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Genetic diversity in populations of *Erica andevalensis*, a vulnerable metallophyte species from the Iberian Peninsula

Manoel Bandeira de Albuquerque, Susana Rodríguez-Echeverría and Helena Freitas

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Erica andevalensis is an endemic vulnerable species that grows in metal-polluted soils from the Iberian Pyrite Belt. The genetic diversity of six *E. andevalensis* populations from Portugal and Spain was studied using PCR Inter-simple sequence repeat (ISSR). The obtained data showed that a) there is a very low genetic diversity within the populations from Portugal, and b) the populations sampled in Portugal are genetically different from the Spanish populations. The lack of genetic diversity in the Portuguese populations suggests the existence of founder effects and subsequent genetic isolation. Plant genetic diversity was also low in the Spanish populations, although the analysis showed that the studied populations were different among them. A Mantel test of the correlation between genetic and geographic distances was significant evidencing the genetic isolation by distance of the studied populations. The distinctiveness and low genetic diversity of the Portuguse populations emphasizes the importance of taking measures for their conservation.

M. Bandeira de Albuquerque, S. Rodríguez-Echeverría (susanare@ci.uc.pt) and H. Freitas, Centre for Functional Ecology, Dept of Botany, Univ. of Coimbra, PT–3000-456 Coimbra, Portugal.

Erica andevalensis is a vulnerable endemic metal-tolerant species, described for the first time in 1980 as a species restricted to the waste dumps around pyrite mines from the Andévalo (Huelva, southwestern Spain), where it can be found sporadically within an area of 1500 km^2 (Asensi et al. 1999). In Portugal, *E. andevalensis* has been found at the historical mining centre of the São Domingos mine (60 km southeast of Beja, Alentejo) where the effects of mining activities can be observed in an area about 50 km^2. At this site, *E. andevalensis* populations appear close to the borders of lakes and streams with acid waters (Capelo et al. 1998). Both mining areas (São Domingos mine and Andevalo region) are part of the Iberian Pyrite Belt, a group of massive sulphide deposits that have been known and exploited since pre-Roman times, mainly as gold, silver and copper ore from the supergene enrichment zones of exposed deposits (Barriga 1990). The main environmental impacts of this long-term exploitation are soil impoverishment, water and soil acidification and high levels of soil contamination by trace metals (Aparício 1995, Abreu et al. 2007).

Although *E. andevalensis* can grow intermixed with other plant species in non-polluted soils from the Iberian Pyrite Belt, the tolerance to different trace metals (Cu, Zn, Al, Pb, Co, Hg, Fe and Mn) allows it to form monospecific patches in disturbed, polluted soils around mines and metal-polluted water streams (Blanca et al. 1999, Rodríguez et al. 2007). Extreme edaphic conditions can act as a selective force that may induce plant speciation, which in some cases can occur in a few generations (MacNair 1993, Rajakaruna 2004). Recent established metal-tolerant populations can also exhibit a reduced genetic variability because of the

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founder effect and selective pressure in this very specific environment (Pollard et al. 2002). There is little information about the genetic diversity of strict edaphic endemic species from metal-enriched environments, such as *E. andevalensis*. Other studies focused on the genetic variation in metal-tolerant and non-tolerant populations have produced contradictory results. Mengoni et al. (2000), using RAPDs, found similar levels of genetic diversity in populations of *Silene paradoxa* growing in contaminated and uncontaminated sites. Similar results were also reported for *Arrhenatherum elatius*, another metal-tolerant species (Ducousso et al. 1990). On the other hand, a reduction of genetic diversity was found in metal-tolerant populations of the Cd-hyperaccumulator *Sedum alfredii* when compared with non-tolerant populations (Deng et al. 2007).

