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Relationship of *Hypochaeris salzmanniana* (Asteraceae, Lactuceae), an endangered species of the Iberian Peninsula, to *H. radicata* and *H. glabra* and biogeographical implications

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Hypochaeris salzmanniana DC. (Asteraceae, Lactuceae) is an endangered species on the Iberian Peninsula, known from only eight coastal populations. Most authors have treated it as a variety, subspecies or simply as a synonym of *H. glabra* L. On the basis of morphological and cytological characters, Talavera recently separated *H. salzmanniana* ($2n = 8$) from *H. glabra* ($2n = 10$). Material of *H. salzmanniana*, *H. glabra* and *H. radicata* was collected from Spain, Italy, Sicily and Tunisia in order to assess taxonomic status and population relationships. Amplified Fragment Length Polymorphism (AFLP) analysis revealed three well-differentiated species. A close relationship between *H. salzmanniana* and *H. radicata* is also confirmed by AFLP analysis and chromosome number ($2n = 8$), morphology, and rDNA localization (FISH, fluorescence *in situ* hybridization). *Hypochaeris salzmanniana* and *H. radicata* share three fixed diagnostic AFLP fragments out of 348 fragments scored. The population structure of *H. salzmanniana* reveals distinct groups in southern Spain that are separated geographically. High differentiation among a western (Conil to Zahara), an intermediate (Punta Paloma and Los Algarbes) and an eastern (Algeciras and La Línea) group may reflect ancient separation. Population sizes and genetic compatibility differ greatly among populations and can be used to explain levels of within-population genetic diversity, together with recent documented loss of habitats resulting from tourist developments. Population structures of *H. radicata* and *H. glabra* show a similar geographical patterning: strongly differentiated populations from the Betic Cordillera and from the Iberian Massif, which are separated at present by the Guadalquivir river. Geological events at the end of the Tertiary (Tortonian–Messinian Miocene) might help explain patterns of differentiation in these three species of sect. *Hypochaeris*.

ADDITIONAL KEYWORDS: AFLP – conservation – genetic variation – Spain.

INTRODUCTION

Hypochaeris salzmanniana DC. was described as a distinct species by A. P. de Candolle (1838: 91). Subsequent authors working on the flora of the western Mediterranean have included *H. salzmanniana* with *H. glabra* L. as a variety (Amo, 1872: 493; Willkomm, 1893: 112), subspecies (Jahandiez & Maire, 1934: 831; Emberger & Maire, 1941: 1164), or simply as a mere synonym of *H. glabra* (De Filipps, 1976). Only recently, Talavera (1980, 1987) resurrected *H. salzmanniana* out of *H. glabra* not only as a good taxon based on morphological and karyological characters, but also for the first time as a species related to *H. radicata* L. rather than *H. glabra*. Förther & Podlech (2003) recently characterized populations from south-west Spain and north-west Morocco (coastal areas) as ssp. *salzmanniana*, being differentiated by conspicuously inflated peduncles

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from ssp. *maroccana* Förther & Podlech in mountains of Morocco and Algeria to 1700 m a.s.l. [= *H. arachnoidea sensu* Oberprieler, 2002, and Oberprieler & Vogt, 2002)].

Morphological, cytological, compatibility and ecological differences exist among *H. salzmanniana*, *H. radicata* and *H. glabra*. *Hypochaeris salzmanniana* has hirsute, rarely glabrous, peduncles, strongly inflated under the capitula during anthesis, and ovate outer involucral bracts with wide scarious margins and setose hairs at the end of the central nerve, and with ligules conspicuously exserted from the involucre (Talavera, 1987). *Hypochaeris glabra* has glabrous, cylindrical peduncles, lanceolate and glabrous involucral bracts, rarely with a few setose hairs, and with ligules barely exserted from the involucre (Talavera, 1987). Field studies in 2002 by Talavera and Stuessy *et al*. have provided new morphological data to suggest recognition of *H. salzmanniana* as a good species. In comparison to its presumptive closest relative, *H. radicata*, the leaves of the former are more undulating at the margin, the leaf rosettes are usually very full, the involucral bracts often have long dark hairs, and the branches are ascending (Fig. 1A). *Hypochaeris glabra* and *H. salzmanniana* are annuals, whereas *H. radicata* is perennial. *Hypochaeris glabra* has $2n = 10$ chromosomes (Adame & Talavera, 1980) whereas *H. salzmanniana* has $2n = 8$ (Talavera, 1981) as does *H. radicata* (Adame & Talavera, 1980). *Hypochaeris radicata* is normally self-incompatible, *H. glabra* self-compatible, and *H. salzmanniana* shows variation in its compatibility system with plants being self-compatible or self-incompatible (M. Á. Ortiz & S. Talavera, unpubl. data).

Hypochaeris glabra and *H. radicata* are widespread in the Mediterranean region and are worldwide weeds. *Hypochaeris glabra* seldom occurs in the same localities as *H. salzmanniana* (e.g. in Barbate and Punta Paloma, see Table 1), and *H. salzmanniana* does not occur with *H. radicata*. *Hypochaeris salzmanniana* is restricted to coastal areas on both sides of the Strait of Gibraltar. The species is reported from Morocco between Tanger and Rabat (Oberprieler, 2002; Förther & Podlech, 2003). In Spain, only eight populations are known along the coast of Cádiz, from Conil to La Línea (Fig. 2; Table 1). Six of these survive among beach developments: the western populations (Conil to Zahara, pops 1–4) and the population of Algeciras (pop. 7), which all live in sand dunes near the coast, and the threatened population of La Línea (pop. 8), which lives at the base of the Rock of Gibraltar. The only populations undisturbed by the tourism industry (Los Algarbes and Punta Paloma, pops 5 and 6) reside in the ecotone constituted by Quaternary dunes with flysch materials on the rocky spur of the Sierra de San Bartolomé (1 km inland).

The reported pattern and size of populations of *H. salzmanniana* raises questions regarding their origins and biogeographical relationships. Because the populations occur in a linear fashion, it would be of interest to test whether or not genetic relationships among these eight populations conform to this linear

Figure 1. *Hypochaeris salzmanniana* (A; *Talavera, Stuessy et al. 5*) and a typical recent dune habitat along the southern Spanish coast (B; Conil de la Frontera: Playa El Palmar).

Table 1. Localities, collectors and number of individuals of *Hypochaeris salzmanniana*, *H. radicata* and *H. glabra* sampled for the AFLP study

Taxa and localities	Collectors and number	No. individuals analysed
H. salzmanniana DC.		
1 Spain, Cádiz, Conil de la Frontera: Playa El Palmar	*Talavera, Stuessy et al. 5-1 to 5-4*	38
2 Spain, Cádiz, Los Caños de Meca	*Talavera, Stuessy et al. 24*	5
3 Spain, Cádiz, Barbate de Franco	*Talavera, Stuessy et al. 14*	5
4 Spain, Cádiz, Zahara de los Atunes	*Talavera & Ortiz 1/03*	5
5 Spain, Cádiz, Punta Paloma: Los Algarbes	*Talavera & Ortiz 2/03*	5
6 Spain, Cádiz, Punta Paloma	*Talavera, Stuessy et al. 32; Talavera & Ortiz 3/03*	19
7 Spain, Cádiz, Algeciras: Palmones	*Talavera, Stuessy et al. 33*	5
8 Spain, Cádiz, La Línea de la Concepción	*Talavera, Stuessy et al. 35*	5
H. radicata L.		
9 Brazil, São Paulo, São Paulo	*Talavera et al. BRA 30*	1
10 Colombia, Bogotá, Bogotá	*Stuessy 31*	2
11 Germany, Nordrhein-Westfalen, Bochum	*Stuessy s.n.*	1
12 Italy, Fòggia, Gargano	*Tremetsberger s.n.*	10
13 Italy, Règgio di Calàbria, Aspromonte	*Tremetsberger s.n.*	6
14 Italy, Règgio di Calàbria, Barriteri	*Tremetsberger s.n.*	2
15 Italy, Règgio di Calàbria, Palmi	*Tremetsberger s.n.*	7
16 Italy, Sicily, Nebrodi	*F. & L. Ehrendorfer 5FE*	4
17 Italy, Sicily, Palermo	*Royal Botanical Garden Madrid 646283* (seeds)	10
18 Mexico, México, Valle de Toluca	*Guevara s.n.*	1
19 Spain, Burgos, Quintanar de la Sierra	*Ortiz s.n.*	1
20 Spain, Cádiz, near Vejer de la Frontera	*Talavera, Stuessy et al. 25*; *Talavera et al. s.n.*	6
21 Spain, Huelva, Aracena	*Talavera et al. s.n.*	5
22 Spain, Huelva, National Park Doñana	*Talavera et al. s.n.*	8
23 Spain, Huelva, Santa Ana la Real	*Talavera, Stuessy et al. 48*	5
24 Spain, Huelva, Valverde del Camino	*Talavera, Stuessy et al. 46*	5
25 Spain, Málaga, Gaucín	*Talavera, Stuessy et al. 40*	3
26 South Africa, Western Cape, near Ceres	*F. & L. Ehrendorfer 83*	1
27 Tunisia, Jendouba, near Aïn Draham	*F. & L. Ehrendorfer s.n.*	9
H. glabra L.		
28 Chile, Región VIII, Ñuble	*Baeza 3924*	3
29 Spain, Cádiz, Barbate de Franco	*Talavera, Stuessy et al. 15*	2
30 Spain, Cádiz, Punta Paloma	*Talavera, Stuessy et al. 31*	2
31 Spain, Canary Islands, Gran Canaria	*Stuessy s.n.*	2
32 Spain, Huelva, Hinojos	*Talavera, Stuessy et al. 43*	2
33 Spain, Huelva, National Park Doñana	*Talavera et al. s.n.*	2
34 Spain, Huelva, Valverde del Camino	*Talavera, Stuessy et al. 45*	2

model. That is, did the populations originate in one location and then spread serially along the coast, or is a more complex explanation required? An attractive hypothesis is that those populations growing on fossil dunes may represent relict populations, perhaps now isolated genetically from the others.

