

When morphometry meets genetics: inferring the phylogeography of *Carabus solieri* using Fourier analyses of pronotum and male genitalia

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Abstract

Population differentiation is a crucial step in the speciation process and is therefore a central subject in studies of microevolution. Assessing divergence and inferring its dynamics in space and time generally require a wide array of markers. Until now however, most studies of population structure are based on molecular markers and those concerning morphological traits are more scarce. In the present work, we studied morphological differentiation among populations of the ground beetle *Carabus solieri*, and tested its congruence with genetic population structure. The shape of pronotum and aedeagus was assessed using Dual Axis Fourier Shape Analysis. MANOVA on Fourier coefficients revealed highly significant morphological variation between populations and a similar geographical pattern of differentiation for both structures. On the whole, morphological and genetic patterns were also found to be congruent. Our analysis confirms the phylogeographical scenario proposing that two entities of *C. solieri* differentiated during the last glaciation events before recolonizing the actual range of the species. It also indicates a large introgression between the two differentiated entities in the centre of the range.

Introduction

Population differentiation is a crucial step in the speciation process (Rice & Hostert, 1993; Foster *et al.*, 1998; Turelli *et al.*, 2001). Recently, several authors have stressed that speciation defined in a broad sense is not restricted to the evolution of reproductive isolation (as assumed under the biological species concept) but includes the diversification of all aspects of the phenotype (see Barton, 2001). This means that it is essential to (i) study the relative influence of the evolutionary forces (e.g. gene flow, natural selection, genetic drift) that interact to produce a given pattern of differentiation and variability before complete reproductive isolation and, (ii) conduct studies with a wide array of markers.

In such a context, studies of morphological differentiation are essential. First, most organismal taxonomy, including intra-specific variation, is based on morphological traits. Thus, one is able to appraise and interpret morphological variation at all levels of integration. Secondly, it is likely that morphological traits are, to a large extent, under polygenic control. Studying differentiation of such characters then provides a good assessment of the amount of divergence between different entities. It has even been reported that morphology could exhibit clear patterns of differentiation where molecular markers failed to detect population structure (Nice & Shapiro, 1999). Thirdly, most morphological traits are the target of selection; their study is central in the evaluation of its strength and its impact in the differentiation process. Finally, the understanding of phylogeographical history of species, or the evaluation of the action of the different evolutionary forces, all need a comparison of the patterns of geographical variation obtained from different markers, for example genetic, morphological,

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physiological or behavioural markers (Long & Singh, 1995; Magniez-Jannin *et al.*, 2000; Drotz, 2003).

In this paper, we study morphological differentiation among populations of the ground beetle *Carabus* (*Chrysocarabus*) *solieri* Dejean (Coleoptera, Carabidae). This species is a suitable model to study forces driving differentiation. Despite its relatively small distribution area in the Southern Alps of France and the Ligurian Alps in Italy (Fig. 1), it exhibits important genetic and morphological variations (Bonadona, 1967; Darnaud *et al.*, 1978; Rasplus *et al.*, 2001). Numerous taxonomic entities (subspecies, races and natis) have been described on the basis of morphological variation, but the precise number varies depending on the authors (Bonadona, 1973; Darnaud *et al.*, 1978; Deuve, 1994). The high level of variation among populations observed in this species can be related to two main factors. First, brachypterous ground beetles have limited dispersal abilities, and genetic and morphological differentiation have often been reported even at a local spatial scale (Assmann & Weber, 1997; Alibert *et al.*, 2001; Rasplus *et al.*, 2001; Brouat *et al.*, 2003; Keller & Laggiadè, 2003). Secondly, in France, the genus *Carabus* was probably affected by the Pleistocene glaciations in Europe, as were other species in this area (Hewitt, 1999). Indeed, it has been proposed recently that *C. solieri* differentiated into two distinct subspecies following isolation in two refuges, one of green colour in Italy and the other of blue colour in the South of France (Rasplus *et al.*, 2001). Post-glacial recolonization then led these entities into secondary contact, where hybridization occurred. A recent study of population genetic structure of *C. solieri* with microsatellites markers allowed the identification of three main groups

of populations (Garnier *et al.*, 2004). The first one occurs in the southernmost part of the distribution area, the second inhabits the north-west and the east, and the third occupies the middle part of the range. The first two could correspond to populations derived from each refuge whereas the exact origin of the third one remains uncertain, all the more so as this group contains both blue and green individuals whereas the first and second groups are represented by blue and green individuals respectively. Moreover, detection of barriers to gene flow suggested two routes of colonization from the Italian refuge. However, the exact location of the secondary contact remains to be determined, as well as the origin of populations at the centre of the range. In such a context, a morphological survey of population differentiation can be very informative because the geographical distribution of morphological differences between two hybridizing entities may reveal the position of the contact zone.

As traditional morphometrics seem to be of limited interest according to the varying number of subspecies defined by authors who have used this approach, we chose to assess the pattern of population differentiation of *C. solieri* using geometrical morphometric methods. Compared with traditional morphometry, they allow description of more complex forms by integrating the complete geometry of objects studied (Bookstein, 1991; Rohlf & Marcus, 1993; Marcus *et al.*, 1996; Lestrel, 1997). In addition, they appraise both shape and size of organisms. Geometric morphometrics methods have proved to be powerful at detecting subtle shape changes even at the intra-specific level (e.g. Baylac & Daufresne, 1996; Adams & Funk, 1997; Alibert *et al.*, 2001; Renaud & Millien, 2001; Bertin *et al.*, 2002). Finally, these

