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Original article

Setting conservation priorities: the case study of *Carabus solieri* (Col. Carabidae)

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Abstract – *C. solieri* is an endangered species of Carabidae which is mostly associated with humid forests of the Alps, in France and in Liguria (Italy). We reanalysed morphological data published by Bonadona and compared the results to the molecular data set presented here. We used partial sequences of the mitochondrial cytochrome *b* gene and seven microsatellite loci to evaluate genetic diversity among and between populations and to propose a phylogeographic scenario. We recognised two basic entities, considered as subspecies, which probably colonised the present distribution range from two main refugia (in France and in Italy). There is no strict agreement between morphological and molecular data and we propose that hybridisation and introgression between the two subspecies have led to the observed patterns of distribution of the characters. Our results show that sampled populations differ significantly in microsatellite allele frequencies even though some populations are < 15 km apart. Gene flow estimates between the 19 sampled populations indicated very restricted exchange. This is in agreement with the low vagility observed for most species of *Carabus*. Consequently, the Solier's Carab is genetically structured on an extremely fine scale, and local population within a definite forest should be considered as management units. In the light of our data on *C. solieri* we discuss the usefulness, when hybridisation occurs, of the two main criteria usually quoted for identifying candidate populations for conservation management (uniqueness and diversity) and give some conservation recommendations for *C. solieri*.

insect / conservation / population genetic / phylogeography / hybridisation

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Résumé – Choix des priorités de conservation : le cas de *Carabus solieri* (Col. Carabidae). *C. solieri* est une espèce de Carabidae en danger d'extinction. Cette espèce est principalement associée aux forêts humides des Alpes françaises et liguriennes (Italie). Nous réanalysons les données morphologiques publiées par Bonadona et comparons les résultats à ceux obtenus à partir des données génétiques présentées ici. Nous utilisons des séquences partielles du gène du cytochrome *b* et sept loci microsatellites pour mesurer la diversité génétique au sein et entre les 19 populations échantillonnées et pour proposer un scénario phylogéographique. Nous reconnaissons deux entités de base, considérées comme sous-espèces, qui ont probablement colonisé l'actuelle aire de distribution à partir de deux refuges glaciaires (en France et en Italie). Il n'existe pas de congruence stricte entre les données morphologiques et moléculaires, et nous proposons l'hybridation et l'introgression entre les sous-espèces comme facteurs expliquant le patron de distribution des caractères observé. Nos résultats montrent que les populations étudiées diffèrent significativement par leurs fréquences alléliques, même pour des populations séparées par moins de 15 kilomètres. Les estimations de flux génique entre les populations indiquent un faible niveau d'échange. Ceci est en accord avec la faible vagilité observée chez la plupart des espèces de *Carabus*. En conséquence le Carabe de Solier est génétiquement structuré à une très fine échelle, en conséquence toute population associée à un bloc forestier peut être considérée comme une unité de gestion. Nos résultats sur *C. solieri*, et en particulier la mise en évidence de l'introgression mitochondriale, nous amènent à discuter les deux principales méthodes (originalité et diversité) utilisées couramment pour identifier les populations susceptibles d'être protégées. Enfin nous donnons quelques considérations générales sur les mesures de protection pouvant être prises.

insectes / conservation / génétique des populations / phylogéographie / hybridation

1. INTRODUCTION

One of the main objectives of conservation biology is the preservation of unique ecological adaptations and the maintenance of evolutionary potential. The major problem faced by conservation biologists is the allocation of scarce resources to an overwhelmingly large number of species or populations in need of preservation efforts. As it is not possible to protect everything everywhere, priorities must be identified and decided upon. At the species level such an assumption means that we have to identify populations that must be protected because their loss will greatly reduce the overall evolutionary potential of the species.

Developing a practical approach to define natural biological units for conservation has been problematic and sometimes contentious. There is no general agreement on the methods and criteria needed to identify which populations should be conserved. Some researchers have questioned the importance of genetic information, stating that ecological or demographic issues may be more

pressing. However, there is a growing body of evidence that both stochastic and deterministic demographic, environmental and genetic factors influence population viability.

Recent advances in molecular techniques have greatly enhanced the importance of genetic information in population management and wildlife conservation. In the field of conservation genetics, molecular markers are currently used, (1) to better define species and subspecies, (2) to detect introgressive hybridisation among closely related taxa, (3) to describe the partitioning of genetic diversity within and among populations, (4) to identify and define natural biological units which must be preserved [36].

Here, we would focus on how genetic studies can contribute to identifying conservation priorities. Two criteria are usually quoted for identifying candidate populations for conservation management: (1) the phylogenetic or genetic uniqueness, (2) the potential for maintaining the evolutionary process [81].

1.1. The phylogenetic or genetic uniqueness and the ESU concept

This criterion is founded on the underlying assumption that populations that are distinct in their morphological traits, ecological requirements and in their DNA markers (mitochondrial and nuclear DNA) should have high conservation status. The concept of Evolutionary Significant Units (ESUs) was developed to provide a rational basis for prioritising taxa for conservation effort [53]. This concept is somehow vague and its merits and limitations have recently been debated [56]. Since its first definition it has not been applied consistently. Ryder [64] introduced the concept to help guide conservation efforts for captive breeding programmes. For Ryder, an ESU is a group of organisms that has been isolated from other conspecific groups for a sufficient period of time to have undergone meaningful genetic divergence from those other groups. Waples [83], subsequently extended this concept to management of wildlife conservation. In an effort to better define subunits of species for conservation purposes, Waples defined ESUs as population or a group of populations that are reproductively isolated and constitute an important component in the evolutionary legacy of the species. Dizon *et al.* [26] suggested that populations should be considered as ESUs if they show significant differences in allele frequencies. Recently, Moritz [53] argued that ESUs could be identified genetically as historically isolated sets of populations which show reciprocal monophyly for mitochondrial alleles and significant divergence of allele frequencies at nuclear loci. Finally, ESUs were likened to species using the phylogenetic species concept [21] by several authors [4, 10, 82]. According to this definition, ESUs are "irreducible clusters of organisms, diagnosably distinct from other such clusters, and within which there is a parental pattern of ancestry and descent" [20]. From a practical point of view, such populations comprise individuals which exhibit attributes not found in other populations. There is no general agreement about the criteria to be used in defining ESUs. Operational definitions rely mostly

on molecular data (mtDNA, allozyme and microsatellite data), but some authors also include ecological, behavioural, biogeographical and morphological data [9,48,53,71,82].

1.2. The potential for maintaining the evolutionary process

This criterion is used to select, for conservation purposes, populations because they exhibit high levels of genetic variability and could preserve the evolutionary potential of the species. The single most significant cause of population decline amongst native fauna is the loss of suitable habitats, resulting in fragmentation, often isolation and the loss of genetic diversity. Frequently, genetic diversity within populations living in remnants is reduced as it becomes increasingly difficult for individuals to disperse between fragments. There is a loss of variability within small populations leading to an inability to evolve through natural selection. From this result, conservationists have concluded that there is a need to maintain genetic diversity in order to increase the short-term viability of fragmented declining populations and to maintain long-term evolutionary potential of individuals, populations and species. Consequently and from a purely genetic perspective, the main goal is to conserve as much as allelic diversity and heterozygosity as possible [19].

Two main components of genetic variability (heterozygosity and allelic richness) have been used to identify natural units which merit conservation effort. Heterozygosity (H) is a measure of allelic diversity which incorporates both number and frequency of alleles. Heterozygosity is thought to have effects on both individual fitness and the potential of a population to change genetically [3]. The use of heterozygosity in conservation management is based on the underlying assumption that a higher level of variation detected at neutral loci enhances individual fitness and consequently the probability of a population's survival over ecological or evolutionary time [8]. Whether or not reduction in genetic diversity compromises long-term fitness is still strongly debated. There are theoretical and empirical reasons to doubt a direct connection between variation at marker loci and those determining fitness [45]. Furthermore, it is likely that most important life history traits associated with fitness are polygenic, quantitative traits [72]. Consequently reservations about the validity of H as a measure of the genetic viability of a population has been expressed by several workers and there are documented cases where extremely high levels of heterozygosity are associated with low allelic richness [39]. However, observed cases of increased susceptibility to pathogens or parasitoids in natural populations that have low levels of heterozygosity indicate the importance of genetic variation (*i.e.* heterozygosity) [65]. There is also some empirical evidence that individual heterozygosity is positively associated with components of fitness in outbreeding species.

Measurement of allelic richness (R) has been proposed to be of obvious interest in conservation genetics and should be given high priority [57]. Allelic richness is more strongly dependent on effective population size than heterozygosity [47]. Furthermore, allelic richness declines more quickly than heterozygosity following a sharp reduction in the population size [2,32] and is considered to be a better indicator than heterozygosity for assessing past demographic changes.

Very few studies have undertaken a "comparative analysis" of the two methods reviewed here, their respective advantages, major flaws and inconsistencies. Furthermore, in most published studies, only one molecular criterion has been thoroughly analysed. Results obtained from one data set have rarely been compared and contrasted with results from other data sets. Our preliminary results on *Carabus solieri*, and those published by Bonadona, provide us with three different types of data (morphological, mitochondrial and nuclear) to compare the two methods.

The aims of the present study were to (1) characterise genetic and morphological variation, within a series of *C. solieri* populations throughout its current distribution. We used two types of molecular markers – mtDNA sequences and microsatellites – and we reanalysed morphological data published by Bonadona [14]; (2) obtain information on the phylogeography of *C. solieri*, and possibly to reveal glacial refuges and postglacial recolonisation routes; (3) identify and give priority to populations which contribute most to the overall diversity; and (4) evaluate the two main methods (based on genetic distinctiveness and genetic variability) used to identify any priority populations for the conservation of genetic diversity within the species.

