

# Geographic differentiation of morphological traits and isozymes in the Mediterranean island endemic *Anchusa crispera*: implications for the conservation of a protected species

A. Quilichini<sup>\*</sup>, M. Debussche, J.D. Thompson

*Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, 1919 Route de Mende, 34293 Montpellier Cedex 5, France*

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## Abstract

Understanding population differentiation and genetic diversity within population is critical to the development of conservation programmes for threatened species. The protected species, *Anchusa crispera* Viv. occurs on coastal sand dunes in three disjunct geographical groups of populations: two populations on the east coast of Corsica, several populations around a single estuary on the west coast of Corsica and at a number of sites in north–west Sardinia, where two different subspecies occur. In this study we quantify differentiation of reproductive traits and isozymes among regions and populations on Corsica and between subspecies on Sardinia. No isozyme variation was detected within any of the studied populations nor among populations from a given geographic region. Two loci showed geographic differentiation between regions on Corsica. On Sardinia, the two subspecies showed no isozyme differentiation, and, depending on the loci examined, are similar to one or other of the two regions on Corsica. Reproductive traits show significant differentiation between geographic regions on Corsica, but little variation among populations within a region. In populations on the west coast of Corsica, flowers are small and have a stigma situated at the same level as the apex of the anthers whereas on the east coast, flowers are larger and have the stigma below the anthers. On Sardinia, *A. crispera* subsp. *crispera* has a floral morphology similar to the Corsican plants, whereas subspecies *maritima* has larger flowers with the stigma positioned above the anthers (approach-herkogamy). Quantitative variation in floral morphology thus agrees with the separation of two taxonomic entities on Sardinia and geographic variation in reproductive traits and isozymes has important ramifications for the sampling of populations for conservation programmes.

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## 1. Introduction

Quantifying and understanding the causes of population differentiation are important goals in conservation biology (Hamrick et al., 1991; Holsinger and Gottlieb, 1991; Ellstrand and Elam, 1993; Wise, 1997; Thompson, 1999). The maintenance of population differentiation depends on the balance between genetic

drift and natural selection which enhance variation among populations, and on gene flow which acts to homogenise variation across the landscape. In small populations, genetic drift can be a major force reducing genetic variation (see for example Richards, 2000; Kéry, 2000). The fragmentation and isolation of small populations which increases genetic drift and reduces gene flow, can thereby cause a reduction of genetic variation within populations and promote the evolution of genetic differentiation between them (Newman and Tallmon, 2001). In this context the extent to which a species may be inbred may have a profound effect on amounts of population differentiation (Charlesworth and Charlesworth, 1995; Hamrick and Godt, 1996; Hendrix and Kyhl, 2000; Barrett, 2002).

<sup>\*</sup> Corresponding author. Present address: Laboratoire Evolution et Diversité Biologique, Université Paul Sabatier, UMR 5174 – Bâtiment 4R3, 118 route de Narbonne, 31062 Toulouse Cedex 4, France.

*E-mail addresses:* [quilichi@cict.fr](mailto:quilichi@cict.fr), [angelique.quilichini@cict.fr](mailto:angelique.quilichini@cict.fr) (A. Quilichini).

Species with low levels of genetic variability are thought to be more vulnerable to environmental changes and to be at greater risk of extinction than species with high genetic variability (Barrett and Kohn, 1991; Huenneke, 1991; Dolan, 1994; but see Soulé and Mills, 1998; Fleishman et al., 2001) since the maintenance of genetic variability is essential for populations to respond to present and future environmental changes (Frankel and Soulé, 1981; Lande, 1995; Lynch et al., 1995; Newman and Pilson, 1997; Soulé and Orians, 2001). To estimate the evolutionary potential of a species it is necessary to quantify the extent to which populations are genetically differentiated for quantitative traits which may facilitate adaptation to environmental variation.

The Mediterranean basin is a major centre of plant endemism (Médail and Quézel, 1997) and population differentiation (Thompson, 1999). The occurrence of high numbers of endemic species, particularly on islands and in mountain ranges in the Mediterranean region, attests to the high levels of geographic differentiation that occurs in the flora. Many species have disjunct distributions such that geographically isolated populations may also exhibit high levels of differentiation. The extent to which such differences among populations compares with differences among what are suggested to be different but closely related endemic taxa in the Mediterranean flora is an issue which has recently attracted attention (Debussche and Thompson, 2002). This issue is particularly important in order to identify and delimit taxa which merit conservation status (Olfelt et al., 2001). In fact, for only a few endemic and protected species do we

have information concerning levels of population differentiation (e.g. Affre and Thompson, 1997; Affre et al., 1997; Fréville et al., 1998; Petit et al., 2001) and amounts of morphological variation among closely related sister taxa (Debussche and Thompson, 2002).