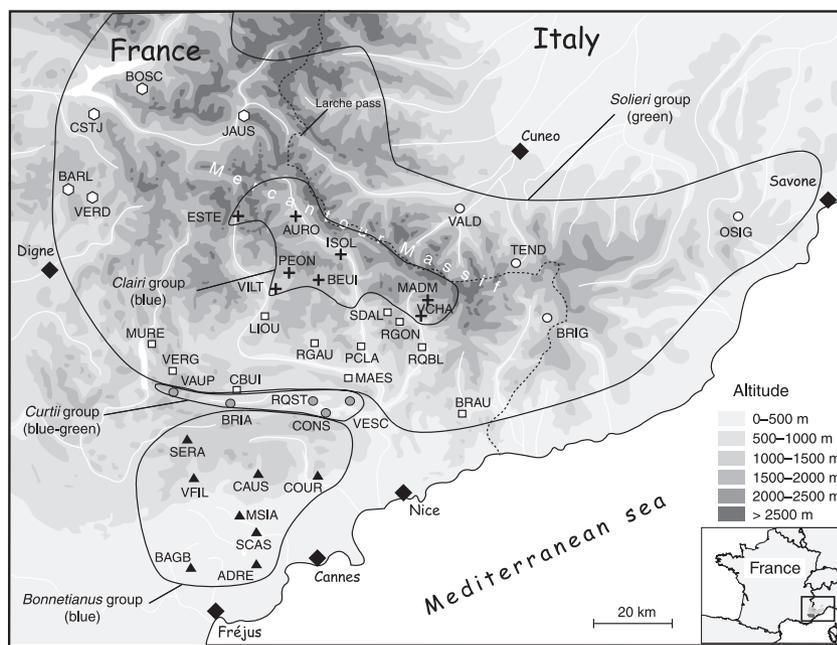


Fig. 1 Distribution area of *C. solieri*, sampling locations, and limits of the groups of populations defined according to the colour of individuals (indicated in parentheses). Sampling sites are represented by distinct symbols according to the group: Bonnetianus group (black triangles), Curtii group (grey circles), Clairi group (crosses), and Solieri group divided into Solieri-C (open squares), Solieri-NW (open hexagons) and Solieri-I (open circles).

methods also allow the direct visualization of shape difference of objects studied. Here, we considered two different morphological structures. One is the pronotum, which is a hardened plate on the dorsal side of the thorax. Leg muscles are attached to this surface. The second is the aedeagus, which is a sclerotized part of the male genitalia. The genital morphology of insects and other animals with internal fertilization has often been used as a discriminant character between closely related species, because of a rapid and divergent evolution (Eberhard, 1985). Both structures are usually considered in systematics of ground beetles and are suitable for morphological study because they are strongly sclerotized and not prone to deformation. As no landmarks are easily identifiable for either structure, their shape was studied using Fourier outline analyses. These analyses fit mathematical functions to outlines, and parameters of these functions are used for statistical appraisal of shape differences (Rohlf, 1990). Our objectives were therefore to (i) test for morphological differentiation within the range of *C. solieri*, (ii) test for congruence between morphological and genetic population structure, and (iii) investigate the implications of congruencies and/or discordances of patterns of population structure from morphological and genetic markers for the phylogeography of *C. solieri*.

Material and methods

Study area and sampling

Carabus solieri is an endangered species distributed in a relatively restricted area in the Southern Alps of France and the Ligurian Alps in Italy (Fig. 1). This ground beetle is mostly associated with coniferous or deciduous humid forests, but it can also occur in Mediterranean dry forests and alpine grasslands. Populations of *C. solieri* are threatened mainly by habitat destruction and fragmentation, particularly in the southern part of its range where habitats are highly anthropized. Moreover, entomologist's trapping can be locally sizeable. This insect is a spring breeder, laying eggs in spring and summer, depending on environmental conditions. Larval development occurs in summer and teneral emerge in late summer or autumn, and over winter in the soil. Mating occurs during the following spring. No precise data on longevity of this species is available. However, Baumgartner *et al.* (1997) reported a lifespan of 5 years for the related species *C. auronitens*.

For convenience in the text, we considered groups of populations defined according to colour of individuals and geographical location. Note that these groups have no taxonomic value, even if they correspond more or less to subspecies described by some authors (Bonadonna, 1973; Darnaud *et al.*, 1978; Deuve, 1994). Bonnetianus group occurs in the most southern part of the range (Fig. 1) and corresponds to the entity differentiated in the French refuge. Clairi group inhabits mountain forests in the

Mercantour massif. Individuals are deep metallic blue in both these groups. Metallic green individuals belonging to the Solieri group occur everywhere else, and we divided these into three geographical subgroups: Solieri-I in Italy, Solieri-NW in the north-west, and Solieri-C in the centre of the distribution area (Fig. 1). Finally, individuals with intermediate colour (blue-green) occur in the contact zone between Bonnetianus and Solieri groups. They constitute the Curtii group, and, while sometimes considered to be a subspecies, members of this group are suspected to be hybrids between the two preceding groups.

Adults were collected with permission using rows of 20 to 60 pitfall traps during spring and summer 1997, 1998, 2000 and 2001. Pitfall traps were checked weekly or every 2 weeks during the adult activity period (from April to August). Pronotum shape analysis was performed for a total of 1094 individuals from 41 sampling sites (Table 1 and Fig. 1). A subsample of 24 populations was considered for studying the shape of male genitalia (310 individuals). Sex ratio was strongly biased in favour of males. However, as most of samples contained <10 females, sex was not distinguished in the analyses. The sex ratio being roughly constant across samples, it is therefore unlikely that shape sexual dimorphism, if any, would introduce a bias in the results.

