Identification of Metalliferous Ecotypes of *Cistus ladanifer* L. using RAPD Markers

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The genetic diversity of *Cistus ladanifer* ssp. *ladanifer* (Cistaceae) growing on ultramafic and non-ultramafic (basic and schists) soils in the NE of Portugal was studied in order to identify molecular markers that could distinguish the metal-tolerant ecotypes of this species. Random Amplified Polymorphic DNA (RAPD) markers were used in order to estimate genetic variation and differences between populations. The RAPD dataset was analysed by means of a cluster analysis and an analysis of molecular variance (AMOVA). Our results indicate a significant partitioning of molecular variance between ultramafic and non-ultramafic populations of *Cistus ladanifer*, although the highest percentage of this variance was found at the intra-population level. Mantel's test showed no relationship between inter-population genetic and geographic distances. A series of RAPD bands that could be related to heavy metal tolerance were observed. The identification of such markers will enable the use of *Cistus ladanifer* in phytoremediation procedures.

Key words: Cistus ladanifer, RAPD Markers, Metal Tolerance

Introduction

Elevated concentrations of heavy metals in soils may occur as a result of natural processes (such as the weathering of ultramafic rocks) or may accumulate due to anthropogenic influences, such as atmospheric deposition from the combustion of fossil fuels, disposal of wastes such as animal manures or sewage sludge, utilization of fertilizers and agrochemicals, and metal industries or mining (Tiller, 1989; McGrath *et al.*, 2002). Existing technologies for remediation of such soils are usually based on civil engineering methods, which are *ex situ* and expensive. Phytoremediation offers a lower cost method for soil remediation and some extracted metals may be recycled for value (Chaney *et al.*, 1997).

Serpentine outcrops and mine deposits are edaphic discontinuities in mainland regions and have been defined as ecological or edaphic islands (Lefèbvre and Vernet, 1990). The evolution of tolerance to the suite of stress factors present in these islands has led to the genesis of serpentine endemics or to the differentiation of metal-tolerant ecotypes from wide-ranging species (Proctor and Woodell, 1975; Kruckeberg, 1984; Brooks, 1987; Linhart and Grant, 1996). These metal-tolerant species or ecotypes are useful tools for performing phytoremediation procedures.

Random Amplified Polymorphic DNA (RAPD; Williams *et al.*, 1990) analysis is a PCR-based technique that allows the amplification of DNA fragments from anonymous genomes (Hadris *et al.*, 1992) and it has been described as a time- and cost-effective method of surveying genetic variation, at least at low taxonomic levels (Wolff and Morgan-Richards, 1999; Kjølner *et al.*, 2004). Despite the dominant nature of RAPD markers, they have been used extensively in population genetic studies (reviewed in Otero-Arnaiz *et al.*, 1997) and RAPD markers linked to Cd-uptake in durum wheat (Penner *et al.*, 1995) and to Cu-tolerance in populations of *Silene paradoxa* (Mengoni *et al.*, 2000) have been identified.

Cistus ladanifer L. (crimson spot rockrose) is a common perennial shrub species occurring, from sea level to an altitude of 1500 m, in the western Mediterranean region (SW of Europe and N of Morocco and Algeria). Of the three subspecies of *C. ladanifer* described in the Flora Ibérica (Muñoz Garmendia and Navarro, 1993), the ssp. *ladanifer* has a wide distribution: it prefers acid soils but has been found growing on serpentine outcrops in the

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NE of Portugal, S of Spain and N of Morocco (Freitas *et al.*, 2004; Kidd *et al.*, 2004; Alados *et al.*, 1999; Ater *et al.*, 2000) and on mine deposits in SW Portugal and SW Spain (Alvarenga *et al.*, 2004; Toro-Gordillo *et al.*, 2001). This woody plant species could be interesting in phytoremediation programs because of its high growth rate and ability to produce considerable biomass in a relatively short time when conditions permit (Núñez *et al.*, 1989).