*Anchusa crispa* Viv. is a protected species in France listed under the Berne Convention 1979 (Olivier et al., 1995). This species is endemic to coastal sand dunes on the islands of Corsica (France) and Sardinia (Italy), in a small number of populations, some of which have only a few tens of individuals (Quilichini, 2001). Two subspecies of *A. crispa* have been described on Sardinia: *A. crispa* Viv. subsp. *crispa* (Vals.) Selvi and Bigazzi and *A. crispa* Viv. subsp. *maritima* (Vals.) Selvi and Bigazzi on the basis of differences in basal leaf form, bract length and calyx divisions (Selvi and Bigazzi, 1998). Populations of *A. crispa* occur in three geographically separate areas: two populations on the east coast of Corsica (Piazza and Paradis, 1988; Thiébaud, 1988; Paradis and Piazza, 1989), five populations on the west coast of Corsica (Conrad et al., 1989) and 14 known populations on the north–west coast Sardinia (Valsecchi, 1976; Selvi and Bigazzi, 1998) (Fig. 1). The total number of plants in each population on Corsica varies from a few individuals (in the east coast populations) to several hundred (in some west coast populations), and the total numbers of plants in the last 10 years has varied from ~800 to ~2300 flowering individuals (see Quilichini, 2001).

Rapidly developing and intense tourist pressure on habitats during summer is probably the most serious

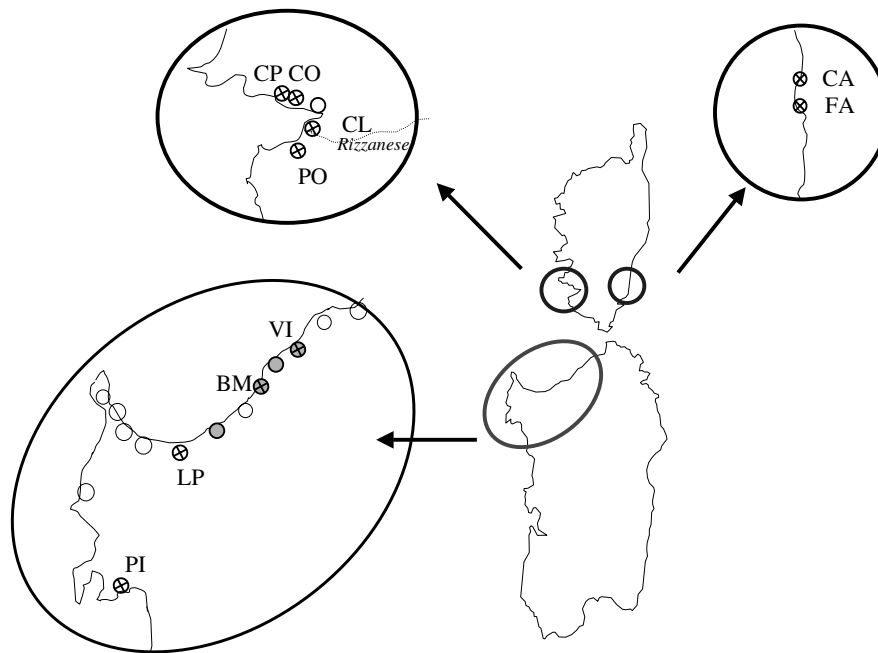


Fig. 1. Geographic location of known populations of *Anchusa crispa*. Studied populations are represented by circles containing a cross. On Sardinia, open circles represent subsp. *crispa* and shaded circles represent subsp. *maritima*. Population codes are: CP, Cala Piscona; CO, Cappiciolo; CL, Capu Laurosù; PO, Portigliolo; CA, Cannella; FA, Favone; VI, Vignolla; BM, Badesi Mare; LP, La Pelosa; PI, Porticciolo.

danger for the persistence of this species in the wild. However, the biology of this species and the isolation of populations are also of particular interest for the development of conservation programmes in protected sites. In a previous study, Quilichini et al. (2001) reported the occurrence of outbreeding depression, i.e., higher fitness of inbred progeny relative to outcrossed progeny, in a two-generation crossing programme. This result indicates that inbreeding is a feature of populations of this species. This finding is particularly pertinent to the conservation management of this species, since it requires that if that reinforcement or reintroduction programmes are to be successful then the source of individuals to be used should be carefully chosen in respect to the site in question.