In order to assess relationships among *H. salzmanniana* and its relatives, *H. glabra* and *H. radicata*, and to determine infraspecific genetic variation within the three species, we used the DNA fingerprinting technique Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995). AFLPs have already been shown efficacious at the population as well as interspecific level for revealing genetic relationships in *Hypochaeris* and other plant groups (e.g. Pfosser *et al*., 2002; Schönswetter *et al*., 2003; Stuessy *et al*., 2003; Tremetsberger *et al*., 2003a). Karyotypic analysis of *H. salzmanniana*, *H. radicata* and *H. glabra* is also provided as a comparison with the AFLP data. The purposes of this paper therefore are

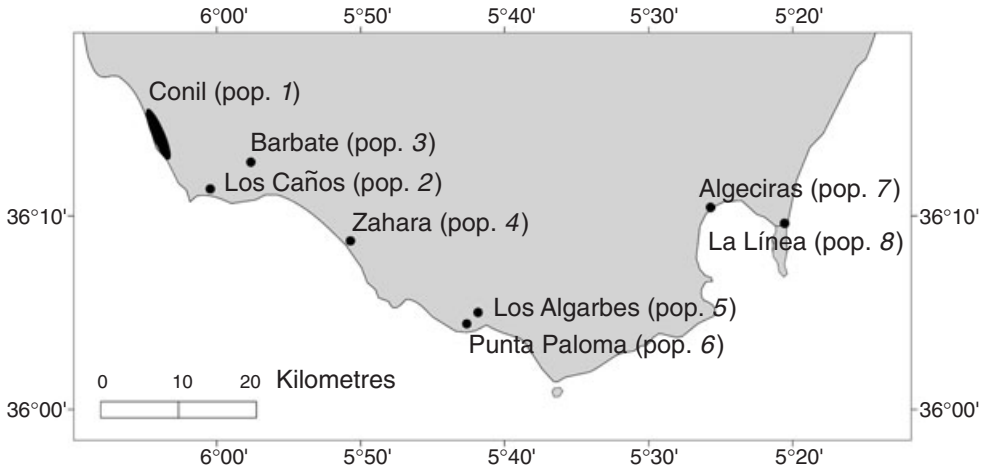

Figure 2. Distribution of *Hypochaeris salzmanniana* in southern Spain, showing all known populations (all sampled; map by ARCVIEW GIS v.3.2; Environmental Systems Research Institute, Inc.).

to: (i) test the specific status of *H. salzmanniana*; (ii) assess its relationships to *H. radicata* and *H. glabra*; (iii) determine infraspecific genetic structures within the three species; and (iv) interpret the observed patterns in the context of biogeographical history and conservation perspectives.

MATERIAL AND METHODS

Sampling

Leaves were sampled in silica gel from individuals in all known populations of *Hypochaeris salzmanniana* from the Iberian Peninsula (Fig. 2, Table 1). Five individuals were analysed in Los Caños, Barbate, Zahara, Los Algarbes, Algeciras, and La Línea (pops 2, 3, 4, 5, 7, and 8). Nineteen individuals were analysed in Punta Paloma (pop. 6). This population exhibited very low genetic variation after analysis of ten individuals sampled in 2002 (*Talavera, Stuessy et al. 32*; inds a to j). Nine additional individuals were analysed after the population had been resampled in 2003 (*Talavera & Ortiz 3/03*; inds k to s). In the large population Playa El Palmar (pop. 1), 38 individuals were analysed from four linear transects from sea to road across the dune, each approximately 1 km apart, so that genetic variation could be related to distance from the ocean. Along these transects each second square-metre quadrant was numbered sequentially from sea to road and one to two individuals, if present, were sampled in quadrants next to the ocean (low numbers: 1–3 in transects 1, 3 and 4; 4–9 in transect 2) and in quadrants far from the ocean (high numbers: 11–14 in transects 1, 2 and 4; 17–19 in transect 3). A total of 87 *H. salzmanniana* plants was investigated.

Populations of *H. radicata* and *H. glabra* were similarly sampled (Table 1), with emphasis on populations of the former taxon from throughout its native range in the Mediterranean region (Spain, Italy, Sicily and Tunisia). Furthermore, a few samples were collected from introduced accessions throughout the world. Fewer populations of *H. glabra* were compared because morphological and karyological data indicated a more distant relationship to *H. salzmanniana*, despite the earlier taxonomic opinions of some authors that they were close relatives (see Introduction). These samples are from Spain, where *H. glabra* is native, the Canary Islands and Chile, where it is introduced and invasive. A total of 87 plants was investigated in *H. radicata* and 15 plants in *H. glabra*. Vouchers of all populations sampled are on deposit at SEV and WU.

Karyotypes

The karyotypes of *H. salzmanniana* [Spain, Cádiz, Playa El Palmar (pop. 1); see Table 1], *H. radicata* (Chile, Région VIII, Lota, *Stuessy et al. 15477*; WU), and *H. glabra* [Spain, Huelva, Valverde del Camino (pop. 34); see Table 1] were determined. Surface-sterilized seeds were germinated on wet filter paper on Petri dishes. Two days after germination, seedlings were pretreated with 0.1% colchicine for 2 h at room temperature and 2 h at 4°C, fixed in 3:1 ethanol/acetic acid for 12 h at room temperature, and stored at –20°C until use. Feulgen staining with Schiff's reagent was carried out according to standard protocol (Weiss *et al.*, 2003). From each species, a preparation with at least 15 well-spread chromosome plates was chosen for analysis.

Chromosomes for fluorescence *in situ* hybridization (FISH) were prepared by enzymatic digestion/squashing as described by Weiss-Schneeweiss *et al.* (2003).

The quality of spreads was checked by phase-contrast microscopy and only those preparations with adequate numbers of well-spread metaphases (10–15) were selected for FISH. Slides were frozen at –80°C, and after cover slip removal, preparations were stored at –20°C. FISH was carried out according to the method of Weiss-Schneeweiss *et al*. (2003). Probes used for FISH were: 18S-25S rDNA from *Arabidopsis thaliana* in plasmid pSK+, labelled with digoxygenin (Roche), and 5S rDNA from *Beta vulgaris* in plasmid pBx1–2, labelled with biotin (Roche). Both probes were labelled by nick translation according to the manufacturer's instruction (Roche). Hybridization was carried out overnight. Stringent washes were performed as in Weiss-Schneeweiss *et al*. (2003). For digoxygenin/biotin detection antidig-FITC solution (Roche)/extravidin Cy3 (Sigma) was applied to the slides at 37°C for 1 h. The preparations were mounted in antifade buffer Vectashield (Vector Laboratories, UK) containing DAPI counterstain (2 μg/mL) and stored at 4°C. Analyses of preparations were made with a ZEISS Axioscope epifluorescence microscope. Images were acquired with a CCD camera (Zeiss), and files were processed using ADOBE PHOTOSHOP software with only those functions that applied equally to all pixels in the image. For rDNA localization, a minimum of 30 well-spread metaphases and prometaphases was analysed for each species.

AFLPs

Total DNA was extracted from dry leaf material according to a modified CTAB-protocol and quality-checked on 1% TAE-agarose gels as in Tremetsberger *et al*. (2003b). The AFLP procedure followed established protocols (Vos *et al*., 1995; PE Applied Biosystems, 1996) with modifications. Restriction-ligation reactions were carried out as in Tremetsberger *et al*. (2003b). The reaction mix contained 1.1 μL 10 × T4 DNA ligase buffer (Promega), 1.1 μL 0.5 M NaCl, 0.55 μL 1 mg/mL BSA (New England Biolabs), 1 μL 50 μM *Mse*I-adaptor, 1 μL 5 μM *Eco*RI-adaptor, 1 U *Mse*I (New England Biolabs), 5 U *Eco*RI (Promega), 1 U T4 DNA ligase (Promega), and *c*. 0.5 μg template DNA, made up with water to a final volume of 11 μL. Ligated DNA fragments were diluted and preselective and selective amplifications performed as in Tremetsberger *et al*. (2003b). The reaction mix for the preselective amplification contained 1.14 μL 10 × RedTaq polymerase buffer (Sigma), 0.2 U RedTaq polymerase (Sigma), 0.22 μL 10 mM dNTPs (Fermentas), 0.58 μL preselective primers (4.8 pmol/μL each of *Mse*I and *Eco*RI preselective primers), and 2 μL diluted product of restriction/ligation, made up with water to a final volume of 10 μL. As selective primers with three and four selective nucleotides were chosen (see below), separate PCRs with one and two selective nucleotides in the *Mse*I primer (*Mse*I-C and -CT; *Eco*RI-A) were performed. The preselective PCR products were checked on a 1.5% TBE-agarose gel and diluted ten-fold with $TE_{0.1}$ buffer. An initial screening of selective primers using 39 primer combinations was performed on five individuals from three populations of *H. radicata* and *H. salzmanniana*. A second screening used 15 primer combinations with clear bands on 13 individuals from eight populations of *H. radicata* and *H. salzmanniana*. From this, we have chosen six selective primer combinations with clear bands evenly distributed over the AFLP profiles for an application to all individuals investigated. These are: *Mse*I-CTCG/*Eco*RI-ATC(Fam), *Mse*I-CAC/*Eco*RI-ACG(Hex), *Mse*I-CTA/*Eco*RI-ACC(Ned), *Mse*I-CTG/*Eco*RI-ACA(Fam), *Mse*I-CTC/*Eco*RI-AGG(Hex), and *Mse*I-CTGA/*Eco*RI-AAC(Ned). The reaction mix for the selective amplification contained 1 μL 10 × RedTaq polymerase buffer, 0.2 U RedTaq polymerase, 0.22 μL 10 mM dNTPs, 0.54 μL of each selective primer (*Mse*I-primer: 5 pmol/μL; *Eco*RI-primer: 1 pmol/μL), and 2 μL diluted product of the preselective amplification, made up to a final volume of 10 μL with water. The fluorescence-labelled selective amplification products were run on a 5% denaturing polyacrylamide gel on an automated sequencer (ABI 377, Perkin Elmer). Before running, 0.8 μL NED- and HEX-labelled, and 0.4 μL FAM-labelled selective amplification products were mixed with 1.2 μL loading dye [containing 64.8 μL deionized formamide, 25.2 μL loading buffer, and 10 μL GeneScan-500 (ROX) size standard], and denatured at 95°C for 2 min. Raw data were scored and exported as a presence/absence matrix as in Tremetsberger *et al*. (2003b) using ABI Prism GENESCAN Analysis Software v.2.1 (PE Applied Biosystems) and GENOGRAPHER (v.1.1.0; Montana State University, 1998, see http://hordeum.msu.montana.edu/genographer/).