Theoretically, rare plants such as edaphic endemics are expected to have low levels of genetic variation both at the species and population levels due to 1) selection under a narrow range of environmental conditions and 2) the small distribution area. Both factors promote genetic drift and inbreeding in small isolated populations (Broadhurst and Coates 2002). The lack of information about the genetic diversity of vulnerable plants can hamper conservation efforts. In fact, the efficient design of conservation strategies for threatened and endangered species depends on the knowledge of the levels of genetic diversity of the target species (Qiu et al. 2005).

Erica andevalensis is considered a vulnerable species because it has a narrow distribution mainly restricted to metal-rich soils. It is also an interesting species for the phytostabilization of mining areas, and conservation could be achieved through the use of this species in restoration projects. Previous studies have found physiological differences between the Spanish and Portuguese populations of *E. andevalensis* (Abreu et al. 2007), but to our knowledge, there is no information on the genetic variability and diversity of this species. The patchy distribution of *E. andevalensis* could contribute to highly differentiated populations, but the production of small seeds easily transported by wind, water or animals could operate in a contrary way. This paper presents a study of the genetic diversity of six populations of *E. andevalensis* from the São Domingos mine (Portugal) and the Andévalo region (Spain) using PCR-ISSR. Our working hypotheses were a) *E. andevalensis* has a low genetic diversity because it is an edaphic endemism, and b) based on the observed physiological differences, the Spanish and Portuguese populations should be genetically different.

Several PCR-based techniques, such as RFLP (Restriction fragment length polymorphism), RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragment length polymorphism) or SSR (Simple sequence repeat, also known as microsatellites), are routinely used to study the genetic diversity of plant populations. Microsatellites are tandemly repeated, short DNA sequence motifs that are ubiquitous components of all eukaryotic genomes and show size-polymorphism within populations. Therefore, several strategies have been developed to use the potential of SSR as population markers. Microsatellite-primed PCR (also called Inter-simple sequence repeat, ISSR) is probably the most widely used of these techniques, and is a powerful tool to investigate the genetic diversity of natural plant populations. ISSR markers generate more reproducible fingerprintings than RAPD amplification due to the longer SSR-based primers, which enables a higher-stringency of the DNA amplifications. However, as a dominant marker, ISSR does not allow to distinguish heterozygotes from homozygous dominant individuals, which prevent a

