Ex situ conservation of Cistus heterophyllus subsp. carthaginensis (Cistaceae) saves the taxon from introgression by Cistus albidus

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Title: *Ex situ* conservation of *Cistus heterophyllus* subsp. *carthaginensis* (Cistaceae) saves the taxon from introgression by *Cistus albidus*

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Abstract: *Cistus heterophyllus* subsp. *carthaginensis* is a critically endangered plant taxon from E Spain. Only two populations, one in La Pobla de Vallbona, Valencian Community (consisting of only one plant) and the other in Llano de Beal, Cartagena municipality, Autonomous Community of the Region of Murcia, are presently known. The low number of individuals and introgression by the closely related *C. albidus* are the major threats to the conservation of *Cistus heterophyllus* subsp. *carthaginensis*. In the years 2007, 2008, 2011, and 2013 seeds were collected from the Murcia population within the efforts to protect this taxon. In this work, we compare the natural population of Murcia with the *ex situ* plants originated from the recollected seeds, the only known sample from Valencia, and a specimen of North Africa of *C. heterophyllus* using genotyping by sequencing. The natural population of Murcia shows clear signals of introgression by *C. albidus* while the *ex situ* plants are much less affected, suggesting that the major part of the introgression of the natural populations took place after the specimens for the *ex situ* collection were taken. The Murcian samples seem not to be very close to the Valencian plant, but the systematic relationships among the studied populations remain unclear. The *ex situ* conservation efforts are a key to the conservation of this taxon.

Keywords: GBS, hybridization, Mediterranean basin, rock-rose, Spain

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Introduction

The Cistaceae are plants highly adapted to Mediterranean climate conditions (Fernández-Mazuecos and Vargas 2018). Within this family, the genus *Cistus* L. forms a monophyletic clade if *Halimium umbellatum* (L.) Spach is included as *C. umbellatus* L. (Guzmán and Vargas 2009a; Guzmán et al. 2009). Molecular data suggest a recent origin of the genus *Cistus*, estimated three million years ago, after typical Mediterranean climatic conditions with dry summers and frequent fires were established in the Mediterranean basin (Guzmán et al. 2009; Fernández-Mazuecos and Vargas 2010). At present, 22 species are recognized (Fernández-Mazuecos and Vargas 2018), which can be grouped in two clades characterized by petal color: white flowers (13 species) and purple flowers (nine species). The only exception is *C. parviflorus* Lam., which belongs to the white flower clade but has flowers with a pale pink color. *Cistus* species can be identified without problems (Fernández-Mazuecos and Vargas, 2018). Nevertheless, hybridization between species is frequently observed in natural habitats (Martín Bolaños and Guinea López 1949; Demoly and Montserrat 1993; Demoly 1996).

Three of the species with purple flowers are present in the Mediterranean basin: *C. albidus* L., *C. creticus* L., and *C. heterophyllus* Desf. The speciation of these three closely related species likely occurred in Northern Africa, where at present all three species are frequent (Fernández-Mazuecos and Vargas 2018). Starting from there, *C. creticus* colonized mainly the central-eastern Mediterranean region, where it reaches Palestine and Turkey, although some isolated locations are known in the Iberian Peninsula (Demoly and Montserrat 1993). On the contrary, *C. albidus* is frequent in most of the western Mediterranean basin. The third species, *C. heterophyllus* (rock-rose) is more restricted to arid conditions and outside Africa, it is only known from two localities in the E of the Iberian Peninsula (Fig. 1). The first Spanish population of this species was discovered near Cartagena in 1903 and initially identified as *C. polymorphus* Willk. (Jiménez Munuera 1903), but later described as new species: *C. carthaginensis* Pau (Pau 1904). In the following years, several authors concluded that the Spanish material should be ascribed to *C. heterophyllus* from North Africa (Vicioso 1946; Martín Bolaños and Guinea López 1949; Warburg 1968). Although initial publications suggested that *C. heterophyllus* was locally abundant near Cartagena (Jiménez Munuera 1903, 1909), the species was later thought to be extinct in Spain (Esteve Chueca 1973). When in 1986 a specimen of this species was found in La Pobla de Vallbona near Valencia, the detailed study of this plant made clear that the Iberian plants of *C. heterophyllus* show slight differences from North African *C. heterophyllus*, which support their recognition at the subspecies level (Crespo and Mateo 1988): *C. heterophyllus* subsp. *carthaginensis* (Pau) M.B. Crespo & Mateo. In 1993, nine plants of *C. heterophyllus* subsp. *carthaginensis* were discovered near the first Iberian site (Robledo et al. 1995). This population was affected by a forest fire in 1998, but thanks to artificial irrigation, which helped with the germination of seeds present in the soil, it recovered and increased to 26 plants (Sánchez-Gómez et al. 2018).

Already Robledo et al. (1995) supposed that some of the initially found nine plants of the rediscovered population near Cartagena showed indications of hybridization with *C. albidus*. Sánchez-Gómez et al. (1998, 2002) became aware of morphological differences between the only plant of La Pobla de Vallbona (Valencian Community), and Llano del Beal (Cartagena municipality, Autonomous Community of the Region of Murcia) plants. Jiménez et al. (2007) and Jiménez Martínez et al. (2018) used molecular markers (RAPD) to study the relations among the populations of Africa, Llano del Beal, and La Pobla de Vallbona and came to the conclusion that the plant of Valencia is closely related to the African specimen they used and that the plants from Murcia are profoundly affected by hybridization with *C. albidus*. These hybrids were formally described as *Cistus*

×*clausonii* Font Quer & Maire nothosubsp. *crespoi* P.P. Ferrer & E. Laguna (Ferrer Gallego and Laguna Lumbreras 2012). Pawluczyk et al. (2012, 2018) further supported the existence of hybrids in the population of Llano del Beal with molecular data.

With only two known localities with a total of fewer than 30 plants in the territory of Spain, *Cistus heterophyllus* was included as "in danger of extinction" (*en peligro de extinción*) in the Spanish and autonomous communities (Region of Murcia and Valencian Community) catalogs of threatened species (Robles Sánchez et al. 2018). In addition to the mere protection of the plants against acts like destroying plants or cutting flowers, the competent authorities at the national and regional level also prepared plans for the conservation and recovering of the species, which included, among other measures, genetic studies, propagation *in vitro*, germplasm banks, the establishment of reference plantations *ex situ*, control of natural populations and eradication of specimens identified as hybrids (Robles Sánchez et al. 2018).

