Population structure of *Hypochaeris salzmanniana* DC. (Asteraceae), an endemic species to the Atlantic coast on both sides of the Strait of Gibraltar, in relation to Quaternary sea level changes

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Abstract

To detect potential Pleistocene refugia and colonization routes along the Atlantic coast, we analysed amplified fragment length polymorphisms (AFLPs) in 140 individuals from 14 populations of *Hypochaeris salzmanniana* (Asteraceae), an annual species endemic to the southwestern European and northwestern African coastal areas. Samples covered the total distributional range of the species, with eight populations in southwestern Spain and six populations in northwestern Morocco. Using nine primer combinations, we obtained 546 fragments in *H. salzmanniana* and its sister species *H. arachnoidea* of which 487 (89.2%) were polymorphic. The neighbour-joining tree shows that the populations south of the Loukos river in Morocco are clearly differentiated, having more polymorphic, private, and rare fragments, and higher genetic diversity, than all the other populations. The southern-most populations in Morocco, south of the river Sebou, form a large panmictic population. They are probably the oldest populations that have been relatively unaffected by stochastic processes resulting from Pleistocene glaciations. Northward migration of populations during this period may have resulted in loss of genetic diversity in specific regions, perhaps due to bottlenecks caused by rise in sea level during interglacial periods, and, in some cases, by changes in the breeding system.

Keywords: AFLP, breeding systems, Compositae, *Hypochaeris*, phylogeography, Pleistocene.

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Introduction

At the end of the Miocene, around 5.33 million years ago (Ma), the Strait of Gibraltar opened, allowing contact between the Atlantic Ocean and the Mediterranean basin, and initiating the end of the ‘Messinian salinity crisis’ (Krijgsman et al. 1999). This event caused the separation of the Iberian Peninsula and African continental areas.

Subsequently, the climatic oscillations caused by Quaternary glaciations and their effects on sea levels, which were lower during glacial periods and higher during interglacial periods, are likely to have had a major influence on the vegetation on either side of the Strait of Gibraltar (Cheddadi et al. 2005). During these glacial events, the sea level was approximately 120–150 m lower than at present (Pou 1989; Yokoyama et al. 2000), thus revealing islands, which could have facilitated biological communication between Africa and Europe in the extreme western Mediterranean area, near the Strait of Gibraltar (Collina-Girard 2001). During interglacial periods, the sea level rose again, submerging the islands, and Europe and Africa were once again physically isolated. Within this dynamic situation, lower sea levels would have favoured the expansion of populations of species into the newly exposed coastland areas and also the migration of populations between Africa and Europe, and vice versa. During interglacial, however, the rise in sea level must have caused the extinction of some populations and a reduction in size of others as a result of the flooding of land previously colonized. For coastal species, river estuaries and the flanks of mountains may have acted as refugial areas during interglacial periods. The Quaternary glacial episodes are likely to have modelled the genetic structure and distribution of present-day coastal species.
Various studies have used diverse molecular genetic markers (ITS, mtDNA, cpDNA-RFLPs, allozymes, and microsatellites) to look at phylogeographical patterns in species that occur on both sides of the Strait of Gibraltar. Most of these have been with animal groups, such as amphibians (Busack 1986; García-París & Jockusch 1999; Fromhage et al. 2004; Martinèz-Solano et al. 2004; Veith et al. 2004), reptiles (Harris et al. 2002; Carranza et al. 2006), scorpions (Gantenbein & Largiadèr 2003; Gantenbein 2004), and beetles (Palmer & Cambefort 2000; Sanmartín 2003). All these groups date back late in the Tertiary and consequently, the Betic crisis at c. 16–14 Ma as well as the Messinian salinity crisis at c. 5.59–5.33 Ma, which provided a land bridge between Iberia and North Africa, are invoked as possibilities for faunal exchange (Palmer & Cambefort 2000; Sanmartín 2003; Veith et al. 2004). The fragmentation of the Betic region at c. 12–10 Ma, the opening of the Betic Strait at c. 10–8 Ma, as well as the opening of the Strait of Gibraltar at c. 5.33 Ma are supposed to have led to vicariance speciation in these genera (Busack 1986; García-París & Jockusch 1999; Gantenbein & Largiadèr 2003; Fromhage et al. 2004; Gantenbein 2004; Martínez-Solano et al. 2004). Moreover, recent dispersal across the Strait of Gibraltar via rafting on vegetation has also been suggested (Harris et al. 2002; Veith et al. 2004; Carranza et al. 2006). Few plant species occurring on both sides of the Strait of Gibraltar have been investigated, including some woody taxa such as Frangula alnus (Hampe et al. 2003), Quercus ilex (Lumaret et al. 2002; Petit et al. 2005), and Abies pinsapo (Garcia et al. 1993), and also various coastal and semicoastal herbaceous taxa, such as Cakile maritima (Clausing et al. 2000; Kadereit et al. 2005), Saxifraga globulifera (Vargas et al. 1999) and Androcymbium gramineum (Caujapé-Castells & Jansen 2003). In the slowly evolving trees, the current genetic structure is thought to reflect population divergence that predates the onset of the Mediterranean climate in the Pliocene (Petit et al. 2005). The Strait of Gibraltar is an effective barrier to gene flow in these taxa (Lumaret et al. 2002; Hampe et al. 2003; Petit et al. 2005). In the Betic-Rifian Saxifraga globulifera (Saxifragaceae), the north African populations are likewise isolated from the Iberian ones (Vargas et al. 1999).