Fourier analysis of outlines

Pronotum as well as aedeagus are particularly smooth and landmarks were quite rare on both structures. We were able to find only landmarks of type 2 (extrema of curvature) and type 3 (extrema of single coordinates) (*sensu* Bookstein, 1991). Because these types of landmarks are not the most accurate in term of measurement error (ME) and homology, and because they were rare we preferred to assess shape of the pronotum and the genitalia from their outline analysis. Outlines studied correspond to the two-dimensional projection of the dorsal view for the pronotum and of the left lateral view for the aedeagus (Fig. 2). A video camera coupled to a binocular stereomicroscope was used to obtain numeric pictures. Then, after manual cleaning of images, outlines were automatically extracted using an image analysis software (Optimas 6.0; Media Cybernetics, Silver Spring, MD, USA). We used Dual Axis Fourier Shape Analysis (Moellering & Rayner, 1981, 1982; Bertin *et al.*, 2002) to decompose periodic signals corresponding to outlines in a sum of trigonometric functions. A total of 128 points equally spaced on the outline were sampled for both morphological structures, and their X , Y Cartesian coordinates were considered as a complex signal, $Z_n = X_n + iY_n$ (with $n = 0-127$). The original outlines were aligned so as to have the same orientation. Starting points ($n = 0$) were defined as the maximum curvature at the right posterior lobe for the pronotum and the maximum curvature on the apex of the aedeagus (see Fig. 2). Using discrete Fourier transforms, 128 harmonics

were calculated, each one characterized by its Fourier coefficient C_k :

$$C_k = \frac{1}{128} \sum_{n=0}^{127} Z_n e^{-\frac{i2\pi kn}{128}},$$

with k the rank of the harmonic. This coefficient can be expressed by two real numbers corresponding to its real and its imaginary part ($C_k = a_k + ib_k$), which are the variables used in statistical analysis of shape (we will thereafter refer to them as real coefficients). Amplitude of harmonic corresponds to the modulus of the Fourier coefficient C_k :

$$A_k = \sqrt{a_k^2 + b_k^2}$$

and thus provides less information than the two real coefficients a and b (the difference corresponding to the phase of harmonics).

A good approximation of the outline is generally obtained with the first few harmonics (Crampton, 1995). However, when harmonics are derived from the complex signal, the conjugates of the first harmonics must also be retained for the outline approximation because of conjugate asymmetry. We thus refer to harmonic pairs consisting of harmonic k and its conjugate, harmonic $128-k$ (for $k \geq 1$). In order to obtain real coefficients independent of size, all the real coefficients were divided by the square root of the structure surface. As the zero harmonic is dependent on translations, it was excluded from the analyses. The number of harmonics to retain was determined on the one hand by assessing ME linked to each harmonic and on the other by estimating visually the quality of a series of inverse Fourier reconstructions using increasing number of harmonics, as suggested by Crampton (1995). Coordinates extraction and Fourier coefficients calculations were performed using the Matlab Toolbox CDFT 2.7 (Dommergues, 2001). Data acquisition was made by a single operator (F. M.-J.) in order to minimize ME sources.

Measurement error

The ME was assessed for three reasons. First, it allowed us to evaluate the reproducibility of our measurements. Secondly, ME can be associated to different geometric scales. Position and parallax error produce effects at a large geometric scale and then possibly affect all harmonics. We verified through preliminary experiments that this source of error was negligible in our case. Besides, as harmonics of increasing rank describe finer and finer details of the outline, ME associated with each one was expected to increase. Then, the rank of the first harmonic displaying high ME can be used to determine the maximum number of harmonic to consider in the analyses. Thirdly, phase of harmonics strongly depends on the orientation and on the starting point, whereas the amplitude does not. As the information contained in the real coefficients (a and b) is

Table 1 Sampling sites and sample size: total number of males and females for pronotum, and number of males for aedeagus.

Site	Locality	Sampling year	Sample size	
			Pronotum	Aedeagus
ADRE	Les Adrets de l'Estérel	1997, 1998	18	11
AURO	Auron	1998, 2001	20	11
BAGB	Bagnols en Forêt	2000	23	14
BARL	Barles	1997, 2001	36	14
BEUI	Beuil	1998	27	–
BOSC	Boscodon	2001	29	14
BRAU	Col de Braus	1998	24	–
BRIA	Briançonnet	2000	30	13
BRIG	La Brigue	1998	20	10
CAUS	Caussols	1998, 2000	26	12
CBUI	Col du Buis	2000	30	18
CONS	Conségudes	2000	29	–
COUR	Courmette	1998	20	–
CSTJ	Col Saint-Jean	2001	15	–
ESTE	Esteng	1998	10	–
ISOL	Isola	1998	21	8
J AUS	Jausiers	2001	25	–
LIOU	Le Liouc	2001	41	14
MADM	La Madone de Fenestre	1998, 2000	30	14
MAES	Malaussène	2000	26	–
MSIA	Montauroux	2001	31	–
MURE	Mure	1998	18	–
OSIG	Osiglia	1997	36	16
PCLA	Pont de Clans	2000	35	16
PEON	Péone	1998, 2000	33	9
RG AU	Rigaud	1998	21	14
RGON	Rigons	2000	30	–
RQBL	Roquebillière	2000	30	13
RQST	Roquestéron	1997, 1998, 2000	26	9
SCAS	Saint-Cassien	1997, 2000, 2001	36	20
SDAL	Saint-Dalmas	2000	16	–
SERA	Séranon	2000	30	–
TEND	Tende	1998	25	9
VALD	Valdieri	1998	25	15
VAUP	Vauplane	1998	24	9
VCHA	Vallon des Châtaigniers	2000	29	–
VERD	Verdaches	2001	35	14
VERG	Vergons	1998	20	–
VESC	Vescous	1997, 1998, 2000	36	13
VFIL	Vallon du Fil	2000	30	–
VILT	Villetalle	2000	28	–
Total			1094	310

Note that effective differences between pronotum and aedeagus do not correspond to female number, as several males were not exploitable for aedeagus shape analysis.

the same as in the amplitude plus the phase, a weak ME on real coefficients allows their use in statistical analyses. However, high ME on real coefficients and weak ME on amplitude reflect high ME on phase, and therefore restrict analyses on the harmonic amplitudes. In this latter case, the information is only partial.