2. MATERIAL AND METHODS

2.1. Biological material and study area

Carabus (Chrysocarabus) solieri Dejean is an endangered species which is restricted to the southern and Ligurian Alps. The species is distributed from Estérel and Tanneron massifs to the north of Briançon, and from the Alpes de Haute Provence (France) to Liguria (Italy) in the east. The outline of the range of *C. solieri,* shown in Figure 1, follows the maps published by Bonadona [14] and Casale and Cavazzuti [17], with some modifications suggested by information published subsequently. The life history of the species is relatively poorly known. *C. solieri* reproduces in spring, mostly laying eggs from March to July, depending on the elevation. Larval development occurs in late spring and summer. Tenerals emerge in late summer and autumn, and overwinter in the soil. Unlike most other species of *Chrysocarabus*, *C. solieri* cannot be collected during winter from their hibernating quarters, probably because the species overwinters deep in the ground. The species is eurytopic, mostly associated with humid, deciduous or coniferous forests. *C. solieri* can

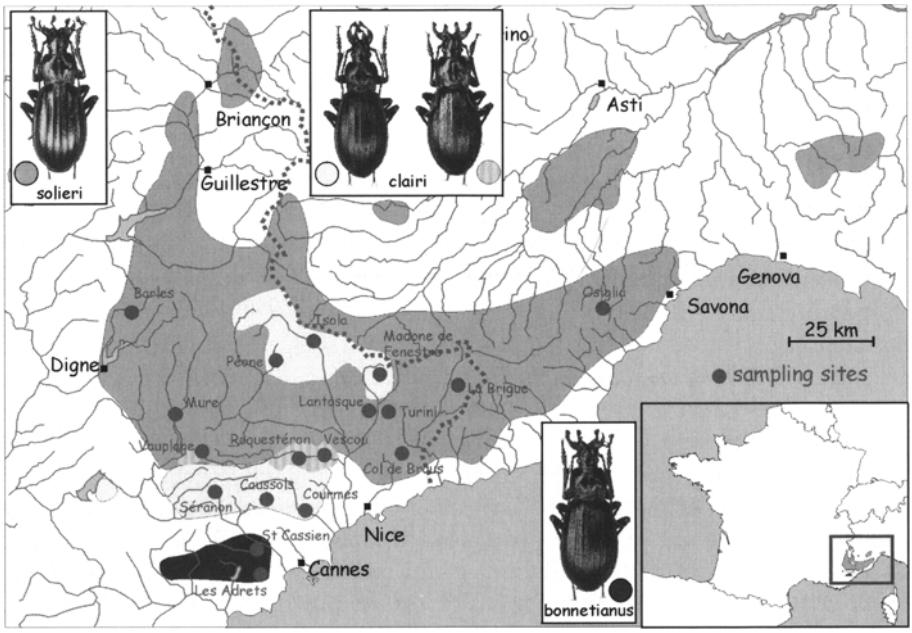

Figure 1. Range of the different subspecies of *C. solieri* and location of the populations sampled for mitochondrial and microsatellite analysis.

also inhabit Mediterranean dry forests and alpine grasslands. In Italy, Casale and Cavazutti [17] found the species associated with forests receiving about 1250-1500 mm of rain/year.

The infraspecific classification of *C. solieri* is unclear. Some authors recognise up to six subspecies, others only three [14, 17, 25, 28, 59, 77, 79]. Here we follow Bonadona [14] who made a thorough morphological study and recognised only the following subspecies: *C. s. solieri*, *C. s. bonnetianus* and *C. s. clairi*. Our choice was only determined by the relative objectivity and simplicity of the hypothesis advocated in Bonadona's study.

C. s. bonnetianus, occurs in the most southern part of the distribution area of the species in the Estérel and Tanneron massifs (Fig. 1). It differs morphologically from the subspecies *C. solieri s.* by different attributes of coloration, pronotum and elytral sculpture: (1) the proportion of the pronotum (pronotum length/pronotal width < 0.78 *versus* > 0.80), (2) the shape and the sculpture of the elytral striation (secondary interstries larger than the tertiary and smooth *versus* secondary and tertiary interstries with the same width and granulation), (3) the colour of the body (deep metallic blue *versus* metallic green).

C. s. solieri has the largest distribution area and has sometimes been subdivided into three subspecies (*vesubiensis*, *liguranus* and *solieri*) based on unreliable morphological characters and distribution.

C. s. clairi has a disjunct distribution range and exhibits morphological characteristics intermediate between *C. s. bonnetianus* and *C. s. solieri*. The southern populations of *clairi*, as defined by Bonadona, have often been considered to belong to *C. s. bonnetianus*, while the northern populations restricted to high mountain forests are sometimes considered as the only true *C. s. clairi* populations. In the contact zone between southern populations of *C. s. clairi* and *C. s. solieri* (green hatching in Fig. 1), populations showing intermediate morphology have often been upgraded to subspecies rank (*C. s. curtii*). It has been suggested that *C. s. clairi* could result from northern postglacial colonisation of *C. s. bonnetianus* which subsequently introgressed by *C. s. solieri* [59].

2.2. Sample collection

During spring and summer 1997 and 1998, adults were collected using rows of 20 to 60 unbaited pitfall traps (without fluids). Pitfall traps were visited weekly during the activity period of the adults. The sampling sites and the taxonomic status of the specimens collected are given in Table I and located in Figure 1. A total of 352 individuals were collected from 19 localities. Some specimens were killed and stored in 100% ethanol at −22 °C until analysis. For other specimens we cut one median leg and stored it under the same conditions.

2.3. Morphology

2.3.1. Data set

We partly used the morphological data published by Bonadona [13, 14] and reanalysed them. To enable comparisons between our data and Bonadona's, we used a sample of the populations he analysed. This sample includes all populations common to both studies (ADR, CAS, CAU, SER, ROQ, VES, TUR, LAN, BRI, MAD, BRA). For six of our populations not present in the Bonadona's data set, we included instead the geographically closest populations he sampled. While these populations could be genetically different, we suppose that the morphological differences they exhibit are not sufficiently divergent to mislead their classification in well supported groups. Consequently, Osiglia, Vauplane, La Mure, Isola, and Barles were respectively replaced by San Giorgio (GIO), Collongues (COL), La Rochette (ROC), St Martin-Colmiane (CLM) and Les Dourbes (DOU), populations which are only a few kilometres distant. Two populations, Courmettes and Péone (COU & PEO), have no equivalent in the data set of Bonadona.

We used all the morphological characters analysed by Bonadona, except for coloration which we consider to be too subjective. Individuals of both sexes (n = 455, ♂ = 265, ♀ = 190) from 16 populations were measured. Numbers of males, females and total number of individuals analysed per population are given in Table III. Five characters were inferred from direct measurements: the

Table I. Sampling sites for the different subspecies of *C. solieri*, with department, altitude, date of collection and population codes.

Taxa	Locality	Population codes	N sampled individuals
C. olympiae	Italy – Valsessera - Elevage J.C. Malausa (8.V.1997)	–	
C. solieri bonnetianus	France (83) – Tanneron – Saint-Cassien des Bois, 150 m (V.1997)	CAS	20
"	France (83) - Les Adrets-de-l'Estérel - Auberge des Adrets, 250 m (7.V.1998)	ADR	19
C. solieri clairi	France (83) - Séranon – Route Forestière des Buissières, 1080 m (VI.1998)	SER	20
"	France (06) - Courmes (V.1998)	COU	20
"	France (06) – Forêt de Caussols (V.1998)	CAU	20
"	France (06) - Toudon - Vescou, Rau. de Ronson, 400 m (V.1997)	VES	20
"	France (06) – Roquestéron-Grasse, bord de l'Estéron, 325 m (V.1997)	ROQ	13
"	France (06) – Péone, 1430 m (VI.1998)	PEO	18
"	France (06) - Isola – Pont St Honoré, 850 m (V.1998)	ISO	20
"	France (06) - Saint-Martin-Vésubie – Route de la Madone de Fenestre, 1400 m (VI.1998)	MAD	20
C. solieri solieri	France (04) – Soleilhas - station de Vauplane, 1560 m (V.1998)	VAU	20
"	France (04) - Barles - Clues de Verdaches, 1150m (VI.1997)	BAR	13
"	France (04) - La Mure-Argens – Clot Mouret, 1380 m (VI.1998)	MUR	18
"	Italy – Savona – Osiglia, 860 m (V.1997)	OSI	20
"	France (06) – Sospel - près Col de Braus, 900 m (VI.1998)	BRA	20
"	France (06) - La Brigue - Notre Dame des Fontaines, 950 m (VI.1998)	BRI	20
"	France (06) – Lantosque - Forêt de Sauma-Longa, parcelle B & C, 550 m (V.1997)	LAB & LAC	20 & 20
"	France (06) - La Bollène-Vésubie, Forêt de Turini, ravin des Issarts, 1200 m (VII.1997)	TUR	11

total length of individuals (Lto *in* [14]), the shape of the pronotum calculated as the ratio length/width (LP/lP), the position of the largest width of the pronotum compared to the length of the pronotum (HP/LP), the shape of the elytrae calculated as the ratio length/width (LE/lE), the position of the largest width of the elytrae compared to the length of the elytrae (HE/LE). Two other morphological characters were used as the proportion of individuals possessing the character within the sampled population: (1) the compared width of the secondary and the tertiary striation (larger *versus* identical), and (2) the sculpture of the tertiary striation (smooth *versus* granulate).

2.3.2. Data analysis

Morphological distances between pairs of populations were calculated using Euclidean distances computed by distance $(x, y) = [\sum_i (x_i - y_i)^2]^{1/2}$. All characters were separately analysed for males and females. Between-population morphological divergence was used to construct a population UPGMA dendrogram [69] in Statistica 6.0. We scaled the tree to a standardised scale, representing the percentage of the range from the maximum to the minimum distance in the data.

2.4. mtDNA sequencing

2.4.1. Sequence production

Total DNA was prepared from the muscles of one leg using standard phenol-chloroform extraction [67]. We amplified ca. 640 bp of the mitochondrial cytochrome *b* (cyt. *b*) gene using PCR. The primers used were CP1: $^{5'}$GATGATGAAATTGGATC$^{3'}$ [37] and CB2: $^{5'}$CTAATGCAATAACTCCTCC$^{3'}$ [41]. Using the Promega Taq package, 30 cycles of amplification were performed as follows in 50 μl reaction volumes: denaturation step at 92 °C for 1 min, annealing at 48 °C for 1 min and 30 sec, and extension at 72 °C for 1 min. PCR products were then purified using the QIAquick PCR purification kit (QIAgen), and directly sequenced on an ABI 373 automated sequencer using TaqFS and dye-labelled terminators (Perkin-Elmer). CP1 and CB2 were both used as sequencing primers.

2.4.2. Data analyses

Sequence alignment was performed using ClustalW software [78]. Analyses were conducted using the distance-matrix method with the Neighbour-Joining (NJ) algorithm [66] with both MEGA (Molecular Evolutionary Genetics Analysis, version 1.01) [44] and PHYLIP 3.57c [31]. Distances were computed by Kimura's two-parameter method [43]. A bootstrap procedure of 1000 iterations

was completed. We also performed a maximum likelihood analysis (quartet puzzling tree search) with PUZZLE 4 [73].