In this study, we have used amplified fragment length polymorphism (AFLP) analyses to study the phylogeography of Hypochaeris salzmanni ana (Lactuceae, Asteraceae), a predominantly self-incompatible species endemic to the maritime sands of the Atlantic coast on either side of the Strait of Gibraltar (Ortiz et al. 2006). Using a molecular clock, Tremetsberger et al. (2005) estimated the divergence between Hypochaeris and Leontodon, the most closely related genus, at 6.6 Ma (95% CI due to uncertainty of branch lengths = 4.0–9.3 Ma); and for sect. Hypochaeris (Tremetsberger, unpublished data) at 0.8 Ma (95% CI due to uncertainty of branch lengths = 0.5–1.4 Ma). Thus, unlike the studied species mentioned above, the evolutionary expansion of H. salzmanniana seems to have occurred during the latest phase of the glacial period. However, due to limitations of the dating method [e.g. transfer of age estimate obtained by molecular clock calculation on an independent tree; see Tremetsberger et al. (2005) for a discussion], these dates are tentative estimates.

The same geographical distribution as in H. salzmanniana is also found in other Lactuceae species, such as Reichardia gudiana Willk., Crepis erythraeus Pau, and Hedypnois arenaria (Schouboe) DC., as well as many other Asteraceae coastal species such as Carduus myriacanthus DC., C. meantiensis Hoffmanns. & Link, and Onopordum dissectum Murb., etc. No matter whether of Pliocene or Pleistocene age, all these species certainly have been affected by glacial and interglacial events during the last phase of the Pleistocene. To date, there is no knowledge about the patterns of genetic structure in species having this particular distributional range.

AFLPs have some advantages over other methods for the analysis of DNA polymorphisms, since the technique requires no prior sequence knowledge, is highly reliable, gives a large number of genomic fragments, and requires only minimal development time (Vos et al. 1995; Mueller & Wolfenbarger 1999). The major disadvantage is that they are dominant markers, therefore precluding any inference of heterozygosity (Mueller & Wolfenbarger 1999).

AFLPs have been established as useful genetic markers in studies of biogeographical patterns that have been shaped by Quaternary glaciations in European alpine species such as Erinus alpinus (Stehlik et al. 2002), Phyteuma globulariifolium (Schönswetter et al. 2002) and Saponaria pumila (Triibsch et al. 2002); and with three South American Hypochaeris species: H. leucaephala, H. palustris, and H. acaulis (Stuessy et al. 2003; Tremetsberger et al. 2003a, b; Mueller et al. 2005). However, none of these studies has focused on the effects of these glaciations on the phylogeography of coastal species.

We focus on the following questions: (i) Did any areas serve as refugia for ancient populations of this species? Because of their long-lasting, uninterrupted existence at the same locality, we expect refugial populations to harbour high allelic richness and possibly also higher genetic diversity than more recently established populations (e.g. Widmer & Lexer 2001). (ii) Are self-compatible populations of H. salzmanniana genetically depauperate in comparison to self-incompatible populations? (iii) Have coastal features such as river estuaries, mountain spurs, and especially the Strait of Gibraltar functioned as significant barriers to gene flow between populations of this species? If the Strait of Gibraltar was an effective barrier prohibiting gene flow in H. salzmanniana as has been postulated for several animal and plant groups (see above), we would expect a strong genetic differentiation between populations in Spain and Morocco. If, on the other hand, river estuaries or mountain spurs have been more effective barriers than
Table 1 Region, population, coordinates, collectors, population size (N), total number of fragments per population (Fragtot), % polymorphic fragments (Fragpol), private fragments (Fragpriv), rare fragments (DW), Shannon diversity (Hst), average gene diversity (Hd), and distance among populations (Fst) of Hypochaeris salzmanniana populations sampled for AFLP. (Ten individuals for overall population; see Materials and methods)