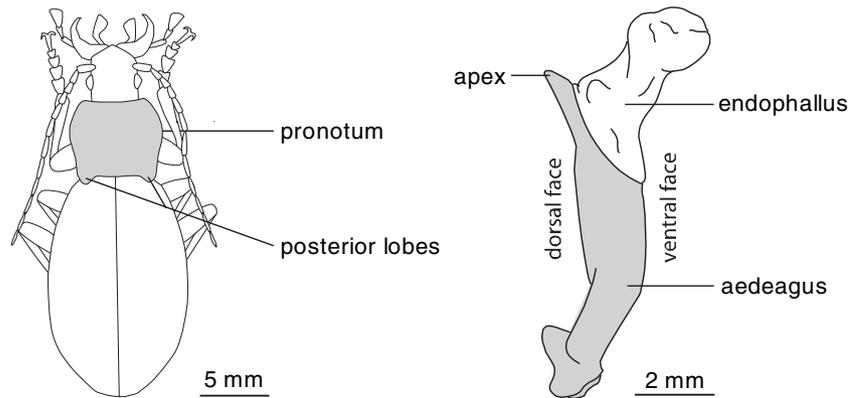


Fig. 2 Schematic representations of a ground beetle (left) and of male genitalia (right). Characters studied (pronotum and aedeagus) are shown in grey.

A subsample of 36 and 20 individuals was measured twice for the pronotum and the aedeagus respectively, in order to assess ME associated to each shape variable. Using model II one-way ANOVAs, with individuals as the categorical factor, the percentage of ME was estimated, for each shape variable separately, as the proportion of the total variance attributable to within-individual variation (Bailey & Byrnes, 1990). For the pronotum, an increase of ME for real coefficients was detected after the fifteenth harmonic pair [ME was <15% before that pair, except for the real coefficients of the first harmonic (ME = 25.2% and 25.6% for coefficients *a* and *b* respectively)]. ME remained roughly constant and <10% for amplitude of the first 16 harmonic pairs, before increasing. Note that in the ME calculations, the measure variability is partitioned into within-individual (ME) and among-individual components. When differences between individuals are moderate, ME increases. Therefore, we considered levels of ME for real coefficients of the first 15 harmonic pairs (i.e. ME < 15%) to be reasonable. As we also verified that 15 harmonic pairs allowed a good outline reconstruction, the real coefficients of these harmonics, i.e. 60 variables, were retained for statistical analyses. Concerning the aedeagus, ME exceeded 40% for at least one real coefficient of the first, the second and the fourth pairs of harmonics. However, ME was <10% for the amplitude of the first 12 harmonic pairs and then gradually increased. This difference of level of ME is due to an important ME for the phase of the harmonics, even for the first ones. Consequently, only amplitudes were considered for the study. As 12 harmonic pairs allowed a good outline reconstruction, harmonic pairs from the thirteenth onward were excluded in further analyses. Male genitalia shape was therefore described with 24 variables.

Morphological appraisal of population structure

Prior to shape analysis we extracted the square root of the surface of the structure studied as an estimator of the size of this structure. Size variation among populations

(populations corresponding to the sampling sites) was tested using ANOVA.

Pronotum shape (described by the set of 60 Fourier coefficients) and aedeagus shape (described by the set of 24 amplitudes) were considered independently in the following analyses. For each structure, a multivariate analysis of variance (MANOVA) was performed on shape variables in order to test the among-population mean difference. Canonical discriminant analyses were also performed with population as the dependent variable, and mean scores of populations were plotted to illustrate the pattern of morphological differentiation in the shape space. Mahalanobis distances (D^2) were also calculated between pairs of populations.

Visualization of shape changes

A major advantage of geometric morphometry is the possibility to visualize shape variation directly on the structure studied. For instance, in the case of outline analyses, the outline can be reconstructed from any set of Fourier coefficients using the inverse Fourier transform. This can be used to describe shape variation associated with a particular direction of shape space, e.g. any multivariate factorial axis. We used multivariate regression (Krzyszowski, 2000) of Fourier coefficients successively upon the two first canonical axes. Parameters of the regression were used to predict values of Fourier coefficients corresponding to theoretical individuals, here the maximum and the minimum projections on the first two canonical axes. As Fourier coefficients *per se* are very difficult to interpret (Kaesler, 1997), this approach allowed to depict outline deformation along canonical axis (Rohlf & Archie, 1984; Monti *et al.*, 2001). Average shape for some populations (which illustrates general tendencies) was also reconstructed using mean values of Fourier coefficients. These populations were chosen to represent main clusters of populations identified. The information born by these reconstructions is complementary to those described above as they summarize shape changes in the whole

shape space (and is not restricted to a particular axis). This last approach was the only one possible with the aedeagus because multivariate analyses were performed on the amplitude of the harmonics, which was insufficient for outline reconstruction through inverse Fourier transform.