To quantify the degree of population differentiation within this protected species we have studied quantitative and qualitative variation in morphological traits and genetic variation at marker loci within and among populations from the different geographic region on Corsica and in both subspecies on Sardinia. This combination of genetic marker diversity and variation in quantitative traits is essential to develop a solid understanding of the implications of population and geographic differentiation for conservation management (Lynch, 1996; Frankham, 1999; Reed and Frankham, 2001). Based on reproductive traits and isozymes we ask the following questions. (1) Do populations in the two different geographic regions on Corsica (east and west coast) show significant differentiation for a range of floral traits and genetic markers? (2) Is there significant variation in reproductive traits and genetic markers between Corsican populations of *A. crispa* and Sardinian populations of *A. crispa* subsp. *crispa*? (3) Do genetic and reproductive traits support the distinction of two different subspecies on Sardinia? In our interpretation of these issues, the conservation implications of differentiation between the two subspecies and among geographic regions are outlined.

## 2. Materials and methods

### 2.1. Study species and sites

*A. crispa* is a short-lived perennial species. Plants have a long tap root and grow to a diameter of 20–30 cm. In the autumn, small new leaves develop at the base of the plant and in the spring they enlarge and form a basal rosette. The plants do not reproduce by vegetative means. Each plant develops several prostrate, scorpioid cymes, each with 10–40 flowers. The flowering period is from March to August and only one or two flowers are in bloom simultaneously on each cyme. Flowers are tubular, pentamerous, actinomorphic and sympetalous, with five papillose scales in the throat, which close the

corolla tube completely. The five stamens are inserted on the corolla tube, below the throat. The stigma is bilobed with crowded papillae (Selvi and Bigazzi, 1998). The flowers are hypogynous with four ovules.

### 2.2. Quantitative floral trait variation

During spring 1997 and 1998, flowers were collected from natural populations on Corsica and Sardinia (Fig. 1). Six populations were sampled on Corsica, four on the west coast [Portigliolo (PO), Capu Laurosus (CL), Cala Pisona (CP) and Cappicciolo (CO)] and two on the east coast [Favone (FA) and Cannella (CA)]. On Sardinia, four populations were studied: two populations of subsp. *crispa* from La Pelosa (LP) and Porticciolo (PI) and two populations of subsp. *maritima* from Badesi Mare (BM) and Vignolla (VI).

One mature flower was collected on 22–30 randomly sampled individuals per population and conserved in 70% ethanol. Five floral traits were measured on each flower using digital calipers (to 0.01 mm): (1) corolla length, (2) corolla diameter, (3) lobe length, (4) anther height (from the base of the ovary to the apex of the anther) and (5) style length (from the base of the ovary to the apex of the stigma). Stigma–anther separation (6) was calculated as the difference between style length and anther height.

Statistical analysis of floral trait variation was performed in one-way ANOVA with PROC GLM (SAS, 1999). Most characters were significantly positively correlated with each other, and interpretation of significant variation in each trait is thus made in this light. Contrasts were made in ANOVA to test for differences between the east and west coasts of Corsica, between Corsica and Sardinia and between subspecies *crispa* and subspecies *maritima* on Sardinia. Scheffé mean tests were carried out for each trait which showed a significant population effect in ANOVA in order to determine which populations were different from one another. To further compare differences among populations, a factorial discriminant analysis was carried out using STAT-ITCF package (ITCF, 1987) and Mahalanobis distances quantified for the 10 populations. Based on this analysis, hierarchical cluster analysis (UPGMA) (Sneath and Sokal, 1973) was used to group populations on the basis of variability in floral traits.

### 2.3. Electrophoresis procedures

Leaf samples were collected from six populations on Corsica (four from the west coast of Corsica (PO, CL, CO, CP) and two from the east coast (CA, FA)) and three populations on Sardinia [one population of *A. crispa* subsp. *crispa* (PI) and two populations of *A. crispa* subsp. *maritima* (BM, VI)]. Twenty-eight to thirty individuals were randomly sampled in each population. Young leaves were removed from each plant and placed

in plastic bags transported on ice to the CEFÉ-CNRS laboratory in Montpellier (France) where electrophoretic analyses were carried out.

Electrophoresis procedures follow Soltis et al. (1983). Enzyme extraction was carried out by grinding the leaf material with a Tris–HCl extraction buffer, pH 7.5 (Lumaret, 1981), with fine-grained sand and 12% PVP. Supernatant from centrifuged homogenate (20 mn at 15.000g) was adsorbed onto filter paper wicks and loaded onto 13% horizontal starch gels. Glasshouse grown plants of *A. crispera* from Corsica were used as marker genotypes on gels. Ten enzyme systems were investigated: glutamate oxalo-transferase (GOT, E.C.2.6.1.1), phosphoglucomutase (PGM, E.C.5.4.2.2), acid-phosphatase (ACP, E.C.3.1.3.2), leucine aminopeptidase (LAP, E.C.3.4.11.1), peroxidase (PRX, E.C.1.11.1.7), esterase (EST, E.C.3.1.1.-), glucose 6-phosphate dehydrogenase (G6PD,

E.C.1.1.1.49), isocitrate dehydrogenase (IDH, E.C.1.1.1.42), malate dehydrogenase (MDH, E.C.1.1.1.37) and phosphoglucoisomerase (PGI, E.C.5.3.1.9).

