	3	0.975	0.988	0.968	0.957	0.986	0.967
	4	0	0	0.016	0	0	0
Apk	1	0	0	0.017	0.026	0	0
	2	1	1	0.966	0.974	1	1
	3	0	0	0.017	0	0	0
Pk1	1	1	1	1	0.957	0.986	0.967
	2	0	0	0	0.043	0.014	0.033
Pk2	1	0.073	0.037	0	0	0	0
	2	0.427	0.537	0.136	0.022	0.086	0.067
	3	0.488	0.402	0.788	0.891	0.900	0.767
	4	0.012	0.024	0.061	0.087	0.014	0.100
	5	0	0	0.015	0	0	0.067