Relationships between morphologic and genetic differentiation

The relationship between morphological differentiation, genetic differentiation and geographical location of populations was assessed by testing the correlation between morphological, genetic and geographical distance matrix. Morphological distances correspond to Mahalanobis distances (D^2). Genetic distances were based on allele frequency data of 10 microsatellite loci. This dataset corresponds to the one used by Garnier *et al.* (2004) to investigate the population genetic structure of *C. solieri*. We used Cavalli-Sforza & Edwards (1967) chord distance D_{CE} as the genetic distance because it has been shown to be one of the most efficient distance measures to obtain correct tree topology from allele frequency data (Takezaki & Nei, 1996). Finally, we considered geographical distances as straight-line distances between all pairs of sampling sites. Simple Mantel tests were performed to test for pairwise relationships between the three distance matrices. However, independent variables may be correlated. Thus, we used partial Mantel tests in order to assess (i) the association between morphological and genetic differentiation while taking into account the effect of geography and (ii) the association between morphological differentiation and geography while taking into account genetic differentiation. Permutation of the residuals of a null model was used because it has been shown to be applicable in most cases (Thorpe *et al.*, 1994; Legendre, 2000; but see Raufaste & Rousset, 2001; Castellano & Balletto, 2002; Rousset, 2002, for a debate). Each test was based on 10^5 permutations.

Results

Size differentiation

Size variation between populations was highly significant (ANOVA, $F_{40,1053} = 36.55$, $P < 0.0001$ and $F_{23,286} = 36.38$, $P < 0.0001$, for the pronotum and the aedeagus respectively). About half of the variation of the population mean size of the pronotum was explained by variation of altitude ($R^2 = 0.49$, $F = 36.89$, $P < 0.001$), whereas this relationship was less evident for the aedeagus ($R^2 = 0.18$, $F = 4.75$, $P = 0.04$). However, there was no clear pattern of size variation according to the six groups of populations considered. Hence, mean size for both characters appeared to be variable among populations of the same group.

Shape differentiation

Pronotum

The MANOVA on the Fourier coefficients indicated a highly significant difference between populations (Wilk's lambda = 5.4×10^{-5} , $F = 4.80$, d.f. = 2400 and 33514.18, $P < 0.0001$). The first 20 canonical axes were statistically significant. However, the first two axes explained 24 and 13.6% of variance while the percentage of variance explained by the following axes was $< 10\%$ and gradually decreased. Moreover, examining the projections onto canonical axes, other than the first two, revealed that they provided no major additional information. Projections of population mean scores onto the first two canonical axes (Fig. 3) showed a clear morphological differentiation between three main groups of populations: the first one corresponding to the Bonnetianus group, the second to the Solieri-NW group and the last one to all the other populations. Projections of population mean values onto the first axis were globally sorted according to latitude, from the Bonnetianus group in the south to the Solieri-I from the north-east and the Solieri-NW in the north-west of the distribution area. The Bonnetianus group appeared to be more heterogeneous than the other groups: morphological variation between its populations was as large as that between all other populations (except the Solieri-NW group), despite the Bonnetianus group occurring in a much smaller geographical area (see Fig. 1).

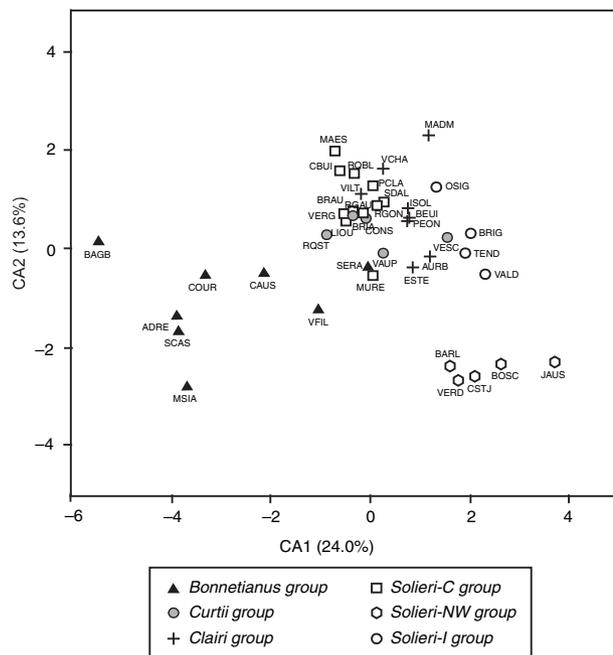


Fig. 3 Plot of the 41 population centroids onto the first two canonical axes (CA1 and CA2) for the pronotum (see text for details about groups and Table 1 for population abbreviations).

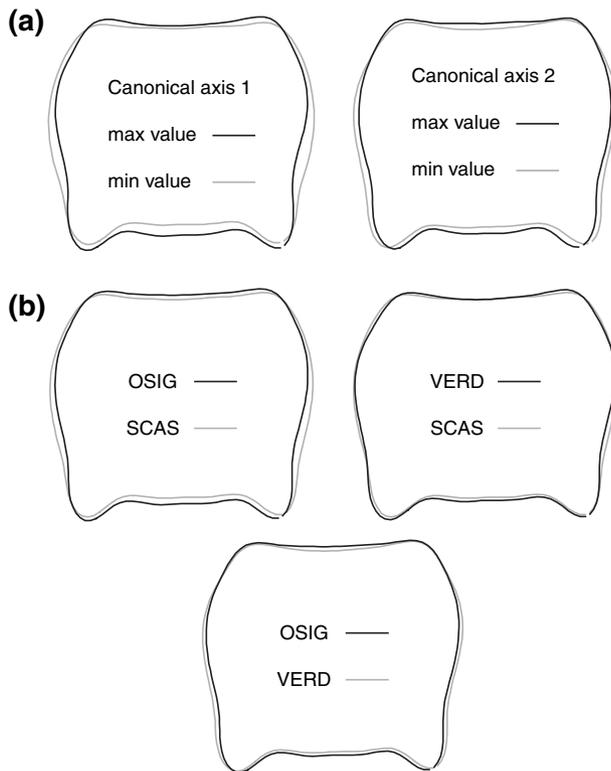


Fig. 4 Pronotum shape variation. Outlines were reconstructed either for minimum and maximum projection values on the first two canonical axes (a) or from average shape for some populations (b), and were superimposed to facilitate the visualization of shape variations.