### 3. Results

#### 3.1. Floral trait variation

All floral traits showed significant variation among the 10 sampled populations of *A. crispera* on Corsica and Sardinia for floral parts (corolla tube length:  $F_{9,272} = 66.05, p < 0.001$ ; corolla lobe length:  $F_{9,272} = 47.26, p < 0.001$ ; corolla diameter:  $F_{9,272} = 59.75, p < 0.001$ ) and sex organ position (style length:  $F_{9,272} = 125.63, p < 0.001$ ; anther height:  $F_{9,272} = 29.88, p < 0.001$ ; stigma–anther separation:  $F_{9,272} = 46.14, p < 0.001$ ).

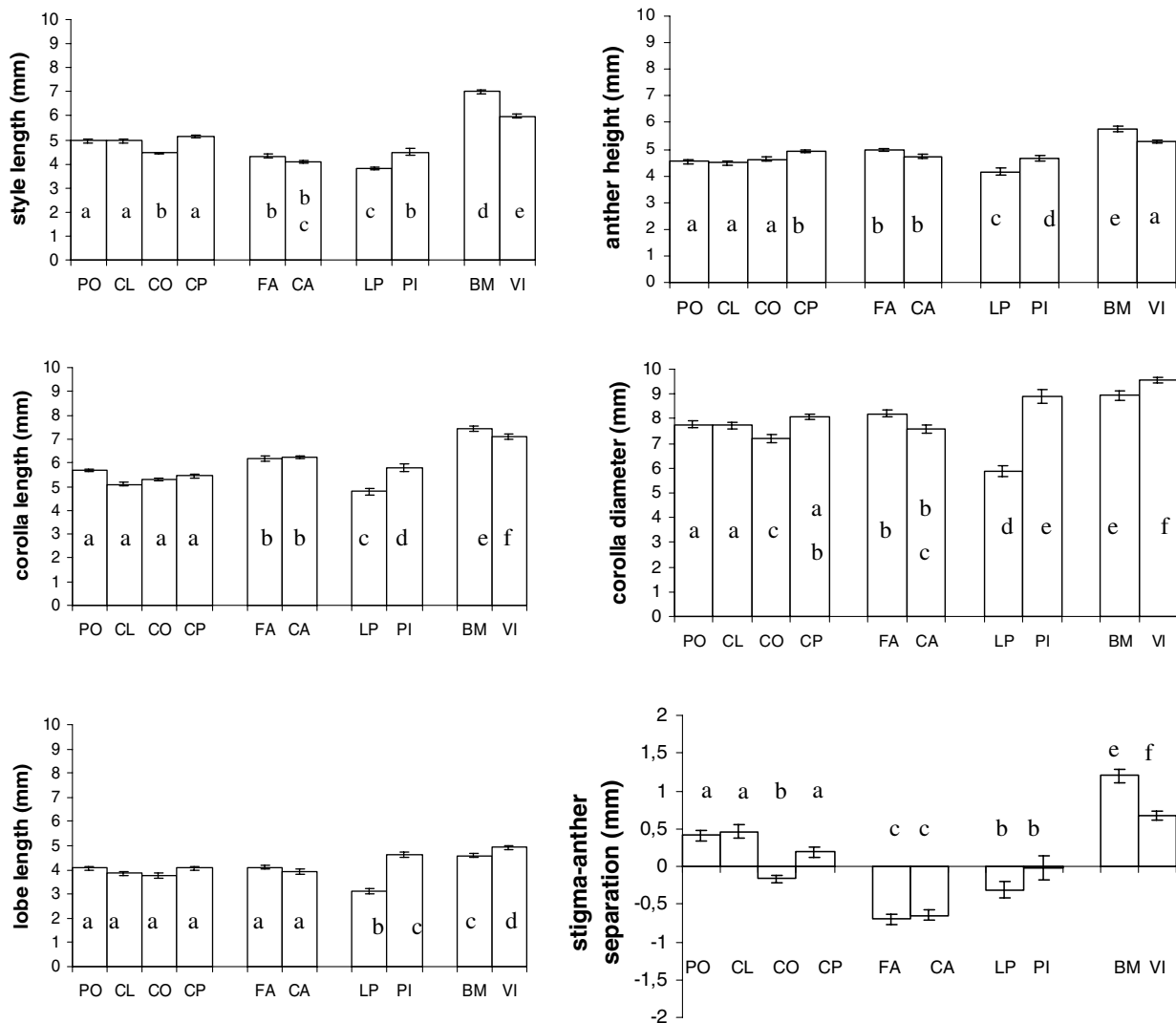


Fig. 2. Mean ( $\pm$  SE) values of six floral traits of *Anchusa crispera* on the west coast (PO, Portigliolo; CL, Capu Lauros; CO, Cappiccio; CP, Cala Piscona) and east coast of Corsica (FA, Favone; CA, Cannella), *A. crispera* subsp. *crispera* on Sardinia (LP, La Pelosa; PI, Porticciolo), and subspecies *maritima* on Sardinia (BM, Badesi Mare; VI, Vignolla). Code letters represent significant differences ( $p < 0.05$ ) following Scheffé means test.