<table>
<thead>
<tr>
<th>Populations</th>
<th>Coordinates (N, W)</th>
<th>Collectors no.</th>
<th>N</th>
<th>Fragpol</th>
<th>Fragpoly</th>
<th>Fragpriv</th>
<th>DW</th>
<th>Hst</th>
<th>Hd</th>
<th>Fst</th>
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<tbody>
<tr>
<td>Spain Barbate river (NB)</td>
<td></td>
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<tr>
<td>Pop. 1. Conil-El Palmar, 10 m</td>
<td>36°13', 6°04'</td>
<td>ST, TS et al. 5/02</td>
<td>&gt; 10^6</td>
<td>256</td>
<td>51.17</td>
<td>6</td>
<td>13.31</td>
<td>32.65</td>
<td>0.0967</td>
<td>0.083***</td>
</tr>
<tr>
<td>Pop. 2. Vejer-Barbate, 50 m</td>
<td>36°12', 5°56'</td>
<td>ST, TS et al. 14/02</td>
<td>100–500</td>
<td>265</td>
<td>51.32</td>
<td>4</td>
<td>10.71</td>
<td>36.88</td>
<td>0.1028</td>
<td></td>
</tr>
<tr>
<td>Pop. 3. Caños de Meca, 10 m</td>
<td>36°11', 6°01'</td>
<td>ST, TS et al. 24/02</td>
<td>100–500</td>
<td>271</td>
<td>52.40</td>
<td>9</td>
<td>14.68</td>
<td>39.40</td>
<td>0.1097</td>
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<tr>
<td>S Barbate river (SB)</td>
<td></td>
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<tr>
<td>Pop. 4. Zahara, 10 m</td>
<td>36°08', 5°51'</td>
<td>ST &amp; MAO 1/03</td>
<td>100–500</td>
<td>209</td>
<td>40.67</td>
<td>1</td>
<td>3.37</td>
<td>21.61</td>
<td>0.0648</td>
<td>0.530***</td>
</tr>
<tr>
<td>Pop. 5. Punta Paloma, 50 m</td>
<td>36°04', 5°41'</td>
<td>ST et al. 32/02; 3/03</td>
<td>&lt; 50</td>
<td>206</td>
<td>31.55</td>
<td>2</td>
<td>5.81</td>
<td>16.10</td>
<td>0.0426</td>
<td></td>
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<tr>
<td>Pop. 6. Los Algarbes, 80 m</td>
<td>36°04', 5°41'</td>
<td>ST &amp; MAO 2/03</td>
<td>2.10^4</td>
<td>208</td>
<td>34.62</td>
<td>1</td>
<td>4.43</td>
<td>16.56</td>
<td>0.0461</td>
<td>0.119***</td>
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<tr>
<td>Algeciras bay (AB)</td>
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<tr>
<td>Pop. 7. Palmones, 10 m</td>
<td>36°10', 5°25'</td>
<td>ST, TS et al. 33/02</td>
<td>600–1000</td>
<td>262</td>
<td>59.16</td>
<td>3</td>
<td>12.98</td>
<td>43.37</td>
<td>0.1231</td>
<td></td>
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<tr>
<td>Pop. 8. La Línea, 10 m</td>
<td>36°09', 5°20'</td>
<td>ST, TS et al. 35/02</td>
<td>100–500</td>
<td>250</td>
<td>56.40</td>
<td>4</td>
<td>10.18</td>
<td>36.41</td>
<td>0.1053</td>
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<tr>
<td>Morocco</td>
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<tr>
<td>N Loukos river (NL)</td>
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<tr>
<td>Pop. 9. c. Tanger, 10 m</td>
<td>35°35', 5°59'</td>
<td>ST, TS et al. 18/03 m</td>
<td>&lt; 50</td>
<td>227</td>
<td>42.29</td>
<td>2</td>
<td>5.16</td>
<td>24.00</td>
<td>0.0702</td>
<td>0.214***</td>
</tr>
<tr>
<td>Pop. 10. Asilah, 10 m</td>
<td>35°29', 6°01'</td>
<td>ST, TS et al. 20/03 m</td>
<td>600–1000</td>
<td>267</td>
<td>54.31</td>
<td>2</td>
<td>8.87</td>
<td>37.56</td>
<td>0.1064</td>
<td></td>
</tr>
<tr>
<td>S Loukos river (SL)</td>
<td></td>
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<tr>
<td>Pop. 11. Larache, 20 m</td>
<td>35°07', 6°09'</td>
<td>ST, TS et al. 31/03 m</td>
<td>100–500</td>
<td>286</td>
<td>55.24</td>
<td>13</td>
<td>24.15</td>
<td>41.61</td>
<td>0.1206</td>
<td>0.156***</td>
</tr>
<tr>
<td>Pop. 12. M.–Bousselhem, 10 m</td>
<td>34°43', 6°15'</td>
<td>ST, TS et al. 51/03 m</td>
<td>100–500</td>
<td>301</td>
<td>59.47</td>
<td>16</td>
<td>25.52</td>
<td>48.39</td>
<td>0.1365</td>
<td></td>
</tr>
<tr>
<td>S Sebou river (SS)</td>
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<tr>
<td>Pop. 13. La Mamora, 37 m</td>
<td>34°15', 6°19'</td>
<td>ST, TS et al. 71/03 m</td>
<td>2.10^4</td>
<td>322</td>
<td>68.32</td>
<td>18</td>
<td>32.43</td>
<td>57.19</td>
<td>0.1590</td>
<td>0.057***</td>
</tr>
<tr>
<td>Pop. 14. c. Kénitra, 46 m</td>
<td>34°13', 6°35'</td>
<td>ST, TS et al. 53/03 m</td>
<td>&gt; 10^7</td>
<td>340</td>
<td>69.41</td>
<td>26</td>
<td>46.40</td>
<td>62.89</td>
<td>0.1675</td>
<td></td>
</tr>
</tbody>
</table>

***P < 0.001.

the Strait, we would expect to find the strongest genetic differentiation between regions separated by these barriers. We will test the genetic differentiation found in groupings defined according to these hypotheses through analysis of molecular variance (AMOVA).

Materials and methods

Study species

Hypochaeris salzmanniana is a species of maritime sands or adjacent woodlands with a restricted distribution along the coast of southwestern Spain (from Conil to Algeciras) and the Atlantic coast of Morocco from Tanger to Kenitra (Fig. 1a). H. salzmanniana is grouped in sect. Hypochaeris. Recent studies on the phylogeny and genetic structure of the four species comprising the monophyletic Hypochaeris sect. Hypochaeris (H. glabra, H. radicata, H. salzmanniana, and H. arachnoidea; Tremetsberger et al. 2004, 2005) demonstrate that H. salzmanniana and H. arachnoidea are sister species, and this clade is sister to H. radicata. Both H. salzmanniana and H. arachnoidea are annual herbs with 2n = 8 chromosomes with allopatric distributions and different habitats. H. arachnoidea grows in dry pastures from 100 to 1800 m above sea level in the mountains of Morocco and Algeria. Both species are interfertile and readily hybridize under experimental conditions (Ortiz, unpublished observations), but natural hybrids have never been found.

A first approach to the population structure of H. salzmanniana in Spain (Tremetsberger et al. 2004) showed that three populations located to the north of the river Barbate (Conil, Barbate and Los Caños) form a panmictic group that is different from those to the south of this river (Zahara, Punta Paloma and Los Algarbes) and also from two populations in Algeciras Bay (Palmones and La Línea).

Subsequently, breeding system studies (Ortiz et al. 2006) on H. salzmanniana plants sampled from the same Spanish populations, and also from populations in Morocco, established that all plants are self-incompatible (SI) with the exception of the populations south of the river Barbate, which consist entirely of self-compatible (SC) plants, and those north of this river, where the populations are semicompatible with SI and SC individuals co-occurring.