In 2007, 2008, and 2011 in the course of management measures seeds were collected from the natural population in Llano del Beal. In 2013, the regional authorities allowed to remove all flower buds of plants that were thought to show evidence of hybridization, and the seeds of "pure" plants were collected afterward. These seeds were used to establish an *ex situ* population maintained in the Center for the Conservation of the Wild Flora of the Autonomous Community of the Region of Murcia (CCFS). In 2019, the seeds taken in 2007, 2008, and 2011 were sown to produce additional plants to select genetically "pure" samples for a renewed reference collection and other aims. The phenotypic characters used for this selection were mainly based on trichome morphology (Navarro Cano 2018). Similar programs were impossible in the case of Valencia because only one plant is known in the wild (Laguna Lumbreras et al. 1998; Güemes et al. 2004; Aguilella et al. 2010; Laguna et al. 2016) and the species of the genus *Cistus* are self-sterile although this autoincompatibility is not 100% effective (Boscaiu and Güemes 2001). Therefore, the Valencian plant was cloned, and the clones maintained in the Center for Forest Investigation and Experimentation (CIEF) of the Valencian Community in Quart de Poblet (Arregui et al. 1993). Later it was shown that these clones were affected by somaclonal mutations (Rosato et al. 2016). With additional measures like artificial irrigation of the wild specimen of La Pobla de Vallbona, finally viable seeds were produced some years (Ferrer-Gallego et al. 2018). Later also artificial hybrids between the descendants of the specimen of Valencia and other specimens of *C. heterophyllus* and even other species (*C. albidus* and *C. creticus*) were created *ex situ* (Ferrer-Gallego et al. 2018).

To evaluate the measures taken to protect and conserve the Murcian population of *C. heterophyllus* subsp. *carthaginensis* and to select the genetically purest specimens of the *ex situ* collection of the CCFS a genetic study was deemed necessary. To achieve this aim we used Genotyping by Sequencing (GBS, Elshire et al. 2011). This technique produces large numbers of genomic fragments created by restriction enzymes from which single nucleotide polymorphisms can be discovered. This way it is possible to obtain an overview of the genetic constitution at the genome level in a cost-effective manner.

Material and Methods

Plant material

For this study, a total of 180 samples were used, distributed as follows: four specimens of *C. albidus* (group number 1), one sample of *C. heterophyllus* subsp. *heterophyllus* from Alhucemas, Morocco, two samples of *C. heterophyllus* subsp. *carthaginensis* from Valencia (clones of the only known plant of this population), one artificial hybrid of the plant from Valencia with a plant from Murcia (these four specimens are considered group number 2), 44 samples from the Murcian natural population of Llano del Beal (group number 3, sampled in 2019), 34 samples of a reference collection held at the CCFS in Murcia, 28 of them from seeds collected in 2013 (group number 4). The mother plants of the 2013 collection are morphologically pure *C. heterophyllus* subsp. *carthaginensis* (plants with labels 9.14, 21, and 32). Furthermore, 28 samples were from seeds collected in 2007 (group number 5), 34 samples from seeds collected in 2008 (group number 6), and 31 plants from seeds collected in 2011 (group number 7). Additionally, one sample of *C. monspeliensis* was included to serve as an outgroup where appropriate (Online Resource).

Nomenclature follows The Plant List (2013).

DNA extraction

Between 50 and 100 mg of leaf material was placed in Eppendorf tubes, frozen in liquid nitrogen, and crushed in a Retsch MM400 mill for 2 min with a vibrational frequency of 30 Hz. We used a modified CTAB protocol (Healey et al. 2014) for DNA extraction. The DNA obtained this way was further purified by applying it to the GeneJET Gel Extraction Kit (ThermoFisher) with the recommendations provided by the manual for high molecular weight DNA. The DNA concentration was estimated with a Qubit 2.0 fluorometer (ThermoFisher) with the dsDNA BR Assay Kit. The DNA concentration of the extracts was then adjusted to 10 ng/µl.

Library preparation

For GBS library preparation, we followed the protocol of Poland et al. (2012). We used a combination of the enzymes PstI (ThermoFisher FD: (CTGCA|G) and Eco47 I (ThermoFisher FD: G|GWCC; W = A or T). 200 ng of genomic DNA were cut in the presence of 10 u PstI, 10 u Eco47 I, 1 mM ATP, 1 u (Weiss) T4 DNA Ligase, 0,5 mM of the Eco47 I Adapter (with GAC overhang) and 0.05 mM barcoded PstI adapters in 40 µ1x FD buffer (ThermoFisher). The adapter sequences were taken from Poland et al. (2012). The enzyme Eco47 I produces two types of overhangs (GAC and GTC), but we used only the GAC overhang for the adapter to decrease the fragment number and this way increase coverage. The restriction/ligation reaction was maintained at 37 °C for 2 h. The temperature was then raised for 20 min to 65 °C to inactivate the DNA Ligase. These reactions were prepared in two 96-well PCR plates (two independent libraries). Four samples of library 2 were replicates of library 1 to ascertain the replicability of the results. For each of the two library preparations, one well was intentionally left without genomic DNA to be able to detect potential errors due to the orientation of the plates. After the inactivation of the DNA Ligase, for each library, 10 µl of each well were united in an Eppendorf tube (a total of 960 µl) and cleaned with the GeneJET Gel Extraction Kit following the protocol of small size DNA fragments. This cleaning step was carried out two times independently for each library. After the cleaning step, a size selection with the ProNex Size Selective Purification System (Promega) following the manufactures indications with 1.1 and 1.6 volumes of the ProNex buffer. The size-selected DNA was eluted from the ProNex beads with 20 µl of H_2O. We prepared four PCR reactions (25 µl) for each clean-up mix (a total of 8 per library)

