Genetic divergence and evolution of *Polyommatus coridon gennargenti* (Lepidoptera: Lycaenidae) in Sardinia

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Electrophoretic analysis of 17 enzyme loci was carried out to assess the genetic differentiation and isolation of the Sardinian *Polyommatus coridon gennargenti* from *P. c. apennina* of peninsular Italy and the related taxon *P. caelestissimus* from central Spain. *P. c. gennargenti* is represented by a small, strongly inbred population, restricted to the central mountains of Sardinia, and probably derived from mainland populations as indicated by the reduction of genetic variation ($P = 17.6$ per cent, $H = 0.024$) with respect to the continental populations ($P > 52$ per cent, $H \geq 0.170$). Absence of gene flow is indicated by the presence of alternative fixed alleles at the *Aat*, *Gpi* and *Pgm* loci and significant differences in allele frequencies at other loci, which distinguish the Sardinian population from *P. c. apennina* and *P. caelestissimus*. The genetic differentiation of *P. c. gennargenti*, as measured using Wright's F_{ST} values and Nei's genetic distances, suggests the evolution of the Sardinian taxon along an independent lineage, facilitated by isolation and the strict dependence of the butterflies on specific biotopes, thus confirming its taxonomic status.

Keywords: electrophoretic markers, genetic differentiation, geographical isolation, *Polyommatus coridon*, speciation.

Introduction

The chalk-hill blue butterfly *Polyommatus* (*Lysandra*) *coridon* is distributed throughout Europe, ranging from the Iberian peninsula across France, Germany and Italy, to Russia and Greece. In recent years the presence of *P. coridon* has been confirmed in the islands of Corsica (Schurian, 1977) and Sardinia (Leigheb, 1987, 1991). Everywhere the butterfly has a patchy distribution in colonies localized in suitable habitats on chalk and limestone. The species is monovoltine, with a prolonged emergence from mid-July to the end of September and can be found at altitudes from 200 to 2500 m. Larval foodplants are various Leguminosae, mostly *Hippocrepis comosa*, *Coronilla varia*, *C. minima* and *Astragalus glycyphyllos*.

The *P. coridon* species complex represents among European butterflies one of the most challenging problems from both a taxonomic and evolutionary standpoint. In fact, the inherent difficulty of sorting out the different populations within the distribution of the complex has spawned a great number of names ranging from species to individual variation (Verity, 1916, 1939, 1943, 1951; Manley & Allcard, 1970; Higgins, 1975; Higgins & Riley, 1980; Higgins & Hargreaves, 1983; de Bast, 1985; Schurian, 1988). The papers by de Lesse (1970, 1971), on the karyology of the complex, show a certain degree of variation with haploid chromosome numbers of *P. coridon* ranging from 87 to 90, which supports the possibility that there is more than one species in the complex. The gene-enzyme electrophoretic technique has proved to be a powerful tool for approaching the systematics of *P. coridon*, allowing Mensi *et al.* (1988) to cluster some populations from Spain and others from southern France and Italy as two separate species.

The Sardinian *P. coridon* is represented by a small population restricted to a few localities of Barbagia around the central Gennargentu massif. The distinct morphological features of both males and females led Leigheb (1987) to propose a subspecific status (*gennargenti*) for the newly found taxon. This author has also suggested the possibility of a close relationship between *P. c. gennargenti* and *P. caelestissimus*

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from central Spain, on the basis of the similarity in the shining blue tinge of the upper surface of the male wing which clearly differentiates these two taxa from other populations of the *P. coridon* group.

In order to assess (i) whether the Sardinian *P. c. gennargenti* represents a genetically isolated population without gene exchange with the continental taxa and (ii) whether morphological differentiation is accompanied by sufficient genetic divergence to justify a change of its taxonomic status, we analysed the genetic variability of the Sardinian taxon of *P. coridon* and determined its phylogenetic relationships with two continental members of the group, based on the electrophoretic analysis of 17 enzyme loci.