Data analysis

To test correspondence of data gathered by each of six primer combinations, Jaccard (1908) similarity matrices on all 189 individuals analysed were constructed for each primer combination separately using R PACKAGE (v.4.0; Casgrain & Legendre, 2000). The similarity matrices were used to compute pairwise correlations among them by a Mantel test (standardized Mantel statistic *r*; R PACKAGE v.4.0; Casgrain & Legendre, 2000). One-tailed test statistic probabilities were obtained through 999 permutations (R PACKAGE v.4.0; Casgrain & Legendre, 2000).

The Jaccard (1908) distance matrix of all primer combinations combined (R PACKAGE; Casgrain & Legendre, 2000) was subsequently imported into PAUP* (v.4.0b8; Swofford, 1998) and used to construct

a UPGMA dendrogram for all individuals analysed, and for individuals of each species separately. A cophenetic correlation coefficient, which measures the goodness-of-fit of a model to the data, was calculated with NTSYSpc (v.2.2h; Applied Biostatistics Inc.). Support for each node was tested by 1000 bootstrap replicates with PAUP* (v.4.0b8; Swofford, 1998) using the UPGMA method in conjunction with Nei & Li's (1979) genetic distances on the original presence/absence matrix, since PAUP* does not feature the Jaccard index. The Nei & Li (1979) distance and neighbour-joining algorithms were also applied to the data and resulted in very similar dendrograms (not shown).

For each species, the total number of fragments, the number of fixed fragments (i.e. those occurring in all individuals analysed of a species), the diagnostic fragments (i.e. those occurring in only one species), and the fixed diagnostic fragments (i.e. those occurring in all individuals analysed of only one species) were assessed.

A Mantel test was used to correlate geographical with genetic distances in *H. salzmanniana*. A Jaccard distance matrix among all pairwise combinations of individuals was computed using the R PACKAGE (v.4.0; Casgrain & Legendre, 2000) and compared to geographical distances between individuals (in km; distances within populations set to 0). The standardized Mantel statistic r is the linear correlation between genetic and geographical distances and was tested for significance with 999 permutations of rows and columns in one (the genetic or geographical) distance matrix to obtain the distribution under the null hypothesis of no correlation.

The percentages of different AFLP phenotypes and of polymorphic fragments were assessed as measures of within-population genetic diversity for each population of *H. salzmanniana* (ARLEQUIN v.2.0; Schneider, Roessli & Excoffier, 2000). The Shannon diversity index was calculated from five randomly chosen individuals in a population as $H_{Sh} = -\Sigma[p_i{*}\ln(p_i)]$, where p_i is the relative frequency of the i^{th} fragment in a population, and correlated with population size (estimated number of individuals) and self-incompatibility (percentage of self-incompatible plants; Ortiz & Talavera, unpubl. data) using a Pearson correlation (1-tailed significance) in SPSS (v.10; SPSS Inc.).

The effects of geographical vicinity within a transect and proximity or distance to the ocean have been tested as factors influencing the partitioning of genetic variation in the large population Playa El Palmar (pop. 1). ARLEQUIN (v.2.0; Schneider *et al.*, 2000) was used to carry out an Analysis of Molecular Variance (AMOVA; Excoffier, Smouse & Quattro, 1992) with 1023 permutations among three hierarchical levels: (i) among transects, (ii) among quadrants next to the ocean and far from the ocean in each transect, and (iii) among individuals within each of the two groups of nearby quadrants (next to the ocean and far from the ocean) in each transect. Furthermore, the differentiation between the four main groups of *H. salzmanniana*, determined by UPGMA analysis, was quantified through pairwise F_{ST} values and tested for significance by 1023 permutations of haplotypes (distance method: pairwise difference; ARLEQUIN v.2.0; Schneider *et al.*, 2000).

RESULTS

A total of 348 unambiguously scoreable AFLP fragments was obtained from analysis of six primer combinations on 189 individuals of *Hypochaeris glabra*, *H. radicata* and *H. salzmanniana*. The primer combinations *Mse*I-CAC/*Eco*RI-ACG(Hex) yielded 71 fragments, *Mse*I-CTCG/*Eco*RI-ATC(Fam) 68, *Mse*I-CTA/*Eco*RI-ACC(Ned) 62, *Mse*I-CTG/*Eco*RI-ACA(Fam) 57, *Mse*I-CTC/*Eco*RI-AGG(Hex) 47, and *Mse*I-CTGA/*Eco*RI-AAC(Ned) 43. A correlation test performed on each pairwise combination of six Jaccard similarity matrices obtained from analysis of each primer combination separately yielded generally high values (Table 2). The lowest value for Mantel's r is 0.859 among *Mse*I-CTG/*Eco*RI-ACA(Fam) and *Mse*I-CTGA/*Eco*RI-AAC(Ned), and the highest is 0.931 among *Mse*I-CAC/*Eco*RI-ACG(Hex) and *Mse*I-CTA/*Eco*RI-ACC(Ned). All values are significant (one-tailed significance = 0.001) after 999 permutations.

DIFFERENTIATION OF *H. GLABRA*, *H. RADICATA* AND *H. SALZMANNIANA*

Comparisons among populations of *H. glabra*, *H. radicata* and *H. salzmanniana* using AFLP show a clear distinction among the three species (Fig. 3; cophenetic correlation = 0.934). Each species consists of a highly supported (100% BS) cluster of populations. *Hypochaeris radicata* and *H. salzmanniana* are more similar to one another (level of divergence 0.327; Fig. 3) than to *H. glabra*, which branches off at a level of divergence of 0.351. However, a strong relationship between *H. radicata* and *H. salzmanniana* is not highly supported (51% BS).

The total numbers of AFLP fragments are 142 in *H. salzmanniana* and 269 in *H. radicata* (accessions from Spain, Italy, Tunisia, and from worldwide introduced accessions). For a direct comparison it seems more appropriate to consider the same geographical area in both species. The Spanish populations of *H. radicata* alone (a total of 33 individuals) have 210 fragments, which is still considerably more than in *H. salzmanniana*. *Hypochaeris glabra* has 124 fragments, although this value might be underestimated, because only 15 individuals were investigated. The

Table 2. Mantel's *r* showing pairwise correlation among six Jaccard similarity matrices obtained from each of six primer combinations used (a total of 348 fragments and 189 individuals). One-tailed significance of all values is 0.001 after 999 permutations (R PACKAGE; Casgrain & Legendre, 2000)

	*Mse*I-CTCG/ *Eco*RI-ATC(Fam)	*Mse*I-CAC/ *Eco*RI-ACG(Hex)	*Mse*I-CTA/ *Eco*RI-ACC(Ned)	*Mse*I-CTG/ *Eco*RI-ACA(Fam)	*Mse*I-CTC/ *Eco*RI-AGG(Hex)
*Mse*I-CAC/ *Eco*RI-ACG(Hex)	0.888				
*Mse*I-CTA/ *Eco*RI-ACC(Ned)	0.889	0.931			
*Mse*I-CTG/ *Eco*RI-ACA(Fam)	0.880	0.871	0.890		
*Mse*I-CTC/ *Eco*RI-AGG(Hex)	0.881	0.886	0.897	0.868	
*Mse*I-CTGA/ *Eco*RI-AAC(Ned)	0.874	0.902	0.927	0.859	0.891

number of fixed fragments is 30 in *Hypochaeris radicata* (41 when only Spanish populations are considered), 63 in *H. salzmanniana*, and 78 in *H. glabra*. Diagnostic fragments are important for species determination, especially when they are fixed. The number of nonfixed diagnostic fragments is 133 in *Hypochaeris radicata*, 26 in *H. salzmanniana*, and 23 in *H. glabra* (Fig. 4A). The number of fixed diagnostic fragments is one in *Hypochaeris radicata*, nine in *H. salzmanniana*, and 17 in *H. glabra* (Fig. 4B).

Shared diagnostic fragments (i.e. those occurring in two of the three species) are important for assessing evolutionary relationships among species. *Hypochaeris radicata* and *H. salzmanniana* share 52 nonfixed diagnostic fragments; *H. radicata* and *H. glabra* 30; and *H. salzmanniana* and *H. glabra* four (Fig. 4A). When shared diagnostic fragments are fixed in all individuals of the two species, the closer relationship of these two species becomes emphasized. *Hypochaeris radicata* and *H. salzmanniana* share three fixed diagnostic fragments; *H. radicata* and *H. glabra* two; and *H. salzmanniana* and *H. glabra* none (Fig. 4B).

Cytological comparisons between *H. radicata* and *H. salzmanniana* (both $2n = 2x = 8$) also reveal their similarity and a clear distinction from *H. glabra* ($2n = 2x = 10$) (Fig. 5). Karyotypically, as analysed by Feulgen-staining and FISH with 5S and 18S–25S rDNA, the former two species are indistinguishable. Their karyotypes have similar length and are symmetrical with metacentric and submetacentric chromosomes (Fig. 5C, E). Analyses of rDNA (5S and 18S–25S rRNA genes) have shown the same number and localization in karyotypes of *H. radicata* and *H. salzmanniana* (Fig. 5D, F). The karyotype of *H. glabra* is also symmetrical, but differs in chromosome number ($2n = 10$ vs. $2n = 8$; Fig. 5A). The number of 5S and 18S–25S rDNA loci is the same as in the other two species, but their localization is different (Fig. 5B, D, F). In *H. glabra*, 5S rDNA is localized distally on the short arm of chromosome 4 (Fig. 5B), whereas in the other two species it is localized in the proximal part of the long arm of chromosome 2 (Fig. 5D, F). The 18S–25S rDNA locus occurs distally on the short arm of chromosome 2 in *H. glabra* (Fig. 5B), and on the short arm of chromosome 3 in the other two species (Fig. 5D, F).

