Plant Soil (2010) 334:161–174
DOI 10.1007/s11104-010-0368-4

REGULAR ARTICLE

Chloroplast microsatellites reveal that metallicolous populations of the Mediterranean shrub *Cistus ladanifer* L have multiple origins

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Received: 3 December 2009 / Accepted: 25 March 2010 / Published online: 14 April 2010

Abstract *Cistus ladanifer* L. (*Cistaceae*) is a Mediterranean shrub covering different kinds of soils in the Western Mediterranean area. This species has colonised several metalliferous areas (serpentine outcrops as well as human-polluted sites) throughout its distribution range, and is therefore an interesting species to study the possible effects on genetic diversity and differentiation produced by the colonisation of areas polluted with heavy metals. The genetic structure of 33 natural populations distributed across its entire natural distribution range (Morocco, Portugal and Spain) and growing on either metalliferous or non-metalliferous soils was investigated using chloroplast microsatellites. Population genetic parameters were estimated and genetic groups were identified using Bayesian inference. In addition, we compared the genetic diversity and differentiation among metallicolous and non-metallicolous populations within each Bayesian-defined group. The cpSSR data suggested that metallicolous populations of *Cistus ladanifer* have arisen through multiple independent evolutionary origins within two different chloroplast lineages. Evidence that the soil type provoked genetic bottlenecks in metallicolous populations or genetic differentiation among metallicolous and non-metallicolous populations was not observed. Historical factors are the main cause of the present genetic structure of *C. ladanifer*. The nature of tolerance to heavy metals as a species-wide trait in this shrub is discussed.

Keywords Chloroplast microsatellites · *Cistus ladanifer* · Genetic diversity · Heavy metals · Ultramafic areas

Responsible Editor: Fangjie Zhao.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-010-0368-4) contains supplementary material, which is available to authorized users.

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Introduction

Sites with high heavy metal contents in soils, either of natural origin (such as weathering of ultramafic bedrocks) or generated by anthropic activities (mining and industrial activities, atmospheric deposition, excessive use of agrochemicals or even highway

traffic) (Padmavathiamma and Li 2007) are interesting areas for plant researchers due to their specific soil conditions and distinctive flora. Although some metals, such as Cu or Zn, are essential for their development, the occurrence of high contents of heavy metals has several toxic effects on plants: binding to proteins and alteration of their structure, displacement of essential elements resulting in deficiency effects or even promoting the formation of free radicals (Hall 2002). Metal toxicity, together with deficiency in nutrient contents, yield areas that are usually shallow with rocky soils and low moisture and with scarce plant cover (Brady et al. 2005). Thus, serpentine outcrops and mine deposits act as edaphic discontinuities in mainland regions, which have been defined as ecological or 'edaphic islands' (Lefèbvre and Vernet 1990).

When colonising these 'islands', plant populations have to cope with several environmental constraints (metals, dryness, isolation, etc.) that can leave their imprints on the genetic structure of plant populations. For instance, it has been proposed that plant populations in metalliferous areas can suffer a founder effect, which would significantly reduce their genetic diversity (Lefèbvre and Vernet 1990). Moreover, in some plant species, populations growing in metalliferous soils have shown significant genetic differentiation with respect to metal tolerance from neighbouring populations in 'normal' soils, even with the occurrence of substantial gene flow (Vékemans and Lefèbvre 1997; Linhart and Grant 1996 and references therein). Deng et al. (2007) using RAPD markers found significant genetic differentiation between mine populations and uncontaminated populations of the pseudometallophyte *Sedum alfredii*.

Plants growing in metalliferous soils can be either exclusive metallophytes (plants restricted to metal-enriched habitats) or facultative metallophytes (also called pseudometallophytes), that is, species having both metallicolous (growing in metalliferous soils) and non-metallicolous populations (Wu 1990). Thus, pseudometallophytes are interesting species when studying the potential influence of environmental constraints on patterns of genetic diversity (Linhart and Grant 1996).

Several works have investigated the distribution of neutral genetic diversity within and between metallicolous populations (M) and non-metallicolous populations (NM) of different pseudometallophyte plant species (mainly herbaceous or undershrubs), using either isozymes (Wu et al. 1975; Ducousso et al. 1990; Westerbergh and Saura 1992; Bush and Barrett 1993; Vekemans and Lefèbvre 1997; Nordal et al. 1999; Nyberg Berglund and Westerbergh 2001) or DNA based markers (Mengoni et al. 2000, 2001, 2006; Pauwels et al. 2005).

In most cases, similar values of within-population diversity were estimated in M and NM populations, although in some works a diversity decrease was observed in M populations of *Deschampsia cespitosa* (Bush and Barrett 1993), *Lychnis alpina* (Nordal et al. 1999) and copper mine (but not serpentine) populations of *Silene paradoxa* (Mengoni et al. 2001).

Another subject that researchers have focused on, is the origin of the M populations. Analyses of diverse pseudometallophytes (Bush and Barrett 1993; Vekemans and Lefèbvre 1997; Mengoni et al. 2001; Pauwels et al. 2005) support the theory that geographically distant M populations (but even at short distances of hundreds of meters; Al-Hiyali et al. 1988) could have evolved independently from neighbouring NM populations; that is, M and NM populations do not constitute different phylogenetic lineages, and genetic distances among N and NM populations are a function of geographic distances between them.

These findings underline the importance of studying historical factors and population-genetic processes in order to dissect the effects of the demographic processes (such as patterns of migration or bottlenecks) from those related to selective processes (Staton et al. 2001).

Cistus ladanifer L. (gum rockrose) is a woody, semi-deciduous shrub growing in a wide range of latitudes, altitudes and climatic conditions in the Western Mediterranean region (South of France, Iberian Peninsula and North of Algeria and Morocco) (Demoly and Montserrat 1993). Its populations constitute early successional stages adapted to disturbances in Mediterranean ecosystems, in particular fires (Bastida and Talavera 2002). It is a pseudometallophyte that has established populations over different types of bedrock material (granites, schists, slates, etc.) and has also colonized different ultramafic areas in N Morocco (Bni Bouchra) (Ater et al. 2000), S Spain (Málaga) (Alados et al. 1999), NE Portugal (Trás-Os-Montes) (Díez Lázaro et al. 2006; Freitas et al. 2004a) and diverse mine tailings

in Central to South-Western Iberian Peninsula (Murciego et al. 2007; Freitas et al. 2004b).