Study populations

We sampled 14 populations (Fig. 1a, Table 1) from throughout the distributional range of H. salzmanniana
Fig. 1 (a) Map of the Atlantic coast from the south of Spain, Strait of Gibraltar, and NW Morocco. The studied populations of *Hypochaeris salzmanniana* are indicated by the numbered circles. The land elevation is shown in metres (75, 150, 225, 300 and 450 m) as well as the sea depth (100 m and 200 m). Map modified from the DMAA Center (compiled 1965, revised 1991) Morocco, TPC G-1D, St Louis, Missouri, USA, (b) NJ dendrogram of the 140 individuals analysed of *H. salzmanniana*, rooted with *Hypochaeris arachnoidea*, based on Nei & Li’s genetic distance. Bootstrap values based on 1000 permutations, if higher than 50% are indicated at each node. For details of populations, see Table 1.
During the spring of 2001 and 2003. Fresh leaves from 10 individuals per population were dried in silica gel to give a total of 140 samples. These samples included eight populations from Spain [three populations from the north side of Barbate river (NB), three from the south of this river (SB) and two from Algeciras Bay (AB)]; and six populations from Morocco [two populations from the north of the Loukos river (NL), two populations from the south of this river (SL), and two from the south side of Sebou river (SS)]. These samples include all known populations of this species with the exception of one from the south of the Loukos river and one from the south of the Sebou river. For purposes of discussion, we initially considered these population samples in six geographical areas: three in Spain (NB, SB and AB), and three in Morocco (NL, SL and SS). Population size was estimated through direct censuses when the number of individuals was lower than 100 individuals and, if the number of individuals was higher, through the estimation of the density of individuals in transects and the extrapolation to the total surface occupied.

To root the tree, we have included as an outgroup in the analysis five individuals of *Hypochaeris arachnoidea* (the species closest to *H. salzmanniana*) sampled from a population at Cap de l’Eau, near Nador (Morocco). Vouchers of all sampled populations are deposited in the Herbarium of the University of Seville (SEV, Spain) and University of Vienna (WU, Austria).

**DNA isolation and AFLP analysis**

Total genomic DNA was extracted from dry leaf material following a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987), and the quality of the extracted DNA was checked on 1% TAE-agarose gels as in Tremetsberger et al. (2004). The average DNA concentration was estimated photometrically (UV 160 A Spectrophotometer, Shimadzu). The AFLP procedure followed established protocols (Vos et al. 1995; PE Applied Biosystems 1996) with modifications (Tremetsberger et al. 2004). We used nine primer combinations, six of which were used by Tremetsberger et al. (2004) for the Spanish populations, and three additional primer combinations were used in this study for better resolution. The nine final primer combinations for the selective polymerase chain reaction (PCR) were: *Mse*I-CTG/*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-CTGA/*Eco*RI-AAC (NED), *Mse*I-CAC/*Eco*RI-ACT (FAM), *Mse*I-CTC/*Eco*RI-ATC (HEX) and *Mse*I-CTG/*Eco*RI-AAC (NED). The fluorescence-labelled selective amplification products were run on an automated sequencer (ABI 377, Perkin Elmer). Raw data were scored and exported as a presence/absence matrix using ABI PRISM GENESCAN Analysis Software 2.1 (PE Applied Biosystems) and GENEOGRAPHER (version 1.6.0, Montana State University 2001; available at http://hordeum.oscs.montana.edu/ genographer/).

The AFLP datamatrix was submitted to TreeBase (study Accession no. SN2812).

**Statistical analyses**

The presence/absence matrix originated with the nine primer combinations was imported into PAUP* (version 4.0b10; Sinauer Associates). To represent overall genetic relationships among all individuals analysed of *H. salzmanniana*, we constructed a dendrogram applying the neighbour-joining method (NJ) in conjunction with Nei & Li (1979) genetic distances with the sister species *H. arachnoidea* used to root the tree. Support for each node was tested by 1000 bootstrap replicates. The UPGMA distance and Jaccard algorithms were also applied to the data matrix and resulted in a very similar dendrogram (data not shown).

As a measure of within-population diversity, we assessed the total number of fragments per population (Fragtot), the percentage of polymorphic fragments (Fragpoly), as well as the number of private fragments (Fragpriv; confined to one population or metapopulation) for all populations of *H. salzmanniana*. We also assessed the number of fragments that were shared exclusively between pairs or groups of populations (shared private fragments), and we looked for ‘rare fragments’, i.e. fragments that were found in less than 10% of the individuals within a population (Stehlik et al. 2002). The latter were used to calculate another index of diversity DW (‘frequency-down-weighted marker values’). For each population, the number of occurrences of each AFLP marker in that population was divided by the number of occurrences of that particular marker in the total data set. Finally these values were summed (Schönswetter & Tripsch 2005).

As another measure of genetic variability, we also calculated the average gene diversity (*H*<sub>DP</sub>; ARLEQUIN version 3.01; Excoffier, Laval & Schneider 2005): 

\[ H_D = 1 - \sum \chi_i^2, \]

where \( \chi_i \) is the population frequency of each ‘allele’ (1 or 0) at locus \( i \). The average gene diversity is the average of this quantity across all loci (Lowe et al. 2004). *H*<sub>D</sub> differences among metapopulations were assessed with a general linear model (GLM; SAS Institute 1989) considering the metapopulation as a fixed effect. The Shannon diversity index is widely used in Ecology and applied also as a measure of genetic diversity: it was calculated as 

\[ H_{SI} = -\sum p_i \ln(p_i), \]

where \( p_i \) is the relative frequency of the \( i \)th fragment in a population (FAMD 1.02, Schlüter & Harris 2006).