Materials and methods

Specimens of *P. c. gennargenti* (Leigheb) were collected in Barbagia Seulo, Nuoro Province, Sardinia (altitude 800–1000 m; 20–25 July 1994), those of *P. c. apennina* (Poda) at Campo Imperatore, L'Aquila Province, Central Italy (altitude 1400–1600 m; 27 July 1994) and those of *P. caelestissimus* (Verity) at Tragacete, Cuenca Province, Central Spain (800 m; 20 August 1994). All specimens were personally carried or sent by express courier in a cool container (15°C) to the laboratory and stored at −70°C for later analysis. Voucher specimens were preserved for reference.

Seventeen enzyme loci were analysed by polyacrylamide gel electrophoresis. Individual butterflies were homogenized in 500 μL of 10 per cent sucrose and 0.02 mM bromophenol blue. Enzymes tested and buffer systems used for each are listed in Table 1. Staining recipes were prepared according to Shaw & Prasad (1970) with slight modifications. Allozymes were ranked alphabetically in order of increasing mobility from the origin. Genetic and statistical analyses were performed using the BIOSYS-1 (Swofford & Selander, 1981) and GENEPOP (Raymond & Rousset, 1995) software. Hardy–Weinberg equilibrium was tested for each locus in all populations using Levene's (1949) correction for small samples. Unbiased exact probabilities were tested using a Markov chain method (Guo & Thompson, 1992). Overall significance was tested by Fisher's combined probabilities method (Fisher, 1970). Genetic differentiation of populations was analysed by Wright's (1978) F statistics and an unbiased estimate of Fisher's exact test on R × C contingency tables was performed using the Markov chain method of Guo & Thompson (1992). Unbiased genetic similarity (I) and distance (D) measures were obtained using Nei's (1972, 1978) formulae. A dendrogram was constructed by the unweighted pair group method (UPGMA) using Nei's unbiased genetic distances.

Table 1 Gene-enzyme systems, EC numbers (N.C.I.U.B.M.B., 1992) and number of loci studied

Abbreviation	Name	EC no.	No. loci	Buffer
AAT	Aspartate aminotransferase	2.6.1.1	1	TBE†
ACO	Aconitate hydratase	4.2.1.3	1	TC‡
AK	Adenylate kinase	2.7.4.3	1	TC
FUM	Fumarate hydratase	4.2.1.2	1	TBE
GPI	Glucose-6-phosphate isomerase	5.3.1.9	1	TBE
GPD	Glycerol-3-phosphate dehydrogenase	1.1.1.8	1	TC
G6PD	Glucose-6-phosphate dehydrogenase	1.1.1.49	1	TC
HK	Hexokinase	2.7.1.1	1	TBE
IDH	Isocitrate dehydrogenase	1.1.1.42	2	TC
LDH	Lactate dehydrogenase	1.1.1.27	1	TC
MDH	Malate dehydrogenase	1.1.1.37	1	TC
ME	'Malic' enzyme	1.1.1.40	1	TBE
MPI	Mannose-6-phosphate isomerase	5.3.1.8	1	TBE
ODH	Octanol dehydrogenase	1.1.1.73	1	TBE
PGM	Phosphoglucomutase	5.4.2.2	1	TBE
PK	Pyruvate kinase	2.7.1.40	1	TBE

†Tris borate EDTA buffer (pH 8.9).
‡Tris citrate buffer (pH 7.1).

Table 2 Allele frequencies at 17 loci in *Polyommatus caelestissimus* from Spain and *P. coridon* from central Italy and Sardinia