C. ladanifer is an entomophyllous, obligatory outcrossing species, bearing a gametophytic mechanism of incompatibility (Talavera et al. 1993). It is the major component of shrublands in oligotrophic acid soils in the western half of the Iberian Peninsula (Rivas-Martínez 1979). Three subspecies have been described based on leaf traits (Demoly and Montserrat 1993). Two subspecies, *Cistus ladanifer* subsp. *ladanifer* and subsp. *africanus*, are widespread and they have colonized M (ultramafic) areas, although only subsp. *ladanifer* is found also in mine tailings from the Iberian Peninsula. Finally *C. ladanifer* subsp. *sulcatus* (formerly *C. palhinhae*) is restricted to limestone derived soils on the coast of the south-western tip of Portugal.

In this work, we have analysed 33 *Cistus ladanifer* populations sampled throughout the species distribution range using chloroplast (cp) DNA markers (microsatellites, SSRs). These markers are of special interest when studying colonisation patterns. Chloroplast DNA is generally maternally inherited in angiosperms, whose dispersion is therefore mediated by seeds only. In addition, the effective population size for haploid cpDNA is smaller than diploid nuclear genes, so the differentiation due to genetic drift can be stronger (Comes and Kadereit 1998) and phenomena like genetic bottlenecks can be more easily detected (Echt et al. 1998). For instance, cpSSR markers detected a reduction in genetic diversity within M populations of *Silene paradoxa* where RAPD markers failed (Mengoni et al. 2001).

These characteristics justify the wide use of cpDNA in order to infer the population history of plant populations (Petit et al. 2003; Magri et al. 2007). Once the phylogeography of the species has been inferred, a better understanding of the effect of metal pollution on the genetic structure of populations is possible, avoiding spurious correlations resulting from historical or demographic processes (Staton et al. 2001). This is especially interesting in *C. ladanifer*, since it colonizes M areas at different latitudes (from N Morocco to NE Portugal) in a region whose physiographic (spatial heterogeneity) and climatic diversity offers complex phylogeographic patterns (Gómez and Lundt 2007).

In the present paper, the following main questions were addressed: Do M populations of *C. ladanifer* have the same origin? Did M populations of *Cistus ladanifer* suffer a reduction in diversity? Do differences exist in the demographic effects of the colonisation of M areas along a latitudinal gradient?

Material and methods

Plant and soil sampling

Thirty-three *Cistus ladanifer* populations covering almost the entire distribution natural range of this species were sampled. The subspecies growing in each site was identified on the basis of morphological traits. We included metallicolous (M) populations from different geographic areas: ultramafic outcrops of Bni Bouchra (N of Morocco), Málaga (SE of Spain) and Trás-os-Montes (NE Portugal), and M populations growing on mine tailings from the centre of the Iberian Peninsula (Table 1). After the analyses of phyto-available trace metals, two populations (EAL and EDE), growing near highways, were included in the M group (see Fig. 1).

In each population, a longitudinal transect was established. Ten plants separated by at least 5 m were selected along the transect and their ripe fruits were collected. Seeds were sown and seedlings grown in a laboratory. One seedling per mother plant was selected for the subsequent analyses. Young plants were frozen in liquid nitrogen and conserved at −20°C until DNA extraction.

In addition, in each site one (or two) soil samples were collected from 5 to 15 cm in depth. Each soil sample was air-dried and sieved through a 2 mm-mesh.

Soil chemical analyses

Sieved soil subsamples were milled in an agatha mortar to achieve homogeneity. Total amounts of Cr, Cu, Mn, Ni, Pb and Zn in soils were quantified in solid subsamples with Energy-Dispersive X-Ray Fluorescence spectrometry (EDXRF). Other subsamples were digested with HNO_3 for the quantification of Co contents with Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) element analysis.

In order to determine the concentration of heavy metals potentially available for plants, 10 g of dried

Table 1 Within-population haplotypic diversity estimates

Population (Code)	Subsp	Longitude	Latitude	Soil Type	Substratum	N Samples	H_E	$r_{(7)}$	D^2_{SH}
Ketama (MKE)	afr	4.64º W	34.95º N	NM	Schists	10	0.38	1.40	0.22
Bni Hadifa (MBH)	afr	4.17º W	35.02º N	NM	Sandstones	10	0.2	0.70	0.1
Bab Tazaa (MBT)	lad	5.24º W	35.08º N	NM	Micaschists	10	0.2	0.70	0.1
El Jebha (MEJ)	afr	4.64º W	35.18º N	NM	Sandstone	10	0	0	0
East Bni Bouchra (MSII)	afr	4.89º W	35.29º N	M (u)	Serpentinised peridotite	12	0.7	2.16	0.58
West Bni Bouchra (MSI)	afr	4.90º W	35.30º N	M (u)	Serpentinised peridotite	10	0.53	1	0.27
Tanger (MTA)	afr	5.93º W	35.78º N	NM	Sandstone	10	0	0	0
Almodóvar (EAL)	lad	5.65º W	36.16º N	M (h)	Clays close to a road	10	0	0	0
Benalup (EBE)	afr	5.73º W	36.32º N	NM	Sandstone	10	0.64	2.33	0.76
Sierra Bermeja (ESB)	lad	5.18º W	36.48º N	M (u)	Serpentinised peridotite	10	0	0	0
Sierra Palmitera (ESP)	lad	5.07º W	36.60º N	M (u)	Serpentinised peridotite	9	0.42	1.56	0.5
Sierra de Tolox (ETO)	lad	4.93º W	36.68º N	M (u)	Serpentinised peridotite	10	0.2	0.70	0.1
Grazalema (EGR)	lad	5.27º W	36.78º N	NM	Decarbonated limestone	10	0	0	0
Sierra de Aguas (EAC)	lad	4.79º W	36.84º N	M (u)	Serpentinised peridotite	10	0	0	0
São Vicente (PSV)	sul	8.98º W	37.03º N	NM	Limestone	10	0.2	0.70	0.1
Burgau (PBU)	sul	8.78º W	37.07º N	NM	Limestone	9	0.39	0.97	0.19
Mazagón (EMA)	lad	6.84º W	37.15º N	NM	Sand deposits	10	0.62	1.87	1.78
Corte Figueira (PCF)	lad	8.03º W	37.39º N	NM	Schists	9	0.39	0.97	0.19
Aljustrel (PAL)	lad	8.18º W	37.88º N	M (m)	Pyrite mine tailing	9	0.64	1.78	0.53
Cardeña (ECA)	lad	4.36º W	38.28º N	NM	Granite	10	0.71	1.93	0.89
Despeñaperros (EDE)	lad	3.51º W	38.39º N	M (h)	Quartzites, close to the highway.	10	0.64	1.70	0.46
El Guijo (EGJ)	lad	4.77º W	38.52º N	NM	Slates	10	0.47	0.99	0.23
La Codosera (ECO)	lad	7.08º W	39.19º N	M (m)	Sb mine tailing	10	0.56	1	0.28
Valdecaballeros (EVC)	lad	5.34º W	39.33º N	NM	Sedimentary material (gravels, clays)	10	0.71	2.39	0.71
Vela (PVE)	lad	7.29º W	40.43º N	NM	Granite	9	0	0	0
Ciudad Rodrigo (ECR)	lad	6.49º W	40.63º N	NM	Quartzites	10	0.36	0.93	0.18
Guadarrama (EGU)	lad	4.10º W	40.68º N	NM	Granite	10	0.2	0.70	0.1
Fuente Saúco (EFS)	lad	5.51º W	41.19º N	NM	Sandstone and conglomerates	10	0.64	1.70	0.46
Macedo dos Cavaleiros (PMC)	lad	6.82º W	41.52º N	M (u)	Serpentinised peridotite	10	0	0	0
Ricobayo (ERB)	lad	5.81º W	41.70º N	NM	Quartzites and filites	10	0.53	1	0.27
Samil (PSA)	lad	6.75º W	41.78º N	M (u)	Serpentinised peridotite	7	0	0	0
Bragança (PBR)	lad	6.87º W	41.85º N	M (u)	Dunite	10	0.53	1	0.27
Monte Furado (EMF)	lad	7.20º W	42.39º N	NM	Schists	10	0	0	0
						Overall mean	0.31 ± 0.27	0.91 ± 0.77	0.27±0.36