The 25 µl reactions contained 1 µl of size-selected DNA, 0.2 mM of each primer (Poland et al. 2012), 0.2 mM each of dNTPs (TAKARA) and 0.5 u Phusion HS II high fidelity DNA polymerase (ThermoFisher) in 1x Phusion HF buffer. The reaction conditions were: initial denaturation of 3 min at 95 °C followed by 20 cycles of 10 s denaturation at 98 °C, 15 s annealing at 65 °C, 15 s extension at 72 °C. The final extension took 5 min at 72 °C. The four PCR reactions that had a common origin in the first clean-up were united and cleaned with the GeneJet Gel Extraction Kit (ThermoFisher) following the protocol for small DNA fragments. The DNA was eluted from the columns in 50 µl elution buffer and size selected with the ProNex Size Selective Purification System (Promega) using the same conditions as mentioned above. The DNA was eluted in 30 µl of H_2O. 5 µl of the final size selected DNA were applied to an agarose gel to check the amplification and the two clean-ups that belonged to the same library were united. The DNA concentration was measured with the Qubit 2.0 fluorometer (ThermoFisher) and the dsDNA HS Assay Kit (ThermoFisher) assuring that the DNA concentration of the library preparations was above the minimum requirements specified by the supplier of the sequencing service. Additionally, the libraries were checked with a Bioanalyzer 2100 instrument (Agilent) to control the size distribution of the fragments of both libraries. The library preparations were sent to Novogene (Cambridge, UK) and sequenced on an Illumina HiSeq 2500 system. The barcode sequences corresponding to each sample are given in the Online Resource.

Data analyses

We used the process_radtags module of Stacks v2 (Rochette et al. 2019) for quality filtering (*-q* option) and demultiplexing. The core Stacks v2 modules were used with the default settings, treating forward and reverse reads independently. The populations module of Stacks v2 calculated population statistics and produced output files for other programs. The *-p* parameter, which sets the number of populations (groups) a locus must be present to process that locus, was set to the total number of groups defined in each case. The *-r* parameter, which indicates the minimum percentage of individuals in a population required to process a locus for that population, was set to 0.85. We used the file output options *--structure* and *–phylip-var-all*. The phylip files were imported into MEGAX (Kumar et al. 2018). Distance matrices, using the Maximum Composite Likelihood, were calculated in MEGAX (Kumar et al. 2018). The distance matrices were then used to calculate neighbor-nets with SplitsTree4 (Huson and Bryant 2006; Huson et al. 2010) and to conduct principal component analyses (PCA) with GenAlEx (Peakall and Smouse 2006; 2012).

Neighbor joining (NJ) and maximum likelihood (ML) trees were calculated with MegaX (Kumar et al. 2018). For the NJ trees, the substitution model was set to *p-distance*, rates among sites to *Gamma distributed with invariant sites*, and gaps/missing data were treated as *pairwise deletion*. For the ML calculations, the best substitution model was identified with MEGAX (Kumar et al. 2018) and the parameters for the phylogenetic tree reconstruction were set accordingly. Additionally, we used Bayesian inference to reconstruct phylogenetic trees with the help of MrBayes 3.7 (Ronquist and Huelsenbeck 2003). MrBayes 3.7 allows sampling across the GTR substitution model space, removing the need for a priori model testing. For this purpose, we used the setting *lset nst = mixed rates = gamma*. The number of generations was set to 1,000,000 and burnin to 200,000. We monitored that the log likelihood values reached stationarity and that the two independent runs converged. The

effective sample size of all relevant parameters was > 200 and the potential scale reduction factor (PSRF) close to 1 (0.99 < PSRF < 1.01).

We used Structure 2.3.4 (Pritchard et al. 2000) to identify individuals possibly affected by hybridization using the admixture model. First, we used three runs for each K between 1 and 6. For these runs, burnin was set to 5,000 followed by 20,000 additional generations. The time series plots of Structure proved that within the first 5,000 generations all monitored parameters reached stationarity. Also, the three independent runs showed highly similar results for all numbers of *K*. We used Structure Harvester (Earl and vonHoldt 2012) to identify the optimal value of *K* with the criteria of Evanno et al. (2005). Once the optimal number for *K* was established five additional runs with this value of *K* but with 5,000 generations for burnin and 50,000 additional generations were run. We used distruct (Rosenberg 2004) to generate the final graphics output of Structure.

Results

When the sample of *C. monspeliensis* was excluded, the sequence alignment of the reads had a length of 482,126 bp with 7,763 variable positions.

When we looked for indications of hybridization with Structure we identified $K = 2$ as the best number for clusters (Fig. 2A). One cluster without indications of hybridization was formed by the samples of *C. albidus*. Surprisingly, the samples from Morocco and Valencia showed indications of admixture with *C. albidus*, although to a low degree. There was also a high number of samples from Murcia with signals of hybridization from the present (2019) natural population of Llano del Beal, many of which show higher similarity to *C. albidus* than to *C. heterophyllus*. The plants of the reference collection and those with origin from the seeds collected in 2007, 2008, and 2011 are much less affected by hybridization according to the Structure results. The exact values of the *C albidus* and *C. heterophyllus* genetic components for each sample, together with their identification label are given in the Online Resource. We also present the results for $K = 3$ (Fig. 2B). In this case, the admixture of *C. albidus* genetic components in *C. heterophyllus* does not change essentially.

In the neighbor-net obtained with SplitsTree4, the samples close to *C. albidus* belong mainly to the natural population of Llano del Beal supporting hybridization (Fig. 3). These results support the data obtained by Structure. The specimens from Morocco and Valencia are separated from the samples from Murcia, except the artificial hybrid from Valencia. The major part of the Murcian samples forms a dense agglomeration in the center of the net.

A similar pattern can be observed in the PCA analysis (Fig. 4). A high number of samples from the natural population is situated close to *C. albidus*. The samples from Morocco and Valencia are close to each other, but their data points are outside the cloud formed by the samples from Murcia, except the artificial hybrid from Valencia. The different groups from Murcia are overlapping, although there seems to be a tendency that separates some of the samples from seeds collected in 2008, 2011, and the reference collection along the second axis.

The genetic distance values between the groups of samples (Table 1) confirm that the different groups from Murcia are close to each other when specimens identified as hybrids by Structure are removed. The group formed by the samples from Morocco and Valencia is differentiated from the samples from Murcia.