In this study, a RAPD procedure was used to identify RAPD bands exclusive to metal-tolerant ecotypes and to analyse the genetic structure and diversity of *Cistus ladanifer* ssp. *ladanifer* populations originating on soils derived from ultramafic and non-ultramafic rocks in the Trás-Os-Montes region of NE Portugal.

Materials and Methods

Population sampling

The plants used were grown from seed collected from four populations of *Cistus ladanifer* ssp. *ladanifer* in the Trás-Os-Montes region (NE Portugal); the populations were selected by Kidd *et al.* (2004). The four sites are of close proximity (linear distances ranged between 3 and 30 km), of similar altitude and climatic conditions, and mainly differed on the basis of the bedrock material and resulting soil properties (Table I). The soils at the four sites were derived from basic (metabasic) rocks (B), ultramafic rocks [ultrabasic (UB) and serpentine (S)] or schists (SC). The sites and their soils were described by Kidd *et al.* (2004). These authors showed ultramafic populations were more Cd- (S), Co- (S and UB), Mn- (S and UB) and Ni-tolerant (S), while the populations B and SC also showed a certain degree of tolerance to Mn (B) and Cu, Zn (SC).

Plant material

Leaves from 24 young plants (6 per population) were used as a DNA source for RAPD analysis. These plants were part of an experiment of heavy metal tolerance carried out in hydroponic solutions by Kidd *et al.* (2004).

DNA extraction

DNA was extracted from leaves following the method of Ziegenhagen and Scholz (1998) for DNA extraction from difficult plant species/tissues. The DNA quality was estimated by visual inspection of bands on 1% agarose gels run in TAE buffer (tris-acetate-EDTA, 1X, pH 8) and stained with ethidium bromide (0.2 μg/ml), and DNA quantification was performed in a fluorometer Ultraspec 2000.

RAPD conditions

RAPD amplifications were carried out on a Hybaid Omn-E thermocycler (Hybaid Ltd., Middlesex, UK) using 25 μl reaction mixture containing 25 ng genomic DNA, 5 pmol of primer, 100 μM of each dNTP (Roche), 1× PCR buffer (10 mM Tris[tris(hydroxymethyl)aminomethane]-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$; Roche) and 0.5 units of *Taq* DNA polymerase (Roche). The reaction mixtures were overlaid with a drop of sterile mineral oil prior to PCR amplification. Since DNA-template concentration has been shown to be the most influential factor in RAPD reproducibility (Ellsworth *et al.*, 1993), prior reactions with different quantities of genomic DNA were performed to determine the most appropriate quantity ensuring a clear and reproducible banding pattern. The PCR schedule was 45 cycles of 1 min at 96 °C, 1 min at 35 °C and 2 min at 72 °C, using the fastest possible transition between each temperature. A negative control was run for each primer. DNA amplification fragments were sepa-

Table I. Mean values of some soil properties of the four study sites: soils developed over basic rocks (B), serpentine rocks (S), schists (SC) or ultrabasic rocks (UB).

Population	pH_{H_2O}*	Mg:Ca (exchangeable)	Co [μg g^{-1}]	Cu [μg g^{-1}]	Mn [μg g^{-1}]	Ni [μg g^{-1}]	Zn [μg g^{-1}]
B	5.6	0.1	94	253	2263	112	118
S	6.5	1.5	178	217	2503	1500	82
SC	4.9	0.6	62	57	2660	78	95
UB	6.0	0.7	94	53	1857	963	72

*1:2.5 soil:water.

rated on 1.4% agarose gels stained with ethidium bromide (0.2 μg/ml) and run in TAE buffer (1×, pH 8). To allow comparison between different gels, a lane with a DNA molecular weight marker (M XVI, 250 bp-ladder, Roche) was included in each electrophoresis. Photographs of gels were performed with a Gel Doc 2000 camera (Molecular Analyst program of Bio-Rad). Amplified bands were designated using the primer code and their size in base pairs, as proposed by Paran *et al.* (1991). Forty 10-mer primers (Operon sets A and B; Operon Technologies, Alameda, CA) were evaluated for suitability in a preliminary screening and 35 primers were selected for the final analyses.