On Corsica, variation among populations within geographic regions was insignificant compared to variation between the two coastal regions (Fig. 2; Table 1). Compared to those of east coast individuals, flowers on the west coast have significantly shorter floral tubes ( $5.41 \pm 0.04$  vs.  $6.23 \pm 0.06$ ), with anthers inserted lower on corolla tube ( $4.65 \pm 0.04$  vs.  $4.88 \pm 0.05$ ) but a longer style ( $4.88 \pm 0.04$  vs.  $4.2 \pm 0.05$ ), a similar corolla diameter and lobe length. Consequently, stigma–anther separation varies significantly between west and east coast populations on Corsica. In west coast populations,

flowers have stigmas situated at the same level or slightly above the anthers (distance between stigma and anther apex =  $0.24 \pm 0.04$ ) while in the east coast populations the stigma is positioned below the anther apex ( $-0.68 \pm 0.05$ ) (Fig. 3). Stigma–anther separation is thus very different between the two regions on Corsica.

Floral traits showed significant variation between subspecies on Sardinia (Fig. 2; Table 1). Subspecies *maritima* has flowers with a longer corolla tube ( $7.26 \pm 0.1$ ), a longer style ( $6.45 \pm 0.1$ ), higher inserted anthers in the corolla tube ( $5.51 \pm 0.07$ ), and a greater

Table 1

F values (and associated probabilities) in ANOVA contrasts of six floral traits among (a) taxonomic entities on Sardinia and (b, c) geographic entities of *Anchusa crispera*

Contrast	df	Style length	Anther height	Corolla length	Corolla diameter	Corolla lobe length	Stigma–anther separation
(a) Subsp. <i>crispera</i> vs. subsp. <i>maritima</i> (Sardinia)	1; 272	742.2***	183.9***	360.9***	262.9***	199.9***	166.2***
(b) Corsica vs. subsp. <i>crispera</i> on Sardinia	1; 272	161.1***	20.6***	85.1***	90.8***	90.7***	55.4***
(c) Corsica: west coast vs. east coast	1; 272	92.6***	11.7***	91.1***	1.8 ns	1.2 ns	142.0***

ns = non-significant. \*\*\*  $p < 0.001$ .

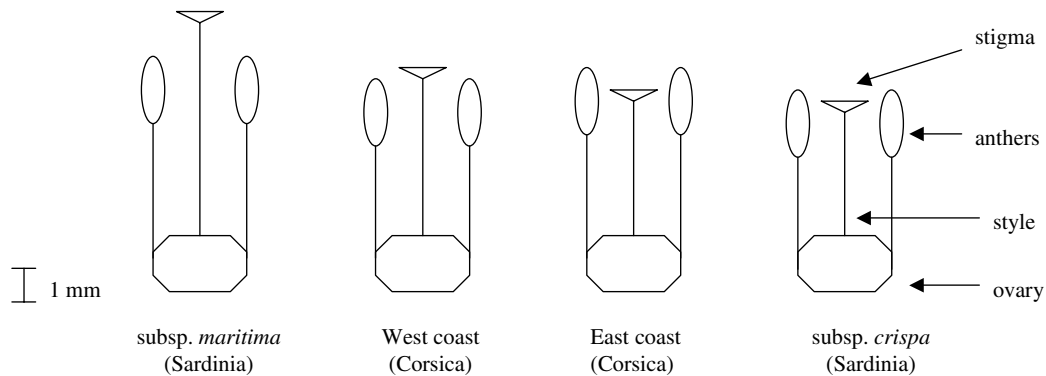


Fig. 3. Schematic representation of mean values of sex organ position in *Anchusa crispera* on Corsica and the two subspecies on Sardinia.