To obtain a tentative idea of the phylogenetic relationships we elaborated phylogenetic trees with a reduced number of samples. In this case, we included *C. monspeliensis* to root the trees. Murcia was represented by 11 samples from different groups, all of them without indications of hybridization according to the Structure results. Because many fragments of the *C. albidus – C. heterophyllus* group did not show sufficient homology with *C. monspeliensis*, the final alignment was reduced to 101,892 bp with 1,228 variable positions. The relationships among the samples from Morocco, Murcia, and Valencia depended on the used method for tree reconstruction. The NJ tree displays the samples from Murcia at the basal position and those from Morocco and Valencia on sister clades (Fig. 5). The ML tree shows the samples from Valencia at a basal position and those from Morocco and Murcia on a sister clade, although the support for the Morocco-Murcia-clade is very low (54 % bootstrap; Fig 6). Finally, the Bayesian analysis shows the samples from Morocco at a basal position, and those from Valencia and Murcia on a sister clade (Fig. 7). In any case, there is a certain distance between the samples from Murcia and Valencia in the phylograms.

The reduced genetic diversity in threatened organisms is a major concern in these species. We compared the genetic diversity of the four specimens of *C. albidus* with the genetic diversity in the samples of *C. heterophyllus* subsp. *carthaginensis* from Murcia after eliminating plants with possible admixture according to the Structure results. The value for the within-group mean distance in *C. albidus* was 0.000415 while the values for the different groups from Murcia were in the range of 0.000278 in the case of the natural population and 0.000417 for the samples proceeding of seeds collected in 2008.

Discussion

Initially, one might think that the populations of Murcia and Valencia should be closely related to each other and more distant from the sample of Morocco. But looking at a map shows that the population from Murcia is located nearer to the African shore (approx. 200 km) than to La Pobla de Vallbona (approx. 220 km), while La Pobla de Vallbona is situated at approx. 375 km from the African coastline. Because at present there is no continuous distribution of *C. heterophyllus* populations in Spain and it is not clear if there ever was, it should not automatically be assumed that the Spanish populations are closely related or represent what is left of a once continuous distribution area. *Cistus* species are not specially adapted to long-distance dispersal, but there is evidence that it occurs occasionally in this genus (Guzmán and Vargas 2009b; Fernández-Mazuecos and Vargas 2010). These authors observed that in *C. monspeliensis* and *C. ladanifer* L., two species located in lowlands and *C. salviifolius* L., a species with wide ecological preferences, maritime barriers like the Strait of Gibraltar do not pose serious limitations to colonization. But in *C. laurifolius* L., a species restricted to highlands, they concluded that distance barrier hypotheses cannot be rejected when analyzing the distribution of haplotypes. As a consequence, in our case, the hypothesis that Iberian Peninsula was colonized two times independently by *C. heterophyllus* (a lowland species) from North Africa seems possible. If this is the case, the taxonomic treatment at the subspecies level might need a revision, because *C. heterophyllus* subsp. *carthaginensis* would no longer be a monophyletic entity. In this case, measures to improve the situation in the Valencia population become

complicated because there would be no plant material ideally suited to rise the genetic diversity and to guarantee the long-time survival of the species in this area. If there were no other possibilities, detailed genetic data would allow selecting individuals with the highest degree of relatedness to the Valencia specimen. In this situation, a detailed taxonomical study, integrating morphological and molecular data and with a good representation of plants from Algeria and Morocco is highly desirable. It seems possible that certain North African populations are more closely related to the Valencia specimen than the population from Murcia and could serve as material to reinforce the Valencia population.

A major concern in threatened species with low effective population size is the reduced genetic diversity and increased inbreeding (Crow and Kimura 1970; Keller and Waller 2002). In our case, the mean genetic distance among the samples from Murcia, excluding specimens affected by introgression from *C. albidus,* is seemingly not much lower than that of the very frequent *C. albidus*. This at first sight surprising result might be explained by the fact that *C. heterophyllus* subsp. *carthaginensis* was much more frequent in Murcia in relatively recent times (Navarro Cano 2018). The area of the original distribution (municipalities Cartagena and La Unión) is probably located near two mountains called Sancti Spiritu and Peña del Águila (Navarro Cano 2018). Especially the landscape of the first one changed drastically when mining in open-air destroyed the natural vegetation completely. In the second mountain, it is supposed that the absence of fire, grassing and other disturbances allowed competing species, most of all *Brachypodium retusum* (Pers.) P. Beauv., to overgrow *C. heterophyllus* (Navarro Cano 2018). But this genetic diversity might soon be lost if no measures are taken to increase the effective population size of the natural populations and reintroduce plants grown in nurseries.

Hybridization has always been a major concern in the planning of the measures to improve the situation of *C. heterophyllus* in Spain. Jiménez et al. (2007) and Jiménez Martínez et al. (2018) concluded in their RAPD analyses that a general introgression of *C. albidus* genes could be observed in the Murcia population. They also concluded that the genetic diversity of the Murcia population was extremely low. Concerning diversity, their data does not show that the diversity is much lower than that of the *C. albidus* samples they included. On the contrary, a figure of their PCA analysis shows that the data points of *C. albidus* are closer together than those of *C. heterophyllus* subsp. *carthaginenis* from Murcia. RAPD bands shared by *C. albidus* and the samples from Murcia may also be caused by incomplete lineage sorting (ILS) of these closely related species. The interpretation of the data of these authors might be biased by the possibility that they expected the samples of Murcia to be more closely related to the plant from Valencia and not a closer relationship of the sample from Valencia with the Algerian plant they included. Pawluczyk (2017) and Pawluczyk et al. (2018), using ITS sequences as markers found a complex pattern with sequences of plants classified as *C. heterophyllus* or as hybrids (*C x clausonii*) close to *C. albidus*. These authors interpreted their data as an indication of frequent hybridization, even in plants morphologically of pure type. But they do not show any evidence that their data cannot be interpreted in terms of ILS. It is not possible to compare the sequences of the plants from Murcia Pawluczyk et al. (2018) used with our data, because they do not identify the individual numbers of each plant.

Our data suggest that introgression might be less common than feared by prior authors and that it is of the highest concern in the plants that arose in the natural population in recent times. Our data also show that the collection of seeds in the years 2007, 2008, 2011, and 2013 by the competent authorities was of critical importance, since without these collections at present there would be a very low number of plants without evidence of introgression in the natural population.