First column includes population name and code. Second column indicates *Cistus ladanifer* subspecies: *afr*: subsp. *africanus*; *lad*: subsp. *ladanifer*; *sul*: subsp. *sulcatus*. Geographic coordinates are given in decimal degrees. *M*: metallicollous population; *(u)*: ultramafic area; *(m)*: mine tailing; *(h)*: highway affected area. *NM*: non-metallicollous population; *N Samples*: number of plants surveyed in each population; N_e: effective number of haplotypes; H_E: Nei's (1987) haplotypic diversity; $r_{(7)}$: haplotypic richness after rarefaction to the uniform sample size of 7 (El Mousadik and Petit 1996); D^2_{SH}: average genetic distances among individuals (Vendramin et al. 1998); *Overall Mean*: mean for the whole set of populations ± standard deviation values

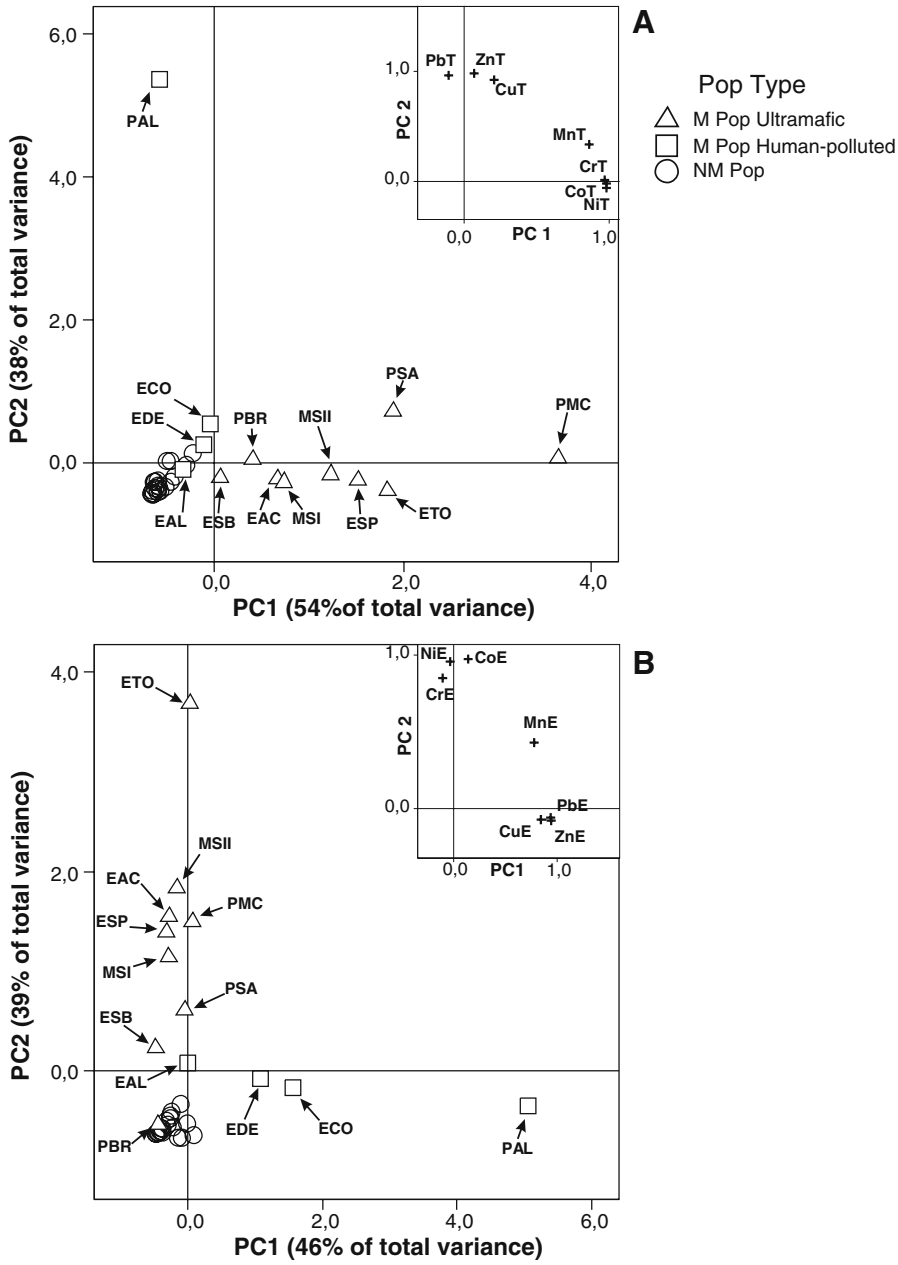

Fig. 1 Principal Component Analysis (PCA) results. Populations are ordinated according to the total (**a**) or Ammonium Acetate/EDTA extractable (**b**) quantities of heavy metals in their soils. The percentage of variance explained by each axis is reported. *Circles* indicate non-metallicolous (NM) populations, whereas *triangles* and *squares* indicate metallicolous (M) populations from ultramafic areas and human-polluted soils, respectively. The codes of M populations are also indicated. In the upper right-hand corner of each graph the loading of each metal on each of the PCs is reported

soil was mixed with an extraction solution (Ammonium Acetate 0.5 M + EDTA 0.02 M + Acetic Acid 0.5 M, buffered at pH 4.65) (Lakanen and Erviö 1971) in a ratio of soil:extraction solution of 1:5. The suspension was shaken for 30 min, after which it was allowed to stand for at least half an hour and was then filtered through paper (Albet DP 145). The filtrate was stored cold to be analysed with an atomic absorption spectrophotometer (AAS). The following available trace metals were determined with AAS: Co, Cr, Cu Mn, Ni, Pb and Zn.