Data analysis

Each PCR product (band) was assumed to represent a single bi-allelic locus. Bands were scored as present (1) or absent (0), and a matrix of the different RAPD phenotypes was assembled. Bands that were monomorphic for the entire data set were not included in the analyses (Huff *et al.*, 1993).

A genetical distance matrix between every pair of individuals was created using $(1-S)$ values, S being the Nei and Li's (1979) Similarity Index. This matrix was produced using the RAPDPLOT program (Black, 1998), and was then used to construct a Neighbour-joining (NJ; Saitou and Nei, 1987) dendrogram by means of the NEIGHBOR program (PHYLIP 3.5c software package; Felsenstein, 1993). The robustness of the RAPD dataset/dendrogram was tested by bootstrap analysis. A total of 500 NJ dendrograms was generated and the CONSENSE program (PHYLIP 3.5c software package) was used to produce a consensus tree. Trees were visualized using the TREEVIEW program (Page, 1996).

The partitioning of molecular variance between edaphic groupings of populations (ultramafic and non-ultramafic), between populations within each group and among individuals within each population was assessed by a nested analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992). Distance measurements used for this analysis were based on Euclidean distances for all pairs of individuals. The significance of the estimated partition of genetic variance was tested with a nonparametric permutational procedure (10,000 random permutations). Pairwise Φ_{st} distances (Excoffier *et al.*, 1992) were calculated among populations and their significance was tested with 3,000 permutations. Matrices of genetic distance and logarithm transformed geographic distances were compared using a Mantel's test (Mantel, 1967), significance was tested with 10,000 random permutations. All analyses were carried out using ARLEQUIN ver 2.001 (Schneider *et al.*, 2000).

Results

RAPD banding pattern

A total of 317 bands were obtained from the RAPD procedure (mean 9.06 bands per primer). Thirty bands, which were shared by all the individuals, were not considered for the statistical analyses. Percentages of polymorphic bands ranged from 67.57 (population B) to 74.15 (population UB), while the global percentage of polymorphism was 90.54 (Table II). Fifty bands were specific to individuals, 13 exclusive to populations (3 in population B, 4 in S, 4 in SC and 2 in UB) and 18 bands shared among plants of the ultramafic (S and UB) populations. These last 31 bands were only observed in plants that showed tolerance to Cd, Co, Cu, Mn, Ni or Zn in hydroponic cultures and they could be related to this metal tolerance (Table III).

Cluster analysis

Dissimilarity $(1-S)$ values (S, Nei and Li's Similarity Index, 1979) ranged from 0.328 (B and SC were the most related populations) to 0.431 (highest distance appeared between the populations SC and UB). Mean values of intra-population differences showed that UB was the most diverse population $[(1-S) = 0.362]$.

The Neighbor-joining dendrogram established two groups, the first of which comprised the ultramafic populations S and UB. The second group was composed of the non-ultramafic populations

Table II. Number of bands, and number and proportion (in parentheses) of polymorphic bands scored from the 35 primers in each population.

Population	No. of bands	No. of polymorphic bands (%)
B	222	150 (67.57)
S	225	160 (71.11)
SC	228	163 (71.49)
UB	236	175 (74.15)
Total	317	287 (90.54)

Table III. Obtained RAPD bands that could be related with tolerance to heavy metals in *Cistus ladanifer*.