A statistical approach to genetic differentiation was the analysis of molecular variance (AMOVA; ARLEQUIN version 3.01; Excoffier, Laval & Schneider 2005) that we undertook with three different groupings of the *H. salzmanniana* populations. The first grouping (a) has two hierarchical levels
and describes differentiation among all populations of the species. Groupings b and c have three hierarchical levels and each describes differentiation among two geographical areas: between Spanish populations north of the Strait of Gibraltar (NB, SB, AB) and Moroccan populations south of the Strait (NL, SL, SS; grouping b); and between Spanish and Moroccan populations north of the Loukos river (NB, SB, AB, NL) and the extension of the Rif mountain in northern Morocco and Moroccan populations south of this geographical barrier (SL, SS; grouping c). In this way, we aimed to test the two different hypotheses (groupings b and c) of which geographical barrier had the largest effect on genetic differentiation in *H. salzmanniana*. The AMOVA-derived fixation index *F*<sub>ST</sub> (Arlequin version 3.01; Excoffier, Laval & Schneider 2005) describes the reduction in heterozygosity within populations relative to the total population (Wright 1951) and is an indirect approach to estimate gene flow. The confidence interval of the *F*<sub>ST</sub> values was determined through bootstrapping (20000 replicates) as implemented in Arlequin version 3.01 (Excoffier, Laval & Schneider 2005).

To evaluate the correlation between *F*<sub>ST</sub> and geographical distance between populations in *H. salzmanniana*, we used two-tailed Mantel tests based on Spearman correlations (on 10<sup>6</sup> random permutations). We also tested whether there was a correlation between the average genetic diversity and the population size using a Spearman nonparametric correlation. For basic statistical analyses (GLM and Spearman correlation) we used JMP 4.0.1 (SAS Institute Inc. 1989) except for Mantel tests where we used XLSTAT-PRO 7.5.3 (Addinsoft).

**Results**

**Population level**

The nine primer combinations employed with samples from 14 populations of *Hypochaeris salzmanniana* and its sister species *Hypochaeris arachnoidea* generated a total of 546 fragments, ranging from 60 to 500 bp, with an average of 60.7 fragments per primer combination, of which 487 (89.2%) AFLP markers were polymorphic. The number of fragments for each primer combination (with percentage of polymorphisms within parenthesis), were: Msel-CTCG/EcoRI-ATC: 52 (90.4%), Msel-CAC/EcoRI-ACG: 66 (93.9%), Msel-CTA/EcoRI-ACC: 93 (91.4%), Msel-CTG/EcoRI-ACA: 59 (89.8%), Msel-CTC/EcoRI-AGG: 72 (93.1%), Msel-CTGA/EcoRI-AAC: 62 (95.2%), Msel-CAC/EcoRI-ACT: 51 (72.5%), Msel-CTC/EcoRI-ATC: 46 (87.0%) and Msel-CTG/EcoRI-AAC: 45 (82.2%). This number of fragments was sufficient to distinguish all individuals as separate phenotypes.

Our analysis of AFLPs for the eight Spanish populations effectively duplicated those made by Tremetsberger et al. (2004), i.e. we did new analyses of AFLP fragments from the same samples from the same populations. Our analyses were made with three extra primer combinations in addition to those used in this previous study. The results show high congruence with, and replicate results for part of the population system of *H. salzmanniana* and provide confidence in the AFLP parameters employed in these studies.

*Hypochaeris salzmanniana* shows a very well-supported large cluster (Fig. 1b) with 85% BS, that separates four populations situated to the south of the river Loukos (SL and SS, from Larache to Kenitra), from all of the rest. The remaining populations form three groups: the first, which is well supported with 78% BS, comprises the populations from Algeciras Bay (AB, 7–8) in Spain; the second consists of a not very well supported cluster with the populations from the north of the Loukos river (NL, 9–10) in Morocco; and the third, weakly supported at 57% BS, includes the populations from the Atlantic coast of Spain. Within the latter, one can distinguish an independent branch with 92% BS that includes the populations at Punta Paloma (5) and Los Algarbes (6), whereas the population at Zahara (4), although well supported with 88% BS, is immersed in the group formed by the three populations from the north of Barbate (NB, 1–3).

The population at Kenitra (14) presents the largest genetic diversity for all parameters, while the population at Punta Paloma (5) is the least diverse. The most diverse population at Kenitra is also the largest population (> 10<sup>7</sup> individuals), and that at Punta Paloma is a very small population (less than 50 individuals) (Table 1). However, over all populations there is no correlation between size and average genetic diversity (Spearman ρ = 0.4045, *P* = 0.1514, *n* = 14).

The Mantel tests, which compared the pairwise distances between populations (in direct line in kilometres) with their respective *F*<sub>ST</sub> values, indicated that there is no correlation between these parameters (*r* = 0.059; *P* = 0.725). This correlation is significant (*r* = 0.466; *P* = 0.004) when removing from the analysis the populations from the south of the river Barbate (SB, 4–6).

**Regional level**

The results of the analyses of molecular variance (AMOVA; Table 2) reveal that the regions separated by the Loukos river and the extension of the Rif mountain in Morocco (grouping c) are more strongly genetically differentiated (AMOVA-derived *F*<sub>ST</sub> = 0.176; 95% confidence interval = 0.140–0.212) than those separated by the Strait of Gibraltar (grouping b; AMOVA-derived *F*<sub>ST</sub> = 0.119; 95% confidence interval = 0.093–0.148). As the upper 1-tailed critical value (*α* = 0.05) for grouping b was of 0.143, genetic differentiation in grouping c (0.176) was significantly larger than in grouping b.