2.5. Microsatellite loci

2.5.1. Isolation and screening

Total DNA was extracted from single individuals following standard phenol-chloroform extractions [67]. Microsatellite screening was performed following Estoup *et al.* [30]. DNA was isolated from one individual of *C. solieri bonnetianus* collected in Saint Cassien des Bois (Var). Two micrograms of total DNA was digested to completion with *Bsp 143I* (Eurogentec). Fragments of 300-800 bp were isolated on a 1.5% agarose gel, ligated to *Bam*HI-digested pUC18 (Pharmacia) and cloned in *Escherichia coli* XL-1 Blue Cells (Stratagene).

We transferred 2621 recombinant clones onto nylon membranes (Amersham). Colonies were screened with six probes (TG_{10}, TC_{10}, $ATCT_6$, CAC_5, $TGTA_6$, CCT_5) linked with digoxygenin. We detected 89 positive clones and 38 were partially sequenced with an automatic sequencer (Perkin Elmer) using the dideoxy chain-termination method on alkaline denatured plasmids. Primer sequences were determined for ten microsatellite loci using the software PRIMER3. All were found to be polymorphic but seven were used in the following study (Tab. II).

PCR amplifications were performed in a total volume of 10 μl using a DNA thermal cycler (MJ research). The reaction mix contained 2 μl diluted genomic DNA (2 ng/μl), 5.46 μl dH_2O, 1 μl 10X *Taq* buffer (50 mM Tris-Hcl pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50% Glycerol, 1% Triton), 0.3 μl 10 mM dNTP mix, 0.48 μl $MgCl_2$ (final concentration = 1.2 mM), 0.4 μl of cold primer 1 (10 μM), 0.064 μl of cold primer 2 (2 μM), 0.046 μl of hot primer 2 (2 μM), 0.2 μl BSA (10 mg/ml), 0.05 μl *Taq* polymerase (5 units/μl) and was amplified in cycleplates (Robbins Scientific Corp). After an initial denaturation at 92 °C for 5 min, we carried out 30 cycles consisting of 30 s denaturation at 92 °C, 30 s at annealing temperature (Tab. II) and 30 s extension at 72 °C, followed by a final extension step for 10 min at 72 °C. PCR products were resolved on urea-polyacrylamide (6%) sequencing gels. Gels were dried and exposed to Kodak BIOMAX MR diagnostic film for one to four days.

2.5.2. Statistical methods

For each locus and each population sample, the genetic diversity was estimated by the number of alleles per locus (A), the observed heterozygosity (H_O) and the unbiased heterozygosity (H_E) [55]. Deviation from Hardy-Weinberg equilibrium, genotypic linkage disequilibrium and differences in allele frequencies were analysed using GENEPOP version 3.1a [62]. Population structure

Table II. Characteristics of seven polymorphic loci in *Carabus solieri*.

Locus	Primer sequences (5′ → 3′)	Annealing temp (°C)	$MgCl_2$ (mM)	Repeat array	Size range (bp)
1122	GAATCCAAAGGTACTCCG	48	1.2	$(AC)_{16}$	142
	CCCGAGTTGTAATTGCG				
5175	CAGGTTTTGTAGACTCGTGC	52	1.2	$(TG)_{10}$	248
	TTACACGACCACCATACTGC				
828A	CAGCAGTTCAAGTAGCATCGG	56	1.6	$(TG)_7(AG)_4$	182
	CACCCGTGCCAAACAGG				
6103	GAATGTTTCCGCTCTCG	52	1	$(GA)_7(GT)_2$	239
	GGTCGTACCAGAAGGTGC				
9170	TATCGGAGTGCCTGTAACC	50	1.5	$(CA)_7$	239
	GTGCCAAGTCTGAACACATC				
1259	CGTGGGATAGAGAAGCAAAG	56	0.8	$(AC)_{12}$	173
	GTATCAGTAAGGGGCGAGAG				
13F	CCACACTGATACGGTTTCG	56	1	$(AC)_8$	168
	GAACTTGGTATATCCGTAGTAGG				

was assessed using the F_{ST} estimator $\hat{\theta}$ of Weir and Cockerman [84]. This estimator performs better when sample size is moderate ($n_s < 50$) and few loci scored (n_l<20) [33]. Permutation procedures ($N = 1000$) were used to test whether values were significantly greater than zero by permuting multilocus genotypes among samples.

Allelic richness was standardised between samples using the rarefaction technique [29, 40]. The contribution of the different populations to total diversity and allelic richness was calculated using CONTRIB, a software written by R. Petit and available at http://www.pierroton.inra.fr/genetics/labo/Software/. The contributions of the k-th population to the total diversity $[C_T(k)]$ and to the total allelic richness $[C_T^r(k)]$ are partitioned in two components, one due to its own diversity $[C_S(k)$ & $C_S^r(k)]$, the other due to its divergence diversity $[C_D(k)$ & $C_D^r(k)]$, see Petit *et al.* [57] for further details.

Neighbour-joining (NJ) [66] trees were constructed using Cavalli-Sforza and Edwards' chord distance (D_{CE}) [18] which shows a higher probability of obtaining the correct topology than other distance measures [76]. Bootstrap values were computed by resampling individuals and loci and are given as percent values for 2000 replications. Trees were reconstructed using a program written by J.M. Cornuet.

3. RESULTS

3.1. Morphology

Morphologically the populations studied by Bonadona [14] cluster into three major groupings, which correspond to the subspecies he defined (Fig. 2). Males and females from the same locality always belong to the same cluster, with the exception of males from La Madonne which cluster within the subspecies *solieri* while the females cluster within *clairi*. We also analysed the whole data set of Bonadona (including 1236 individuals belonging to 38 populations) and found similar groupings (not shown).

C. s. bonnetianus populations are, morphologically, the most distant populations, clearly differing from other populations in the shape of the pronotum, that of elytrae, and the sculpture and size of the tertiary elytral striation. *C. s. clairi* and *C. s. solieri* are morphologically more closely related. Nevertheless they are distinct and, with the exception of MAD (m), these two groups also match, with differences in body colour (blue for *C. s. clairi* and green for *C. s. solieri*), a character not included in the analysis.

Some populations of *C. s. clairi* (*e.g.* CAU, SER, MAD) exhibit polymorphism in the sculpture and size of the tertiary striation. In this respect there are intermediate between *C. s. bonnetianus* and *C. s. solieri*, which show contrasting patterns of elytral sculpture.

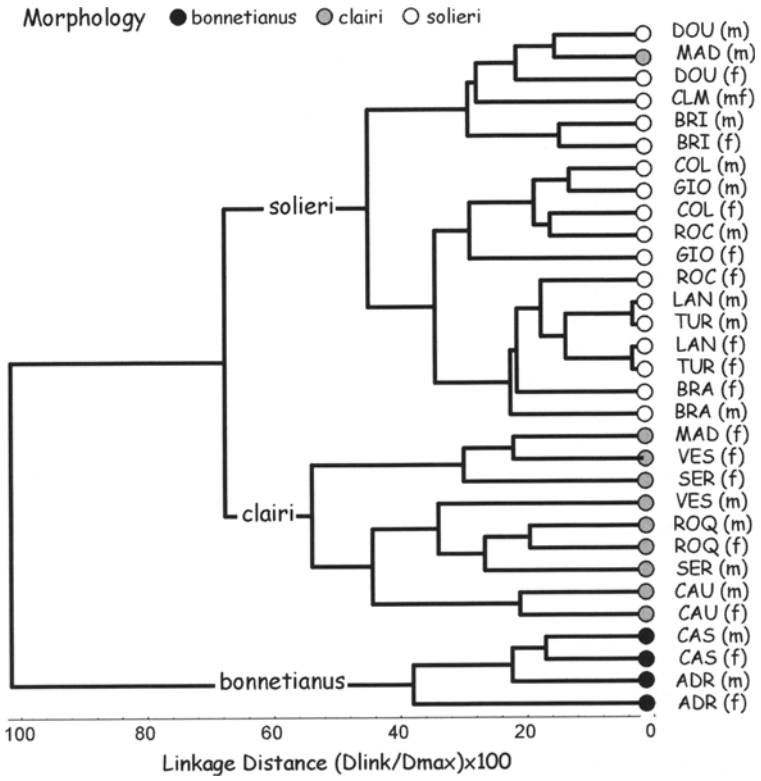

Figure 2. UPGMA phenogram based on Euclidian distance calculated from seven morphological characters. Codes refer to populations identified in Table I, San Giorgio (GIO), Collongues (COL), La Rochette (ROC), St Martin-Colmiane (CLM) and Les Dourbes (DOU) [substitutive populations used for this analysis]. Males (m) and females (f) are analysed separately. The horizontal axis is scaled in percentages as dlink/dmax $\times$ 100.

3.2. mtDNA

Nucleotide sequences 642 bp in length were obtained from the mitochondrial DNA cyt. *b* gene for 45 *C. solieri* specimens representing 15 distinct populations. *C. olympiae*, another endangered species, lives in a restricted habitat in the Italian Piedmont, and was used as outgroup. Morphology [51], cross-breeding experiments [1] and mitochondrial sequences (Rasplus *et al.*, in preparation) clearly showed that *C. olympiae* is the sister species of *C. solieri*. The sequences of the three individuals sequenced per population were identical except in Barles where we found slight differences for one individual (Fig. 3). However, the differences are not ambiguous enough to suspect that a nuclear

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                        111 2222233333 3334444445 5555566
                  1366679066 1346700002 2460025662 2789902
                  2337900867 6731903675 7532439562 8911461
solu5000  (VES)   TTATATCTCC TCATCTAAAG ATCCCCAAAC CCATTAG
solv7000  (TUR)   .......... .......... .......... .......
solc2300  (ISO)   .......... .......... .......... .......
solc3200  (PEO)   .......... .......... .......... .......
sols1600  (VAU)   .......... .......... .......... .......
soll1200  (BRA)   .......... .......... .......... .......
solc4002  (MAD)   .......... .......... ......G... .......
soll6510  (OSI)   C......... .......... .......... .......
sols2005  (MUR)   ....G..... ..G......A .C........ .......
sols3500  (BAR)   ....G..... ..G....... .C.....GG. .......
sols3505  (BAR)   ....G..... ..G....... .C........ .......
solbo100  (ADR)   ....G..... .TG....... .C........ .......
solbo147  (CAS)   ....G..... .TG....... .C........ .......
solb3700  (SER)   .......... .......... .......... .......
solb4300  (CAU)   .......... .......... .......... .......
solb4400  (COU)   .......... .......... .......... .......
olymp149          ..GC.CTCTA CT.ATCGCC. GCATTTC..A AT.CATA
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Figure 3. Sequence variation within the partial cytochrome b for the seven haplotypes detected among 45 *C. solieri* sampled in 15 populations. Only variable positions are shown.

homologue was being-coamplified with the mtDNA cyt. *b*. Only one individual per population was used for the tree reconstruction, with the exception of Barles where the two haplotypes found were used.

