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A new conservation unit in the butterfly *Erebia triaria* (Nymphalidae) as revealed by nuclear and mitochondrial markers

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Priorities for conservation of biological units should ideally combine ecology and genetics. The European butterfly *Erebia triaria* (Nymphalidae: Satyrinae) has disappeared from several sites in Europe during the 20th century. In order to assess the conservation values of this species in NW Iberia, we screened the genetic variability and differentiation of four nuclear microsatellite markers in five populations from this area. We used a Pyrenean population as an outgroup. One particular population (Xistral, NW Iberia) was significantly different from the others. Thus, the nuclear results fully agreed with the pattern found using mitochondrial DNA sequences, and the hypothesis of incipient speciation of this population, due to an ancient isolation event, gained additional support. By combining our genetic findings with morpho- and ecological data, we argue that this population be considered a distinct unit for conservation.

Introduction

The European butterfly *Erebia triaria* de Prunner 1798 (Nymphalidae: Satyrinae) inhabits different Iberian and Alpine mountain ranges. It has an annual cycle and flies in bushy and grassy patches in rocky mountain areas (usually above 1000 m). It is a generalist feeder that lays eggs on different

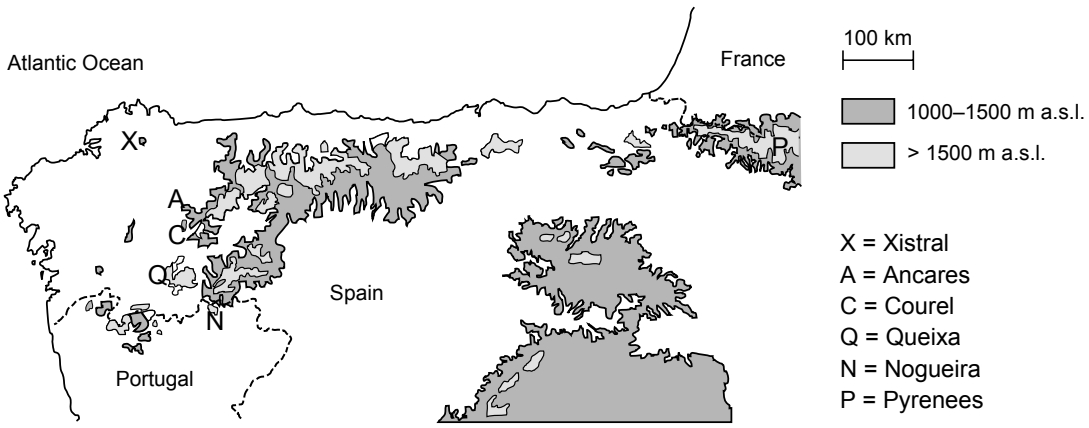

Fig. 1. Map of northern Iberia showing the sampled locations. a.s.l. = above sea level.

species of grasses (e.g., *Poa* and *Nardus*). The species has suffered local extinctions (Warren 1936, cf. Van Swaay & Warren 1999, Kudrna 2002). In fact, the Swiss populations of *Erebia triaria* were classified as endangered (Duelli 1994). The aim of our current study was to assess whether any NW Iberian population warranted the status as an evolutionary significant unit (Moritz 1994). This goal arose from the fact that recent mitochondrial DNA (mtDNA) analyses revealed that the Xistral population (Fig. 1) was as genetically divergent from the nearest NW Iberian population as the Pyrenees and Alpine populations were. Such a differentiation might have been caused by a longer stage of isolation of the Xistral population, leading to an incipient speciation process. This hypothesis is supported by the fact that Xistral was the only monophyletic population, and therefore suggesting completion of its lineage sorting (Vila *et al.* 2005).

Our study case posed an interesting scenario, as the intraspecific taxonomy of *Erebia triaria* is rather controversial. There are at least twelve morphological subspecies of *E. triaria* (reviewed by Vila 2004). While the population from Xistral has been defined as a morphologically different subspecies (*Erebia triaria pargapondalense*) (Fernández Vidal 1984), there is little consensus over the taxonomic status of the other NW Iberian populations: they were variously classified as *E. t. evias* or *E. t. hispanica*. Interestingly, the Pyrenean populations were unambiguously identified as *E. t. evias* (reviewed by Fernández Vidal 1984).