DNA extraction

DNA was extracted from 100 mg of frozen leaves using Dneasy® Plant Mini Kit (QIAGen), following the manufacturer's indications. In several cases an additional wash with 500 μl of absolute ethanol was

needed in order to remove secondary compounds from the DNA extracts.

Microsatellite analysis

In an initial screening, universal cpSSR primers ccmp1 to ccmp10 (Weising and Gardner 1999) and Fagaceae cpSSR primers cmcs 1 to 14 (Sebastiani et al. 2004) were tested on a subset of 30 samples from 6 geographically distant populations. Only primers ccmp1, ccmp2, ccmp3, ccmp5, ccmp10 and cmcs1 yielded consistent amplifications, of which ccmp1, ccmp5, ccmp10 and cmcs1 were monomorphic. The two polymorphic cpSSRs were then used to amplify all samples of the 33 populations. Amplification reactions were performed in 12.5 µl total volume using 10 ng of template DNA, 1× reaction buffer (Promega, Madison, WI, USA) containing 1.5 mM of $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, 1% of bovine serum albumin, and 0.5 U of GoTaq® DNA Polymerase (Promega). DNA was amplified with the following thermal profile: one denaturation cycle of 4 min at 95°C, followed by 25 cycles each consisting of 95°C for 30 s, 50°C for 30 s, 72°C for 30 s with a final extension step at 72°C for 8 min. PCR products were loaded on a capillary automatic sequencer MegaBACE 1000 (GE Healthcare Biosciences). MegaBACE ET400 (GE Healthcare Biosciences) was used as size standard. Fragment lengths were determined using the MegaBACE FRAGMENT PROFILER software version 1.2 (GE Healthcare Biosciences).

Data analysis

A Principal Component Analysis (PCA) was performed in order to aggregate populations according to (1) total contents of metals Co, Cr, Cu, Mn, Ni, Pb and Zn in soils (referred here as CoT, CrT, CuT, MnT, NiT, PbT and ZnT) or (2) Ammonium Acetate/EDTA metal extractable contents (referred here as CoE, CrE, CuE, MnE, NiE, PbE and ZnE). Values below detection limits were recorded as 0.1 µg g^{-1} for statistical analysis. With the PCA, we reduced the dimensionality of the data, retaining, in our case, two first Principal Components (PCs) that contribute most to the variance of soils. A Varimax rotation was applied to the PCAs in order to simplify the interpretation of the extracted Principal Components.

Phylogenetic relationships among haplotypes were inferred with NETWORK 4.5 (Fluxus Technology Ltd. at www.fluxus-engineering.com) using the median joining (MJ) method (Bandelt et al. 1999). We applied the three criteria (frequency, topology and geography) proposed by Pfenninger and Posada (2002) in order to remove loops or ambiguities in the haplotype network.

Different parameters of genetic diversity within populations were estimated: (1) the haplotypic diversity $\left(H_E = [n/(n-1)]\left[1 - \sum p_i^2\right]\right)$, (where n is the number of individuals analysed in a population and p_i is the frequency of the i-th haplotype in a population, Nei 1987)), (2) the haplotypic richness $r_{(n)}$, which is obtained after rarefaction to a uniform sample size of n (in our study, the value of n was fixed at 7, the lowest size of the analysed populations), as described in El Mousadik and Petit (1996), and (3) the D^2_{SH} measure, as defined by Vendramin et al. (1998), which takes into account the difference in the number of repeats among the different cpDNA haplotypes considered.

In addition, the distribution of the pairwise cpSSR repeat length differences among individual plants, totalled over all two cpSSR loci within an individual plant, was plotted to compare different patterns.

Genetic differentiation among populations was estimated by the analysis of molecular variance (AMOVA, Excoffier et al. 1992) using Arlequin software (version 3.11; Excoffier et al. 2005). The significance of the values was computed by a permutation test from 10,000 permutated matrices. The AMOVA was based on distances between cpSSR haplotypes calculated from the sum of the squared number of repeat differences between two haplotypes: $d_{xy} = \sum \left[a_{xi} - a_{yi}\right]^2$ (where a_{xi} and a_{yi} are the number of repeats for the ith locus in haplotype x and y). This gives Φ_{ST}, an analogue of Slatkin's R_{ST} (Slatkin 1995) for population differentiation (Michalakis and Excoffier 1996).

The Cavalli-Sforza and Edwards distances based on haplotype frequencies were used to construct a Neighbour Joining (NJ, Saitou and Nei 1987) dendrogram with the Populations software (O. Langella, UMR de Génétique Végétale, Ferme du Moulon, Gif/Yvette, France). To test for node robustness, bootstrapping was performed on individuals using 1,000 resamplings.

In order to infer population genetic structure, Bayesian analysis using a spatial clustering model

implemented in BAPS software version 5.2 was performed (Corander et al. 2008). These authors have shown that the spatial model improves the statistical power to detect the underlying population structure when dealing with a low number of loci.

The spatial clustering of groups model was run using each population, with known coordinates, as the unit to be clustered. We initially fixed k (the number of clusters) from 2 to 25. We then selected the value of k that had the minimum log marginal likelihood and re-ran the analysis 100 times to obtain the optimal partition of populations. A neighbour-joining tree was then constructed (Saitou and Nei 1987) with the Kullback–Leibler divergence matrix provided as output with BAPS. This matrix can be used as a measure of relative genetic distance between the BAPS-identified clusters (BAPS 5.2 manual distributed with the program).

Taking into account the grouping performed by BAPS, we (1) compared the levels of intra-population diversity of M and NM populations using an analysis of variance (ANOVA) and (2) tested the differentiation of M and NM populations with additional separate AMOVA analyses (Excoffier et al. 1992). With this two step-approach (BAPS and ANOVA/AMOVA) we tried to extract first the effect of phylogeography and then analyse the effect of colonisation of metallicolous areas within phylogeographic homogeneous groups of populations, thus avoiding confounding effects of phylogeography on the effect of metal pollution over population genetic structure.