Attempts to reconstruct the phylogeny of the haplotypes were limited by the low level of variation. However, genealogies estimated by either the maximum likelihood or the neighbour-joining method yielded similar topologies. For this reason only the NJ reconstruction with bootstrap values above 50% was presented (Fig. 4). When analysed, the sequence divergence estimates among the seven haplotypes fell into two groups. The distribution of *C. solieri* mtDNA haplotypes was relatively highly structured geographically but did not correspond to the infraspecific subdivision suggested by the morphology. Group 1 haplotypes were found (1) in the western part of the distribution range of the subspecies *solieri* (Alpes de Haute Provence), and (2) in the two sampled populations of *C. s. bonnetianus*. By contrast, group 2 haplotypes were widespread (Fig. 5B) and encountered in all other sampled populations belonging to subspecies *clairi* and *solieri*.

Analysis of sequence variation revealed 29 variable sites between *C. solieri* and the outgroup. Among the fifteen sampling locations for *C. solieri*, only nine were variable, of which four were phylogenetically informative (Fig. 3). The averaged Kimura two-parameter distance between the two groups of haplotypes was 0.6%, while the averaged distance between *C. solieri* and *C. olympiae* was

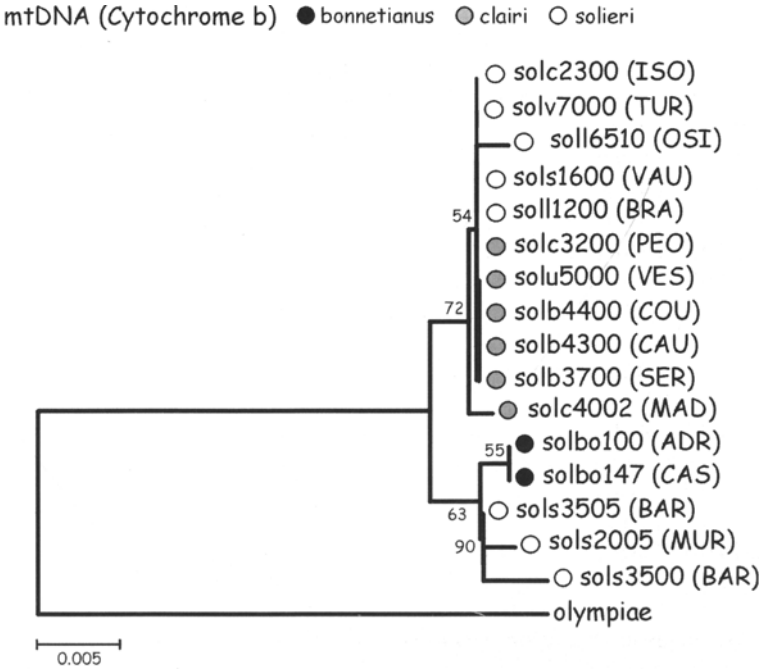

Figure 4. Phenogram reconstructed from the sequences of cytochrome b gene using neighbor-joining analysis. Numbers along the branches indicate the percentage of bootstrap replications (out of 1000) in which a node is supported. Only nodes with bootstrap values above 50% are presented. Codes refer to populations identified in Table I.

4.7%. Averaged pairwise sequence divergence within groups of haplotypes was clearly higher in group 1 (0.7% *vs.* 0.06%).

A molecular clock with a rate of approximately 2% per myr per pair lineages [15, 42] then implies divergence ca. 600000 years ago. Recently, Prüser and Mossakowski [60] calibrated mtDNA (ND1) in west Mediterranean *Carabus* using the disintegration of the Gibraltar bridge at the end of the Messinian event (5.3 myr). If we applied the low rates they found (0.6%), then the two *C. solieri* lineages are estimated to have diverged ca. 2 million years ago.

3.3. Microsatellite loci

3.3.1. Genetic variability

Genetic diversity was measured by allelic diversity and heterozygosity. The total number of alleles per microsatellite locus ranged from five (L13F) to 26 (L5175) with an average of 12.6. Three loci (L5175, L1122, L1259) are highly variable with respectively 18, 18 and 26 alleles, while the four other loci exhibit relatively low allelic variability (ranging from 5 to 8 alleles). The three more

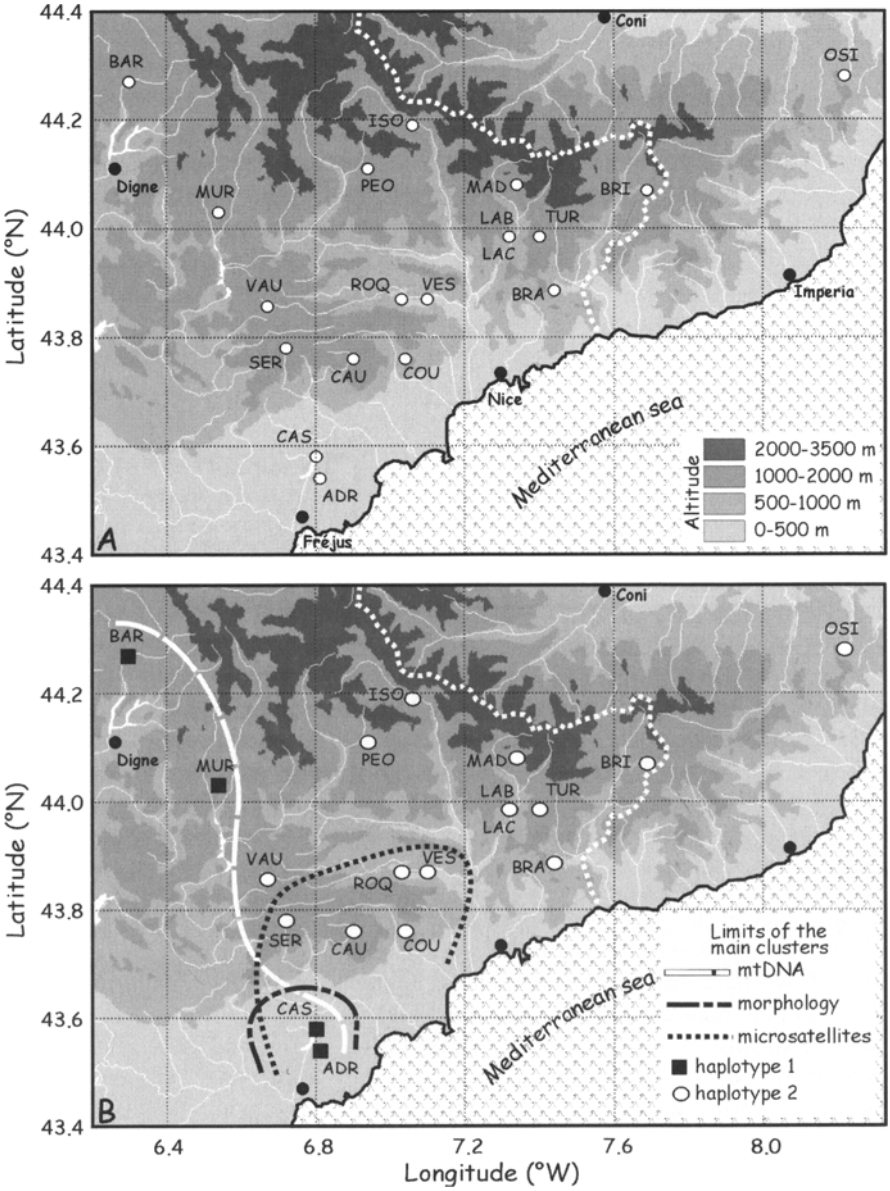

Figure 5. A) Map showing the sampling sites and the altitudinal range of the species. B) Geographical location of the limits between the two main groups of *C. solieri* based on morphological differences, mtDNA haplotypes and microsatellite frequencies.

variable loci showed disjunct distributions as allele length classes were separated by more than one repeat unit.

Allelic diversity (see Tab. IV) ranged from 2.00 (±1.41) for St Cassien to 6.57 (±3.51) for La Brigue with marginally higher values in the eastern part of the range. Three loci were monomorphic in the sample from St Cassien, where the lowest average number of alleles per locus was observed. When corrected for differences in sample size, allelic richness decreased from east to west and south-west (Fig. 6B). Allelic richness is positively correlated with observed heterozygosity ($r = 0.87$, see Fig. 7).

Expected mean heterozygosity within samples ranged from 0.253 (±0.298) for the St Cassien sample (Tanneron massif) to 0.710 (±0.163) for the La Brigue sample (Italian border). Heterozygosity also shows a clear pattern of decreasing values from east to west of the distribution range (Fig. 6A). Allele frequencies for the seven loci across the 18 populations sampled are given in Table IV.

For each population, we calculate the contribution to the total diversity and to the total allelic richness (Tab. III). The population which contributes the most to the total diversity and to the total allelic richness is Osiglia [C_T(OSI) = 4.20 and C_T^r(OSI) = 6.76]. This is due to its relatively strong divergence and to its diversity. The other populations contribute much less than Osiglia to the total allelic richness. Among them, La Brigue and Turini are the two populations which contribute more. Nearly all the eastern populations of *C. s. solieri* contribute positively to the total allelic richness while all populations of *C. s. bonnetianus* and *C. s. clairi* populations, apart from PEO and ISO, have negative contributions (populations which exhibit lower allelic richness than the mean have a net contribution to allelic richness which is negative).

3.3.2. HW equilibrium and linkage disequilibrium

Exact test for genotypic linkage disequilibrium depicted 11 significant adjusted P values out of 311 (3.5%) comparisons, a proportion which is lower than expected by chance alone (15). No significant P value was observed for given pairwise comparison across populations, suggesting that there was no evidence of linkage between loci and therefore independent assortment was assumed.

One locus (L5175) showed significant deviations from the genotype expected according to the Hardy-Weinberg equilibrium for several populations ($P<0.01$). Deficiency of heterozygotes was found in the following populations: Les Adrets, Barles, La Brigue and Osiglia. These heterozygote deficits could be due to: non-random mating, selection against heterozygotes, inbreeding, the Wahlund effect or the presence of a null allele.