Shape changes associated with the first axis mainly affect lateral edges of the pronotum (Fig. 4a): they appear more convex for negative projections (Bonnetianus group) and S-shaped for positive projections (Solieri-NW and Solieri-I groups). In addition, the ratio length/width seems to be higher, and the anterior edge looks more concave for positive projections. For the second canonical axis, the ratio length/width increases for positive projections but this change seems to mostly involve a decrease in the width of the posterior part of the pronotum (Fig. 4a). The anterior edge of the pronotum also looks more concave for negative projections. All these shape variations are congruent with those expressed on average shape for some populations (Fig. 4b).

The highest values of D^2 occurred between populations from the Bonnetianus group on the one hand, and populations from the Solieri-I and Solieri-NW groups, on the other. The values of D^2 are not shown here but this trend is visible on Fig. 5.

Aedeagus

Morphological differentiation between populations was highly significant, as shown by the result of the MANOVA

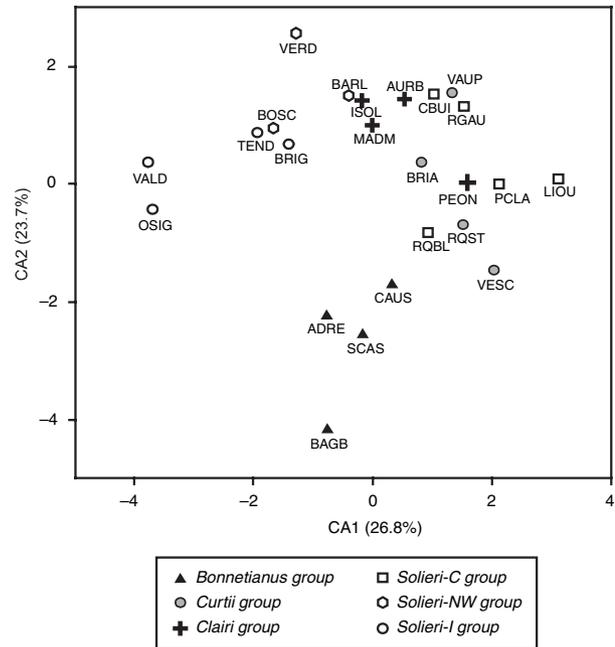


Fig. 5 Plot of the 24 population centroids onto the first two canonical axes (CA1 and CA2) for the aedeagus (see text for details about groups and Table 1 for population abbreviations).

performed on the amplitude of the first 12 harmonic pairs (Wilk's lambda = 3.2×10^{-4} , $F = 5.03$, d.f. = 552 and 4468.34, $P < 0.0001$). While the first 12 canonical axes were statistically significant, only the first two were retained. Indeed, the percentages of variance explained by these axes were 26.8 and 23.7%, whereas the other axes did not exceed 11%. The scatterplot of population mean scores on the first two canonical axes showed three groups of populations that were not completely separated (Fig. 5). The first one corresponds to the Bonnetianus group, the second includes the Solieri-I and Solieri-NW groups and the last one included populations belonging to the three other groups (Curtii, Clairi and Solieri-C).

Reconstruction of average outline of some populations showed only subtle differences which were not easy to interpret (Fig. 6). The three populations compared (SCAS, OSIG and RGAU) represented a general trend for shape changes among the three groups identified from the projections onto the first canonical plan. Aedeagus apex is thicker for RGAU, especially when compared with OSIG. In the ventral face, the zone of eversion of the endophallus is more convex and the corresponding dorsal part is more concave for OSIG, than for the two other populations. Finally, the basal part of the aedeagus appears thinner for SCAS.

In the whole shape space, the highest values of D^2 corresponded to population pairs implicating either one population from the Bonnetianus group and one from

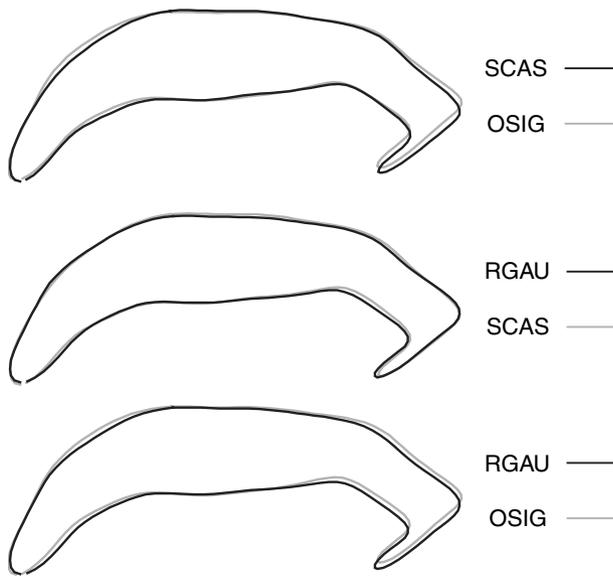


Fig. 6 Aedeagus shape variation, illustrated by superimposed outlines corresponding to average shape of three populations.

Table 2 Correspondence between morphological distances (Morpho, dependent variable), and genetic (Genet) or geographic (Geo) distances (independent variables), measured either by correlation coefficient (r) or partial correlation coefficient (r').