The south Sebou populations (13–14) had the highest significant average gene diversity (Tukey-Kramer HSD
test, \( q^* = 2.85; \alpha = 0.05 \). The average gene diversity of south Loukos populations (11–12) was not statistically different from that of the north Barbate and from the Algeciras Bay populations, and the average gene diversity of the south Barbate populations (4–6) was not statistically different from north Barbate populations (1–3) and north Loukos populations (9–10) (Fig. 2).

With respect to the numbers of shared private fragments between regions, the region to the south of the Sebou river (SS) is the only one to share fragments with all other regions (Table 3), followed by north Barbate (NB), which shares private fragments with all populations except those of the north Loukos (NL).

The group of populations that possesses most private fragments are south Sebou and south Loukos (54 and 32 fragments, respectively), and those with the least number of private fragments are south Barbate and north Loukos (6 and 7 private fragments, respectively).

The values of the pairwise fixation index \( (F_{ST}) \) between regional groups (Table 3) indicate that the following populations are most related: south Sebou and south Loukos \( (F_{ST} = 0.09) \), north Barbate and south Barbate \( (F_{ST} = 0.11) \), north Barbate and north Loukos \( (F_{ST} = 0.14) \), and Algeciras Bay and north Loukos \( (F_{ST} = 0.14) \). South Barbate is the metapopulation that is least related to the others.

### Discussion

The neighbour-joining dendrogram (Fig. 1b) shows that the populations of *Hypochaeris salzmanniana* are genetically structured into two geographical areas: those to the south of the Sebou river at La Mamora and Kenitra (populations 13–14) have a high affinity with those to the south of the river Loukos at Larache and M. Bousselham (populations 11–12; Fig. 1b, Table 1), while in contrast, the Moroccan populations at Tanger and Asilah (9–10) link with all Spanish populations (Algeciras Bay, and south and north of the Barbate river). Clustering among Spanish populations corresponds to that obtained by Tremetsberger *et al.* (2004) with six primer combinations, but in this study using nine primer combinations, the groups are more strongly supported. Possibly, the extension of the Rif mountain that reaches the Atlantic coast in the region of Larache (see Fig. 1a) may have acted as a natural frontier separating the coastal areas to its north (populations 1–10; territories of the Betic-Rifian Cordillera) from those to its south (populations 11–14; valleys of the rivers Loukos and Sebou) during interglacial periods.

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**Table 2** Results of analyses of molecular variance (amova) of AFLP data (squared Euclidean distance) from 14 populations of *Hypochaeris salzmanniana*. Groupings b and c were used to test the effectiveness of geographical barriers in *H. salzmanniana* by maximizing the percentage of variation among regions. For abbreviations of populations and regions see Fig. 1 and Table 1; d.f. degrees of freedom; SS, mean sum of squares.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>N</th>
<th>Source of variation</th>
<th>d.f.</th>
<th>SS</th>
<th>Variance components</th>
<th>Percentage of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>a [1–14]</td>
<td>14</td>
<td>Among populations</td>
<td>13</td>
<td>2065.293</td>
<td>13.34695</td>
<td>34.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Among individuals</td>
<td>126</td>
<td>3200.300</td>
<td>25.39921</td>
<td>65.55</td>
</tr>
<tr>
<td>b [NB, SB, AB], [NL, SL, SS]</td>
<td>2</td>
<td>Among groups</td>
<td>1</td>
<td>468.805</td>
<td>4.89687</td>
<td>11.93</td>
</tr>
<tr>
<td>Strait of Gibraltar</td>
<td></td>
<td>Among populations</td>
<td>12</td>
<td>1507.880</td>
<td>10.76414</td>
<td>26.22</td>
</tr>
<tr>
<td>c [NB, SB, AB, NL], [SL, SS]</td>
<td>2</td>
<td>Among groups</td>
<td>1</td>
<td>557.413</td>
<td>7.55573</td>
<td>17.58</td>
</tr>
<tr>
<td>Extension of the Rif mountain in Morocco</td>
<td></td>
<td>Among populations</td>
<td>12</td>
<td>1507.880</td>
<td>10.02575</td>
<td>23.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Among individuals</td>
<td>126</td>
<td>3200.300</td>
<td>25.39921</td>
<td>59.09</td>
</tr>
</tbody>
</table>
Effectively, the populations to the south of the Loukos river valley share the highest parameters of genetic diversity, particularly with regard to the number of private fragments and rare fragments found in them. On the assumption that such fragments accumulate through time and are therefore a measure of population antiquity (Stehlik et al. 2002; Schönswetter & Tribsch 2005), we can assume that the populations south of the Sebou river (SS) and south of the Loukos river (SL) are the oldest populations in this species system. That the Sebou populations are the only ones to have some pairwise ‘shared private fragments’ with all other populations located in northern Morocco and the Iberian Peninsula also supports this view. This reasoning is similar to that used to support the antiquity proposed for Hypochaeris palustris in the Coastal Cordillera of Chile (Muellner et al. 2005), and for eastern populations of Saponaria pumila in the European Alps (Tribsch et al. 2002).