In terms of practical use of the data, we suggest that the competent authorities eliminate the plants of the natural population with a high degree of similarity to *C. albidus*. It is planned to use the data to establish a new reference collection in the plant nursery of the local government of Murcia (CCFS). To this aim, in addition to avoiding plants with evidence of introgression, care should be taken to represent as much genetic diversity as possible. The same is true for the reintroduction of plants in the natural habitat near the original population.

This study shows that GBS is a cost-effective method to assess the genome-wide diversity of populations of endangered species. The results we obtained are a step beyond the limitations of prior studies that relied on DNA sequences of a low number of markers or RAPD markers. As already mentioned, future research should include a good representation of North African samples if possible due to the political situation, especially in Algeria

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FIGURE CAPTIONS

Fig. 1 Distribution of *Cistus heterophyllus*. The species is frequent in the NE of Morocco and the NW of Algeria. In Spain, there are only two known localities, one in Llano del Beal (Cartagena municipality, Autonomous Community of the Region of Murcia) and the other with only one plant in La Pobla de Vallbona (Valencian Community). The distance between the Spanish localities and between these and the nearest point in North Africa are indicated

Fig. 2 Structure results for $K = 2$ (A) and $K = 3$ (B). The genetic component of *Cistus albidus* is of green color. The natural population is heavily influenced by hybridization, but also the plants from Morocco and Valencia show a minor influence of *C. albidus*. The samples proceeding of seeds collected in 2007, 2008, and 2011 and the reference collection show a much lower signal of introgression than the present natural population. The values for each individual are given in the Online Resource

Fig. 3 Neighbor-net obtained with SplitsTree4 based on the sequence data. The samples from Morocco and Valencia (to the left) are situated away from the samples from Murcia (center). Several samples from Murcia occupy positions in the upper direction where *Cistus albidus* is situated. The color code indicates the major genetic component of the samples according to $K = 3$ in the Structure analysis: green *C. albidus*, blue *C. heterophyllus* cluster 1, red: *C. heterophyllus* cluster 2 (compare with Fig. 2)

Fig. 4 PCA analysis of the sequence data. The colors codify the groups indicated in the legend the samples belong to. The general outcome confirms the results obtained with the neighbor-net. The samples from Morocco and Valencia are close to each other and separated from the samples from Murcia. The natural population of Murcia harbors several specimens that are near *Cistus albidus*

Fig. 5 NJ tree with selected samples. *Cistus monspeliensis* was chosen as an outgroup. The samples from Morocco and Valencia share a clade sister to the samples from Murcia. The two samples of *C. heterophyllus* subsp. *heterophyllus* M, *C. heterophyllus* subsp. *carthaginensis* ISB2 and *C. monspeliensis* are replicates included in different GBS library preparations. The very similar sequences show the high reproducibility of our analyses

Fig. 6. ML tree with selected samples. In this case, the samples of Valencia occupy the basal position within *Cistus heterophyllus* and the samples of Morocco and Murcia share a common clade, although with very low bootstrap support. Two replicates were used for *C. heterophyllus* subsp. *heterophyllus* M, *C. heterophyllus* subsp. *carthaginensis* ISB2 and *C. monspeliensis* as in Fig. 5

Fig. 7 Bayesian analysis of the sequence data. In this case, the sample from Morocco occupies the basal position, with the samples from Valencia and Murcia on sister clades, although with some distance in between. Two replicates were used for *C. heterophyllus* subsp. *heterophyllus* M, *C. heterophyllus* subsp. *carthaginensis* ISB2 and *C. monspeliensis* as in Fig. 5

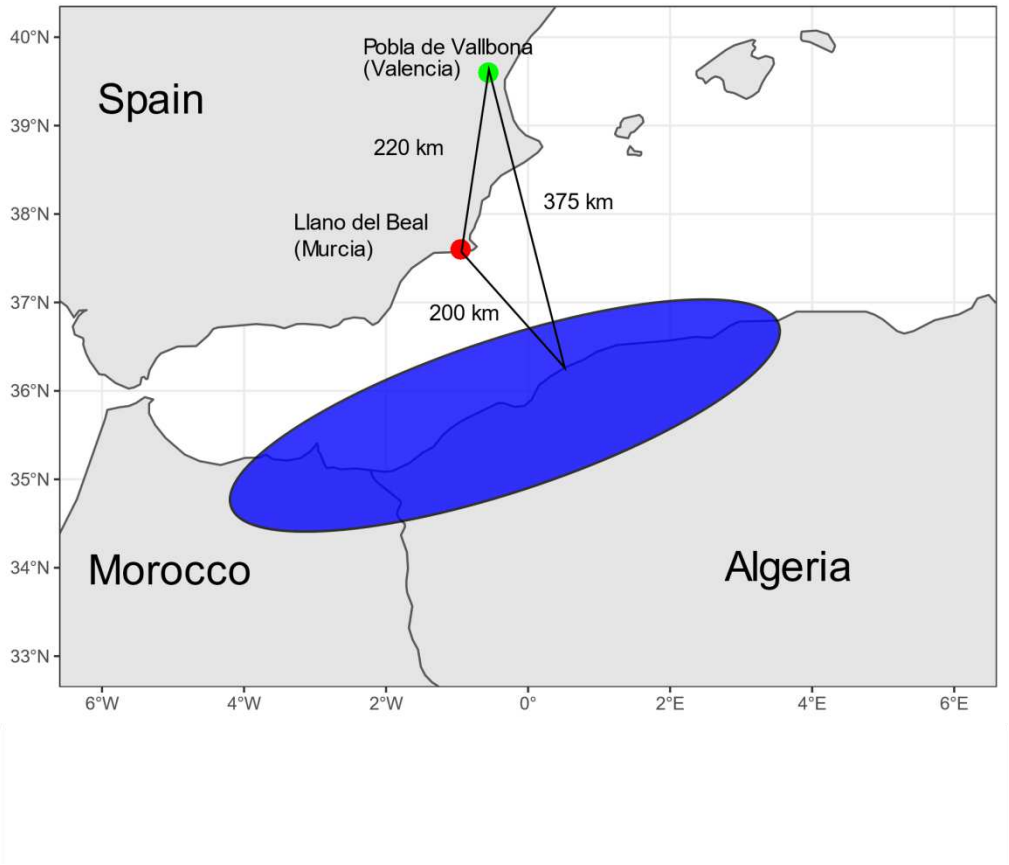
Pobla de Vallbona
(Valencia)
Spain
220 km
375 km
Llano del Beal
(Murcia)
200 km
Morocco
Algeria
40°N
39°N
38°N
37°N
36°N
35°N
34°N
33°N
6°W
4°W
2°W
0°
2°E
4°E
6°E