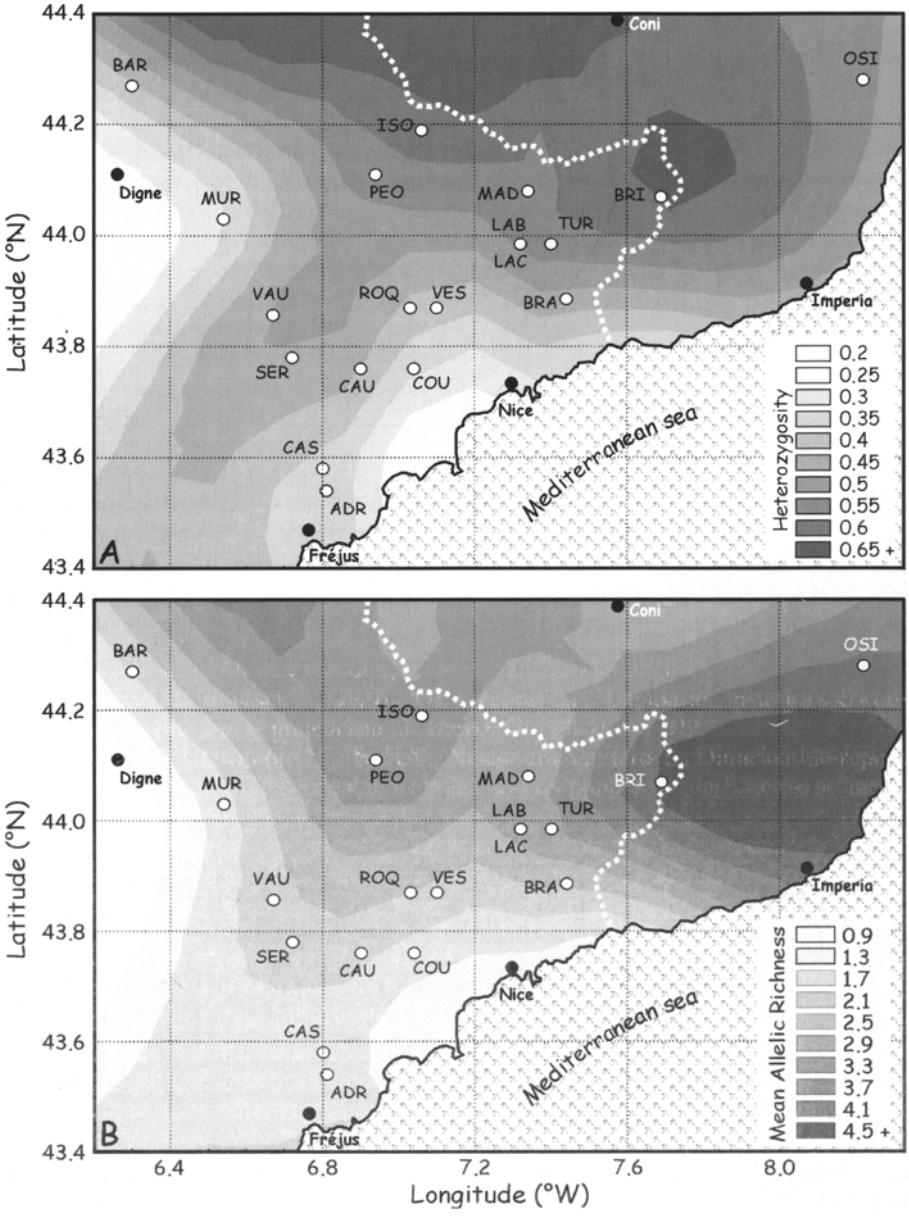

Figure 6. Geographical distribution of A) the observed heterozygosity and B) the allelic richness. A surface was fitted to the XYZ co-ordinate data (Latitude/Longitude/ Heterozygosity or Allelic Richness), according to a distance-weighted least squares smoothing procedure and then projected onto a 2-dimensional plane. Codes refer to populations identified in Table I.

Table III. Number of individuals (♂,♀) measured in the morphological analysis and measures of the genetic diversity (heterozygosity, corrected allelic richness and contributions) for each *C. solieri* populations based on microsatellite data. Population names in brackets refer to substitutive populations studied in the morphology analysis. Ho = Mean heterozygote proportion, $r(20)$ = Corrected allelic richness, C_T = Contribution to the total diversity, C_S = Contribution to total diversity due to diversity, C_D = Contribution to total diversity due to divergence, C_T^r = Contribution to the total allelic richness, C_S^r = Contribution to total allelic richness due to diversity, C_D^r = Contribution to total allelic richness due to divergence.

	Morphology			Microsatellites							
Populations	n	n♂	n♀	**Ho**	$r(20)$	C_T	C_S	C_D	C_T^r	C_S^r	C_D^r
ADR	6	4	2	0.381	1.82	0.21	0.22	–0.05	–0.50	–0.23	–0.27
BAR (DOU)	15	9	6	0.391	1.71	1.38	0.94	0.44	0.86	0.05	0.81
BRA	7	4	3	2.07	0.421	–0.06	–0.16	0.10	1.00	–0.30	1.30
BRI	42	27	15	0.618	4.34	1.10	1.95	–0.85	2.23	2.90	–0.67
CAS	21	15	6	0.250	0.97	0.08	–0.08	0.16	–0.52	–0.69	0.17
CAU	84	59	25	0.479	2.00	0.42	0.36	0.10	–1.02	–0.08	–0.94
COU				0.307	1.52	0.49	–0.10	0.59	–0.95	–0.11	–0.84
ISO	10	6	4	0.574	2.93	0.73	1.39	–0.66	1.11	1.44	–0.33
LAB				0.507	-	-	-	-	-	-	-
LAC (LAN)	51	30	21	0.521	2.54	0.07	0.83	–0.76	–0.04	0.62	–0.66
MAD	14	9	5	0.500	2.33	0.76	0.30	0.46	–0.36	0.39	–0.75
MUR (ROC)	16	8	8	0.255	1.41	–0.21	–0.28	0.07	–0.86	–0.70	–0.16
OSI (GIO)	16	8	8	0.514	3.80	4.20	2.49	1.71	6.76	3.16	3.60
PEO				0.542	3.65	0.34	1.21	–0.87	1.16	2.30	–1.14
ROQ	61	32	29	0.440	2.71	–0.60	0.35	–0.95	–1.33	0.88	–2.21
SER	16	13	3	0.429	1.94	–0.45	–0.08	–0.37	–0.94	0.29	–1.23
TUR	39	20	19	0.606	3.48	1.38	1.80	–0.42	2.63	2.11	0.52
VAU (COL)	29	13	16	0.493	2.00	–0.67	0.33	–1.00	–1.72	–0.11	–1.61
VES	28	8	20	0.236	1.17	0.12	–0.02	0.14	–2.05	–0.85	–1.20

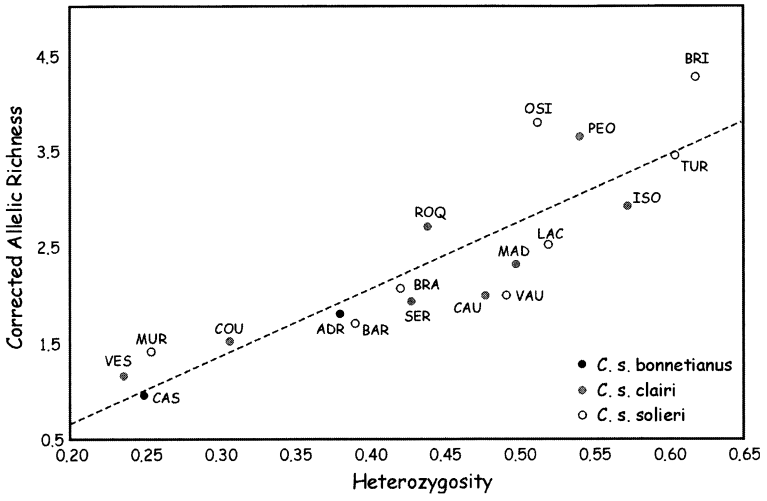

Figure 7. Scatterplot of heterozygosity against corrected allelic richness.

3.3.3. Population differentiation and structure

Log-likelihood (G)-based exact tests for differentiation of all pairwise combinations of population at each locus ($n = 1170$ tests) were performed. Most of these comparisons (1023 out of 1170) yielded significant differences. 68.5 % of the non-significant results were at two of the least variable loci (L13F and L828A).

Geographical population structure was also estimated by multilocus F_{st}. We calculated $\hat{\theta}$ (F_{ST}) values to describe the overall differences between pairs of populations. The mean $\hat{\theta}$ averaged across all pairwise population comparisons were 0.312. All $\hat{\theta}$ values were significantly different from zero with the exception of the value between the populations sampled in the forest of Lantosque (LAC and LAB), which are separated by less than one kilometre. The estimates of $\hat{\theta}$ ranged from relatively low values (0.09–0.12) between geographically close populations (separated by less than 15 km of more or less continuous forests), to very high values (0.656) for the comparison between St Cassien and Mure populations. The observed values indicate a high overall level of differentiation for all the studied populations except the two populations sampled in the forest of Lantosque.

The average $\hat{\theta}$ value calculated between five populations from the southern part of the distribution range (SER, CAU, COU, CAS and ADR) is significantly higher (0.353), than the average value calculated between five populations (BRI, MAD, BRA, LAC and TUR) separated by equivalent distances and belonging to the *solieri* subspecies (0.183).

3.3.4. Genetic distance

Cavalli-Sforza and Edwards' distance was calculated for each pairwise comparison for the 19 sampled populations, and a phenogram was constructed from the distance matrix using the NJ algorithm. Genetically the populations cluster into two well-supported groups (Fig. 8), which fit neither with the main groups delimited by the morphological study nor with the two groups of haplotypes shown by the mtDNA analysis. Group 1 comprises all the populations belonging to the subspecies *solieri* (respectively BAR, MUR, VAU; OSI, BRI, BRA; LAC, LAB, TUR) plus northern isolated populations of *clairi* (PEO, ISO, MAD). Four subgroups are rather well supported and correspond mostly to the geographic location of the populations. The only exception is Barles, located on the western border of the distribution, which is fairly clustered with Osiglia and La Brigue, two populations located more than 110 km eastward. One of these subgroups, strongly supported, contains all the sampled populations within the northern range of the subspecies *clairi*. Group 2 clusters all the populations belonging to the subspecies *bonnetianus* (namely ADR, CAS) and the southern populations of *clairi* (CAU, COU, SER and ROQ, VES) (Fig. 5B). The southern populations of *C. s. clairi* are positioned as sister taxa to *C. s. bonnetianus*.