These southern Moroccan populations also represent the largest extant populations of *H. salzmanniana* (> 10⁷ individuals), and it is probably significant that they are located in *Quercus suber* woodlands, adjacent to the sea coast (see Fig. 1a), a habitat similar to that of its Moroccan sister species, Hypochaeris arachnoidea. The ancestor of *H. salzmanniana* (and also of *H. arachnoidea*) may have been an annual herb of *Quercus* woodlands in northern Africa. During the earlier glacializations of the Pleistocene, these forests, mainly *Quercus suber*, expanded to occupy the newly emerged lowlands, as a consequence of the decrease of the sea level. In these new habitats, the Hypochaeris populations neighbouring to the sea coast could expand and diversify. Such events could have originated the present-day *H. salzmanniana*. Subsequently, during the marine transgressions in the interglacial periods, many of these coastal populations of *H. salzmanniana* would have been extinguished, while others became isolated in riverine estuaries and the slopes of the Betic-Rifian Mountains. Consecutive glacial and interglacial periods would favour new expansions, reductions and local extinctions of some populations, and migrations in other cases; these all helped to model the genetic structure and breeding system of this species. The fact that in south Sebou populations, we found a higher genetic diversity and higher number of private and rare fragments in populations that live in *Quercus* woodlands supports the view that such woodlands may be the primitive habitat of *H. salzmanniana*.

These factors lead us to believe that these southern populations are older than those of the northern group. Moreover, *H. arachnoidea* shares more fragments with the south Sebou populations (16 shared private fragments) than with the other regions (11 with south Loukos, 1 with north Loukos, 4 with Algeciras Bay, 1 with south Barbate, and 8 with north Barbate), and this further supports the view that this southern Moroccan area is probably ancestral for both *H. salzmanniana* and its sister taxon *H. arachnoidea*.

Based on Tremetsberger et al. (2005), we consider that the period for active evolutionary expansion of *Hypochaeris* sect. *Hypochaeris* most probably occurred during the more recent glaciations of the Pleistocene. We hypothesize therefore that the species *H. salzmanniana* originated in southern Morocco, as indicated by fragment data, and migrated northwards along the shifting coastline to the area of the Strait of Gibraltar. At glacial maximum, with a much lower sea level prevailing, islands were established in the area of the present Strait (see Fig. 1a), thus providing ‘stepping stones’, and we can assume that *H. salzmanniana* migrated from north Africa into southern Spain via these temporary

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**Table 3** Pairwise shared private fragments (values below diagonal), and pairwise fixation index (AMOVA-derived $F_{ST}$) with 95% confidence interval between regions (values above diagonal) and private fragments (Frag$_{priv}$) of each region, in *Hypochaeris salzmanniana* based on analysis of 490 AFLP fragments (excluding the outgroup *Hypochaeris arachnoidea*).

<table>
<thead>
<tr>
<th></th>
<th>N Barbate</th>
<th>S Barbate</th>
<th>Algeciras Bay</th>
<th>N Loukos</th>
<th>S Loukos</th>
<th>S Sebou</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Barbate</td>
<td>—</td>
<td>0.11</td>
<td>0.19</td>
<td>0.14</td>
<td>0.28</td>
<td>0.27</td>
</tr>
<tr>
<td>S Barbate</td>
<td>2</td>
<td>0.18</td>
<td>(0.12–0.25)</td>
<td>0.16</td>
<td>0.28</td>
<td>0.27</td>
</tr>
<tr>
<td>Algeciras Bay</td>
<td>1</td>
<td>1</td>
<td>—</td>
<td>0.14</td>
<td>0.25</td>
<td>0.21</td>
</tr>
<tr>
<td>N Loukos</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.18</td>
<td>0.18</td>
<td>0.21</td>
</tr>
<tr>
<td>S Loukos</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.13–0.24</td>
<td>0.16–0.25</td>
</tr>
<tr>
<td>S Sebou</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>Frag$_{priv}$</td>
<td>20</td>
<td>6</td>
<td>13</td>
<td>7</td>
<td>32</td>
<td>54</td>
</tr>
</tbody>
</table>
land connections and further migrated along the much more extensive southwestern Spanish coastline.

The extant north Moroccan populations (NL) and Spanish populations (AB, SB and NB) of *H. salzmanniana* all show much lower values than the other populations for private and rare fragments (Table 1), which indicates that the northwards migratory history of this species may have been affected adversely by diverse genetic bottlenecks and founder effects. Such population expansions and reductions were also accompanied in some areas by changes in breeding system (Ortiz et al. 2006).

We consider below in more detail the possible factors that may have affected the genetic diversity in these northerly populations.

**North Loukos and Algeciras Bay populations**

The populations NL (Asilah and Tanger, 9–10) and AB (Palmones and La Línea, 7–8) are small, with only 50–1000 individuals. However, as in the southern Moroccan populations (SL, 11–12; SS, 13–14), NL and AB comprise only self-incompatible plants (Ortiz et al. 2006), i.e. with obligate outbreeding. We might expect these outcrossing populations to show high levels of genetic diversity. In fact, while average gene diversity is reasonably high (mean $H_D = 0.088$ and 0.114, respectively), in comparison with the southern Moroccan populations (overall mean $H_D = 0.146$), values for private and rare fragment numbers are much lower than for SL and SS. On the other hand, the values for polymorphic fragments and Shannon diversity index are relatively high. These characteristics indicate that both the NL and AB populations are probably old populations that have suffered bottleneck events and fragmentation since the peak expansion of *H. salzmanniana*, perhaps in part due to historic changes in the coastline, with rising sea levels allowing mountain uplands to have greater influence, and possibly in part due to more recent human perturbations.

**South Barbate populations**

These three populations, Zahara (4), Punta Paloma (5), and Los Algarbes (6), occupy an area bounded by the river Barbate and an extension of the Sierra de San Bartolomé near to Tarifa, the latter being the most southerly extension of the Iberian Peninsula. All three populations are genetically depauperate, suggesting that they might have originated through recent colonization. However, the NJ dendrogram (Fig. 1b) and the fixation index ($F_{ST} = 0.37$) calculated for these two populations, Punta Paloma (5) and Los Algarbes (6), is surprisingly high considering that they are only 1 km distant from each other.