Genetic data complementing morphological differentiation is needed to design appropriate conservation policies, as the use of morphology to define subspecific status may lead to inappropriate decisions with regard to conserving genetic diversity within the species (Zink 2004). Knowledge of the genetic structure of populations is desirable to prioritize conservation units (Moritz 1994, Crandall *et al.* 2000). Mitochondrial DNA (mtDNA) is commonly used in phylogeographic studies in animals to assess relationships among populations. However, the whole mitochondrial genome is transmitted as one single unit, and therefore the evolutionary relationships may be oversimplified (Rosenberg & Nordborg 2002). The combination of mitochondrial and nuclear genetic markers usually provides a more general view of the processes shaping a given genetic pattern. A widespread technique for assessing nuclear DNA variation in population genetics surveys is the amplification of neutral hyper variable co-dominant DNA markers, e.g. microsatellite loci (Zhang & Hewitt 2003). A large number of loci are desirable in general terms. However, as few as three or four loci may be sufficient to answer particular questions (e.g., Wilson & Bernatchez 1998, Smith *et al.* 2000, Solano *et al.* 2000, Hoelzel *et al.* 2002, Krafsur 2002, Curtis & Taylor 2004, Pamilo *et al.* 2005). This is important in the light of the relative difficulty to isolate and characterize microsatellite markers in Lepidoptera (Meglécz *et al.* 2004, Zhang 2004). Thus, we compared the extent of genetic structuring yielded by four nuclear

microsatellites and mitochondrial sequences in six Iberian populations of this species. Genetic data allowed us to calculate the actual degree of differentiation within the NW Iberian populations of *Erebia triaria*. This was of great value to determine if there was any population worth a separate management.

Material and methods

Population sampling

We analysed 105 individuals from six Iberian populations: Xistral n = 20, Ancares n = 12, Courel n = 20, Queixa n = 20, Nogueira n = 12, and Pyrenees n = 21 (Fig. 1). All these specimens were adults collected during flight season, stored in 95% ethanol, and sequenced for two mitochondrial fragments by Vila *et al*. (2005), who provided geographic coordinates and further collection details.

Laboratory procedures

We screened the seven microsatellite loci available for *Coenonympha hero* (Cassel 2002). Three of them were polymorphic for *Erebia triaria*: hero4, hero13 and hero17. We present a new locus (hero18, accession number AY396747), polymorphic in *E. triaria*. This locus was also initially developed for *C. hero* using the same procedures as for the others. It did not reveal any polymorphism until tested in *E. triaria*. The microsatellite of the cloned fragment was 113 bp long (including the flanking regions) and it had the repeat motif $(CT)_2(TC)_{10}$. The primer sequences are 5´CCGTCGTATTATGTTACCG3´ and 5´GGTAGTTGTATGGCAGCAC3´.

We extracted DNA from thorax tissue with Chelex or DNeasy Tissue Kit (Qiagen). PCR were undertaken in 10 μl volumes using approximately 10 ng DNA, 0.3 mM of each dNTP, 0.5 units AmpliTaq DNA polymerase, 1 μl 10X PCR Buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1.5–2.5 mM $MgCl_2$ (all reagents by PE Biosystems), and 0.5 mM of each primer. PCR amplifications consisted of 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C followed by extension for 30 s at 72 °C, and ending with 7 min at 72 °C. PCR products were run on 6% polyacrylamide gels and visualized using silver staining.

Data analysis

Analyses of Hardy-Weinberg (HW) segregations were performed for all loci and all populations. We used the score test (U-test, Rousset & Raymond 1995) in order to evaluate whether the observed deviations from HW were due to heterozygote excess or deficit. An unbiased estimate of the exact P value was computed using the Markov Chain Monte Carlo Method (Guo & Thompson 1992). Genotypic linkage disequilibria among loci were tested from contingency tables and an unbiased estimate of the exact probability was obtained by using the Markov Chain Monte Carlo Method (Raymond & Rousset 1995a). All these calculations were computed as implemented in the software GENEPOP (http://wbiomed.curtin.edu.au/genepop/index.html) developed after Raymond and Rousset (1995b). Expected heterozygosity was calculated in BIOSYS-2 (Swofford *et al*. 1997) and using Levene's correction. We calculated genic and genotypic differentiation among populations using a contingency table. Significance was tested with an unbiased estimate of the P value of a log-likelihood (G) based test (Goudet *et al*. 1996). Weighted F-statistics were calculated using the estimators described by Weir and Cockerham (1984). F-statistics were calculated in FSTAT (Goudet 1995), a program also used to compute the standard error for each statistic through jackknifing over populations. The proportion of null alleles was calculated following Brookfield (1996). We estimated the relatedness (r) of individuals within each sampled population using the program DELRIOUS (Stone & Björklund 2001). This estimate uses the method devised by Lynch and Ritland (1999), which calculates relatedness as the probability of sharing alleles by common descent conditional on the distribution of alleles in the population. The correlation between genetic and geographic distance measures was examined using the Mantel test (1000 permutations, Mantel 1967). The geographic distances