Isolation-by-distance patterns between populations were tested considering all populations and then considering M and NM populations separately. A Mantel test with 10,000 random permutations was performed with the matrix of pairwise genetic differentiation between populations, using $\Phi_{ST}/(1 - \Phi_{ST})$, and a matrix of the logarithmically (ln) transformed geographic distance. AMOVA and Mantel tests were computed with the Arlequin software version 3.11 (Excoffier et al. 2005).

Results

Soil characteristics

The results of the analyses of total and Ammonium Acetate/EDTA extractable metals in soils for each population are presented in the Online Resource 1. On the basis of these data, we conducted two Principal Component Analyses (PCA).

The PCA performed on total metal contents (Fig. 1a) extracted two principal components (PCs) that explained 92% of total variance. The first Principal Component (PC1; 54% of total variance) was mainly composed of the total contents of Co, Cr, Ni, and Mn, suggesting that this axis is related to the ultramafic nature of soils. The second PC (PC2; 38% of total variance) was related to the degree of pollution due to human activities, since the metals with higher loadings were Cu, Pb and Zn.

The PCA based on extractable metals showed a similar pattern (Fig. 1b). Two PCs explaining 85% of total variance were extracted. In this case, the first PC (PC1; 46% of total variance) was related to human pollution (extractable Cu, Pb and Zn were the main contributors to this PC) whereas the second PC (PC2; 39% of total variance) reflected the ultramafic origin of soils, since the extractable contents of Co, Cr and Ni contributed significantly in explaining the observed variance. The manganese content showed similar loadings on both axes.

According to the defined Principal Components, in both cases (total or extractable metal contents) NM populations were plotted in a dense swarm placed mainly in the negative values on both axes, whereas the M populations showed high scores along one of the PCs, depending on their nature (ultramafic area or human-polluted site) (Fig 1a and b).

On the other hand, three populations (EAL, EDE and PBR) showed differences between the two PCAs. Population PBR, whose bedrock material are dunites (a type of peridotite) is clearly separated from the NM populations based on total metal contents. Thus, it is plotted along the axis of ultramafic populations in Fig 1a. In contrast, in Fig 1b (PCA based on extractable metal contents) this population is plotted with the NM populations. This population was treated as M because of the high total contents of metals Cr, Mn and Ni (944, 1,578 and 1,151 $\mu g.g^{-1}$, respectively) in the analysed soil samples.

The two other populations (EAL and EDE) were initially considered as NM (taking into account bedrock material and total metal contents, see Online Resource 1 and Fig. 1a). Nevertheless, when performing the PCA on Ammonium Acetate/EDTA

extractable metal contents, these two populations were separated from the group of NM populations (Fig 1b). These populations, affected by highway traffic, were consequently classified as M. EDE has an extractable Pb of more than 100 $\mu g.g^{-1}$, whereas EAL possesses in its soil moderate extractable contents of all the analysed metals.

Genetic diversity and structure

The two polymorphic microsatellites ccmp2 and ccmp3 yielded 3 and 5 size variants, respectively. According to the nature of these microsatellites (both are mononucleotide repeats; Weising and Gardner 1999) the sizes of alleles varied by one base-pair.

The variants found in each microsatellite locus combined into 10 different haplotypes (see Online Resource 2 for the definition of each haplotype). They are connected through a complex haplotype network, with 3 loops and no missing haplotypes (Fig. 2).

Only two haplotypes (H3 and H7) are present both in the Iberian Peninsula and North of Morocco. Haplotypes H2 to H4 are distributed across the Iberian Peninsula, whereas haplotypes H8 to H10 are found in North of Morocco and H6 is restricted to south-eastern Spain (Betic Area) (Fig. 3). Three haplotypes (H1, H5 and H10), found on the tips of the network, were exclusive to one population type (M or NM) (Fig. 2). Two of these haplotypes (H5 and H10) were singletons, whereas H1 is exclusive to NM population EMA from south-western Spain.

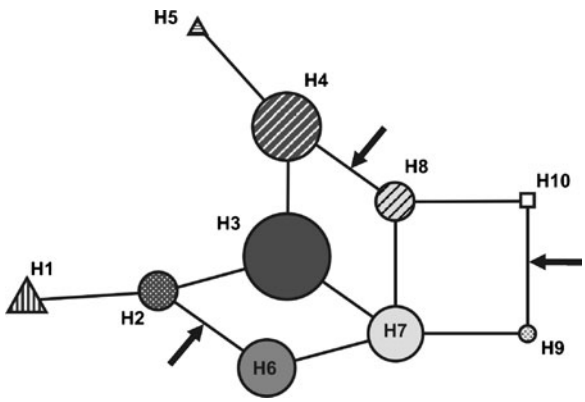

Fig. 2 Median Joining (MJ) network of *Cistus ladanifer* cpSSR haplotypes. Haplotypes are identified with the same colours as in Fig. 3. *Circles*: haplotypes present both in M and NM populations. *Triangles*: haplotypes exclusive to NM populations. *Square*: haplotype present only in M population. Symbols' sizes are proportional to the absolute haplotype frequency in the whole sample. *Arrows* indicate the connections between haplotypes that can be removed following the criteria of Pfenninger and Posada (2002)

The geographic distribution and the frequency of haplotypes, together with a criterion of topology (Pfenninger and Posada 2002) were employed to remove one of the edges in each of the 3 loops inferred in the haplotype network and thus resolve the uncertainties in the network (Fig. 2).

Bayesian analysis yielded an ideal grouping with 8 clusters (Fig. 4), of which clusters 1, 3, 4, 6, and 7 included both M and NM populations. The NJ tree grouped these 8 clusters again in two diverging lineages of populations. The first lineage (hereafter referred to as "South lineage") comprises populations in which H6 to H10 haplotypes are dominant (clusters 7, 8, 6 and 2), whereas the other lineage (hereafter referred to as "North lineage") includes those populations with a high frequency of H1 to H5 haplotypes (clusters 4, 1, 3 and 5). These lineages have a taxonomic support, since the "south lineage" comprises populations of *C. ladanifer* subsp *africanus*, together with populations of *C. ladanifer* subsp. *ladanifer* growing in the Betic area, whereas the "north lineage" is composed of populations of *C. ladanifer* subsp. *sulcatus* and *C. ladanifer* subsp *ladanifer* from the Iberian Peninsula, plus one population of subsp. *ladanifer* from the N of Morocco.