	Pronotum		Aedeagus	
	r	P	r	P
Simple Mantel tests				
Morpho – Geo	0.46	0.0001	0.40	0.0150
Morpho – Genet	0.69	<0.0001	0.59	<0.0001
	r'		r'	
Partial Mantel tests				
Morpho – Geo–Genet	0.09	0.1800	0.12	0.1700
Morpho – Genet–Geo	0.58	<0.0001	0.48	<0.0001

Partial Mantel tests considered for the correspondence between the first two matrices while controlling for the third. All tests were one-tailed and based on 100 000 permutations. Significant correspondences are indicated in bold. Tests were performed across 41 populations for the pronotum and 24 populations for the aedeagus.

another group, or one population from the Solieri-C group and one from the Solieri-I group (not shown).

Relationships between morphologic and genetic differentiation

Distance matrix correspondence tests provided similar results for the pronotum and for the aedeagus (Table 2). Morphological and geographical distances were positively and significantly correlated, as were morphological and genetic distances. However, partial Mantel tests showed that geographical proximity has no impact on

morphological differentiation once genetic differentiation has been accounted for. Conversely, genetic differentiation carried significant additional information about morphological differentiation even when geographical proximity had been accounted for (Table 2).

Discussion

Pattern of morphological differentiation

We found highly significant morphological differentiation between populations of *C. solieri*. Overall, patterns of morphological changes assessed from the shape of the pronotum and the aedeagus are congruent, although analyses of the latter structure, performed from reduced sample sizes, should be considered more cautiously. Previous studies on morphological differentiation in this species have reported a rather different spatial pattern of variation. Bonadona (1967, 1973) studied several morphological characters based on traditional measurements on the pronotum and the abdomen and on the elytral sculptures. From his results, he defined three subspecies. The first one – *Chrysocarabus solieri bonnetianus* – corresponds to the most southern populations of the Bonnetianus group defined in the present work. The second one – *C. s. clairi* – occurs in two distinct areas, one corresponding to the north of our Bonnetianus group plus the Curtii group and the other corresponding to the Clairi group. Finally, the third subspecies – *C. s. solieri* – matches the Solieri group as defined here. Rasplus *et al.* (2001) reanalysed data from the studies of Bonadona (1967, 1973) and, not surprisingly, obtained similar results. The major discordance between our findings and previous studies concerns the Solieri-C group. Our results show that this group is not clearly differentiated from the Clairi and the Curtii groups, whereas Bonadona (1967, 1973) clustered the latter into the same subspecies (*C. s. clairi*) and considered populations of the Solieri-C group as a distinct subspecies (*C. s. solieri*). Several features in the studies of Bonadona (1967, 1973) could explain this disagreement: for example the sampling scheme which was restricted to some part of the range and the lack of estimation of ME. In addition, measures (length, width...) used by Bonadona (1967, 1973) concerned a rather limited part of the geometry of the structure studied in contrast to outlines appraisal through Fourier transforms. Finally, subspecies defined by Bonadona (1967, 1973) match the colour pattern of populations, contrary to our morphological groups. In *C. solieri* however, colour is structural and probably produced by multilayer reflectors (Neville, 1977; Parker, 2000). If there exist a strong covariation between elytral striation and cuticle microstructure (and thus colour), and if other morphological characters studied by Bonadona (1967, 1973) are weakly, if not, discriminant, then subspecies defined by Bonadona (1967, 1973) would mainly mirror variations of colour rather than variations

of individuals' shape. Unfortunately, we have no idea of the weight of each character in Bonadona's (1967, 1973) results. The study of elytral sculpture would help to test this hypothesis, even if an objective and quantified assessment of this character seems difficult.

Rapid and divergent evolution of male genitalia is one of the most widespread patterns of animal evolution (Eberhard, 1985), and three main hypotheses, i.e. the lock-and-key hypothesis, the pleiotropy hypothesis, and the sexual selection hypothesis (see Arnqvist, 1997 for a review) have been proposed to explain this pattern. In our case, it is noteworthy that the shape of male genitalia does not appear more divergent than shape of the pronotum. A first explanation could be linked to incomplete assessment of the information about the shape of aedeagus because of the technique used. For example, the three-dimensional assessment of the shape could be more relevant than the two-dimensional projection. A second hypothesis concerns the functionality of the aedeagus. Not all genital parts have the same functional importance for purpose of genital coupling and sperm transfer (e.g. Goulson, 1993), and hence are not subjected to the same evolutionary forces. In fact, aedeagus is just partly inserted into female genitalia during copulation, and other genital parts such as the copulatory piece (a chitinized apophysis on the endophallus of the male) have been reported to be functionally very important for genital coupling (Sota & Kubota, 1998). However, whereas this piece exists in some *Carabus* subgenus, it is absent in the subgenus *Chrysocarabus* and therefore in *C. solieri* (Deuve, 1994).

Morphology vs. genetics

On the whole, morphological and genetic patterns of differentiation were correlated, independently of the influence of geographical proximity (Table 2). A previous, related, study using a Bayesian clustering analysis of genotypes at 10 microsatellites loci, identified three main clusters of populations in the distribution area of *C. solieri*, and barriers to gene flow between them (Garnier *et al.*, 2004). The first one corresponds to the Bonnetianus group plus three populations (CONS, RQST, VESC) of the Curtii group. The second cluster is constituted by the Clairi and the Solieri-C groups plus two populations from the Curtii group (BRIA and VAUP). Finally, the third cluster can be further divided into two parts: one matching the Solieri-I group and the other the Solieri-NW group (except population VALD which clusters with the Solieri-NW group). Overall, the morphological characters studied allow the identification of the same clusters of populations, even if less differentiated. Morphological variation seems therefore to reflect the underlying population genetic structure of *C. solieri*.