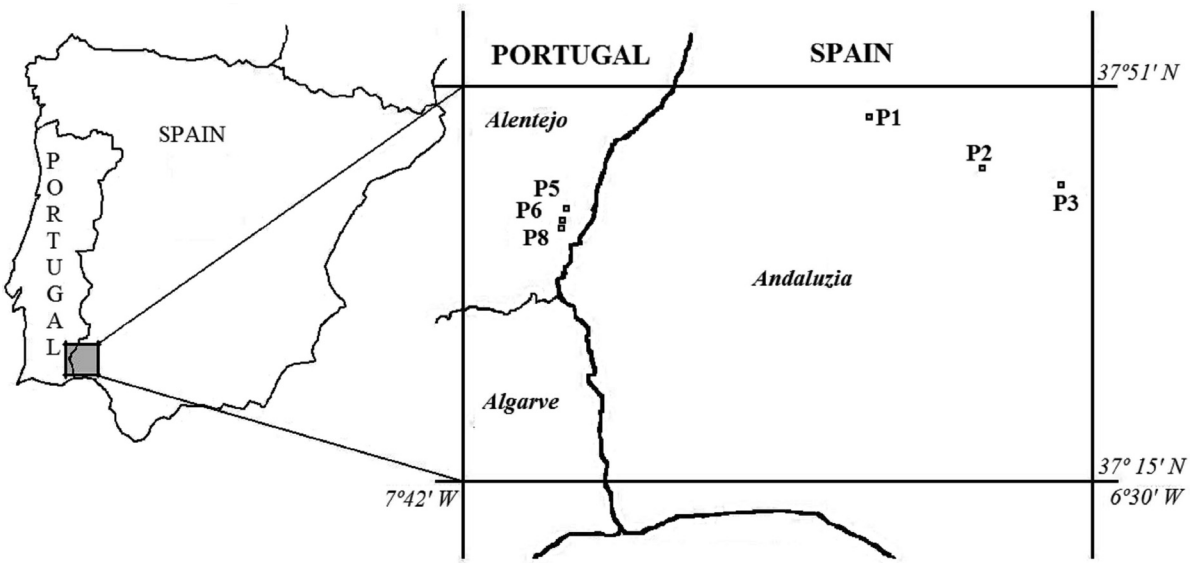

Fig. 1. Location of the sampling sites of *E. andevalensis* populations in Portugal and Spain.

Table 1. A brief description of the sampled populations.

	Location	Habitat	Soil pH	Coordinates
P1	El Lomero	sandy soil with influence of acid drain waters	3.35 ± 0.18	37°48′12″N 6°55′34″W
P2	Odiel river	sandy river terrace	3.75 ± 0.04	37°43′38″N 6°42′27″W
P3	Nerva village	xeric mine tailing	3.91 ± 0.11	37°42′3″N 6°33′21″W
P5	São Domingos	edge of a stabilization lake for acid drain waters	3.61 ± 0.21	37°39′59″N 7°30′5″W
P6	São Domingos	xeric mine tailing	3.15 ± 0.15	37°38′52″N 7°30′36″W
P8	São Domingos	ephemeral metal-polluted stream	3.48 ± 0.08	37°38′4″N 7°30′50″W

direct estimate of inbreeding. This technique was chosen for this study because of its robustness, reproducibility and low cost, and because it does not require previous information about the genome (Meekins et al. 2001, Ge et al. 2005a, 2005b, McRoberts et al. 2005, Li and Jin 2007).

Material and methods

Study area and sampling

Six different populations of *E. andevalensis* were selected in December 2004; three populations from the Andévalo region in Spain and three populations close to the São Domingos mine in Portugal (Fig. 1, Table 1). Young stems and leaves of 12 adult individuals were collected from each population and stored at –20°C for subsequent DNA extraction.

DNA isolation and PCR-ISSR protocol

The extraction of DNA was carried out with a Plant genomic DNA mini-prep kit following the protocol provided with the kit, except that incubation at 65°C was performed for 3 h. After checking their quality by electrophoresis on 0.8% (w/v) agarose gels according to Sambrook et al. (1989), all DNA samples were stored frozen at –20°C until use.

Ten different primers (and six different combinations among primers) manufactured by Sigma-Genosis and previously used in our laboratory for genetic fingerprinting of plants and fungi were screened for the DNA amplification of *E. andevalensis*. All reactions were carried out in a final volume of 25 µl containing 2.5 µl 10× PCR buffer, 200 µM dNTP mix, 0.8 mM primer, 1.5 U Taq polymerase and 1 µl of each DNA extract. The conditions of the PCR were an initial denaturation step of 2 min at 95°C, 35 cycles of 60 s at 95°C, 60 s of annealing (anneling temperatures for each primer, Table 2) and 60 s at 72°C, followed by a single step of 10 min at 72°C. All PCRs were performed in a GeneAmp PCR 9700 and included a negative control reaction without DNA.

An aliquot of the PCR products was loaded on 1.6% agarose gel and electrophoresed at 100 mV for 1 h in 1×TBE buffer. The PCR products were visualized using ethidium bromide staining and UV illumination.

Table 2. Primers, annealing temperatures (Tm) used in this study and PCR results (+ = successful DNA amplification, – = primer failed to amplify *E. andevalensis*).