The AMOVA analysis performed on the whole sample of populations indicate that most of the molecular variation is found among populations (Φ_{ST}=0.69, Table 2a), that is a value similar to the mean value for angiosperms (Petit et al. 2005). Differentiation between edaphic types were not significant either when the whole set of populations (33 pops) was considered (Table 2b), nor when separate AMOVA analyses within each of the lineages (North and South, Table 2c and d) were performed.

The NJ tree further confirmed that M and NM populations did not constitute distinct genetic groups (Fig. 5). M populations appear dispersed among NM populations, forming clusters that are partially congruent with BAPS results. In most cases, M populations are clustered with geographically close NM populations and were genetically distant to M populations from other geographic areas; on the other hand populations PMC, PSA and PVE (from the NE and

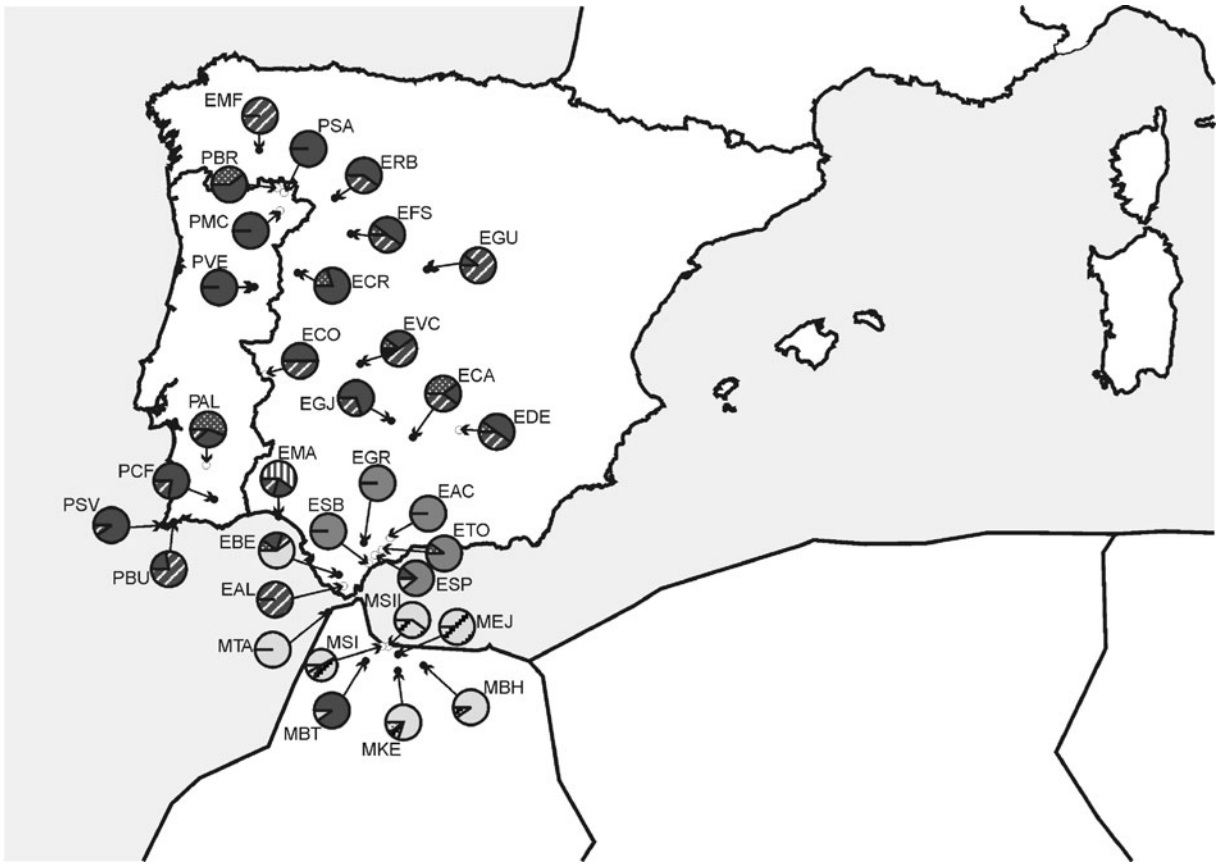

Fig. 3 Geographic distribution of *Cistus ladanifer* cpSSR haplotypes. Colour patterns used are the same as in Fig. 2. In order to obtain a proper view of haplotype distribution, radial graphs were displaced from their original position (*arrows* connect them with their original position). Next to each graph the population code is indicated (see Table 1). Metallicolous (M) populations are noted as *white dots*, non-metallicolous (NM) ones as *black dots*

C of Portugal) were clustered with population MBT from the N of Morocco.

No isolation-by-distance pattern was detected by Mantel's tests, irrespectively of whether we considered M populations (P=0.12), NM populations (P=0.23) or the whole set of populations (P=0.23).

The number of haplotypes per population ranged from 1 to 4. The different estimators of within-population diversity (H_E, $r_{(7)}$ and D^2_{SH}) displayed overall mean values of 0.31±0.27, 0.91±0.77 and 0.27±0.36, respectively (Table 1). The Analysis of Variance (ANOVA) showed no significant differences of diversity among M and NM populations considering either the whole set of populations or the lineages defined by BAPS (North, South) separately (Table 3). In addition, there were no qualitative differences among mismatch distributions from M and NM populations (see Online Resource 3).

Discussion

Over the last 50 years pseudometallophytes have been studied as models of microevolution and of regional differentiation among plants. The main aim of these works has been, on the one hand, to test whether the colonisation of areas with heavy metals may result in genetically depauperated populations (as a result of bottlenecks and genetic drift), and on the other, to determine whether the metalliferous populations of a particular species arose from a single micro-evolutionary event, or whether they resulted from processes of regional differentiation. Our study on the Mediterranean shrub *Cistus ladanifer*, which encompasses almost all of its natural distribution, provides evidence for the colonisation routes of metalliferous areas by this species.