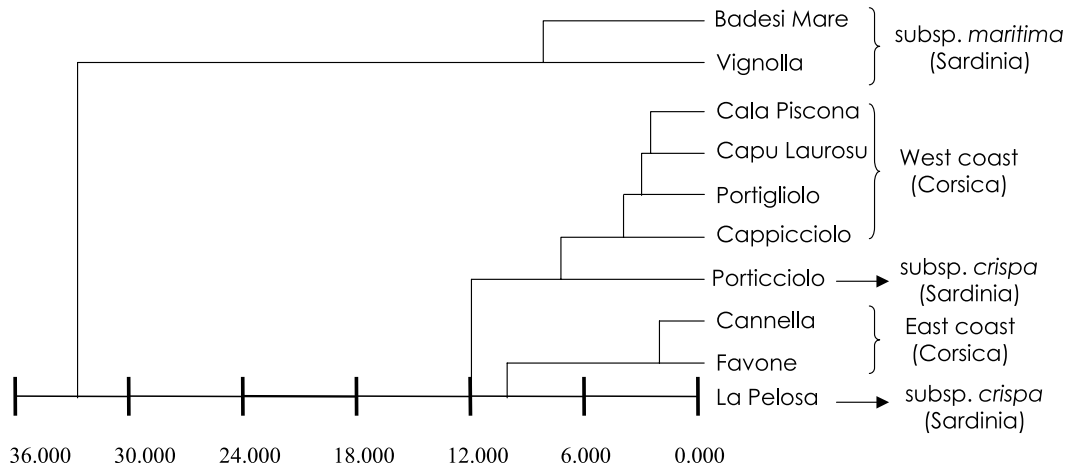


Fig. 4. UPGMA phenogram (Mahalanobis distances) based on six floral traits (corolla length, corolla diameter, lobe length, stamen length, style length and stigma–anther separation) of *Anchusa crispera* on Corsica and two subspecies on Sardinia.

stigma–anther separation ( $0.94 \pm 0.1$ ) than subspecies *crispa* (Fig. 3). Corolla lobe length and diameter showed no significant variation between the two subspecies on Sardinia. Subspecies *crispa* on Sardinia has flowers with a morphology more similar to populations of the species on Corsica, with smaller flowers and shorter stigmas than subspecies *maritima* (Fig. 2). The similarities and differences between the two regions on Corsica and Sardinian populations of subsp. *crispa* depend on the character examined (Figs. 2 and 3; Table 1). Flowers of subspecies *crispa* on Sardinia have a style length and lobe length similar to east coast populations on Corsica and corolla tube length more similar to west coast flowers on Corsica. Stigma–anther separation is very similar in subspecies *crispa* in east coast populations on Corsica and in populations on Sardinia ( $F_{1,272} = 55.43$ ,  $p < 10^{-5}$ ) (Fig. 2) with significant differences between populations on Sardinia and west coast populations on Corsica ( $F_{1,272} = 142.01$ ,  $p < 10^{-5}$ ).

The first and second canonical axes in the factorial analyses explained 69.1% and 18.9% (a total of 88%) of the among-population variation. Mahalanobis distances among the 10 populations show several patterns which confirm the analysis of individual traits (Fig. 4). First, the floral morphology of subspecies *maritima* is distinct from subspecies *crispa* on Sardinia and Corsica. Second, subspecies *crispa* on Sardinia is more similar to the species on Corsica than to subspecies *maritima* on

Sardinia. Third, east and west coast populations on Corsica show marked geographic differentiation in floral morphology. Whereas east coast populations have a morphology similar to the La Pelosa population on Sardinia, west coast populations have a floral morphology similar to the Porticciolo population.

### 3.2. Electrophoretic analysis

Of the 10 enzyme systems investigated, four systems, which encode nine enzyme loci, showed interpretable results: *GOT-A*, *GOT-B*, *PGM-A*, *PGM-B*, *ACP-A*, *ACP-B*, *LAP*, *PRX-A* and *PRX-B*. All loci were monomorphic for a single allele within all populations and among the populations of each geographic region. The total amount of genetic diversity within populations, and among populations within geographic regions, for these markers is thus estimated to be zero. However, two loci (*GOT-A* and *PGM-B*) showed differentiation among geographic regions (Fig. 5). Populations on the west coast of Corsica and Sardinian populations of both *A. crispa* subsp. *crispa* and *A. crispa* subsp. *maritima* were monomorphic for the same *PGM-B* allele. In contrast, for the *GOT-A* locus, east coast populations on Corsica shared the same allele as Sardinian populations of the two subspecies.