Primers	Tm (°C)	PCR
$(GACA)_4$	51	+
$(TCC)_5$	55	+
$(GGAT)_4$	55	+
888	52	+
$(GTG)_5$	57	–
$(AAC)_8$	36	–
$(CAT)_5$	42	–
$(GA)_8$	43	–
$(GATA)_4$	40	–
$(TGTC)_4$	48	–
$(GGAT)_4 + (GACA)_4$	53	–
$(TCC)_5 + (GACA)_4$	52	–
$(TCC)_5 + (GGAT)_4$	55	–
$888 + (GACA)_4$	52	+
$888 + (TCC)_5$	53	+
$888 + (GGAT)_4$	52	–

Data analysis

Digital images of the gels were processed with the software BIO-1D. Only bands that were unambiguously scored across all samples, i.e. clear bands in the gel, were included in the analysis.

The obtained binary data matrix was analysed using POPGENE ver. 1.32 (Yeh and Boyle 1997) to calculate the following parameters: the observed number of bands (N), percentage of polymorphic bands (P%), Nei's gene diversity (h) (Nei 1973), Shannon diversity index (Ho) (Shannon and Weaver 1949), expected heterozigosity in the total population (H_T), and mean expected heterozigosity within populations (H_S). The Shannon's diversity index was also used to calculate the average (Hpop) and total (Hsp) genetic diversity and the partitioning of the diversity within populations (Hpop/Hsp) and between populations (Hsp–Hpop)/Hsp.

An analysis of molecular variance (AMOVA) was performed to further explore the genetic structure of the studied populations. AMOVA works with the squared Euclidian genetic distances between all individuals and allows the partitioning of variance among and within populations. AMOVA was performed using the program ARLEQUIN ver. 3.11 (Excoffier et al. 2005).

Using the Tools for population genetic analyses software (TFPGA ver. 1.3), a dendrogram was built based on the Nei's genetic distance (Nei 1972) matrix and unweighted pair group method arithmetic average (UPGMA) to explore the relationship among populations. The topology of the dendrogram was confirmed by a bootstrap test with 1000 replicates. A Mantel test was also performed using TFPGA to test the correlation between geographical distance between pairs of populations and Nei's genetic distance (Nei 1972) (computing 1000 permutations).

Results

Among the 10 primers and six primer combinations tested for ISSR amplification of the *E. andevalensis* total DNA, only four primers and two combinations generated interpretable and reproducible bands. From a total of 57 unambiguous bands, polymorphic bands were detected only for three primers (Table 3).

Table 3. Total number of detected bands, band size range and percentage (%) of polymorphic bands obtained in the six analysed populations of *E. andevalensis*.

Primer	Bands	Size range (bp)	Polymorphic n (%)
$(GACA)_4$	13	1650–250	3 (23.1)
$(TCC)_5$	12	3400–650	2 (16.7)
$(GGAT)_4$	5	1300–500	2 (40.0)
888	10	1250–300	0
888 + $(GACA)_4$	7	900–300	0
888 + $(TCC)_5$	10	1350–250	0

Table 4. Genetic diversity within populations of *E. andevalensis* from Spain and Portugal. N = total number of usable loci, P% = percentage of polymorphism, h = Nei's gene diversity and Ho = Shannon's index.

Population	N	P%	h	Ho
P1	57	7.02	0.0287	0.0419
P2	57	8.77	0.0280	0.0433
P3	57	8.77	0.0234	0.0379
P5	57	0.00	0.0	0.0
P6	57	0.00	0.0	0.0
P8	57	0.00	0.0	0.0
At species level	57	12.28	0.0483	0.0708

The percentage of polymorphic loci at the population level was low in the Spanish populations (ranging from 7.0–8.8%), and non-existent in the Portuguese populations (0%, Table 4). For the Spanish populations, the Nei's gene diversity (h) ranged from 0.0234 to 0.0287 and the Shannon's index (Ho) ranged from 0.0379 to 0.0433. As a consequence of the lack of polymorphic bands in the Portuguese populations, the Nei's gene diversity and the Shannon's index were 0.00. At the species level, the genetic diversity was higher but still with relatively low values (P% = 12.3%, h = 0.0483, Hsp = 0.0708).