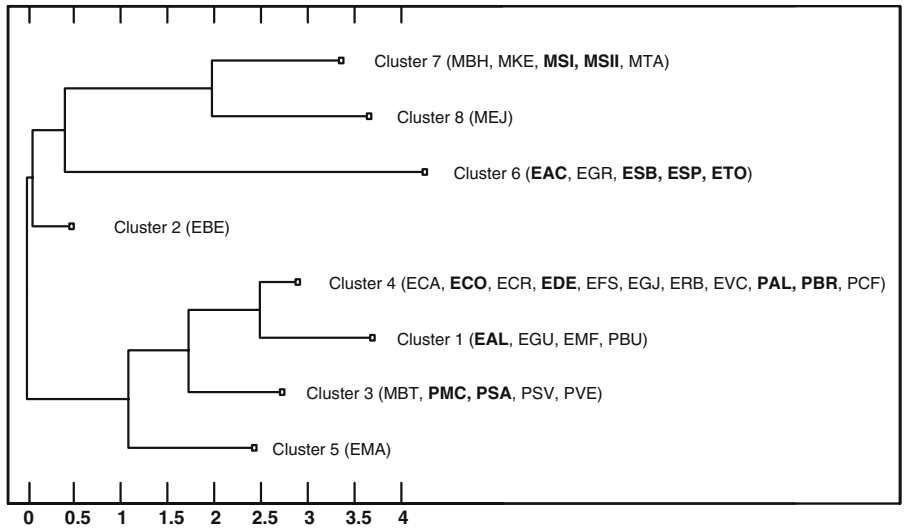

Fig. 4 BAPS-based clustering and relationships among clusters based on the Kullback–Leibler divergence matrix. Clusters of populations were constructed with a spatial clustering model (Corander et al. 2008). *Codes in brackets* indicate populations included within each cluster. Population codes are the same as in Table 1. M populations are indicated in *bold type*. *Horizontal scale bar* indicates Kullback–Leibler distances among clusters

Using Bayesian methods, we infer 2 population lineages with different haplotype compositions showing partial taxonomic significance ('South' lineage made up of the *C. ladanifer* subsp. *africanus* and subsp. *ladanifer* from the Betic area; 'North' lineage, made up of populations of *C. ladanifer* subsp. *ladanifer* and subsp. *sulcatus*).

The three *C. ladanifer* subspecies have been classified on the basis of leaf traits (leaf shape, nerve type and length of petiole, Demoly and Montserrat 1993). Our results showed an incongruence between molecular information and morphological traits: in fact populations of subspecies *ladanifer* from Betic area are placed within the cluster of subspecies *africanus*. This incongruence not only refers to chloroplast markers, but also to AFLP markers analysed on the same 33 populations included in this work (manuscript in preparation), thus suggesting that additional studies are needed to determine the taxonomic status for these subspecies.

In addition to the taxonomic considerations, several evidences indicate that the edaphic type does not influence the occurrence of the lineages: haplotypes specific to one single type of soil only appear on the tips of the haplotype network. Moreover, within the 'North' and 'South' lineages, as well as in the clusters defined by NJ dendrogram, both M and NM populations are found.

The two lineages detected in this study are congruent to those found by Guzmán and Vargas (2009) analysing the sequences of several chloroplast regions of *Cistus ladanifer*: these authors inferred two main chloroplast lineages, referred to as 'European' and 'African', and found that populations from the Betic area display an exclusive haplotype which belong to the 'African' lineage. Likewise, the two lineages we detected have the same geographical distribution as reported by Guzmán and Vargas (2009); moreover haplotype H6, which is exclusive to populations from the Betic area, is connected to haplotype H7, common in the North of Morocco.

Thus, the 'North' and 'South' lineages seem to be more related to the existence of diverse glacial refugia for *Cistus ladanifer* located in the N of Morocco and in the Southeast (Betic area) and Southwest of the Iberian Peninsula, areas indicated as refugia for several other Mediterranean taxa (Médail and Diadema 2009).

Therefore, the extant M populations of *Cistus ladanifer* have arisen through distinct foundation events in different geographical regions and within different postglacial recolonisation lineages.

The independent origin of the M populations of a given species seems to be common in pseudometallophytes (Mengoni et al. 2001; Nyberg Berglund and Westerbergh 2001; Vekemans and Lefèbvre 1997), even at a scale of hundreds of metres (Al-Hiyali et al. 1988). Interestingly, we found M populations within two chlorotype lineages while, for example, in *Arabidopsis halleri*, a model pseudometallophyte, all M populations, of independent origins, come from one chlorotype lineage only (north of the Alps) (Pauwels et al. 2005, 2008).

Table 2 Analysis of molecular variance (AMOVA) of *Cistus ladanifer*

Source of variation	d.f.	SS	Variance components	% Total Variance	Φ statistics	Sig.
(a) *Whole data set*						
Among populations	32	191.15	0.58	68.69	Φ_{ST}=0.69	<0.0001
Within populations	291	77.14	0.26	31.31		
Total	323	268.29	0.85			
(b) *M vs NM*						
Among groups	1	10.16	0.03	3.23	Φ_{CT}=0.03	n.s.
Among pops within groups	31	180.99	0.57	65.97	Φ_{SC}=0.68	<0.0001
Within populations	291	77.14	0.26	30.80	Φ_{ST}=0.69	<0.0001
Total	323	268.29	0.86			
(c) *South cluster*						
Between groups (*M vs NM*)	1	6.93	0.03	4.59	Φ_{CT}=0.05	n.s.
Among pops within groups	10	50.32	0.48	71.62	Φ_{SC}=0.75	<0.0001
Within populations	109	17.51	0.16	23.80		<0.0001
Total	120	74.76	0.67			
Among pops irrespective of groups					Φ_{ST}=0.76	<0.0001
(d) *North cluster*						
Between groups (*M vs NM*)	1	0.36	−0.02	−4.63	Φ_{CT}=-0.04	n.s.
Among pops within groups	19	48.89	0.23	43.41	Φ_{SC}=0.41	<0.0001
Within populations	182	59.63	0.33	61.22		
Total	202	108.88	0.53			
Among pops irrespective of groups					Φ_{ST}=0.40	<0.0001

We considered (a) all the populations (Φ_{ST}), (b) hierarchical analysis of variance among populations within edaphic types (Φ_{SC}) and between edaphic types (Φ_{CT}) and (c) (d) separate hierarchical analyses considering the clustering obtained by BAPS. d.f. = degrees of freedom, SS = sum of squared deviation, Sig. = probability of obtaining a more extreme component estimate by chance alone. *n.s.*, not-significant (α *value= 0.05*)

Most of the works on genetic structure of pseudometallophytes did not reveal evidence of genetic bottlenecks in M populations (for review, see Vekemans and Lefèbvre 1997; Mengoni et al. 2000). These results were mainly based on nuclear markers (isozymes or RAPDs), so the possible founder effect at nuclear loci may be eroded by subsequent pollen flow from neighbouring NM populations which could increase genetic variation in M populations. In contrast, chloroplast markers are (generally) maternally inherited, so they will only reflect the effect of seed flow. To our knowledge, only two other studies have analysed pseudometallophytes using chloroplast markers, obtaining contrasting results: Mengoni et al. (2001) detected a founder effect in *Silene paradoxa* populations growing on copper mine deposits, whereas Pauwels et al. (2005) did not find any difference in genetic diversity between M and NM populations of *Arabidopsis halleri*.