4. DISCUSSION

4.1. Phylogeography

The mitochondrial variants fell into two basic lineages (*bonnetianus* and *solieri* lineages) that do not exactly fit either the subspecies classification based on morphological characters or the genetic partition observed using microsatellite markers. However, despite some discrepancies, it seems more likely that *C. solieri* is subdivided into two distinctive entities which can be considered as subspecies: *C. s. bonnetianus* and *C. s. solieri* [61]. The maximum extent of the Alpine ice sheet and its periglacial influence may have influenced the present-day break in the distribution of between-population variability between the two subspecies of *C. solieri*. The estimated age of the two main subspecific genomes of *C. solieri* is roughly the same as the time of effective separation between the subspecies of the grasshopper *Chorthippus parallelus* [74]. This would indicate that *C. solieri* last entered its southern refugia no more than five ice ages ago. However, estimates of divergence time between the two subspecies are strongly speculative and give different results according to whether the estimation is calculated from microsatellite data (12000 years) [61] or from mitochondrial DNA (0.6–2 Mya). The two refugia of *C. solieri* could have been located in Italy, as suggested by high heterozygosity and allelic richness of microsatellite loci, and in France, probably in the Estérel or Maures massifs, as suggested by morphology and mtDNA. Retreat of the ice sheet enabled

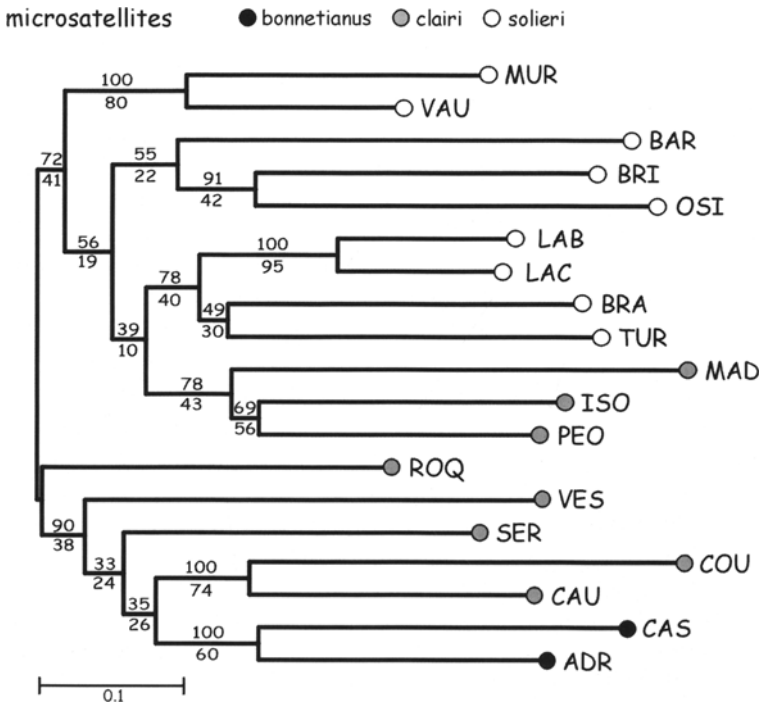

Figure 8. Cavalli-Sforza and Edwards' (1967) genetic distance phenogram calculated from microsatellite data. Codes refer to populations identified in Table I. Percentages of replication of the observed topology based on 2000 bootstraps on loci (above the branch) and individuals (under) are given. Tree is rooted at mid distance.

northward recolonisation from these refuges. *C. s. solieri* probably recolonised from its north-central Italian refuge while *C. s. bonnetianus* recolonised from its French refuge close to the Mediterranean sea. The paraphyly of mtDNA between the two main groups of *C. solieri* with monophyletic nuclear alleles and distinct morphology, strongly suggests that one or more episodes of historical hybridisation between them have occurred. Here, we use the term hybridisation in its broader sense, referring to mating by individuals belonging to different populations, subspecies or species [63].

Sampled populations of the southern part of the range of *clairi* all have a fixed mtDNA that belongs to the *solieri* lineage, while they exhibit characteristics intermediate between *C. s. solieri* and *C. s. bonnetianus* on the basis of morphology (Fig. 1) and nuclear markers. These characteristics strongly suggest that the most likely explanation for this discordant pattern is mtDNA introgression from *C. s. solieri* into *C. s. bonnetianus*, following contact and hybridisation between the two subspecies. mtDNA could have flowed persistently across the contact zone between the subspecies and largely in one direction. mtDNA for the *solieri* lineage therefore replaced the mtDNA of *bonnetianus*

in its northern distribution range (SER, CAU, COU, VES, ROQ). Typical *C. s. bonnetianus* mtDNA only maintains in the most southern populations of *C. s. bonnetianus* (Tanneron and Estérel massifs) which are geographically isolated by non forested habitats (grasslands) from northern populations. This asymmetric mtDNA introgression is also supported by experimental crosses showing that it is difficult to obtain descent in crossing female *bonnetianus* with male *solieri* [49]. It is also likely that northern populations of *C. s. clairi* are the result of a range expansion of *C. s. bonnetianus* that occurred subsequent to the Alpine glacier retreat and prior to the range expansion of *C. s. solieri.* However, more results are needed to confirm this assumption as traces of hybridisation have disappeared in most data sets except in the morphology.

Interestingly, an mtDNA variant, belonging to the *bonnetianus* lineage, seems to have been fixed in the western populations (BAR and MUR) which by microsatellites and morphology clearly belong to *C. s. solieri.* Since this sample represents the extreme western edge of *C. s. solieri* and are adjacent (MUR) or relatively close (BAR) to non-sampled locations occupied by *C. s. clairi* (*e.g.* Grand Margès Forest in Var), they may reflect the effects of past introgression events from *C. s. bonnetianus.* The current distributional patterns may be the result of the history of postglacial colonisation, selection in habitat or resource mosaic or a combination of these factors. BAR and MUR populations, which share the presence of *bonnetianus*-like mitotypes and essentially *solieri*-like morphologies, may constitute proof of northward colonisation of *C. s. bonnetianus* followed by extensive introgression by *C. s. solieri.* However, more results are needed to confirm this scenario.

4.2. Population structure of *C. solieri*

Our results lead us to conclude that all *C. solieri* populations, surveyed in this study, were highly divergent relative to the extent of genetic differentiation observed. They are genetically distinct, each comprising reproductively isolated groups. Genetic diversity analysis indicated that a high component of genetic diversity was attributable to differences among populations as opposed to within them. The sampling design employed in this study was geared toward a broad-scale survey and it is possible that on a finer geographic scale populations would cease to differ significantly in allele frequencies at the most variable loci. However, even geographically close populations exhibited significant differences in allelic frequencies. This is particularly true in the distribution range of the subspecies *bonnetianus* and in the southern part of the range of the subspecies *clairi* where some populations show low levels of genetic diversity and fixation of alleles. Fst calculated among the "*bonnetianus* – southern *clairi*" populations was consequently much greater than among *solieri* populations, suggesting that there is some subdivision among the southern populations, probably because of their small population size and isolation. Although generalisations are difficult, there appears to be agreement with the general expectation and patterns

observed in most vertebrates and the few invertebrates, that genetic differentiation among populations increases following fragmentation whereas genetic diversity within populations decreases. This is consistent with the assertion that human activities (forest clearing for agriculture, urban development, fire) have resulted in fragmentation and loss of habitats such that southern *C. solieri* populations are small and isolated, leaving them vulnerable to further random perturbations. Consequently, populations inhabiting one continuous forest area should be considered as the fundamental component in the genetic structuring of *C. solieri* and as the basis for the identification of management units. Thus Solier's Carab populations separated by a non-forested area of more than a few kilometres (5–10) should be treated as separate management units.

4.3. Setting priorities for conservation

4.3.1. Applying the high level of genetic variability method

From our analysis we can conclude that few populations exhibit high levels of genetic variability at microsatellite loci. More precisely, only eastern and northern populations belonging to the subspecies *C. s. solieri* make large contributions to the total diversity and to the total allelic richness. As a consequence, protection of only two populations (BRI and OSI) will enable conservation of 58 (65.9%) of the known alleles of the species ($n = 88$). However, these two populations only maintain two close haplotypes belonging to the *solieri* group and one morphotype. Furthermore, they are both associated with humid mountain forests and represent only part of the ecological adaptative ability of the species.

This example shows that strict application of the method developed by Petit *et al.* [57] will not enable optimal preservation of the overall variability of the species. It also emphasises the fact that methods based on allele frequency data and allelic richness can mislead conservation programme [34].

For the microsatellite loci analysed in our study, *C. s. bonnetianus* showed very few private alleles. Some of the populations supposed to be of hybrid origin (ISO or PEO) cumulate alleles that are mostly encountered, on one side, in *C. s. bonnetianus* and in southern *C. s. clairi* populations and, on the other side, in Italian *C. s. solieri* (see L1122 in Tab. IV). Hybridisation of *bonnetianus* and *solieri* have probably led to an increasing allelic diversity of some populations.

The low level of genetic variability in the subspecies *bonnetianus* could result from the relative isolation and small size of the studied populations due to habitat fragmentation. However, in the light of our results, it cannot be ruled out that this low level of genetic variability has an historical origin. During the last glaciations, *C. solieri* populations which were restricted to the French refuge, bordered on the south by the Mediterranean sea, may have suffered greater reduction in population size than the Italian populations which could retreat in the forests of the central Italian peninsula.