Fig.1

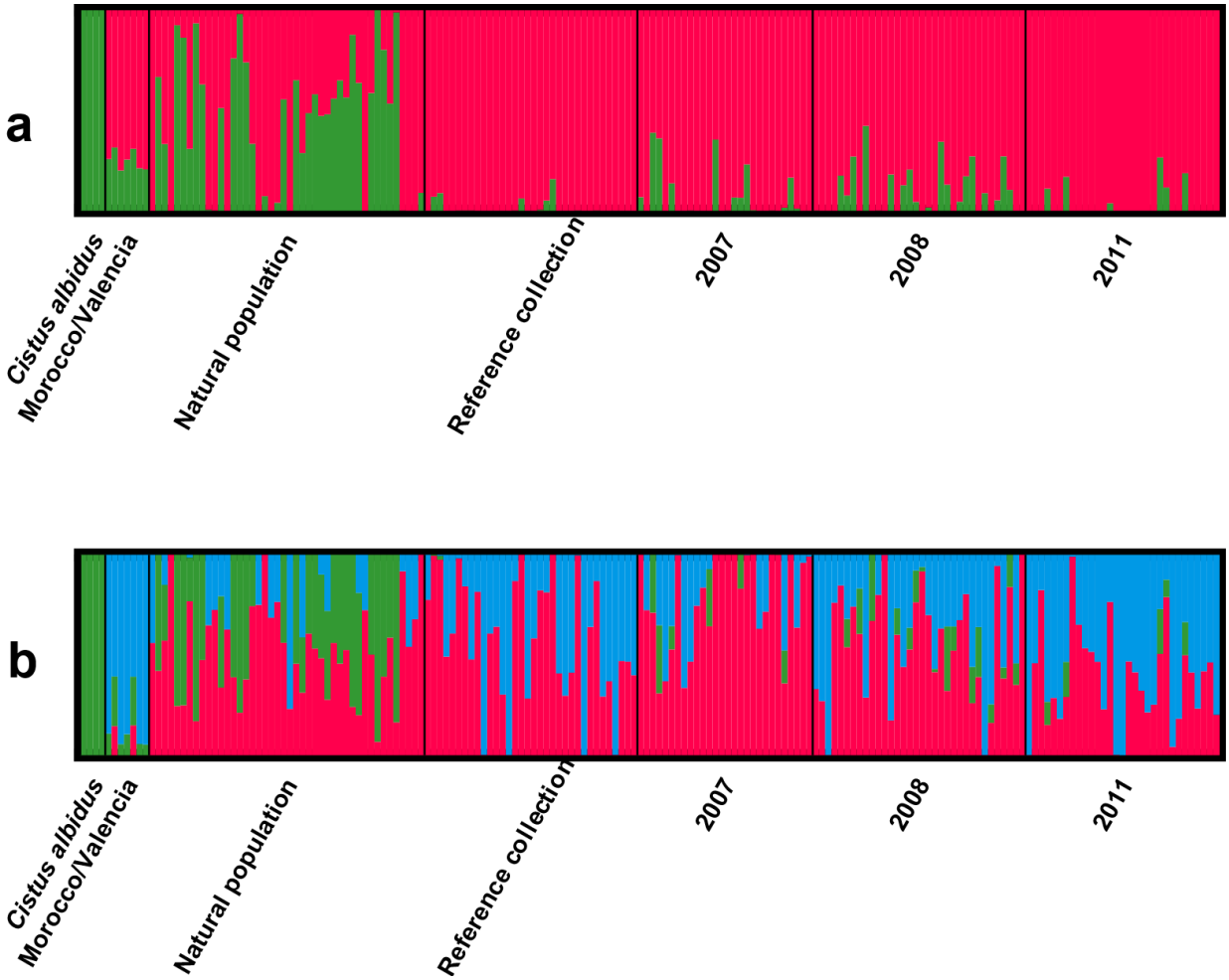
a
Cistus albidus Morocco/Valencia
Natural population
Reference collection
2007
2008
2011
b
Cistus albidus Morocco/Valencia
Natural population
Reference collection
2007
2008
2011

Fig.2

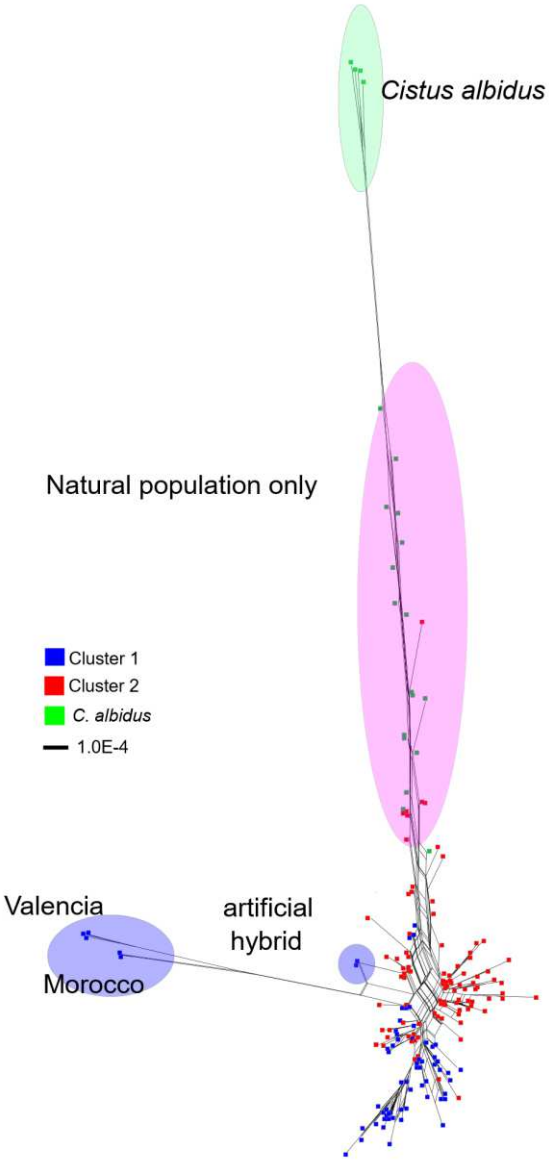
Cistus albidus
Natural population only
Cluster 1
Cluster 2
C. albidus
1.0E-4
Valencia
Morocco
artificial
hybrid