To explain these different patterns of diversity between *S. paradoxa* and *A. halleri*, Pauwels et al. (2005) proposed that the colonisation of metal-polluted environments is associated with a genetic bottleneck in species with populational tolerance (that is, species in which metal tolerance is found only in those populations growing on metalliferous soils), whereas in species with constitutive (or "species-wide") tolerance (such as *A. halleri*) the effect of a bottleneck may not be detected.

In the case of *Cistus ladanifer*, once we had extracted the phylogeographic effect through the Bayesian analysis, we did not detected any significant differentiation nor significant differences in the genetic diversity between edaphic types, even in the M populations of more recent origin, i.e. populations growing on mine tailings from the Iberian Peninsula.

These inferences may suggest that M populations were founded recently by a high number of individ-

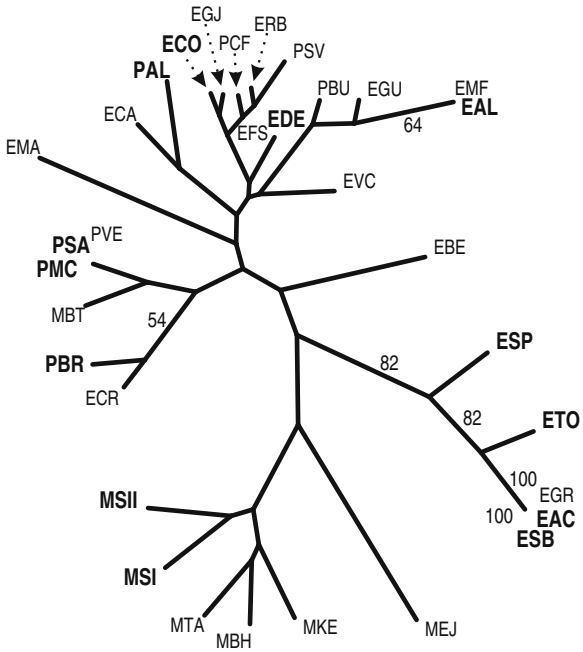

Fig. 5 Neighbour-Joining consensus tree of populations. Cavalli-Sforza and Edwards distances between populations were used. Only bootstrap values higher than 50 are reported. **M** populations are indicated in *bold type*

uals, or that the foundation events are antique but in the presence of a significant seed flow (cpDNA is maternally inherited in *Cistus ladanifer*; Guzmán and Vargas 2009) from neighbouring NM populations that masked the effect of genetic bottlenecks and limited genetic differentiation between edaphic types.

Both hypotheses assume that the metalliferous areas do not exert selective pressures on *Cistus ladanifer*; however, it should be stressed that variation of neutral markers, like cpSSRs, cannot be related to variation of adaptive traits such as tolerance to heavy metals (Le Corre and Kremer 2003), as it occurred in *Thlaspi caerulescens* (Jiménez-Ambriz et al. 2007). Thus, genetic differentiation between M and NM populations at genes related to metal-tolerance can be significantly underestimated.

Nevertheless, as discussed by Vekemans and Lefèbvre (1997), after a bottleneck the number of alleles at neutral loci increases as a result of new mutations, when population size increases. This phenomenon can be observed in the pseudometallophyte *Silene paradoxa* (Mengoni et al. 2001): 8 populations (5 serpentine outcrops, 2 copper mines, 1 non-metallicolous) separated up to 205 km showed 13 cpSSR haplotypes exclusive to one population (of 27 haplotypes detected) and no haplotypes were shared by all populations. In contrast, in the case of *C. ladanifer* only a singleton was exclusive of M populations. This fact suggests that the inferred levels of diversity cannot be caused by mutations after a bottleneck following the colonisation of metalliferous areas.

To conclude, following Pauwels et al. (2005) and considering the multiple origins of M populations and the lack of bottlenecks, we propose that the tolerance to heavy metals could be a characteristic of *Cistus ladanifer*, even though this plant is more commonly found in non-metalliferous areas, as already observed in *Thlaspi montanum* (Boyd and Martens 1998) or in *Andropogon virginicus* (Gibson and Risser 1982). Indeed, our hypothesis is supported by the results obtained by Kidd et al. (2004), who observed that non-metallicolous populations from North of Portugal showed high tolerance to Cu and Zn in hydroponic cultivation.

Taking into account our findings on *Cistus ladanifer* and those obtained by other authors, we believe that this plant could be very useful in the recovering of degraded soils in the Mediterranean region, also con-

Table 3 ANOVA results. The table presents the mean values for M (Metallicolous) and NM (Non-Metallicolous) of within-population diversity indexes for each cluster of populations (defined after Bayesian analysis), and for the whole set of populations

		H_E			$r_{(7)}$			D^2_{SH}		
		M	NM	Sig.	M	NM	Sig.	M	NM	Sig.
Cluster defined by BAPS	South	0.308	0.203	n.s.	0.903	0.738	n.s.	0.242	0.180	n.s.
	North	0.339	0.387	n.s.	0.783	1.061	n.s.	0.220	0.371	n.s.
Whole data		0.325	0.332	n.s.	0.838	0.964	n.s.	0.230	0.314	n.s.

H_E: Nei's (1987) haplotypic diversity. $r_{(7)}$: allelic richness (El Mousadik and Petit 1996) with a fixed rarefaction size of 7. D^2_{SH}: average genetic distances among individuals (Vendramin et al. 1998). Sig.: significance value. n.s.: not-significant (α *value= 0.05*)

sidering that it has other interesting traits (Frérot et al. 2006). First, *C. ladanifer* is adapted to water stress (Nuñez-Olivera et al. 1996) so it can cope with a long, dry summer season. Second, it has a high growth rate and productivity (Patón et al. 1998) when conditions permit, developing dense root and shoot systems that can limit soil erosion. And third, it is a native species of Western Mediterranean flora; therefore, its use in phytoremediation would not imply any potential threats to ecosystems deriving from the use of alien species (Méndez and Maier 2008; and references therein), an important issue in the biodiversity-rich Mediterranean area.

Acknowledgements We thank Olivier Langella for providing us with a compiled version of the Populations software; and Dr. Margarida Ribeiro and Dr. Rita Costa for their help in the first phases of this work. Alessio Mengoni and two anonymous referees provided useful comments that improved this paper.

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