Infraspecific variation within *H. salzmanniana*

The AFLP data also reveal similarities and distances among individuals of *H. salzmanniana* in southern Spain (Fig. 6). Four main clusters were found. The first cluster includes the geographically neighbouring populations Conil, Los Caños and Barbate (pops 1–3; with the exception of individual 2c clustering apart). The individual plants from the three locations are completely intermingled in the UPGMA dendrogram, suggesting a large panmictic group. The second cluster consists of population Zahara (pop. 4) and is most similar to the first cluster (pops 1–3). The third cluster comprises the geographically closely neighbouring populations Los Algarbes and Punta Paloma (pops 5 and 6). In fact, two collections in Punta Paloma (pop. 6) in two subsequent years (2002: *Talavera, Stuessy et al. 32*, inds a to j; 2003: *Talavera & Ortiz 3/03*, inds k to s) are separated by pop. 5 with the exception of ind. 6s collected in 2003 clustering with the 2002 collection. The fourth cluster comprises populations Algeciras and La Línea (pops 7 and 8), which grow in geographical proximity on the eastern side of the Strait of Gibraltar. The highest differentiation is between pops (5, 6) and (4) ($F_{ST} = 0.662$) and pops (5,6) and (7,8) ($F_{ST} = 0.644$; Table 3). The lowest differentiation is between pops (1–3) and (4) ($F_{ST} = 0.318$) and

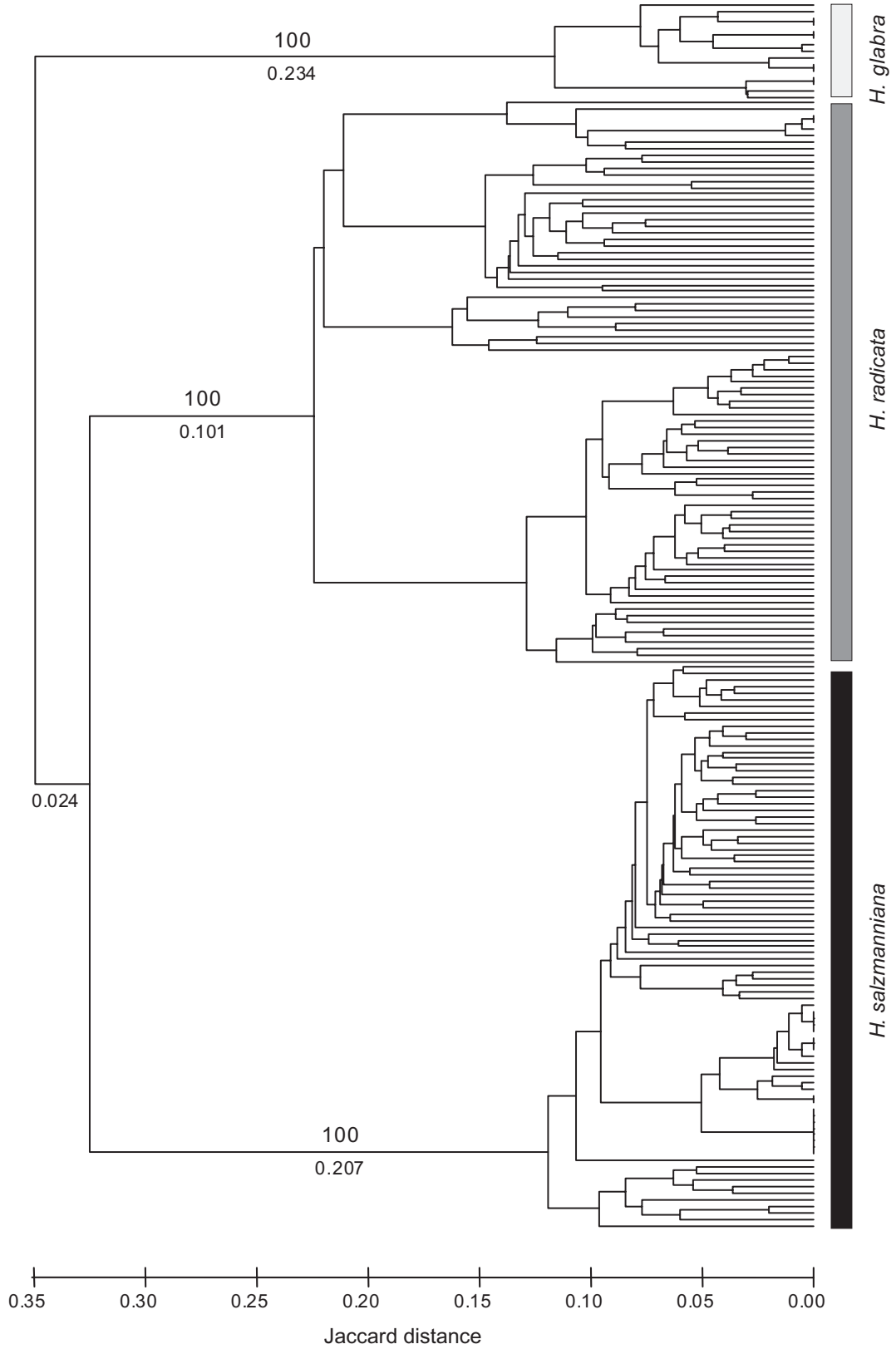

Figure 3. UPGMA dendrogram of Jaccard distances (based on 348 AFLP fragments) showing general relationships among individuals of *Hypochaeris salzmanniana*, *H. radicata*, and *H. glabra*. Cophenetic correlation coefficient 0.934. Bootstrap values for the three species (1000 replicates) above branches; Jaccard distances below branches.

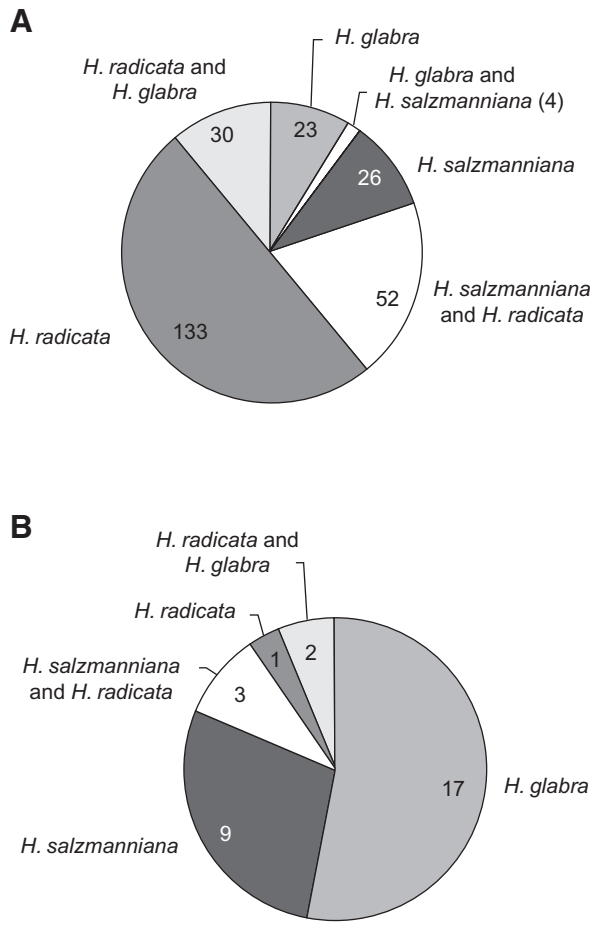

Figure 4. Pie diagrams of nonfixed (A) and fixed (B) diagnostic AFLP fragments (i.e. fragments occurring in only one or in only two species of *Hypochaeris salzmanniana*, *H. glabra*, and/or *H. radicata*).

between pops (1–3) and (7, 8) (F_{ST} = 0.364). The Mantel test revealed an overall positive, albeit low, correlation between geographical distances in km and genetic distances measured by the Jaccard index among all individuals investigated by Mantel's r = 0.625 (one-tailed significance = 0.001 after 999 permutations; R PACKAGE, Casgrain & Legendre, 2000).

Levels of within-population genetic variation vary among populations (Table 4). The large population Conil (pop. 1; *c*. 2 000 000 plants) has the largest variation (16.7% polymorphic fragments, Shannon diversity 9.52), and the 38 individuals analysed in this population harbour 125 of 142 fragments entirely found in *H. salzmanniana*. The population Los Algarbes (pop. 5) has the lowest variation (2.0% polymorphic fragments, Shannon diversity 2.31), and the five individuals analysed in this population harbour 96 fragments. Individuals 5b and 5d share the same AFLP phenotype. Identical AFLP phenotypes are also found in Punta Paloma (pop. 6), in which three (b–d) and four (f, h–j) individuals of the 2002 collection

Table 3. Pairwise F_{ST}s between four main groups of *Hypochaeris salzmanniana* (determined through UPGMA analysis, Fig. 6). Significance of each value is 0.000 after 1023 permutations (ARLEQUIN v.2.0; Schneider *et al*., 2000)

	Pops 1–3	Pop. 4	Pops 5, 6
Pops 4	0.318		
Pops 5, 6	0.418	0.662	
Pops 7, 8	0.364	0.510	0.644

Table 4. Population size (estimated no. of individuals in population) and genetic diversity in populations of *Hypochaeris salzmanniana* based on 142 AFLP fragments (ARLEQUIN v.2.0; Schneider *et al*., 2000). None of the populations has fixed private fragments

	Population size	% different AFLP phenotypes	% polymorphic fragments	Shannon diversity	No. of private fragments
Conil (pop. 1)	2 000 000	100	16.7	9.52	5
Los Caños (pop. 2)	500	100	9.8	9.90	1
Barbate (pop. 3)	250	100	7.8	7.82	–
Zahara (pop. 4)	200	100	4.0	4.15	2
Los Algarbes (pop. 5)	20 000	80	2.0	2.31	–
Punta Paloma (pop. 6)	50	47	4.3	3.44	3
Algeciras (pop. 7)	600	100	6.3	6.97	2
La Línea (pop. 8)	250	100	8.9	9.00	2