A major issue of studies of differentiation and speciation is the relative importance of drift and natural or sexual selection in the evolution of reproductive isolation (Coyne, 1992). The role of natural selection in promoting

speciation through ecological processes becomes more and more documented (Orr & Smith, 1998; Schluter, 2001), even if genetic drift has also been shown to be a possible important factor promoting divergence (Wlasiuk *et al.*, 2003). Interestingly, the morphological divergence between populations within the Bonnetianus group is associated with a strong genetic differentiation between populations and a weak genetic diversity within populations (Rasplus *et al.*, 2001; Garnier *et al.*, 2004). Populations of Bonnetianus groups are isolated from each other because of the fragmentation of the forest habitat, mainly because of anthropogenic activities and a high frequency of forest fires. Ground beetles have often been shown to be very sensitive to habitat fragmentation (Assmann & Weber, 1997; Keller & Largiadèr, 2003) because of their weak dispersal power. Alibert *et al.* (2001) reported a clear morphological differentiation between two forests located 45 km apart and a correlation between morphological and geographical distances in the species *C. auronitens*. Differentiation of neighbouring populations has also been revealed from molecular markers (Brouat *et al.*, 2003). In our case, the quite low level of gene flow and the local fixation of alleles observed in the Bonnetianus group area (Garnier *et al.*, 2004) suggest that drift could play a significant role in the morphological divergence between populations of the Bonnetianus group. Indeed, local environmental conditions do not appear to be very different among sites; at least they are less different than they are between sites of Solieri-C and Clairi groups which nevertheless appear less differentiated morphologically. Here for instance, the area of the sampling site of population BAGB was particularly limited, i.e. a few hundred metres of humid grove within a dry area, and not surprisingly, this population was highly distinct from the others according to both morphological characters studied and to its very low genetic diversity (Garnier *et al.*, 2004). However, we cannot exclude natural selection as a process promoting a part of the morphological variation observed. Comparison of genetic variance components (within and among populations) between neutral markers and morphological characters could help to resolve the question.

Size did not exhibit a clear pattern of differentiation between groups of populations, in contrast to shape. It is often argued that size can also significantly depend on environmental conditions (Patton & Smith, 1989; Adams & Funk, 1997; Tatsuta *et al.*, 2001). The correlation reported here between size of both morphological structures and altitude could illustrate this potential contribution of environmental factors to size. However, the relative contribution of genetic and nongenetic factors to size is clearly impossible to estimate from our data.

Implications for phylogeography of *C. solieri*

The phylogeographic scenario proposed by Rasplus *et al.* (2001) postulates that during the last glacial events,

C. solieri differentiated into two refuges, involving one blue and one green subspecies, in the south of France and in Italy respectively. The results of the study of pronotum and aedeagus shape are consistent with this hypothesis as Bonnetianus and Solieri-I (the groups supposed to derive from refuge areas) are morphologically the most differentiated. Postglacial re-colonization occurred westward for the Italian entity, probably following two routes (Garnier *et al.*, 2004). The first one was in the north of the Mercantour massif, through the Larche pass (Fig. 1). In this context, the genetic proximity of populations from Solieri-I and Solieri-NW groups (Garnier *et al.*, 2004) is in agreement with their proximity according to aedeagus shape, but contrasts with their strong differentiation according to pronotum shape. Two explanations can be proposed for this apparent discordance. First, a low level of genetic diversity in populations from the Solieri-NW group relative to those of the Solieri-I group could have resulted from successive founder effects during the expansion process (Garnier *et al.*, 2004). Indeed, both theoretical and empirical studies suggest that colonization events are often characterized by one or several founder effects resulting in a loss of genetic diversity (Le Corre & Kremer, 1998; Hewitt, 1999). Neutral or weakly selected morphological characters could have been more affected by these founder effects. As compared with aedeagus, this could be the case for the shape of the pronotum. Second and unlike the first hypothesis, the pronotum shape may be under selection. In this context, variation between Solieri-I and Solieri-NW groups could result from adaptation to different local environmental conditions acting directly or indirectly on the shape of the pronotum. However, to our knowledge, until now there is no study reporting (or suggesting) selection on this morphological structure.

The second re-colonization route from Italy occurred in the south of the Mercantour massif and led to a secondary contact with the entity differentiated in southern France, which probably expanded northward. Whereas a first hypothesis proposes that this contact could correspond to the Curtii group range, a second hypothesis postulates that the initial contact zone corresponds to the transition zone between Solieri-I group on the one hand and Solieri-C plus Clairi groups on the other (Rasplus *et al.*, 2001; Garnier *et al.*, 2004). In this second case, the Clairi group would reflect the northernmost expansion of the blue subspecies preceding the spread in the centre of the range of the entity differentiated in Italy. Our results could give arguments in favour of this second hypothesis as they show that Clairi and Solieri-C groups are relatively little differentiated and appear morphologically intermediate between Solieri-I and Bonnetianus groups. This is therefore in accordance with the idea that Clairi and Solieri-C groups could originate from hybridization and introgression between the two original subspecies. The case of *C. solieri* would

then add to the few cases where introgression between differentiated entities has been reported over large geographic zones (e.g. Largiadèr *et al.*, 1994; Sota *et al.*, 2000; Turgeon & Bernatchez, 2001).

Conclusions

Outline analysis of the shape of the pronotum and of the aedeagus provided a clear pattern of morphological differentiation within the range of *C. solieri*, even at a local scale. However, this pattern is different to that found in previous studies. Outline reconstructions show that the shape changes implicated were subtle, particularly for the aedeagus. The use of powerful methods such as geometric morphometrics, but more importantly the use of different approaches and markers (morphological and molecular markers), enable us to clarify the phylogeographic history of *C. solieri*. Not only does this 'multi-marker approach' allow a better evaluation of the divergence between different entities, but it also allows the possibility of assessing evolutionary forces involved in the history and the dynamic of divergence.

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