were logarithmically transformed and analysed together with values of $F_{ST}/(1 - F_{ST})$, as suggested by Rousset (1997). We used BIOSYS-2 (Swofford *et al*. 1997) to compute the chord distance of Cavalli-Sforza and Edwards. The relationships between the populations were determined by constructing a Neighbor-joining (NJ) tree, based on those chord distances, using PHYLIP (Felsenstein 1995). Its robustness was evaluated after 1000 bootstrap replicates.

Results

Mean number of alleles per locus within populations ranged from 2.0 at Queixa to 2.8 at Xistral and Ancares (Table 1). The number of alleles per locus ranged from three to four (Table 2). Expected heterozygosity ranged between populations and loci, although the average values over all loci were very similar (Table 1). Private alleles were only found in Xistral (hero4) and the Pyrenees (hero18) (Table 1). The proportion of null alleles was estimated to be > 0.1 for locus hero18 (Ancares, 0.18; Courel, 0.21), hero4 (Xistral, 0.14) and hero13 (Queixa, 0.12).

There was no indication of linkage among any of the four loci ($P > 0.05$). There was a significant excess of homozygotes when tested over all populations and all loci (HW exact test: $P < 0.001$). We found significant deviations from HW in 3 out of 24 tests (loci within each population). Heterozygote deficit was significant at Courel, Queixa and Pyrenees (Table 1). Accordingly, F_{IS} values were significantly positive in Courel and Pyrenees. We found no excess of heterozygotes.

Despite the low number of loci, there were significant differences among all populations both at the level of allele frequency and genotype (*G*-test: $P < 0.001$). This resulted in $F_{ST} = 0.07$ over all loci and populations ($P < 0.001$). The result was consistent for all loci but hero13, which had the lowest *F* values (Table 2).

There was no sign of kin groups in any of the studied populations. The relatedness values obtained for the six populations were determined to be insignificantly different from what would be expected on the basis of simulations involving unbiased mating.

The NW Iberian populations did not follow the expected isolation-by-distance model (Mantel

Table 1. Genetic diversity within populations of *Erebia triaria* as from four microsatellite loci. Mean *n* = average number of individuals analysed, A = mean number of alleles per locus, PA = number of private alleles, HWE = deviations from Hardy-Weinberg equilibrium, H_{exp} = expected and H_{obs} = observed heterozygosities. NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Standard errors are given in parentheses.

Pop	Mean *n*	*A*	PA	HWE	H_{exp}	H_{obs}	F_{IS}
Xistral	18.5 (0.6)	2.8 (0.5)	1	NS	0.31 (0.09)	0.28 (0.09)	0.12 NS
Ancares	12.0 (0)	2.8 (0.3)	0	NS	0.35 (0.06)	0.27 (0.02)	0.24 NS
Courel	18.8 (0.3)	2.5 (0.3)	0	**	0.38 (0.09)	0.27 (0.12)	0.31 **
Queixa	19.3 (0.5)	2.0 (0.0)	0	*	0.34 (0.09)	0.23 (0.06)	0.32 NS
Nogueira	11.3 (0.5)	2.0 (0.0)	0	NS	0.38 (0.10)	0.34 (0.09)	0.10 NS
Pyrenees	19.8 (0.6)	2.3 (0.6)	2	**	0.26 (0.14)	0.21 (0.12)	0.18 **

Table 2. Single and multilocus genetic estimates. Calculations performed across the six studied populations of *Erebia triaria*. *N* = number of alleles per locus, HWE = deviations from Hardy-Weinberg equilibrium. NS = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Standard errors for *F*-statistics are given in parentheses.