Bands	Present in population	Possibly related with tolerance to
$OPA06_{2400}$, $OPA09_{1700}$, $OPB13_{1000}$	B	Mn
$OPA02_{840}$, $OPA02_{910}$, $OPA09_{2900}$, $OPB14_{2060}$	S	Cd, Co, Mn, Ni
$OPA02_{1420}$, $OPA03_{570}$, $OPA08_{670}$, $OPA11_{1000}$, $OPA16_{650}$, $OPA16_{1260}$, $OPA18_{1040}$, $OPA18_{640}$, $OPB03_{1770}$, $OPB03_{1370}$, $OPB03_{1190}$, $OPB04_{600}$, $OPB04_{1900}$, $OPB04_{1450}$, $OPB06_{1780}$, $OPB10_{800}$, $OPB10_{700}$, $OPB14_{970}$	S, UB	Co, Mn
$OPA16_{1670}$, $OPA17_{1700}$, $OPA19_{900}$, $OPB04_{850}$	SC	Cu, Zn
$OPA07_{810}$, $OPB19_{820}$	UB	Mn

B and SC (Fig. 1a). Within each group, however, populations were not clearly defined, since some individuals from different populations appeared intermixed. These results were reinforced by the consensus tree, obtained from the 500 bootstrapped dendrograms (Fig. 1b).

Partition of variance

The nested AMOVA analysis showed significant differences between the ultramafic and non-ultramafic population groups (this factor accounted for 19.13% of total molecular variance), while the percentage of variation contributed by intra-group differences was only 6.41. The highest percentage of genetic diversity was found at the intra-population level (74.46%) (Table IV). Genetic distances among populations obtained from the AMOVA (Φ_{st} values between pairs of populations) were significant, showing that the smallest separation did not correspond with the populations of closest proximity (Table V). The Mantel's Test showed that there was no relationship between genetic and log-transformed geographic distances among populations ($r = -0.026$; $P > 0.05$, after 10,000 random permutations).

Table V. Relationship between matrices of genetic and log-transformed geographic (*italics*) distances (expressed in km) using Mantel's correlation test (Mantel, 1967). Genetic distances are expressed as Φ_{st}. * Significant Φ_{st} values ($P < 0.05$, after 3,000 permutations).

	B	S	SC	UB
B	–	0.281*	0.068*	0.251*
S	*1.20*	–	0.243*	0.090*
SC	*1.16*	*1.48*	–	0.247*
UB	*0.58*	*1.11*	*1.25*	–

Discussion

To our knowledge this is the first report estimating genetic variation in populations of *C. ladanifer*. The RAPD technique was shown to be useful, since we obtained 317 different amplification products with no prior knowledge of any genome sequence of this species.

The cluster and AMOVA analyses performed on the RAPD data showed similar patterns of genetic structure in the four populations of *C. ladanifer* studied.

Table IV. Nested analysis of molecular variance (AMOVA) for 24 individuals of *Cistus ladanifer* from 4 populations, employing 287 RAPD markers. Statistics include: d.f., degrees of freedom; SSD, sum of squares; Sig., probability of obtaining a more extreme component estimate by chance alone (after 10,000 random permutations) and Percentage of Variation contributed by each component. Φ_{ct} is the correlation of random pairs of haplotypes drawn from a group relative to the correlation of pairs of random haplotypes drawn from the whole population. Φ_{sc} is the correlation of random pairs of haplotypes drawn from a population relative to the correlation of pairs of random haplotypes drawn from the whole group, averaged over all populations. Φ_{st} is the correlation of random pairs of haplotypes drawn from within sub-populations relative to the correlation of pairs of random haplotypes drawn from the whole population.

Source of Variation	d.f.	SSD	Variance components	Sig.	% of variation	Φ Statistics
Between groups	1	119.667	6.68403 Va	< 0.001	19.13	$\Phi_{ct} = 0.19129$
Between populations within groups	2	78.917	2.24028 Vb	< 0.001	6.41	$\Phi_{sc} = 0.07928$
Within populations	20	520.333	26.01667 Vc	< 0.001	74.46	$\Phi_{st} = 0.25541$