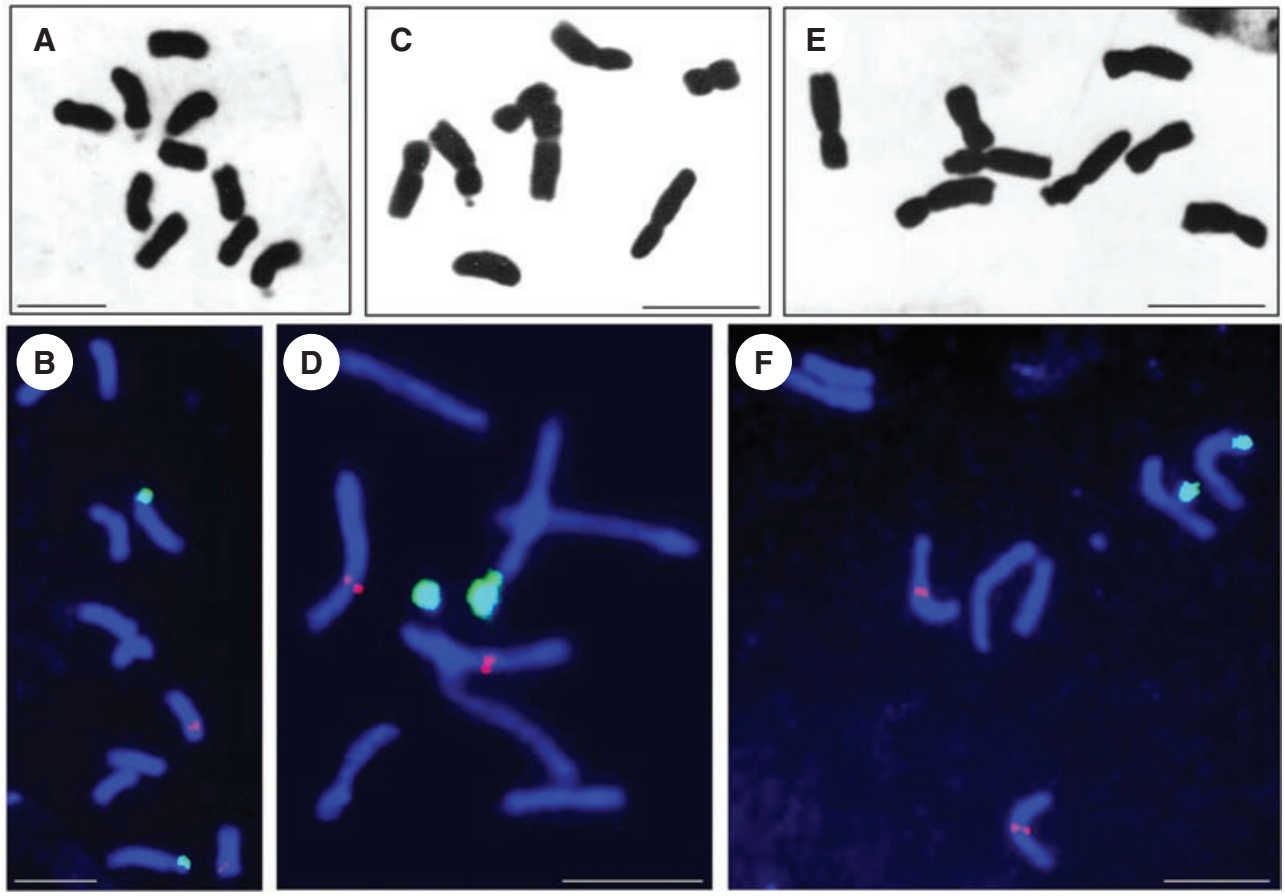

Figure 5. Chromosome morphology (A, C, E) and localization of 5S and 18S–25S rRNA genes (B, D, F) in chromosomes of *Hypochaeris glabra* (A, B), *H. radicata* (C, D), and *H. salzmanniana* (E, F). 5S rDNA marked red; 18S–25S rDNA marked green; blue, DAPI counterstaining. D, F, satellites (carrying 18S–25S rDNA) detached from their chromosomes. Scale bars = 5 μm.

share the same AFLP phenotype, as well as eight individuals (k–r) of the 2003 collection (Fig. 6). All individuals investigated in other populations have a unique AFLP phenotype. Considering only eight cases, we found a nonsignificant correlation between Shannon diversity and population size (Pearson's $r = 0.389$; one-tailed significance = 0.171) and between Shannon diversity and self-incompatibility (Pearson's $r = 0.620$; one-tailed significance = 0.050).

The number of private fragments in a population or cluster is a good indicator of its degree of isolation. Conil (pop. 1) has five private fragments; Punta Paloma (pop. 6) three; Zahara, Algeciras and La Línea (pops 4, 7, and 8) two each; and Barbate and Los Algarbes (pops 3 and 5) none. The four main clusters derived from UPGMA analysis (Fig. 6) also have private fragments: 11 in the first cluster (pops 1–3); two in the second cluster (pop. 4); six in the third cluster (pops 5 and 6); and five in fourth cluster (pops 7 and 8). None of the populations or clusters has fixed private fragments.

Within the large population Conil (pop. 1), the effects of geographical vicinity within a transect and proximity or distance to the ocean have been tested as factors that might influence the partitioning of genetic variation. An analysis of molecular variance (Table 5) carried out on three hierarchical levels revealed 15% genetic variation among the four transects, 0.4% among quadrants next to the ocean and far from the ocean in each transect, and 85% among individuals within each of the two groups of nearby quadrants (next to the ocean and far from the ocean) in each transect.

Differentiation within *H. radicata* and *H. glabra*

Cluster analyses among populations within *H. radicata* and *H. glabra* (Figs 7 and 8) reveal a clear geographical patterning. In *H. radicata*, the largest divide (level of divergence 0.226) is between Spain and worldwide introduced accessions, on the one hand,

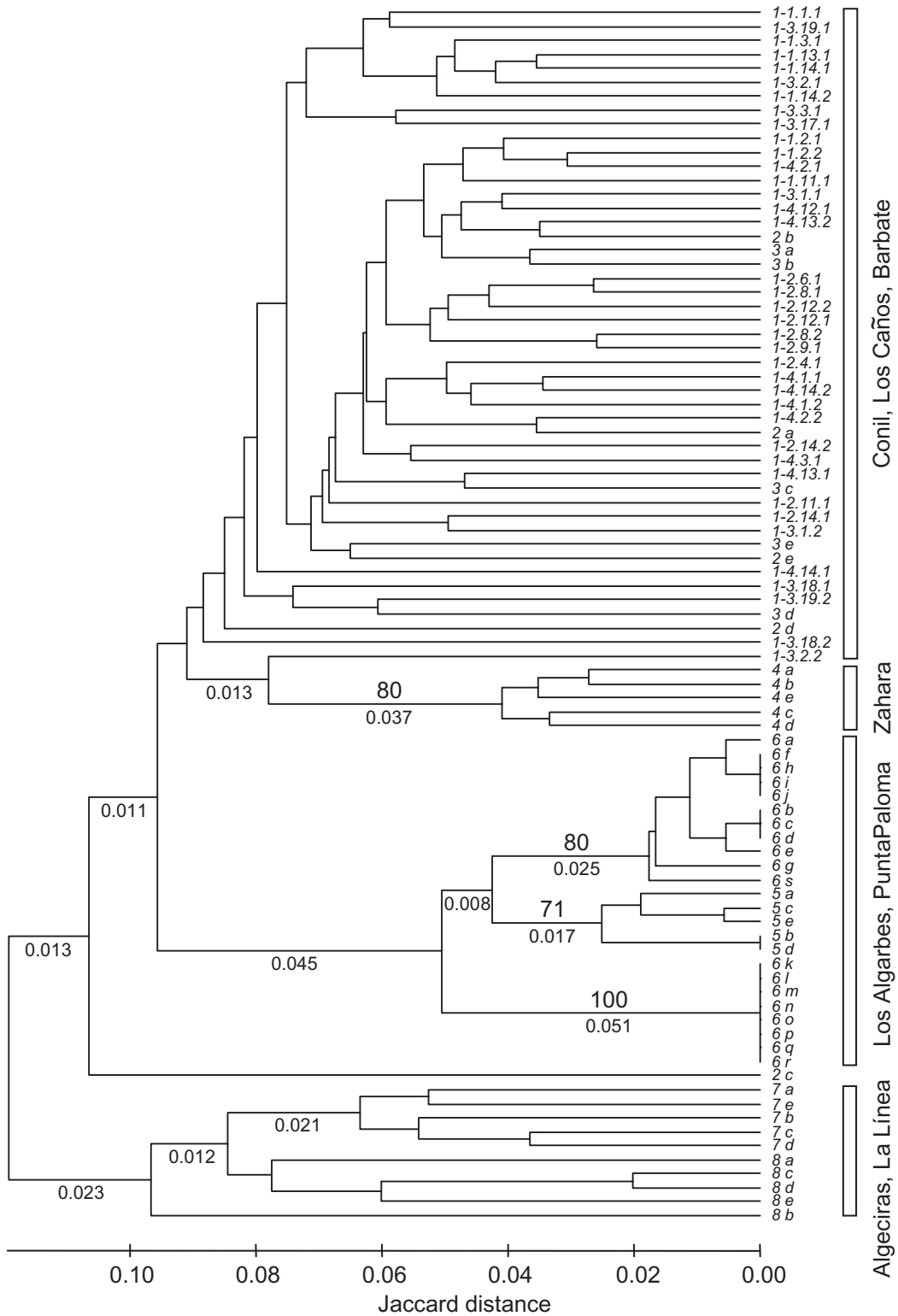

Figure 6. UPGMA dendrogram of Jaccard distances (based on 142 AFLP fragments) showing relationships among individuals of *Hypochaeris salzmanniana*. Only bootstrap values >70% (1000 replicates) shown above branches; Jaccard distances below branches. Numbers refer to populations (see Table 1). Letters in pops 2–8 refer to individual plants. In the large population Conil (pop. 1) the second number refers to the transect (1–4), the third number to the quadrant within the transect, and the fourth number to the individual within the quadrant.