Locus	Allele	Population		
		Spain	Central Italy	Sardinia
Aat		*n* = 12	*n* = 29	*n* = 40
	A	0.000	0.000	1.000
	B	0.958	0.914	0.000
	C	0.000	0.034	0.000
	D	0.042	0.052	0.000
Aco		*n* = 8	*n* = 18	*n* = 19
	A	1.000	0.944	0.000
	B	0.000	0.056	1.000
Ak		*n* = 7	*n* = 15	*n* = 21
	A	1.000	1.000	1.000
Fum		*n* = 12	*n* = 29	*n* = 40
	A	1.000	1.000	1.000
Gpi		*n* = 7	*n* = 15	*n* = 21
	A	0.071	0.033	0.000
	B	0.857	0.867	0.000
	C	0.000	0.000	1.000
	D	0.071	0.100	0.000
Gpd		*n* = 10	*n* = 23	*n* = 29
	A	1.000	1.000	1.000
G6pd		*n* = 10	*n* = 23	*n* = 29
	A	0.900	1.000	0.000
	B	0.100	0.000	1.000
Hk		*n* = 10	*n* = 22	*n* = 29
	A	0.700	0.818	1.000
	B	0.300	0.182	0.000
Idh-1		*n* = 10	*n* = 23	*n* = 29
	A	0.950	0.609	0.931
	B	0.050	0.391	0.069
Idh-2		*n* = 10	*n* = 23	*n* = 29
	A	0.000	0.022	0.000
	B	1.000	0.935	1.000
	C	0.000	0.043	0.000
Ldh		*n* = 5	*n* = 10	*n* = 11
	A	0.000	0.450	0.000
	B	1.000	0.550	1.000
Mdh		*n* = 9	*n* = 23	*n* = 29
	A	0.889	0.261	0.948
	B	0.111	0.696	0.052
	C	0.000	0.043	0.000
Me		*n* = 9	*n* = 22	*n* = 32
	A	0.000	0.023	0.000
	B	0.889	0.955	0.000
	C	0.111	0.023	1.000

Table 2 *Continued*

Locus	Allele	Spain	Central Italy	Sardinia
Mpi		*n* = 12	*n* = 29	*n* = 40
	A	0.000	0.103	0.000
	B	0.000	0.103	0.000
	C	0.000	0.017	0.000
	D	0.250	0.190	0.000
	E	0.000	0.017	0.000
	F	0.000	0.034	0.000
	G	0.708	0.534	1.000
	H	0.042	0.000	0.000
Odh		*n* = 11	*n* = 29	*n* = 40
	A	0.091	0.000	0.000
	B	0.864	0.983	1.000
	C	0.000	0.017	0.000
	D	0.045	0.000	0.000
Pgm		*n* = 12	*n* = 29	*n* = 40
	A	0.000	0.000	1.000
	B	0.000	0.000	0.000
	C	0.250	0.121	0.000
	D	0.500	0.810	0.000
	E	0.250	0.052	0.000
	F	0.000	0.017	0.000
Pk		*n* = 10	*n* = 23	*n* = 29
	A	1.000	0.978	0.914
	B	0.000	0.022	0.086

n, no. individuals tested for each population.

Results

Allelic frequencies at the 17 loci analysed are reported in Table 2. A total of 50 alleles were identified at all loci. Two loci, *Ak* and *Gpd*, were found to be monomorphic in all samples based on the 95 per cent criterion. All the other loci were polymorphic in at least one population. More than 50 per cent of loci were polymorphic in the continental *P. c. apennina* and *P. caelestissimus* whereas most loci were monomorphic in the Sardinian *P. c. gennargenti* (*P* = 17.6 per cent) (Table 3). The mean heterozygosity of the Sardinian sample was significantly lower (*H* = 0.024, *P*<0.01) than the continental samples (*H* = 0.185 − 0.170). Alleles at all loci were in Hardy–Weinberg equilibrium (*P*>0.05) except for *Hk* and *Mdh* in *P. c. apennina* and *Odh* in *P. caelestissimus* because of an excess of homozygotes. However, χ^2 values for these loci were not significant at the 0.01 probability level. Moreover, Hardy–

Weinberg equilibrium was not rejected when testing across loci within each sample (*P*>0.7) and with the overall test for all loci in all populations (*P*>0.9).

Alternative fixed alleles at the *Aat*, *Gpi* and *Pgm* loci were diagnostic for the Sardinian *P. c. gennargenti*. Allelic frequencies at the loci *Aco*, *Me* and *Mpi* were also significantly different in Sardinian and continental samples (*P*<0.0005). These loci gave the major contribution to the distinction between samples as shown by F_{ST} values ranging from 0.923 for *Aco* to 0.129 for *Mpi* (Table 4). The mean F_{ST} value of 0.521 indicates that at least 52 per cent of the total variability in the samples is attributable to divergence among populations. The high value of F_{ST} was attributable mainly to the Sardinian population because removing it from the analysis substantially decreased the F_{ST} value (F_{ST} = 0.120).