7 APPENDIX (*TABLE IV*)

Locus 1122	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		32	40	40	40	40	26	40	24	34	40	40	40	20	40	40	40	36	40	40	
Allele Number		5	5	6	4	6	8	1	4	4	7	4	5	7	5	8	5	10	10	5	5.737
	118	0.625	0.225	0.250	0.450	0.150	0.038	-	-	-	-	-	-	-	-	0.175	-	-	-	-	
	120	-	-	-	-	-	0.577	1.000	0.167	0.765	0.550	0.450	0.400	0.100	0.275	0.150	0.125	0.083	0.075	0.100	
	121	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	-	
	122	-	-	-	-	0.050	0.115	-	-	-	-	0.075	0.125	0.400	0.275	0.225	0.200	-	-	-	
	124	-	-	-	-	-	-	-	0.458	-	0.075	-	-	-	-	0.250	0.600	-	0.075	-	
	126	-	-	-	-	-	0.038	-	0.125	-	-	-	-	-	-	0.075	-	0.056	0.050	-	
	128	0.094	0.225	0.325	0.375	0.100	0.077	-	-	0.088	0.050	0.300	0.275	0.050	-	0.025	-	0.056	0.100	0.075	
	130	-	-	0.225	-	0.600	0.077	-	-	-	0.075	0.175	0.175	0.250	0.125	-	0.025	0.250	0.050	0.725	
	132	-	-	-	0.075	0.025	-	-	-	0.029	0.150	-	-	0.050	0.300	-	0.050	-	-	-	
	134	-	-	-	0.100	-	-	-	-	-	0.025	-	-	0.100	0.025	0.050	-	-	-	-	
	136	-	-	0.075	-	0.075	-	-	-	-	-	-	0.025	-	-	-	-	0.167	0.300	0.075	
	138	0.094	0.150	-	-	-	0.038	-	-	-	-	-	-	0.050	-	-	-	-	0.025	0.025	
	140	0.094	0.300	0.100	-	-	-	-	0.250	-	-	-	-	-	-	-	-	0.028	0.075	-	
	142	0.094	0.100	-	-	-	0.038	-	-	-	-	-	-	-	-	-	-	-	-	-	
	144	-	-	-	-	-	-	-	-	-	0.075	-	-	-	-	-	-	0.083	0.225	-	
	146	-	-	0.025	-	-	-	-	-	-	-	-	-	-	-	-	-	0.111	-	-	
	148	-	-	-	-	-	-	-	-	0.118	-	-	-	-	-	-	-	0.028	0.025	-	
	156	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.139	-	-	
Heterozygote Proportion		0.563	0.800	0.900	0.550	0.700	0.615	0	0.583	0.294	0.750	0.800	0.800	0.700	0.750	0.800	0.650	0.778	0.800	0.500	0.650
Gene Diversity		0.593	0.796	0.785	0.658	0.614	0.662	0	0.714	0.405	0.672	0.688	0.736	0.789	0.762	0.844	0.596	0.881	0.847	0.464	0.658

Locus 1259	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		38	40	40	40	40	26	40	22	34	40	40	40	22	40	38	40	36	40	40	
Allele Number		5	2	3	2	5	5	4	6	5	4	7	8	6	5	11	9	5	4	5	5.316
	161	-	-	0.400	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	163	0.026	-	0.225	-	0.025	-	0.025	-	0.176	0.500	0.100	-	-	-	-	-	0.556	0.250	0.700	
	165	0.132	-	-	0.025	-	0.192	-	-	-	-	0.075	0.175	0.091	0.500	0.053	0.050	-	-	0.050	
	167	0.421	0.250	0.375	0.975	0.800	0.654	0.225	-	0.206	0.250	-	0.075	0.182	0.300	0.079	0.475	0.167	0.200	0.125	
	169	0.395	-	-	-	0.100	-	-	0.227	-	-	-	-	0.136	0.075	-	0.075	0.167	0.425	-	
	171	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.026	0.025	-	-	-	
	173	0.026	0.750	-	-	0.050	-	0.025	0.091	-	-	0.050	-	-	-	0.026	0.150	0.083	0.125	-	
	175	-	-	-	-	0.025	0.038	0.725	0.091	0.412	0.200	0.075	0.125	0.136	-	0.105	0.075	-	-	-	
	177	-	-	-	-	-	0.077	-	-	0.176	-	-	-	-	-	0.237	-	-	-	-	
	179	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.053	0.075	-	-	-	
	181	-	-	-	-	-	-	-	0.182	-	-	0.350	0.225	-	-	0.158	0.050	-	-	-	
	183	-	-	-	-	-	-	-	0.182	0.029	-	0.225	0.200	-	0.100	0.105	-	0.028	-	-	
	185	-	-	-	-	-	-	-	0.227	-	0.050	0.125	0.150	0.364	-	0.105	-	-	-	0.025	
	187	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.053	-	-	-	0.100	
	191	-	-	-	-	-	-	-	-	-	-	-	0.025	0.091	-	-	-	-	-	-	
	193	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	0.025	-	-	-	
	195	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	-	-	
	197	-	-	-	-	-	0.038	-	-	-	-	-	-	-	-	-	-	-	-	-	
Heterozygote Proportion		0.789	0.300	0.600	0.050	0.350	0.615	0.300	0.818	0.882	0.750	0.700	0.900	0.545	0.750	0.526	0.500	0.722	0.800	0.500	0.599
Gene Diversity		0.666	0.385	0.665	0.050	0.355	0.548	0.433	0.853	0.747	0.662	0.808	0.855	0.818	0.660	0.893	0.747	0.646	0.719	0.494	0.631

Locus 13F	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		38	40	40	40	40	26	40	26	34	40	40	40	22	38	40	40	36	40	40	
Allele Number		2	2	2	2	3	1	2	1	1	1	2	2	4	2	5	3	2	2	2	2.158
	164	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	-	
	166	0.184	0.100	0.525	0.950	0.025	-	0.425	-	-	-	0.225	0.150	0.364	0.105	0.300	0.050	0.056	0.150	0.850	
	168	0.816	0.900	0.475	0.050	0.825	1.000	0.575	1.000	1.000	1.000	0.775	0.850	0.273	0.895	0.550	0.675	0.944	0.850	0.150	
	170	-	-	-	-	-	-	-	-	-	-	-	-	0.273	-	0.075	-	-	-	-	
	172	-	-	-	-	0.150	-	-	-	-	-	-	-	0.091	-	0.025	0.275	-	-	-	
Heterozygote Proportion		0.263	0.200	0.550	0.100	0.250	0	0.550	0	0	0	0.250	0.100	0.636	0	0.600	0.250	0	0.300	0.200	0.223
Gene Diversity		0.309	0.185	0.512	0.097	0.304	0	0.501	0	0	0	0.358	0.262	0.745	0.193	0.614	0.478	0.108	0.262	0.262	0.273

Locus 5175	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		38	40	40	40	40	26	40	22	36	40	40	40	22	40	40	40	36	38	40	
Allele Number		5	2	7	3	3	8	4	3	3	5	5	6	5	6	11	11	12	9	4	5.895
	236	-	-	-	-	-	-	-	-	-	-	0.050	0.025	-	-	-	-	-	-	-	
	240	-	-	0.050	0.275	-	0.192	-	-	-	-	-	-	-	0.025	0.025	-	0.139	0.132	0.500	
	242	0.395	-	-	-	-	0.115	-	-	-	-	-	-	0.136	-	-	-	-	-	-	
	244	0.132	0.275	0.075	-	0.775	0.462	0.100	0.818	0.694	0.450	0.675	0.650	0.636	0.725	0.050	0.025	0.028	-	0.125	
	246	0.105	-	-	-	-	-	-	-	-	-	-	-	0.045	0.150	0.050	0.025	0.056	-	-	
	248	0.158	0.725	0.650	0.550	-	0.038	-	-	-	-	-	-	-	-	0.200	0.050	-	-	-	
	250	0.211	-	0.050	-	0.025	-	-	-	-	-	-	-	-	-	0.075	0.050	0.139	0.316	0.300	
	252	-	-	0.075	0.175	0.200	0.038	0.225	-	-	0.175	-	-	-	-	0.100	0.300	0.139	0.132	0.075	
	254	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.375	0.400	0.083	-	-	
	256	-	-	0.075	-	-	-	-	-	-	-	-	-	-	-	0.050	0.050	0.083	0.079	-	
	258	-	-	-	-	-	-	-	-	-	-	-	-	0.136	-	0.025	0.025	-	0.026	-	
	260	-	-	-	-	-	-	0.050	-	-	-	0.100	0.025	-	-	-	-	0.083	-	-	
	262	-	-	0.025	-	-	0.077	0.625	-	0.194	0.300	0.025	0.150	-	-	-	0.025	0.056	0.158	-	
	264	-	-	-	-	-	0.038	-	-	0.111	0.050	0.150	0.125	-	-	-	-	0.083	0.026	-	
	266	-	-	-	-	-	0.038	-	-	-	0.025	-	0.025	-	-	-	-	0.083	-	-	
	268	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	-	-	
	270	-	-	-	-	-	-	-	-	-	-	-	-	0.045	-	-	-	-	-	-	
	272	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	
	276	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	-	
	280	-	-	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	-	-	
	282	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	-	-	
	284	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.079	-	
	290	-	-	-	-	-	-	-	0.091	-	-	-	-	-	-	-	-	-	-	-	
	294	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	0.028	-	-	
	296	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.053	-	
	298	-	-	-	-	-	-	-	0.091	-	-	-	-	-	-	0.025	-	-	-	-	
Heterozygote Proportion		0.737	0.450	0.550	0.700	0.400	0.538	0.450	0.182	0.444	0.800	0.450	0.500	0.727	0.500	0.600	0.350	1.000	0.895	0.700	0.576
Gene Diversity		0.767	0.409	0.569	0.606	0.368	0.754	0.560	0.329	0.481	0.691	0.522	0.551	0.580	0.459	0.814	0.758	0.925	0.846	0.655	0.612

Locus 6103	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		38	40	40	40	40	26	40	26	36	40	40	40	22	40	40	40	36	36	40	
Allele Number		2	1	2	3	2	3	2	2	1	2	2	2	3	2	4	4	3	4	3	2.474
	231	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	-	
	233	-	-	-	-	-	0.231	-	-	-	-	0.300	0.275	0.182	0.250	0.225	0.275	0.194	0.722	0.425	
	235	-	-	-	-	-	-	0.075	0.308	1.000	0.550	0.700	0.725	0.773	0.750	0.550	0.575	0.722	0.083	0.500	
	237	0.684	-	-	0.100	0.300	0.115	-	0.692	-	0.450	-	-	0.045	-	0.200	0.125	0.083	0.167	0.075	
	239	0.316	1.000	0.775	0.150	0.700	0.654	0.925	-	-	-	-	-	-	-	-	0.025	-	0.028	-	
	241	-	-	0.225	0.750	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Heterozygote Proportion		0.316	0	0.350	0.400	0.600	0.385	0.150	0.308	0	0.700	0.500	0.250	0.364	0.400	0.700	0.550	0.333	0.222	0.450	0.367
Gene Diversity		0.444	0	0.358	0.415	0.431	0.526	0.142	0.443	0	0.508	0.431	0.409	0.385	0.385	0.622	0.592	0.446	0.456	0.578	0.398