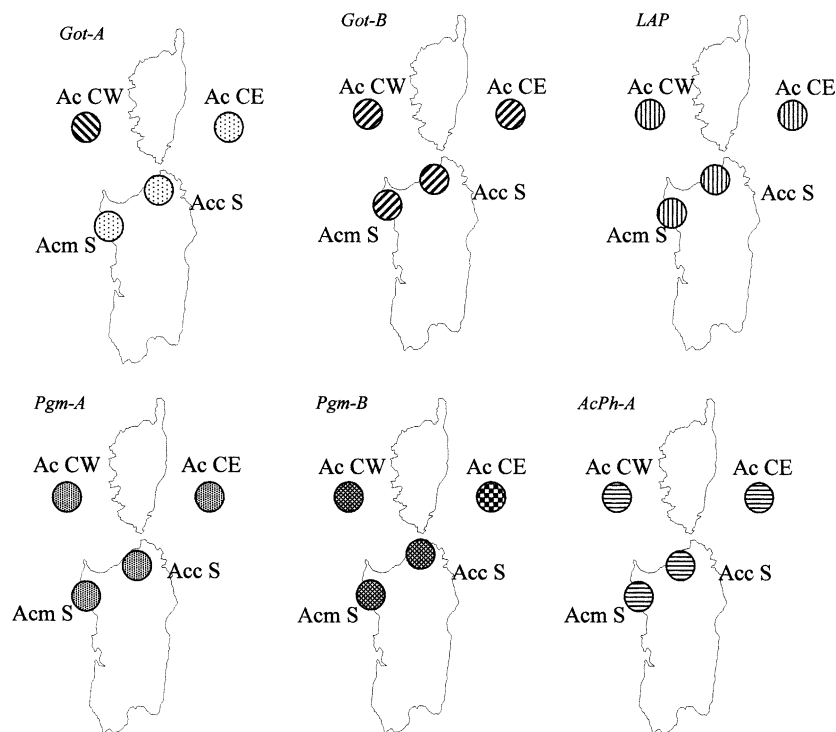


Fig. 5. Allele frequency variation for six loci (four enzyme systems) for *Anchusa crispa* on Corsica and the two subspecies on Sardinia ( $n = 30$  for each population on Corsica, number of individuals sampled,  $n = 28$  for each population on Sardinia). Codes are: A.c. CW, *A. crispa* on the west coast of Corsica; A.c. CE, *A. crispa* on the east coast of Corsica; A.c.c. S, *A. crispa* subsp. *crispa* on Sardinia; A.c.m. S, *A. crispa* subsp. *maritima* on Sardinia.

#### 4. Discussion

This study presents two important results concerning differentiation among populations of a protected species. First, there is a clear-cut pattern of differentiation in reproductive traits and isozyme variation among disjunct populations in the different parts of the range of *A. crispa*. Populations within each portion of the range of this taxon show insignificant levels of differentiation compared to that among regions. Second, on Sardinia, *A. crispa* subsp. *crispa* and subsp. *maritima* show marked differences in floral morphology but no differentiation at the isozyme loci studied.

##### 4.1. Geographic differentiation within *A. crispa*

Isozyme and floral trait variation concord to show a similar pattern of geographic differentiation among disjunct populations of *A. crispa*. An intriguing feature of this differentiation is that while the two coastal regions where this taxon occurs on Corsica show consistent geographic differentiation, both for floral traits and isozymes, each of these two regions has more similarities to populations of subspecies *crispa* on Sardinia than they do to one other. The construction of a phenogram based on differences in floral traits among populations (Fig. 4) and comparisons of isozyme frequency at different loci (Fig. 5) illustrate these patterns. Two potential causes can be invoked to explain the similarities between Sardinian populations and one or other of the geographic entities on Corsica.

First, populations in the two coastal regions on Corsica may have a very long history of isolation. This could explain the high levels of geographic differentiation among regions and the low levels of variation among populations within regions. Such differentiation would be promoted in this taxon due to its capacity to self-pollinate in the absence of pollinators – flowers set as many seed per flower on autonomous selfing when pollinators are excluded as they do following manual cross-pollination (Quilichini et al., 2001). This capacity for autonomous self-pollination is no doubt promoted by the proximity of stigmas and anthers within individual flowers. The lack of inbreeding depression in this species (Quilichini et al., 2001) will further allow inbreeding to enhance genetic drift and thus the genetic divergence of isolated populations. Seeds of this species can float for several hours and some are still capable of germinating after a week's submersion in sea water (Quilichini and Debussche, 2000) hence dissemination among populations around single bays may occur – on the west coast the different populations all occur around a single large estuary. Such seed dispersal among populations at different sites in a single estuary may limit differentiation among local populations.

Second, populations on Corsica may be derived from independent colonisation events on each coast. A genetic bottleneck during each colonisation event, and subsequent rapid genetic drift in a highly selfing species such as this would facilitate this differentiation between coastal regions. This scenario would also cause the different populations within each region to be genetically similar as they are likely to have resulted from secondary dispersal from an initial site of colonisation in the same region. This interpretation does not require that the populations on each coast are particularly ancient and concurs with the finding that each coastal region contains floral traits and isozyme frequencies that are more similar to a (different) population on Sardinia than they are between the east and west of Corsica.

The lack of any genetic diversity within populations should be treated with some caution. Although allozyme electrophoresis provides a means of estimating levels of intra- and interpopulation genetic variation as well as the amount of genetic differentiation between populations, isozymes show lower variation than more powerful DNA-based markers (reviewed in Parker et al., 1998). Our results with isozymes thus need to be confirmed by the use of highly variable DNA-markers such as AFLPs, microsatellites or simple sequence repeats (SSRs). For example, Gitzendanner and Soltis (2001) used new molecular markers (AFLPs and single-strand conformation polymorphisms (SSCPs)) to show how species with small populations show less genetic variation than their widespread congeners.