Fig.3

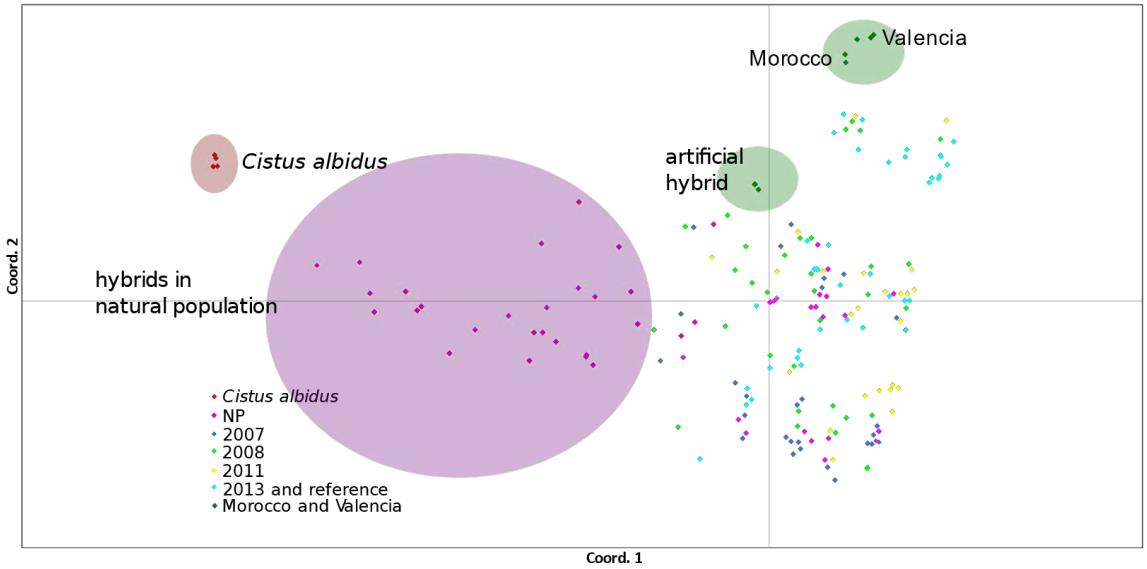
Valencia
Morocco
Cistus albidus
artificial
hybrid
hybrids in
natural population
Cistus albidus
NP
2007
2008
2011
2013 and reference
Morocco and Valencia
Coord. 2
Coord. 1

Fig.4

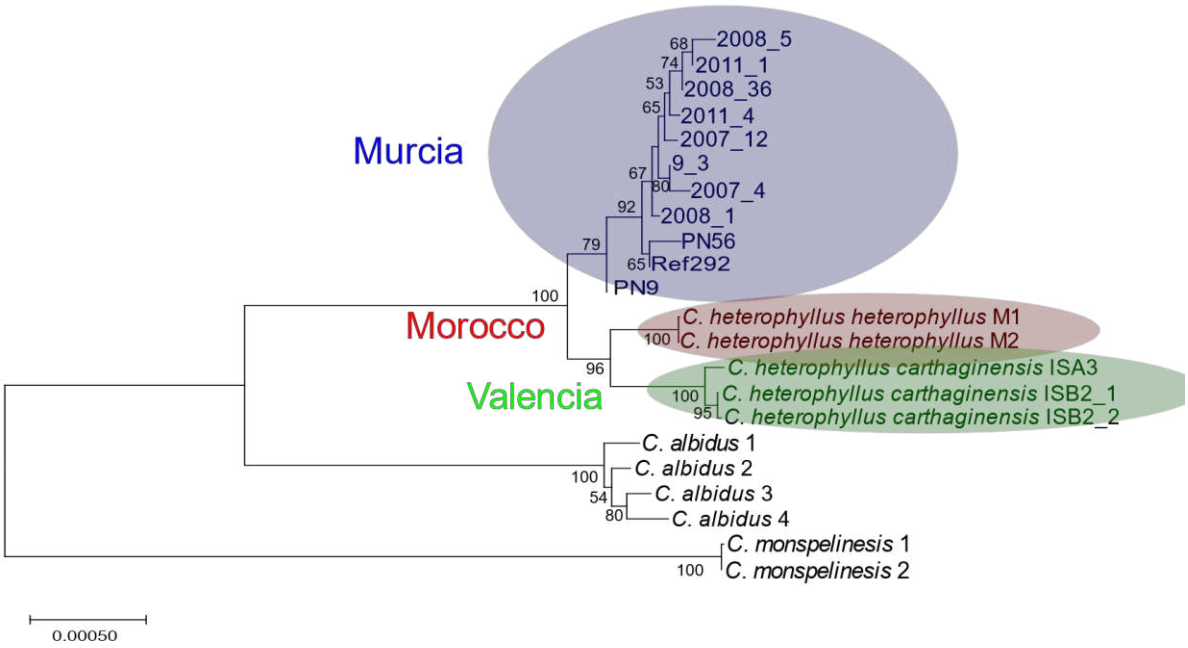
Murcia
Morocco
Valencia
2008_5
2011_1
2008_36
2011_4
2007_12
9_3
2007_4
2008_1
PN56
Ref292
PN9
C. heterophyllus heterophyllus M1
C. heterophyllus heterophyllus M2
C. heterophyllus carthaginensis ISA3
C. heterophyllus carthaginensis ISB2_1
C. heterophyllus carthaginensis ISB2_2
C. albidus 1
C. albidus 2
C. albidus 3
C. albidus 4
C. monspelinesis 1
C. monspelinesis 2
68
74
53
65
67
80
92
79
65
100
100
96
100
95
100
54
80
100
0.00050