Locus 828A	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		38	40	40	40	40	26	40	26	32	40	40	40	22	40	40	40	34	40	40	
Allele Number		1	1	1	1	1	2	1	2	1	2	2	2	2	2	2	7	2	3	3	2
	178	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	
	180	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.350	-	0.075	0.100	
	182	1.000	1.000	1.000	1.000	1.000	0.846	1.000	0.423	1.000	0.900	0.850	0.750	0.636	0.950	0.700	0.075	0.882	0.750	0.800	
	184	-	-	-	-	-	0.154	-	0.577	-	0.100	0.150	0.250	0.364	0.050	0.300	0.275	0.118	0.175	0.100	
	186	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	
	192	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.150	-	-	-	
	194	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.075	-	-	-	
Heterozygote Proportion		0	0	0	0	0	0.154	0	0.692	0	0.200	0.300	0.300	0.545	0.100	0.400	0.700	0.235	0.400	0.400	0.233
Gene Diversity		0	0	0	0	0	0.271	0	0.508	0	0.185	0.262	0.385	0.485	0.097	0.431	0.785	0.214	0.412	0.349	0.231

Locus 9170	Allele	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone	Means
Gene Number		38	40	40	40	40	26	40	26	36	40	40	40	22	40	40	40	36	40	40	
Allele Number		1	1	2	3	4	2	2	2	2	2	4	4	5	3	5	3	3	3	4	2.895
	235	-	-	-	-	0.100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	238	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	
	239	1.000	1.000	0.500	0.175	0.400	0.385	0.900	0.077	0.083	0.225	0.375	0.400	0.273	0.150	0.200	0.425	0.472	0.450	0.550	
	241	-	-	0.500	0.725	0.325	0.615	0.100	0.923	0.917	0.775	0.300	0.200	0.182	-	0.350	0.400	0.500	0.475	0.125	
	243	-	-	-	0.100	-	-	-	-	-	-	-	-	0.091	-	0.300	0.175	0.028	-	-	
	245	-	-	-	-	0.175	-	-	-	-	-	0.125	0.150	0.409	0.225	0.125	-	-	0.075	0.300	
	247	-	-	-	-	-	-	-	-	-	-	0.200	0.250	0.045	0.625	-	-	-	-	-	
	249	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-	-	
Heterozygote Proportion		0	0	0.400	0.350	0.700	0.769	0.200	0.154	0.167	0.250	0.550	0.800	0.727	0.450	0.700	0.600	0.722	0.600	0.750	0.468
Gene Diversity		0	0	0.513	0.445	0.712	0.492	0.185	0.148	0.157	0.358	0.732	0.733	0.749	0.550	0.750	0.645	0.541	0.581	0.606	0.468

All loci	Adrets	Cassien	Caussols	Courmes	Séranon	Roquest	Vescous	Barles	Mure	Vauplane	LantosqB	LantosqC	Turini	Braus	Brigue	Osiglia	Péone	Isola	Madone
Mean Allele Number	3.000	2.000	3.286	2.571	3.429	4.143	2.286	2.857	2.429	3.286	3.714	4.143	4.571	3.571	6.571	6.000	5.286	5.000	3.714
Allele Number S.D.	1.915	1.414	2.289	0.976	1.718	2.911	1.254	1.676	1.618	2.138	1.890	2.340	1.718	1.718	3.505	3.109	4.071	3.162	1.113
Mean Heterozygote proportion	0.381	0.250	0.479	0.307	0.429	0.440	0.236	0.391	0.255	0.493	0.507	0.521	0.606	0.421	0.618	0.514	0.542	0.574	0.500
Heterozygotes proportion S.D.	0.325	0.299	0.275	0.267	0.258	0.276	0.212	0.308	0.325	0.331	0.199	0.316	0.132	0.290	0.131	0.163	0.356	0.269	0.185
Mean Gene Diversity	0.397	0.253	0.486	0.325	0.398	0.465	0.260	0.428	0.256	0.439	0.543	0.562	0.650	0.444	0.710	0.657	0.537	0.589	0.487
Gene Diversity S.D.	0.309	0.298	0.252	0.272	0.230	0.254	0.236	0.300	0.294	0.270	0.206	0.220	0.168	0.240	0.163	0.112	0.310	0.226	0.142

Despite its low level of genetic variability, *C. s. bonnetianus* exhibits distinctive morphology, private mitochondrial haplotypes (close to but different from those found in the two western populations of *C. s. solieri*) and original ecological particularities (mostly associated with relatively dry forested habitats). All these characters, our hypothesis on the phylogeography of the subspecies, and the existing examples of apparently viable small populations with a high degree of inbreeding strongly, are strong arguments in favour of protection of habitats where *C. s. bonnetianus* occurs.

4.3.2. Applying the ESU concept

As pointed out by Moritz [53], the ESU concept must be applied with common sense. One of the failures of the concept is its inability to recognise separate entities when natural hybridisation occurs, whatever the taxonomic level of the hybridising entities. In the case of *C. solieri*, mtDNA alleles cannot be sorted between taxa that can be easily separated on the basis of other characters (morphology, biology, ecology). DNA characters offer a wealth of discrete character information for identification of conservation units. However, mtDNA cannot always be reliable when interspecific or infraspecific mitochondrial introgression or complete transfer occurs, accompanied or not by traces of nuclear introgression, which is likely to be the rule in several insect groups. Mitochondrial DNA can be a source of valuable characters that may be useful for the characterisation and conservation of entities. Combination of both morphological and mtDNA character analysis may prevent misinterpretation due to flow of mtDNA through species or subspecies boundaries. The prerequisite condition of monophyletic mtDNA for designation of ESU, could be ruled out when hybridisation occurred between taxa. There is growing evidence of frequent hybridisation between animal species, and possible secondary contact between infraspecific entities which reached some level of mtDNA differentiation during the last ice ages. This limits the usefulness of mtDNA in selecting populations that must be protected (but also in recognising taxa on the basis of phylogenetic species concept). These results also argue for the need to include other sets of characters in the recognition of the most important population components for the optimal conservation of endangered species.

Nevertheless, natural hybridisation may lead to adaptative evolution and evolutionary diversification and therefore, far from being detrimental, may be an important part of the evolutionary process. Hybridisation may result in genetic enrichment of endangered forms or of populations that would otherwise lose their diversity through genetic drift [5, 6]. This addition of genetic variability could produce new recombinant genotypes with fitness as high as or higher than both of their parents [35, 68]. Hybrid genotypes that possess increased fitness in certain environments can enable the species to colonise new habitats by widening the range of environmental parameters that a single phenotype can tolerate. Furthermore, hybridisation can act as the starting point

for further evolutionary diversification in animals [23, 27] and particularly in insects [7, 16, 54, 70].

Consequently, hybrid zones formed when differentiated genomes meet each other could be of great significance for conservation purposes. As many as 37% of the known hybrid zones in Europe and North America have developed as a result of Pleistocene disjunction, range expansion following the retreat of the glaciers and subsequent secondary contact [11]. In Europe, the southern peninsulas of Iberia, Italy, the Balkans and Greece are considered to be distinct major refugia in the last ice age [38] and contact areas are mostly localised in the Pyrenees and the Alps [38, 75]. The Maritime and Ligurian Alps are both considered as a contact area between taxa differentiated in different refuge zones [12, 46, 58, 75] and an area with high species richness and endemism for plants [52, 80] and insects [22].

None of the two main methods supposed to be useful for recognition of populations to be protected seem appropriate in recognising populations of *C. solieri* that contribute more to the overall diversity of the species. This is probably due to secondary contact between the two constitutive subspecies of *C. solieri*. Furthermore, the two subspecies have experienced different histories which have resulted in markedly different levels of genetic diversity within each subspecies. *C. s. bonnetianus* shows lower levels of genetic variability and fewer diagnostic alleles than any other populations belonging to *C. s. solieri*. Our study clearly showed that genetic data in isolation cannot always give straightforward answers to conservation managers. However, its careful use in conjunction with other parameters such us morphological, demographic, and economic information may help in optimising management decisions.

Nevertheless, our study of *C. solieri* enables us to propose some conservation rules which could help to preserve the species, especially in its southern range, where the species seems greatly endangered. The strong genetic structure found at the population level corroborates field studies which indicate that, following local extinction due to fire or habitat destruction/transformation etc., *Carabus* are not likely to recolonise forest patches separated by inhospitable habitats of more than a few kilometres [24]. As for most other endangered species of insects, habitat degradation or destruction is the single most important and widely recognised agent of beetle decline. Conservation efforts, therefore, should focus on maintaining as many populations as possible across as many geographical regions as possible within both *C. solieri* lineages because it is at this level that the majority of molecular (nuclear and mitochondrial) and morphological variation resides.

Management practices such as translocation may have profound consequences for the maintenance of genetic diversity in *C. solieri*. Translocations have become a prominent strategy in efforts to conserve threatened and endangered species, but the strong genetic partitioning in the Solier's Carab indicates that translocations could compromise the integrity of genetic differences that have accumulated over thousands of years. Consequently, translocation, which is

considered by entomologists as a promising conservation practice [50], must be avoided in *C. solieri*. Habitat change that can generate contact and, consequently, can lead to the mixing of distinct genomes must be also avoided. For example, construction of a permanent corridor (*e.g.* forested area) allowing movement of the northern populations of *C. s. bonnetianus* (mtDNA *solieri*-like) into the range of the southern populations of *C. s. bonnetianus* (characterised by original mtDNA haplotype) may endanger their genetic uniqueness.

To preserve *C. solieri* populations successfully, management practices must concentrate on reserve selection, especially in the part of the distribution range where *C. solieri* is greatly endangered (low level of genetic diversity, fixed alleles). Habitat preservation and restoration could have a greater impact than the legislative protection of the species. Some of the surveyed habitats are located in protected areas (*e.g.* Mercantour National Park, Haut Verdon Regional Park, Courmettes Natural Reserve). However, very few of the southern habitats are protected. In Tanneron and Estérel, the loss of the few suitable habitats for *C. solieri bonnetianus* probably signals the loss of one of the most distinctive entities (ecology, morphology, mtDNA). Most of these habitats are disappearing under an ongoing wave of urban expansion, forest fire and habitat fragmentation. Our study emphasised the need for legal protection of some of the forested habitats that still maintain populations of *C. s. bonnetianus*.

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