Nei's coefficient of genetic distance (*D*) between the Sardinian and the central Italian *P. c. apennina* was 0.434, and between the Sardinian and Spanish

Table 3 Genetic variability at 17 loci in the three samples of *Polyommatus*

Taxon	Sample size per locus	Mean no. alleles per locus	% polymorphic loci	Heterozygosity (mean ± SE)
P. coridon	22.6	2.5	58.80	0.185 ± 0.045
P. c. gennargenti	29.8	1.2	17.60	0.024 ± 0.113
P. caelestissimus	9.6	1.8	52.90	0.170 ± 0.051

A locus is considered to be monomorphic if the frequency of the common allele is greater than 0.95.

Table 4 Summary of *F*-statistics at all loci of *Polyommatus*

Locus	F_{IS}	F_{IT}	F_{ST}
Aat	−0.061	0.829	0.839
Aco	−0.059	0.919	0.923
Gpi	−0.121	0.668	0.704
G6pd	−0.111	−0.034	0.069
Hk	−0.090	0.033	0.113
Idh-1	0.163	0.308	0.174
Idh-2	−0.053	−0.017	0.034
Ldh	−0.010	0.346	0.353
Me	−0.097	0.782	0.802
Mpi	−0.105	0.037	0.129
Mdh	0.209	0.544	0.423
Odh	0.548	0.576	0.061
Pgm	0.075	0.564	0.529
Pk	−0.079	−0.037	0.039
Mean	0.024	0.532	0.521

Table 5 Nei's genetic identity (above diagonal) and distance (below diagonal)

	1	2	3
1 *Polyommatus coridon apennina*	—	0.648	0.947
2 *P. c. gennargenti*	0.434	—	0.714
3 *P. caelestissimus*	0.054	0.337	—

taxa it was 0.337 (Table 5). Nei's identity values were 0.648 and 0.714, respectively. The genetic distance between the two continental populations was 0.054. UPGMA cluster analysis based on Nei's unbiased genetic distances (cophenetic correlation coefficient = 0.96) confirmed the divergence of the Sardinian taxon from the two continental taxa (Table 5, Fig. 1).

Discussion

The Sardinian *P. c. gennargenti* is characterized by a high proportion of monomorphic loci and very low heterozygosity, with all loci in Hardy–Weinberg equilibrium. From the analysis of allozymic data this taxon appears as an isolated, strongly inbred population, with a very low level of genetic variation when compared to the continental *P. c. apennina* and *P. caelestissimus*. Reduction of genetic variability has been observed in peripheral isolated colonies of the

montane butterfly, *Parnassius mnemosyne* in southern France (Descimon & Napolitano, 1993) and, generally, it is a common feature of peripheral populations with respect to the source area of the species. In this perspective, *P. c. gennargenti* has probably derived from mainland populations of *P. coridon*.

The presence of diagnostic alleles and significant differences in allele frequencies at several loci indicates lack of gene flow between the island and the continental populations, suggesting that the Sardinian taxon is evolving independently from the mainland taxa. This conclusion is in agreement with the taxonomic distinction of the Sardinian taxon from the continental *P. coridon* proposed by Leigheb (1987, 1991) in his original description of *P. c. gennargenti* and based on morphological differences in wing coloration. Genetic drift probably plays a major role in the genetic differentiation of the Sardinian taxon favoured by geographical isolation and strong inbreeding within a small and subdivided population occupying a limited habitat area, probably not exceeding 20 km² (Crnjar, unpublished observation). Moreover, genetic isolation is not likely to be affected by migration, because *P. coridon* is generally a poor flyer and shows behaviour typical of sedentary butterflies (Eitschberger *et al.*, 1991).

Values of Nei's genetic distances between the Sardinian *P. c. gennargenti* and the continental taxa, ranging from 0.337 to 0.434, are higher than those found at intraspecific level, among geographically
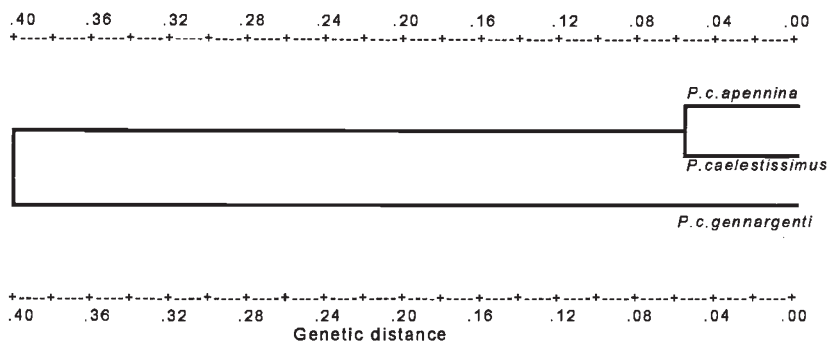
Fig. 1 UPGMA dendrogram using unbiased Nei's genetic distances.