The genetic differentiation of the studied populations was analysed using estimates based on both Nei's and Shannon's indices (Table 5). These values indicated a higher level of genetic differentiation between populations than within populations. The AMOVA also revealed that most of the genetic variation (65.2%) detected for *E. andevalensis* occurs among populations with lower values of variation within each population (34.8%, Table 6).

The UPGMA dendrogram based on the matrix of genetic distances separated the six populations into two

Table 5. Coefficients of genetic diversity and differentiation within and between populations of *E. andevalensis.* Hpop = average population genetic diversity, Hsp = total genetic diversity genetic diversity, Hpop/Hsp = partitioning of the diversity within populations, (Hsp–Hpop)/Hsp = partitioning of the diversity between populations, H_S = mean expected heterozygosity within populations, H_T = expected heterozygosity in the total population.

Shannon's index		Nei's gene diversity	
Hpop	0.0205	H_S	0.0133
Hsp	0.0708	H_T	0.0483
Hpop/Hsp	0.290		
(Hsp–Hpop)/Hsp	0.710		

Table 6. Analysis of molecular variance (AMOVA) of six populations of the *E. andevalensis* from Spain and Portugal.

Source of variation	DF	Sum of squares	Variance	% of variation	F_{ST}
Among populations	5	59.764	0.954	65.21	0.652
Within populations	66	33.583	0.509	34.79	
Total	71	93.347	1.462		

groups (Spanish and Portuguese populations). In the Spanish populations a closer relationship between P1 and P3 was detected, while the Portuguese populations were clustered into one unique population (Fig. 2).

The Mantel test showed a significant correlation between the matrix of genetic distances and the corresponding matrix of geographical distances ($r = 0.76$, $p < 0.05$, Fig. 3).

Discussion

The observation of low levels of genetic variation within and between the *E. andevalensis* populations analysed in our work is consistent with the general thought about the genetic diversity of rare endemic species (Broadhurst and Coates 2002, Kothera et al. 2007). Also, geographically distant metalliferous genotypes may be differentially adapted to local conditions (Pauwels et al. 2008). *Erica andevalensis* is a self-compatible species without described auto-pollination events for which insects are essential for pollination. This species also produces a high number of viable fruits and seeds under natural conditions (Aparício and García-Martin 1996). These seeds are very small (0.3–0.4 mm) and could be easily transported by wind, water and animals. It would be expected that the abovementioned factors could promote a larger dispersion of this species in the Iberian Pyrite Belt. However, the strong edaphic preference of *E. andevalensis* to wet, acidic and metal-enriched environments (Nelson et al. 1985, Aparício and García-Martin 1996, Blanca et al. 1999) limits its distribution area and has probably affected the populational genetic structure.

The genetic markers used in the present study revealed low levels of genetic diversity between and within the studied populations from Spain and did not detect polymorphism within the populations from Portugal. Although more genetic markers are needed to conclude that the Portuguese populations are genetically homogeneus, the results obtained in this study indicated that a) there is indeed a very low genetic diversity within these populations from São Domingos, and b) the populations sampled in Portugal are genetically different for the Spanish populations.

Taking into account the reproductive biology of *E. andevalensis*, the genetic homogeneity found for the Portuguese plants might be explained by a founder effect due to deliberate planting of this species in the area and subsequent genetic isolation. The founder effect is the result of establishing a new population by a small number of individuals, carrying only a small fraction of the original population's genetic variation (Mayr 1963). In situations of geographic isolation, gene exchange with external populations is precluded and inbreeding can occur, leading to the reduction of genetic diversity within those isolated populations (He-

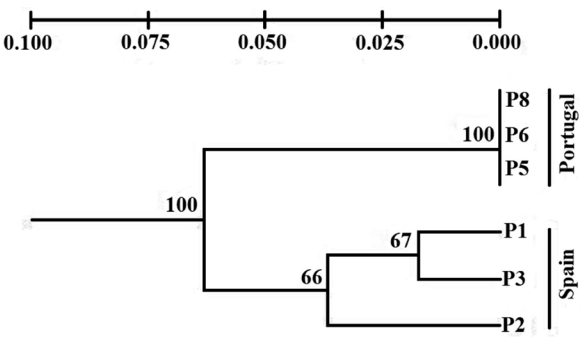

Fig. 2. UPGMA cluster analysis using Nei's genetic distance calculated for six populations of *E. andevalensis*. Portuguese and Spanish populations are indicated.