Locus	*N*	HWE	F_{IS}	F_{ST}	F_{IT}
Hero4	4	**	0.287 (0.064)	0.083 (0.095)	0.344 (0.073)
Hero13	4	**	0.042 (0.176)	0.044 (0.038)	0.081 (0.156)
Hero17	3	NS	0.015 (0.217)	0.098 (0.052)	0.099 (0.16)
Hero18	4	***	0.344 (0.089)	0.071 (0.081)	0.390 (0.092)
ALL	–	***	0.226	0.071	0.280

test: $P > 0.05$), not even when the Pyrenean population was included ($P = 0.07$). The NW Iberian population of *Erebia triaria* occurring at Xistral was at least as much genetically different from the other NW Iberian populations (e.g., Xistral-Courel, $F_{ST} = 0.161$, 119 km apart) as a geographically distant population was (e.g., Pyrenees-Xistral, $F_{ST} = 0.136$, 720 km apart; Pyrenees-Courel, $F_{ST} = 0.104$, 718 km apart, Table 3). Furthermore, the detection of one private allele (Table 1), and the phylogenetic tree (Fig. 2) supported the distinctiveness of Xistral from the other NW Iberian populations.

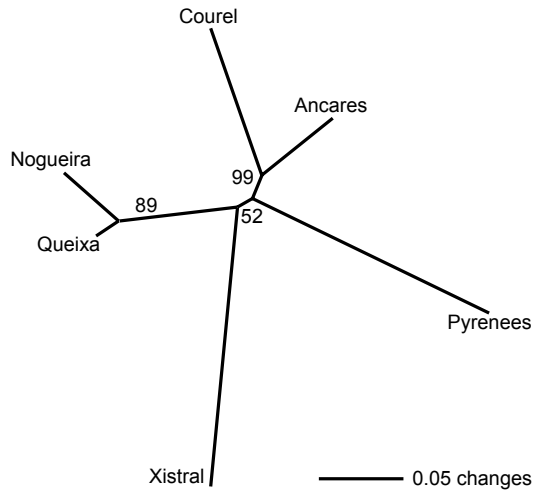

Fig. 2. Phylogenetic relationships of six Iberian populations of *Erebia triaria* obtained from microsatellite data. Neighbor-joining dendogram from Cavalli-Sforza and Edwards chord genetic distances. Bootstrap values higher than 50% after 1000 replicates are displayed beside branches.

Discussion

Conservation genetics

Both the microsatellite and mtDNA analyses (Vila *et al.* 2005) revealed the genetic distinctiveness of the population of *Erebia triaria* from Xistral (Fig. 2 and Table 3). If distinct genetic units do not show ecological exchangeability, they should be managed separately and may even be treated as different species (Crandall *et al.* 2000). Despite the lack of experimental studies, an ecological distinctiveness of Xistral (endemic subspecies *Erebia triaria pargapondalense*) is indicated by the following facts: (i) Xistral is the only location in the entire distribution area where the species can be found as low as 700 m above sea level (Fernández Vidal 1984), (ii) Atlantic climate features at Xistral are different from the other areas inhabited by the species (Martínez Cortizas & Pérez Alberti 2000), which is reflected by the delayed flight period for the species in this particular area (M. Vila unpubl. data), (iii) adult *Erebia triaria* from Xistral are morphologically different in body size and wing pattern (Fernández Vidal 1984), (iv) floristic criteria established a clear distinction between the vegetation present in Xistral and the other areas where the species appears (reviewed by Izco Sevillano 2001), and (v) the area between Xistral and its closest population (Ancares) has been unsuitable for the species since, at least, the last glacial maximum (González & Saa 2000, Santos *et al.* 2000). Based on these observations and our genetic analyses, we recommend that *Erebia triaria pargapondalense*, endemic to the

Table 3. Pairwise F_{ST} values for one Pyrenean and five NW Iberian populations of *Erebia triaria*. Below diagonal: from the microsatellite dataset. Above diagonal: from mtDNA data (Vila *et al.* 2005). Significance of the F_{ST} estimates from microsatellite data was obtained after 300 permutations. (*) Significant values after sequential Bonferroni correction.

	Xistral	Ancares	Courel	Queixa	Nogueira	Pyrenees
Xistral		0.85*	0.74*	0.77*	0.81*	0.92*
Ancares	0.086		0.092	0.136	0.75*	0.66*
Courel	0.161*	–0.005		0.875	0.53*	0.45*
Queixa	0.065*	0.001	0.034		0.60*	0.56*
Nogueira	0.074	0.053	0.057	–0.023		0.87*
Pyrenees	0.136*	0.004	0.104*	0.0534*	0.135*	

NW Iberian mountain range of Xistral (43°27´N, 7°30´W), be managed separately, bearing in mind that this is a species sensitive to habitat loss and fragmentation (reviewed by Vila 2004).