isolated populations of *P. coridon* from peninsular Italy ($D \leq 0.1$), and at interspecific level, between *P. coridon* and the closely related *P. albicans* species complex ($D \approx 0.25$) (Mensi *et al.*, 1988). In another taxonomically controversial species group of the same Papilionidea superfamily, the *Pieris napi* complex, interspecific genetic distances were estimated to be around 0.35 or lower (Geiger & Scholl, 1985). However, if the biological species concept is applied, electrophoretic data alone do not allow us to propose a taxonomic status higher than subspecies for the Sardinian taxon. On the other hand, although reproductive isolation cannot be tested among these allopatric populations, our estimates of genetic identities between *P. c. gennargenti* and the two continental taxa ($I = 0.648$ and 0.714) are below the 0.85 value proposed as discriminating separate species (Thorpe, 1982), thus pointing to the genetic isolation of the Sardinian *P. coridon*. The diagnostic differences at morphological and biochemical levels would then favour the hypothesis of species status from at least the phylogenetic point of view (Wiley, 1978). Similar conclusions have been drawn for other allopatric taxa of Lepidoptera (Geiger & Scholl, 1985).

Calculation of divergence time from genetic distances gives estimates ranging from 6 to 1.6 Myr depending on the calibration used, $D = 1$ equivalent to 18.9 Myr (Wilson *et al.*, 1977) or $D = 1$ equivalent to 5 Myr (Nei, 1972), respectively. The cladogenetic event leading to the Sardinian lineage would have occurred after the separation of the Sardo-Corsican microplate from the continental landmass (about 30 Myr ago), probably after one of the successive marine regressions, from late Miocene to Pliocene, that brought 'Cor-Sardinia' in contact with northern Italy and southern France 5.7–0.23 Myr ago (Arias *et al.*, 1980; Cita, 1980).

Given the morphological similarities between males and the relative incidence of blue-form

females (absolute for *gennargenti* and significantly higher for *caelestissimus* as compared to other taxa in the *coridon* group), Leigheb (1987, 1991) speculated that *gennargenti* could belong to *caelestissimus*, thus confirming the faunal affinities of the Iberian Peninsula with Sardinia and Corsica. However, the differences in Nei's genetic distance between *P. c. gennargenti* and the two continental taxa, *P. caelestissimus* ($D = 0.337$) and *P. c. apennina* ($D = 0.434$) are not significant ($P > 0.05$) and similarities between the Sardinian and Spanish taxa could be the result of morphological convergence.

The genetic distance between *P. caelestissimus* and *P. c. apennina* is very low ($D = 0.054$), indicating a high level of gene flow between the two. This last result is quite different from that reported by Mensi *et al.* (1988) based on an electrophoretic study of 16 enzyme loci. They found significant differences in allele frequency at the *G6pd* locus and an alternative allele at the *Pk-2* locus between nine Spanish populations (from northern and central Spain) attributed to *P. caelestissimus* and six populations of *P. coridon* from southern France and peninsular Italy. The genetic distance between the two groups was 0.430, far higher than that reported here. A possible explanation of the discrepancy could be the origin of the populations sampled. In fact, all Spanish populations but one investigated by Mensi and co-workers came from different areas of Spain outside the distribution range of *P. caelestissimus*, which is limited to the Montes Universales in central Spain. Moreover, experimental bias caused by the small sample size cannot be excluded. Considering the wide distribution and relic characteristics of this species group, additional investigation will be necessary to clarify the phylogenetic relationship among all the member of the *P. coridon* complex.

Finally, it should be noted that the low level of genetic variation and the biological and ecological characteristics of *P. c. gennargenti* could eventually

impair the survival of this butterfly following environmental and climatic changes, thus making the Sardinian taxon vulnerable to extinction.

Acknowledgements

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