It is significant that the three populations south of the Barbate river are the only fully self-compatible (SC) populations found in *H. salzmanniana* (Ortiz et al. 2006). A selfing mating system might arise as a consequence of bottleneck or founder events, which dramatically reduce the number of individuals in a population. This reduction in population size could enhance the probability that a low-frequency self-compatibility allele of the S-locus might become homozygous and eventually become fixed in the populations by chance. Moreover, a selfing mating system would explain the depauperate genetic structure of these populations regardless of their age and origin. Low levels of genetic diversity as a consequence of selfing have been reported in the SC species *Lasthenia maritima* (Heliantheae, Asteraceae), a species derived from the self-incompatible (SI) *Lasthenia minor* (Crawford et al. 1985), and in SC populations of *Leavenworthia crassa* (Brassicaceae) in comparison with SI populations of this species (Liu et al. 1999). Effectively, the probable consequences of the selfing breeding system in these south Barbate populations means that we do not have reliable indicator genetic parameters (private and rare fragment values, etc.) to hypothesize whether they are all relatively recent in origin, or whether some are recent and others are old.

**North Barbate populations**

The populations 1–3 (NB) of *H. salzmanniana* from Conil, Vejer and Caños de Meca are particularly interesting. The population at Conil (1) is large (over 106 individuals) with the other two being small satellite populations. The NJ dendrogram (Fig. 1b) and the fixation index (0.083) (Table 1) indicate that effectively, these three are subpopulations of a single population that may have been recently fragmented, possibly due to urban expansion in this coastal area (Ortiz et al. 2003). Despite the very large population in the NB region, the values for rare and private fragments are surprisingly much lower than those found in the only other large population of *H. salzmanniana* at Kenitra (population 14). Even if the NB populations had passed through a bottleneck event, as it expanded to its present large size, one would expect genetic heterozygosity to regain previous levels, although perhaps with a drastic reduction in the number of alleles (Nei et al. 1975; Allendorf 1986) and rare and private fragments.
Again, the breeding system is important. All of these north Barbate populations (1–3) of H. salzmanniana are ‘semicompatible’ in that they comprise a mix of SI and SC individuals in a 1:1 proportion (Ortiz et al. 2006). This fact was unknown to us when these populations were originally sampled for leaf material, but by random sampling, one would expect approximately half of our 30 leaf samples to come from SC plants and the other half from SI individuals. A consequence of this population structure would be a mixed mating system that would lead to a marked diminution in heterozygosity and in the parameters used to estimate genetic diversity (Fontdevila & Moya 1999). Since H. salzmanniana is predominantly an SI species, as its sister H. arachnoidea is (Ortiz et al. 2006), it is likely that selfing is a recent development in these north Barbate populations.

**The Strait of Gibraltar: how effective as a barrier?**

Analysis of molecular variance (AMOVA) with three hierarchical levels revealed that the genetic differentiation between the areas to the north and south of the extension of the Rif mountains in northern Morocco explain 17.6% of the variance encountered, whereas the differentiation between those populations delimited just by the Strait of Gibraltar, explain only 11.9% (see Table 2). This indicates that when the coastal migration pathway in western Morocco, and island ‘stepping stones’ across the Strait were eliminated by rising seas, mountain ranges became important barriers that must be considered explaining the phylogeography of H. salzmanniana.

If we assume that H. salzmanniana is of Pleistocene age (Tremetsberger, unpublished data; see also Tremetsberger et al. 2005), it is likely that one of the glaciations of this epoch facilitated expansion of Moroccan populations across the Strait of Gibraltar into the Iberian Peninsula. During each of the Pleistocene glaciations, the sea level in the western Mediterranean region was lower than today (Pou 1989; Yokoyama et al. 2005), it is likely that one of the glaciations of this epoch facilitated expansion of Moroccan populations across the Strait of Gibraltar into the Iberian Peninsula. During each of the Pleistocene glaciations, the sea level in the western Mediterranean region was lower than today (Pou 1989; Yokoyama et al. 2000), thereby reducing the distance between European and African coasts; moreover, emergent islands that were present periodically during successive glacial periods in the extreme west of the Mediterranean, in the Strait of Gibraltar area, must have favoured contact between the two continents (Collina-Girard 2001). The Strait of Gibraltar therefore would not have been a major geographical barrier as it is at present.

Once established in the southern Iberian Peninsula, the populations of H. salzmanniana seem to have undergone further vicissitudes. With retreat of the ice sheets, and rising sea levels, the populations north and south of Barbate (1–6) on the one hand, and in Algeciras Bay (7, 8) on the other hand, are likely to have been separated by the steep barrier of the Betic Cordillera (Fig. 1a). This could account for the similarities in genetic structure between the Algeciras Bay and north Loukos populations vis-a-vis those farther north. Moreover, the extension of the Sierra de San Bartolomé seems to have effectively isolated the populations at Punta Paloma (5) and Los Algarbes (6), thereby leading to decrease in population size, genetic depauperation, and self-compatibility. While the Rif and Betic mountain ranges seem to have formed important barriers, this does not seem to have been the case for rivers. Our results show that the populations on both sides of the river Sebou (NB and SB; see Table 3). Therefore, these rivers do not seem to have been insurmountable barriers to gene flow.

In conclusion, our results suggest that (i) H. salzmanniana originated in Morocco from its common ancestor with H. arachnoidea; (ii) the species spread northwards, i.e. from Morocco to Spain; (iii) during this expansion, a loss of within-populational genetic variability occurred, in part probably due to bottleneck events; (iv) self-compatibility in SB populations (4–6) is also associated with a loss of genetic variability; and (v) historically, the Strait of Gibraltar was not a major barrier for H. salzmanniana.

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**References**


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