Table 5. Analysis of Molecular Variance (AMOVA; Excoffier *et al*., 1992) in *Hypochaeris salzmanniana* from Playa El Palmar (pop. 1) with *c*. 2 000 000 estimated plants; one-tailed test statistic probability obtained from 1023 permutations of the original presence/absence matrix

Source of variation	Degrees of freedom	Sum of squares	Variance components	% of variation	Test statistic probability
Among the four transects	3	50.930	1.100	14.61	0.006
Among quadrants next to the ocean and far from the ocean in each transect	4	26.225	0.033	0.44	0.419
Among individuals within groups of nearby quadrants (two in each transect)	30	191.950	6.398	84.95	0.000
Total	37	269.105	7.532		

and Tunisia, Sicily and Italy, on the other. The level of divergence is higher in the Spanish and worldwide groups than in the Tunisian and Italian–Sicilian groups. Accessions from the Betic Cordillera (Cádiz and Málaga) differ to a high degree from accessions from the Iberian Massif (Huelva and Burgos) and worldwide introduced accessions (level of divergence 0.221). Within Huelva, there is also a high differentiation, namely between the accession from Doñana National Park (pop. 22) and other accessions from Sierra Morena [Aracena, Santa Ana, and Valverde (pops 21, 23, 24); level of divergence 0.212]. An accession from Burgos (pop. 19) clusters within the larger Sierra Morena (Huelva) group (pops 21, 23, 24). Worldwide introduced accessions from Brazil, Colombia, Germany, Mexico and South Africa (pops 9, 10, 11, 18, 26) are grouped together and cluster with the larger Iberian Massif (Huelva and Burgos) group (pops 19, 21, 23, 24; level of divergence 0.149).

Hypochaeris glabra (Fig. 8) shows a geographical patterning similar to that of *H. radicata*. The largest divide (level of divergence 0.117) is between the Betic Cordillera (Cádiz), on the one hand, and the Iberian Massif (Huelva) and worldwide introduced accessions, on the other. Accessions from Chile and the Canary Islands (pops 28, 31) cluster completely within the Iberian Massif group.

DISCUSSION

TAXONOMY

AFLP data have proven to be very helpful in defining specific limits in *Hypochaeris* sect. *Hypochaeris*. The data support recognition of *H. salzmanniana* as a good species based on 100% BS support in the UPGMA dendrogram and the possession of nine fixed diagnostic fragments, following the hypotheses of Talavera (1980, 1987). The population systems of *H. radicata* and *H. glabra* are also justifiably recognized as distinct species. Among the three species, *H. salzmanniana* is clearly more closely related to *H. radicata* than to *H. glabra*. Evidence for this is provided by the greater genetic similarity between *H. salzmanniana* and *H. radicata* in the UPGMA dendrogram (although this is not highly supported) and the distribution of shared diagnostic fragments among the three species. *Hypochaeris glabra* as a sister group to *H. radicata* and *H. salzmanniana* is consistent with DNA sequence data of the nuclear Internal Transcribed Spacer (ITS; Samuel *et al.*, 2003; K. Tremetsberger, unpubl. data). The similarity of karyotypes of *H. salzmanniana* and *H. radicata* also strongly supports their close relationship and distinctness from *H. glabra*. The application of molecular cytogenetic techniques further emphasizes the similarity of karyotypes between *H. salzmanniana* and *H. radicata*.

BIOGEOGRAPHY

At the end of the Tertiary, increase in aridity and the rise of mountain ranges in the course of the Alpine orogeny might have been factors promoting biodiversity in the Mediterranean Basin (see Myers *et al*., 2000), particularly of the flora (Bocquet, Widler & Kiefer, 1978; Blanca, 1993). The postulated evolutionary history of sect. *Hypochaeris*, in accordance with genetic and karyotypic data, is differentiation of the ancestor of *H. radicata* and *H. salzmanniana* (with $2n = 8$ chromosomes) from a common ancestor with *H. glabra* (with $2n = 10$ chromosomes), followed by subsequent differentiation of *H. salzmanniana* and *H. radicata*.

The infraspecific genetic structure of *H. salzmanniana* may be interpreted in the context of different geological ages of their habitats. Generally, isolation-by-distance, as initially presented by Wright (1943), is found in *H. salzmanniana*. However, the correlation is weak, because the entire population system is subdivided into four main groups. The coast from

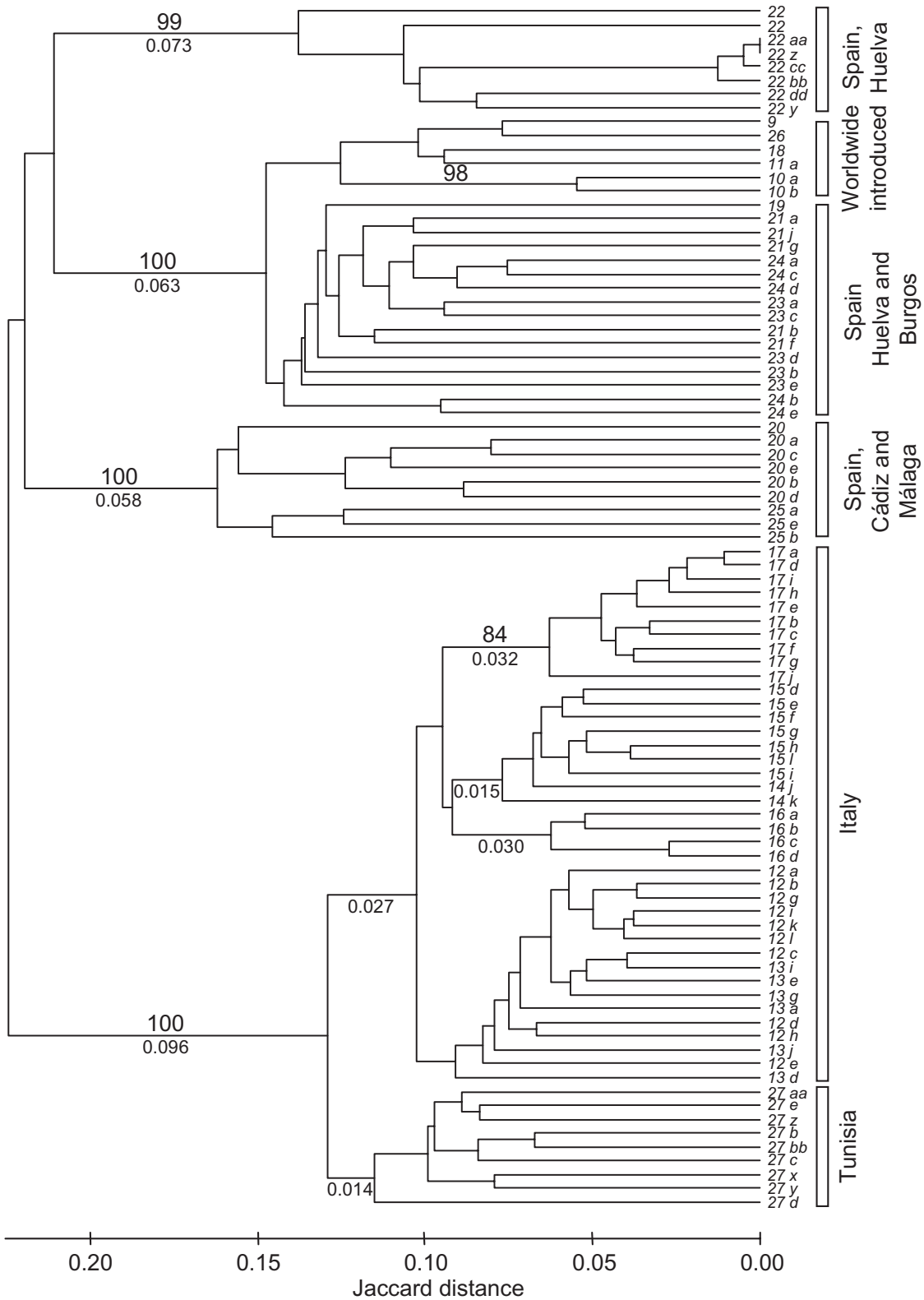

Figure 7. UPGMA dendrogram of Jaccard distances (based on 269 AFLP fragments) showing relationships among individuals of *Hypochaeris radicata*. Only bootstrap values >70% (1000 replicates) shown above branches; Jaccard distances below branches. Numbers refer to populations (see Table 1). Letters refer to individual plants.

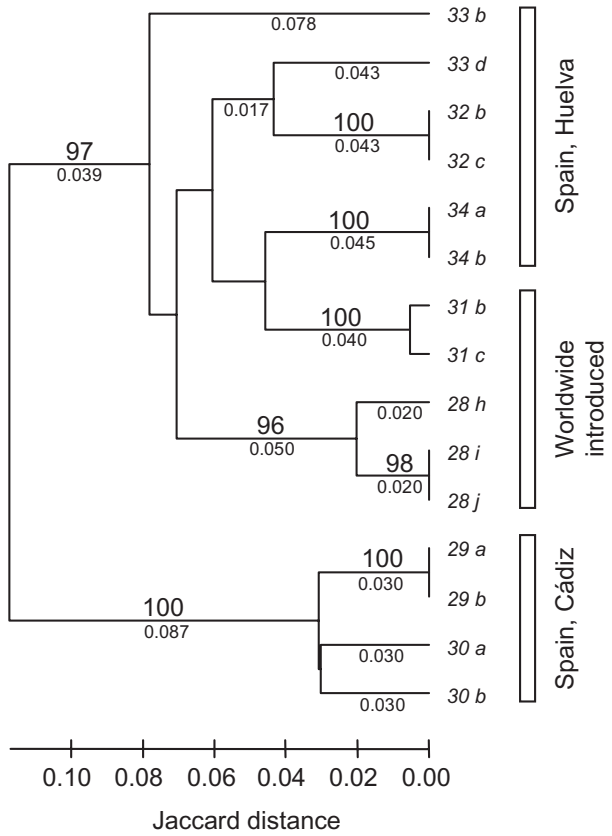

Figure 8. UPGMA dendrogram of Jaccard distances (based on 124 fragments) showing relationships among individuals of *Hypochaeris glabra*. Only bootstrap values >70% (1000 replicates) shown above branches; Jaccard distances below branches. Numbers refer to populations (see Table 1). Letters refer to individual plants.