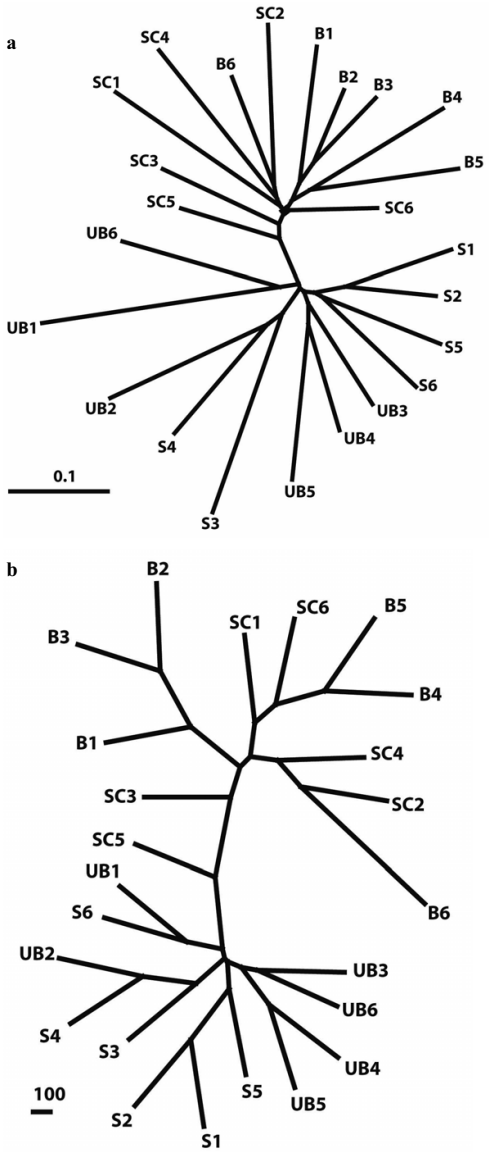

Fig. 1. (a) Neighbour-joining unrooted dendrogram (NJ; Saitou and Nei, 1987) based on Nei and Li's (1979) Similarity Index showing genetic relationships within and among populations. (b) Consensus tree based on 500 NJ dendrograms showing genetic relationships within and among populations. The populations are originated on soils derived from four rock types: basic (B), serpentine (S), schists (SC) and ultrabasic (UB). Bar indicates the genetic distance between individuals.

The Neighbor-joining dendrogram showed a clear separation between edaphic groups (ultramafic and non-ultramafic), whereas differences between populations (S/UB and B/SC) were less clear. These findings were supported by the results of the AMOVA: 19.13% of the total molecular variance was found between edaphic groups, and only 6.41% of the genetic variation was contributed by inter-population/intra-group differences.

The obtained Φ_{st} value (0.255) showed that genetic diversity is higher within *C. ladanifer* populations than between populations. This falls within the expected range for an outcrossing species (Loveless and Hamrick, 1984, and Hamrick and Godt, 1990, from protein data; Bartish *et al.*, 1999; Bussell, 1999, from RAPD data). Relatively high values of the estimates of inter-population divergence indicate a strong genetic structure. This is supported by the values of Φ_{st} distances (significantly different from 0) between pairs of *C. ladanifer* populations, indicating evidence of genetic isolation.

The lack of correlation between the matrices of genetic distances and log-transformed geographic distances in the Mantel's test indicates that geographical isolation (or isolation-by-distance) is not the main process accounting for the distribution of genetic variation among the *C. ladanifer* populations. Unfortunately, with the obtained RAPD data it is not possible to determine wether the origin of this population structuring could be related to phylogeopraphy of this species or if the genetic similarity between S and UB populations could be a convergence produced as result of the influence of the different stress factors present in ultramafic areas (high heavy metal content, nutrient deficiencies, low Ca/Mg ratio, high temperature, low moisture).

The existence of RAPD molecular markers distinguishing metal-tolerant ecotypes of this species would enable the use of *C. ladanifer* in phytoremediation procedures. However, further research will be necessary in order to confirm the validity of these markers.

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