The striking geographic differentiation of isozymes on Corsica has nonetheless important ramifications for the development of conservation programmes for *A. crispa* – a protected species in France. Fitness comparisons over two generations of controlled self- and outcross-pollination on plants from a single population on the west coast of Corsica have shown the existence of outbreeding depression (a reduced fitness of progeny produced by outcrossing relative to those from selfing) on a very local scale, i.e. between patches within a “population” (Quilichini et al., 2001). This phenomenon may arise because of sharp and highly localised adaptation or due to the co-adaptation of different genes, which become disrupted when outcrossing occurs in a highly inbred lineage. Given the high levels of geographic differentiation between east and west coast populations, it will now be most important to ascertain the interfertility of east and west coast populations of *A. crispa*. Such work would contribute to better understand the degree of genetic differentiation among geographic regions and provide important data for conservation and reinforcement programmes in this species.

The patterns of geographic differentiation we present illustrate the importance of conserving populations in both coastal regions on Corsica. The two populations on the east coast are currently subject to intense human

activity associated with the passage of four by four trucks, sand collection and other beach activities. In situ conservation to reduce these impacts is a necessary first step. As our paper highlights, the preservation of the evolutionary potential of this species, in terms of a representative sample of different geographic entities conserved in the wild are clearly necessary and this includes samples from the small and highly threatened east coast populations. In addition, ex situ conservation will require the constitution of distinct seed accessions based on achenes sampled in each region. Any future reintroduction programme should carefully use different seed sources in relation to the sites where potential introductions are to occur.

#### 4.2. Variation in floral morphology among subspecies on Sardinia

Our results further illustrate the distinctness of *A. crispa* subsp. *crispa* relative to subsp. *maritima* on Sardinia, an important issue for the conservation of taxa whose genetic delimitation is questionable (e.g. Olfelt et al., 2001). In *A. crispa* the two subspecies do not show variation at isozyme loci to the same extent as shown by east and west coast populations on Corsica. The similarity of isozyme profiles for the two subspecies on Sardinia may result from a shared evolutionary history and/or recent hybridisation between them, a distinct possibility given the proximity of populations of the two taxa (Fig. 1). If it is true that populations of *A. crispa* subsp. *crispa* on Sardinia have been subject to genetic introgression with *A. crispa* subsp. *maritima* then sampled seeds from these populations should be kept distinct from populations of this taxon from its protected range in France.

In contrast to isozymes, floral traits of *A. crispa* subsp. *crispa* show a clear distinction from *A. crispa* subsp. *maritima* on Sardinia. *A. crispa* subsp. *crispa* populations are composed of homostylous flowers with stigmas and anthers at roughly the same height in the flower. In contrast, in subspecies *maritima*, flowers display approach-herkogamy, i.e. the stigma is positioned above the anthers. Such differences in floral morphology may be associated with different amounts of outcrossing, since the approach-herkogamy in subsp. *maritima* may allow for greater rates of outcrossing (e.g. Belaousoff and Shore, 1995). The latter taxon may thus maintain higher levels of genetic variation within its populations than the protected taxon on Corsica, an issue worth future investigation.

The floral morphology of both subspecies also differs from that in more widespread species in the genus which appear to show polymorphic variation in style length and a well-developed self-incompatibility system (Dulberger, 1970; Philipp and Schou, 1981; Selvi et al., 1996;

Selvi, 1998). The phylogenetic position of *A. crispa* in terms of its highly selfing mating system would merit thus future attention. The existing variation suggests that *A. crispa* may be a recently evolved selfing taxon whose ancestral taxa were probably more outcrossed.

Finally, from a conservation point of view, our results show that it is important to consider taxonomic rank, even for a well-described species as *A. crispa*, and underline the problem of endangered species with uncertain taxonomic ranks. Since many imperfectly described species requiring conservation effort exist in the world, botanists and conservationists should collaborate to resolve biodiversity-related problems as recently proposed by Lowry and Smith (2003) and Golding and Timberlake (2003). For example, the Convention on International Trade in Endangered Species does not take subspecies into account in its lists. In France, Olivier et al. (1995) have pointed out the taxonomic limits and the choice of strict nomenclatural references accepted at an international level for threatened taxa, so that French Red Data book contains several subspecies. However, it is still difficult to estimate the rank of a studied organism as floras are not frequently updated (see examples in Molina et al., 1999). Our study shows that there are clear-cut differences between subspecies, which merit full consideration in the elaboration of conservation programmes which aim to conserve a representative sample of genetic diversity in *A. crispa*.

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