Punta Paloma to Gibraltar is bordered by the Betic Cordillera (also called in this area Campo de Gibraltar or Unidad de Algeciras). The base of this unit comprises Cretaceous to middle Miocene basic flysch (formed 135–22 Mya), which emerged from the sea bottom during lower and middle Miocene (25–16 Mya). Above the flysch layer are sandy acidic materials called 'Aljibe Sandstones' or 'Numidic Sandstones'. These materials are allochthonous turbiditic sands that migrated from Tunisia to the Strait of Gibraltar, where they sedimented in the furrows of the older substrates (flysch) (Pendón, 1978; Gutiérrez *et al*., 1991). When the Alpine orogeny ceased and the Strait of Gibraltar opened up, the low littoral zones were covered with Pliocene and recent Quaternary sands (Fernández-Palacios *et al.*, 1988). The populations of Conil, Los Caños, Barbate, Zahara, and Algeciras (pops 1–4, 7) are in sand dunes near the coast. The population of Barbate, as an exception, is found on a fossil dune of the marine transgression of Flandrian (11 000 y BP). The populations of Los Algarbes and Punta Paloma (pops 5, 6) occur in the ecotone of the Quaternary dunes with the flysch materials belonging to the rocky spur of the Sierra de San Bartolomé (Cretaceous). The population of La Línea (pop. 8) is at the base of the Rock of Gibraltar, in the loam-sandstone mica layer of the flysch (Gutiérrez *et al*., 1991) of the Lower Miocene or Aquitanian (*c*. 20 Mya). The high differentiation between the three groups of populations, [(1, 2, 3) (4)], [5, 6], and [7, 8] therefore may reflect an ancient separation of populations.

The coinciding population structures of *H. radicata* and *H. glabra* are also most interesting. These two species independently show a clear differentiation between accessions from the Betic Cordillera (Cádiz and Málaga) and those from the Iberian Massif (Huelva and Burgos) in southern Spain. *Hypochaeris radicata* in the Betic Cordillera is also differentiated morphologically from its conspecific neighbour in the Iberian Massif and has been separated as *H. radicata* ssp. *platylepis* (Boiss.) Maire (Galán de Mera, 1995). This taxon is differentiated from ssp. *radicata* by involucral bracts with a wide scarious margin.

A possible explanation for the differentiation between populations in the Betic Cordillera and the Iberian Massif lies in the geological history of the region. The Guadalquivir river, which flows through Andalusia from east to west, separates the Precambrian and Palaeozoic terrains (north of the river, Iberian Massif) from the Mesozoic and Neogene terrains (south of the river, Betic Cordillera). During the middle and the beginning of the upper Miocene (until lower Tortonian, 9 Mya), the Betic–Rifian bow (today's Betic Cordillera and Rif Mountains) was united and separated as a unit from the Iberian Massif (northwards) by the Betic Strait and from North Africa (southwards) by the Rifian Strait. The Mediterranean Sea was then connected with the Atlantic Ocean through two straits (Orszag-Sperber *et al*., 1993). Between 8.5 and 7.3 Mya, the eastern side of the Betic Strait, and *c*. 7.16 Mya, the Rifian Strait disappeared under silt (Guerra-Merchán & Serrano, 1993; Soria, Fernández & Viseras, 1999; Meulenkamp *et al*., 2000; Seidenkrantz *et al*., 2000; Braga & Aguirre, 2001; Sánchez-Almazo *et al*., 2001). These events, in addition to strong volcanic activity in the western Mediterranean, led to the Messinian salinity crisis and the subsequent desiccation of the Mediterranean Sea between 5.96 and 5.33 Myr (Riding *et al*., 1998; Krijgsman *et al*., 1999; Duggen *et al*., 2003). Intercontinental bridges appeared between southern Europe and North Africa (Estabrook, 2001) enabling the migration of numerous groups of animals and plants between the two continents (Bocquet *et al*., 1978). At the end of the Messinian (5.33 Mya), the Strait of Gibraltar opened,

land bridges between Europe and Africa were flooded, and water from the Atlantic Ocean refilled the dry Mediterranean basin (Lonergan & White, 1997; de Jong, 1998). Direct land connection between both continents disappeared therefore and the Strait of Gibraltar definitively separated the Betic Cordillera in southern Spain from those (nowadays the Rifian ones) in North Africa (de Jong, 1998). The south Guadalquivir river material emerged from the sea bottom during the Alpine orogeny between the lower Miocene (Aquitanian, 23.8–22 Mya) and Pliocene, 5 Mya (Lonergan & Johnson, 1998; Sánchez-Gómez *et al*., 2002). During the Pliocene (5–3 Mya), a deep fluvial drainage was still present in today's Guadalquivir river basin (de Jong, 1998; García-París & Jockusch, 1999), which could have maintained isolation between populations from the Iberian Massif and those from the Betic Cordillera. The ancient separation of the two mountain chains is also reflected in the distribution of other animal and plant groups (Asensi & Díez-Garretas, 1987) and in their genetic structures (e.g. haplotypes of different genera of amphibians; Busack, 1986; García-París, Alcobendas & Alberch, 1998; García-París & Jockusch, 1999).

CONSERVATION

Because *H. salzmanniana* is a restricted and endangered species in the vascular flora of Spain, conservation implications from analysis of genetic variation within and among populations are important to highlight. The species is now known from only eight populations in Spain, all of which have been sampled in the present study.

A conspicuous result is that the first group consisting of Conil, Los Caños, and Barbate (pops 1–3) actually behaves as one large panmictic population with individuals of the three populations being strongly intermingled. The population of Conil, Playa El Palmar, is nearly 10 km long and separated from Los Caños by only 6.5 km, and this from Barbate by another 6.7 km (a total of about 20 km). Two possible explanations might account for this situation: (i) that there is actually high gene flow between these populations; or (ii) that the populations were interconnected not long ago and were only recently interrupted through destruction of geographically intermediate populations by human activity. Investigations from herbarium records (M. Á. Ortiz & S. Talavera, unpubl. data) show that *H. salzmanniana* occupied a continuous territory along the Atlantic coast from Chiclana de la Frontera to Punta Camarinal until the 1970s. The remaining populations of this formerly continuous distributional area number only four: Conil, Los Caños, Barbate and Zahara. In contrast, historical records (back to the 1890s) reveal that *Hypochaeris salzmanniana* was only locally abundant from Punta Camarinal to Gibraltar. Suitable habitats are scarce along the steep coast of the Betic Cordillera.

Population sizes in *H. salzmanniana* explain 15% of the observed variation in Shannon diversity. The population Los Caños (pop. 2) and the large population Conil (pop. 1; *c*. 2000 000 plants) have the highest genetic diversity. This stresses the importance of preserving not only populations of the four major groups, but also of population Conil in its entirety, because it harbours the majority of genetic diversity within the species. Within this population, there is not only high genetic differentiation between neighbouring plants within a transect (a few metres distant), but also differentiation between transects (at *c*. 1 km spacing). Proximity or distance to the ocean does not influence partitioning of genetic variation. As long as this dune system is not destroyed (shown in Fig. 1B), the species should continue to thrive as it does not seem bothered by relatively high levels of human disturbance.

The compatibility systems vary greatly among populations of *H. salzmanniana* (M. Á. Ortiz & S. Talavera, unpubl. data) and contribute 38% to the explanation of the observed variation in Shannon diversity. Plants in Conil and Los Caños (pops 1, 2) are 33% self-incompatible; those in Barbate (pop. 3) are 53% self-incompatible; those in Algeciras (pop. 7) are all self-incompatible; those in La Línea (pop. 8) are 86% self-incompatible; whereas plants in Zahara, Los Algarbes and Punta Paloma (pops 4–6) are all self-compatible (M. Á. Ortiz & S. Talavera, unpubl. data). Self-compatibility and possibly high levels of inbreeding in Zahara, Los Algarbes and Punta Paloma (pops 4–6) correlate with the low within-population genetic diversity and many identical AFLP phenotypes in these populations.

There is an urgent need to preserve the genetic variation harboured in each of the four groups. Los Algarbes and Punta Paloma are presently under no threat, being situated along a small road away from any human development. This cannot be said for Algeciras and La Línea, however, which are on coastal land that is being heavily developed. Algeciras (pop. 7) is a relatively large population broken into patches by houses and streams feeding into Bahía de Algeciras. La Línea (pop. 8) is even more endangered, surviving along the coast in the town of La Línea de la Concepción just adjacent to the fence that separates it from Gibraltar. This small dune area is already seriously disturbed and not likely to remain into the future.

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REFERENCES

Adame P, Talavera S. 1980. Números cromosómicos para la flora española: 130–131. *Lagascalia* **9:** 256–257.

Amo M. 1872. *Flora fanerogámica de la Península Ibérica 4.* Granada.

Asensi A, Díez-Garretas B. 1987. Andalucía Occidental. In: Peinado-Lorca M, Rivas-Martínez S, eds. *La vegetación de España*. Alcalá de Henares: Universidad de Alcalá, 199–230.

Blanca G. 1993. Origen de la Flora Andaluza. In: Valdés B, ed. *Introducción a la flora Andaluza*. Sevilla: Junta de Andalucía, 19–35.

Bocquet G, Widler B, Kiefer H. 1978. The Messinian Model – a new outlook for the floristics and systematics of the Mediterranean area. *Candollea* **33:** 269–287.

Braga JC, Aguirre J. 2001. Coralline algal assemblages in upper Neogene reef and temperate carbonates in Southern Spain. *Palaeogeography, Palaeoclimatology, Palaeoecology* **175:** 27–41.