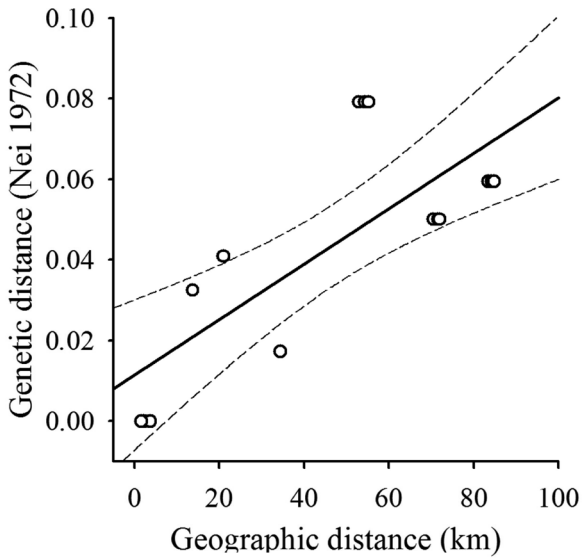

Fig. 3. Genetic distance among *E. andevalensis* populations as a function of geographic distance: $y = 0.0115 + 0.0007\ x$, $r^2 = 0.59$, $p < 0.01$. Dotted lines show 95% confidence limits of regression.

drick 2000, Weising et al. 2005). This effect may be most pronounced in species that form small patch-like populations and are self-compatible like *E. andevalensis*. The high values of population genetic differentiation obtained with either Shannon's indices (0.710) or AMOVA (Fst = 0.652) also confirm the studied populations as distinct groups. The genetic isolation of the Portuguese plants from the Spanish studied populations is further corroborated by the results of the Mantel test, which revealed a significant correlation between the geographical and genetic distances of the sampled *E. andevalensis* populations. These results are not unexpected if we consider the discontinuous spatial distribution of this Ericaceae, a result of its high habitat specificity.

Measures of conservation for *E. andevalensis*, such as the conservation of Gossan rocks, the stabilisation of mine tailings and the avoidance of drastic interventions on the Tinto and Odiel rivers, have already been suggested by the Andalusian Regional Government (Spain) (Blanca et al. 1999). Nevertheless, in Portugal, and to our knowledge, there are no official measures of conservation recommended for *E. andevalensis*. Considering that this plant species only grows in the São Domingos mine, the forementioned measures of conservation should clearly be adopted in this area. Because São Domingos mine is a zone of historic interest with a strong turistic potential, the conservation of *E. andevalensis* could add value to this area from an environmental point of view.

Despite the low genetic diversity found between the studied *E. andevalensis* populations, the geographic isolation might have produced locally adapted genotypes to the soils of São Domingos mine. In fact, Abreu et al. (2007) reported that the range of soil pH in which *E. andevalensis* grows in the São Domingos mine is narrower than that in Spain, suggesting that the Portuguese populations might be different from the Spanish ones. This study provides genetic evidence to support the distinctiveness of the Portuguese populations and stresses the importance of their preservation.

Taking into consideration the singularities of the mining disturbed soils where *E. andevalensis* plants are found we suggest that one effective way to maintain this plant species will be its use as a possible phytostabilizer of mining soils. This active approach could contribute not only to the conservation of *E. andevalensis* but to the amelioration of metal contaminated soils where plant growth is usually impaired.

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