Four microsatellite loci were sufficient to reveal the significant distinctiveness of *Erebia triaria* at Xistral (i.e., subspecies *pargapondalense*). However, these four loci were not conclusive regarding population structuring among the other NW Iberian locations. The failure of Nogueira and Ancares to be significantly different from other populations (Table 3) may be due to their small sample sizes (n = 12). MtDNA analyses demonstrated that Nogueira was significantly different from Ancares and Courel (Table 3). The lack of a correlation between genetic (microsatellite) and geographic distance may imply that the populations in this study are not connected by current gene flow and that genetic drift has caused the pattern of divergence. In any case, the population from Nogueira (NE Portugal) was not genetically distinguishable from the NW Spanish population of Queixa. Hence, we encourage coherent conservation policies between both countries.

Homozygote excess

There was a deficiency of heterozygotes in all populations and loci. A recurrent excess of homozygotes has also been found in several other microsatellite studies on Lepidoptera and some authors were able to identify null alleles (reviewed by Meglécz *et al*. 2004, Prasad *et al*. 2005, Van't Hof *et al*. 2005). We cannot exclude that the excess of homozygotes in our dataset is also due to the presence of null alleles. We are aware of the fact that the four microsatellite markers used in the present study were originally developed for *Coenonympha hero* (Cassel 2002), and there are indications of null alleles in that species (A. Cassel Lundhagen unpubl. data). Family material was not available to test for the presence of null alleles. Still, we did not detect any new allele when redoing amplifications of failed and homozygote individuals at lower annealing temperatures. Even if null alleles existed, they probably did not play a major role in our results because (1) separate analyses excluding locus hero18, hero4, and hero13 showed congruent results (data available under request); (2) regardless of the origin of homozygote excess, the estimates of relatedness did not significantly differ from expectations under unbiased mating, and (3) the population differentiation pattern inferred from microsatellites did not markedly differ from that based on mtDNA (Table 3, cf. Vila *et al*. 2005).

An excess of homozygotes might also be due to other processes such as allele drop-out (Björklund 2005 and references therein), unintentional mixing of separate breeding units (i.e., the Wahlund effect), under-dominant selection, assortative mating, or inbreeding. Our data did not allow us to reject any of these hypotheses. The Wahlund effect is unlikely because each population was collected in plots less than one hectare in size and over one or two consecutive days. Further, significant homozygote excess was found in three out of the four studied loci and we consider it unlikely that selection has driven three independent loci in the same direction. For the same reason, we argue that assortative mating (i.e., mate selection, not including close relatives) did not cause the observed pattern. Such a behavioural trait is unlikely to be linked to three out of four microsatellite loci, as these markers were in linkage equilibrium themselves. The final alternative, inbreeding, is unlikely because it should have affected equally to all markers, causing equal F_{IS} values across loci. Our data did not support the inbreeding hypothesis, as F_{IS} values ranged between 0.015 and 0.344 (Table 2), and relatedness did not depart from expected under random mating in any of the studied populations. Even so, data on effective population size and mating patterns within local populations are needed before drawing further conclusions about a putative inbreeding process.

Resolution of the markers

The number of unique alleles and number of alleles per locus was lower than expected for populations hypothetically isolated since the last deglaciation. Had the studied populations been actually isolated since then, the mutation rates of these markers would have been on

the level of allozymes (10^{-9}–10^{-4} mutations per gene and generation) rather than microsatellites (10^{-5}–10^{-2}) (Jarne & Lagoda 1996). A possible explanation for this is that variability tends to be lower in those markers originally developed for another species (Jarne & Lagoda 1996), as was the case here. If the presented low variability was due to cross-amplification from a different genus, it is not surprising that these four loci had enough resolution to reveal the differentiation of highly divergent populations such as Xistral and Pyrenees, but were not sufficient to show the putative structuring between more related populations (e.g., Queixa-Ancares).

To summarise, both mitochondrial and nuclear markers agreed in revealing Xistral as a highly divergent NW Iberian population, i.e., Xistral differed as much from the other NW Iberian populations as the Pyrenean population did. Therefore, the hypothesis of a longer isolation of Xistral versus the other NW Iberian populations, postulated from mtDNA data (Vila *et al*. 2005), gained additional support. This historical value enhances the definition of *Erebia triaria pargapondalense* as a new conservation unit to be separately managed from the other NW Iberian populations of the species.

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