Busack SD. 1986. Biochemical and morphological differentiation in Spanish and Moroccan populations of *Discoglossus* and the description of a new species from Southern Spain (Amphibia, Anura, Discoglossidae). *Annals of Carnegie Museum* **55:** 41–61.

de Candolle AP. 1838. *Prodromus Systematis Naturalis Regni Vegetabilis 7.* Paris.

Casgrain P, Legendre P. 2000. *The R package for multivariate and spatial analysis, v.4.0. (development release 2).* Québec: University of Montréal.

De Filipps RA. 1976. 158. *Hypochoeris* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA, Chater AO, De Filipps RA, Richardson IBK, eds. *Flora Europaea*, *Vol. 4: Plantaginaceae to Compositae (and Rubiaceae).* Cambridge: Cambridge University Press, 308–310.

Duggen S, Hoernle K, Van den Bogaard P, Rüpke L, Morgan JP. 2003. Deep roots of the Messinian salinity crisis. *Nature* **422:** 602–606.

Emberger L, Maire R. 1941. *Catalogue des plantes du Maroc 4.* Algiers: Imprimerie Minerva.

Estabrook GF. 2001. Vicariance or dispersal: The use of natural historical data to test competing hypotheses of disjunction on the Tyrrhenian coast. *Journal of Biogeography* **28:** 95–103.

Excoffier L, Smouse P, Quattro J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction site data. *Genetics* **131:** 479–491.

Fernández-Palacios A, Fernández-Palacios C, Gil BJ. 1988. *Guias de naturalistas de la Provincia de Cádiz. I. El Litoral*. Cádiz: Diputación de Cádiz.

Förther H, Podlech D. 2003. Die Gattung *Hypochaeris* L. sect. *Hypochaeris* (Compositae) im westlichen Afrika. *Sendtnera* **8:** 35–43.

Galán de Mera A. 1995. Contribución a las floras de la provincia de Cádiz y de la Península Tingitana. *Lagascalia* **18:** 55–62.

García-París M, Jockusch EL. 1999. A mitochondrial DNA perspective on the evolution of Iberian *Discoglossus* (Amphibia: Anura). *Journal of Zoology* **248:** 209–218.

García-París M, Alcobendas M, Alberch P. 1998. Influence of the Guadalquivir River Basin on mitochondrial DNA evolution of *Salamandra salamandra* (Caudata: Salamandridae) from Southern Spain. *Copeia* **1998:** 173–176.

Guerra-Merchán A, Serrano F. 1993. Tectosedimentary setting and chronostratigraphy of the Neogene reefs in the Almanzora corridor (Betic Cordillera, Spain). *Geobios* **26:** 57–67.

Gutiérrez JM, Martin A, Domínguez S, Moral JP. 1991. *Introducción a la geología de la Provincia de Cádiz*. Cádiz: Universidad de Cádiz.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. *Bulletin de la Société Vaudoise de Sciences Naturales* **44:** 223–270.

Jahandiez E, Maire R. 1934. *Catalogue des plantes du Maroc, 3.* Algiers: Imprimerie Minerva.

de Jong H. 1998. In search of historical biogeographic patterns in the Mediterranean terrestrial fauna. *Biological Journal of the Linnean Society* **65:** 99–164.

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS. 1999. Chronology, causes, and progression of the Messinian salinity crisis. *Nature* **400:** 652–655.

Lonergan L, Johnson C. 1998. Reconstructing orogenic exhumation histories using synorogenic detrital zircons and apatites: an example from the Betic Cordillera, SE Spain. *Basin Research* **10:** 353–364.

Lonergan L, White N. 1997. Origin of the Betic-Rif mountain belt. *Tectonics* **16:** 504–522.

Meulenkamp JE, Sissingh W, Ilyina LB, Kovac M, Barrier E. 2000. Late Tortonian (8.4–7.2 Ma). In: Dercourt J, Gaetani M, Vrielynck B, Barrier E, Biju-Duval B, Brunet MF, Cadet JP, Crasquin S, Sandulescu M, eds. *Atlas Peri-Tethys, Palaeogeographical maps*. Paris: CCGM/CGMW.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403:** 853–858.

Nei M, Li W-H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences, USA* **76:** 5269–5273.

Oberprieler C. 2002. *Hypochaeris*. In: Valdés B, Rejdali M, Achhal El Kadmiri A, Jury SL, Montserrat JM, eds. *Catalogue des plantes vasculaires du Nordiska du Maroc, incluant des clés d'identification, 2.* Madrid: Consejo Superior de Investigaciones Científicas, 686–689.

Oberprieler C, Vogt R. 2002. *Hypochaeris arachnoidea* Poir., a hitherto neglected species in NW Africa. *Willdenowia* **32:** 231–236.

Orszag-Sperber F, Butterlin J, Clermonte J, Colchen M, Guiraud R, Poisson A, Ricou LE. 1993. Tortonian (11.5–6 Ma). In: Dercourt J, Ricou LE, Vrielynck B, eds. *Atlas Tethys palaeoenvironmental maps*. Paris: Gauthier Villars.

PE Applied Biosystems. 1996. *AFLP™ plant mapping protocol*. Foster City, CA: PE Applied Biosystems.

Pendón JG. 1978. *Sedimentación turbidítica en las unidades del Campo de Gibraltar*. Granada: Universidad de Granada.

Pfosser MF, Guzy-Wróbelska H, Sun B-Y, Stuessy TF, Sugawara T, Fujii N. 2002. The origin of species of *Acer* (Sapindaceae) endemic to Ullung Island, Korea. *Systematic Botany* **27:** 351–367.

Riding R, Braga JC, Martín JM, Sánchez-Almazo IM. 1998. Mediterranean Messinian Salinity Crisis: constraints from a coeval marginal basin, Sorbas, southeastern Spain. *Marine Geology* **146:** 1–20.

Samuel R, Stuessy T, Tremetsberger K, Baeza C, Siljak-Yakovlev S. 2003. Phylogenetic relationships among species of *Hypochaeris* (Asteraceae, Lactuceae) based on ITS, plastid *trnL* intron, *trnL-F* spacer, and *matK* sequences. *American Journal of Botany* **90:** 496–507.

Sánchez-Almazo IM, Spiro B, Braga JC, Martín JM. 2001. Constraints of stable isotope signatures on the depositional palaeoenvironments of upper Miocene reef and temperate carbonates in the Sorbas Basin, SE Spain. *Palaeogeography, Palaeoclimatology, Palaeoecology* **175:** 153–172.

Sánchez-Gómez M, Balanyá JC, García-Dueñas V, Azañón JM. 2002. Intracrustal tectonic evolution of large lithosphere mantle slabs in the western end of the Mediterranean orogen (Gibraltar arc). *Journal of the Virtual Explorer* **8:** 23–34.

Schneider S, Roessli D, Excoffier L. 2000. *Arlequin*, Version 2.000*: a software for population genetics data analysis*. Geneva: University of Geneva.

Schönswetter P, Tribsch A, Schneeweiss GM, Niklfeld H. 2003. Disjunctions in relict alpine plants: phylogeography of *Androsace brevis* and *A. wulfeniana* (Primulaceae). *Botanical Journal of the Linnean Society* **141:** 437–446.

Seidenkrantz M-S, Kouwenhoven TJ, Jorissen FJ, Shackleton NJ, Van der Zwaan GJ. 2000. Benthic foraminifera as indicators of changing Mediterranean-Atlantic water exchange in the late Miocene. *Marine Geology* **163:** 387–407.

Soria JM, Fernández J, Viseras C. 1999. Late Miocene stratigraphy and paleogeographic evolution of the intramontane Guadix Basin (Central Betic Cordillera, Spain): implications for an Atlantic-Mediterranean connection. *Palaeogeography, Palaeoclimatology, Palaeoecology* **151:** 255–266.

Stuessy TF, Tremetsberger K, Müllner AN, Jankowicz J, Guo Y-P, Baeza CM, Samuel RM. 2003. The melding of systematics and biogeography through investigations at the populational level: examples from the genus *Hypochaeris* (Asteraceae). *Basic and Applied Ecology* **4:** 287–296.

Swofford DL. 1998. *PAUP*: phylogenetic analysis using parsimony (*and other methods)*, v.4.0b8. Sunderland, MA: Sinauer Associates.

Talavera S. 1980. Nota taxonómica sobre el género *Hypochoeris* L. en Andalucía Occidental. *Lagascalia* **9:** 232–234.

Talavera S. 1981. Números chromosómicos para la flora española: 182–256. *Lagascalia* **10:** 225–256.

Talavera S. 1987. *Hypochaeris*. In: Valdés B, Talavera S, Fernández-Galiano E, eds. *Flora vascular de Andalucía Occidental, 3.* Barcelona: Ketres Editoria, 102–105.

Tremetsberger K, Stuessy TF, Guo Y-P, Baeza CM, Weiss H, Samuel RM. 2003a. Amplified Fragment Length Polymorphism (AFLP) variation within and among populations of *Hypochaeris acaulis* (Asteraceae) of Andean southern South America. *Taxon* **52:** 237–245.

Tremetsberger K, Stuessy TF, Samuel RM, Baeza CM, Fay MF. 2003b. Genetics of colonization in *Hypochaeris tenuifolia* (Asteraceae, Lactuceae) on Volcán Lonquimay, Chile. *Molecular Ecology* **12:** 2649–2659.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23:** 4407–4414.

Weiss H, Stuessy TF, Grau J, Baeza CM. 2003. Chromosome reports from South American *Hypochaeris* (Asteraceae). *Annals of the Missouri Botanical Garden* **90:** 56–63.

Weiss-Schneeweiss H, Stuessy TF, Siljak-Yakovlev S, Baeza CM, Parker J. 2003. Karyotype evolution in South American species of *Hypochaeris* (Asteraceae, Lactuceae). *Plant Systematics and Evolution* **241:** 171–184.

Willkomm M. 1893. *Supplementum Prodromi Florae Hispanicae.* Stuttgart.

Wright S. 1943. Isolation by distance. *Genetics* **28:** 114–138.