Fig.5

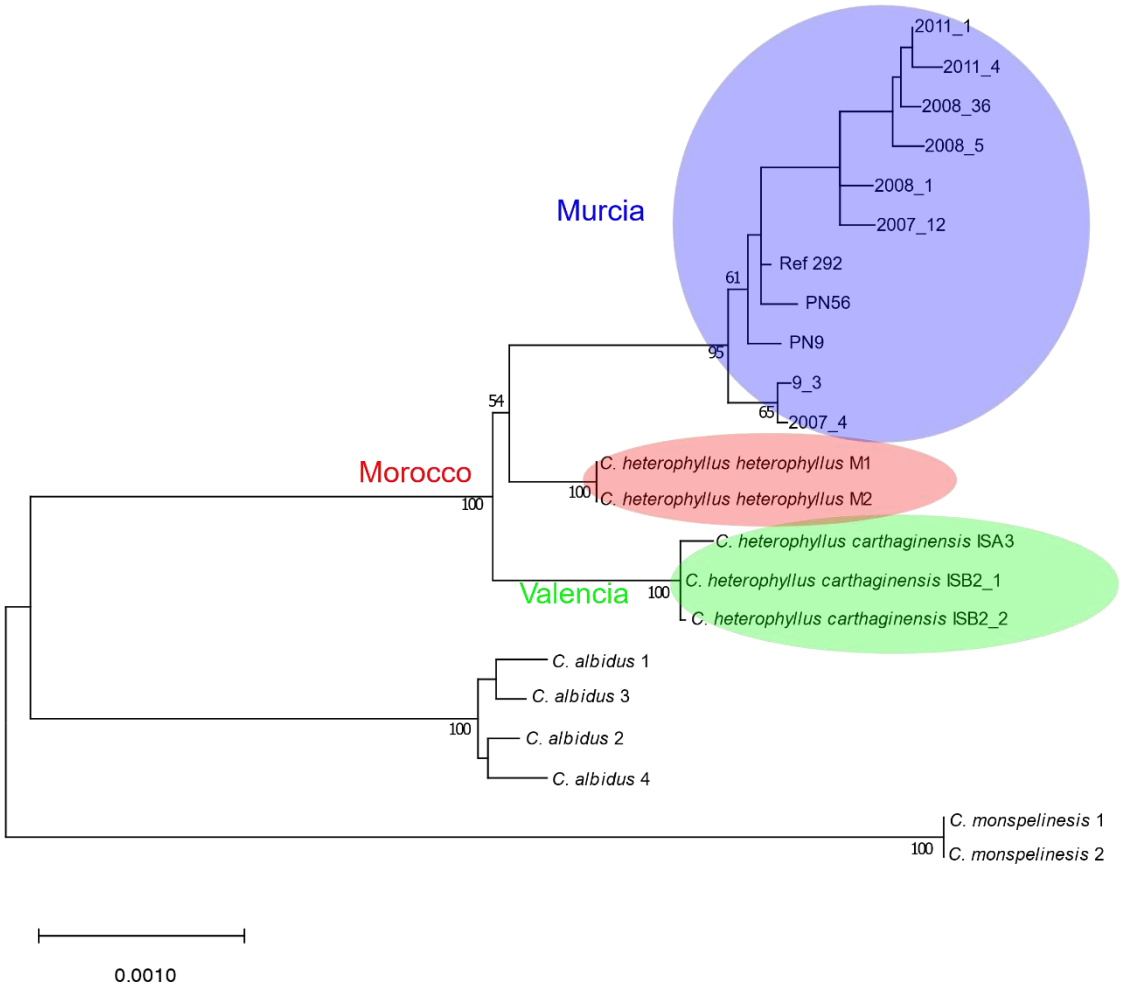
2011_1
2011_4
2008_36
2008_5
2008_1
2007_12
Murcia
Ref 292
PN56
PN9
9_3
2007_4
61
95
54
65
Morocco
C. heterophyllus heterophyllus M1
C. heterophyllus heterophyllus M2
100
100
C. heterophyllus carthaginensis ISA3
C. heterophyllus carthaginensis ISB2_1
C. heterophyllus carthaginensis ISB2_2
Valencia
100
C. albidus 1
C. albidus 3
C. albidus 2
C. albidus 4
100
C. monspelinesis 1
C. monspelinesis 2
100
0.0010

Fig.6.

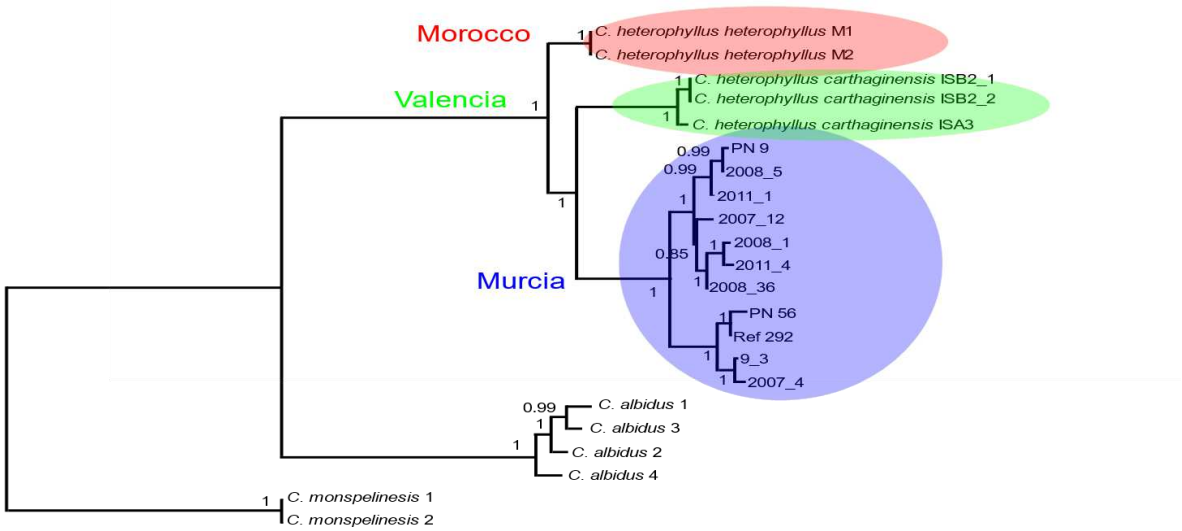
Morocco
Valencia
Murcia
C. heterophyllus heterophyllus M1
C. heterophyllus heterophyllus M2
C. heterophyllus carthaginensis ISB2_1
C. heterophyllus carthaginensis ISB2_2
C. heterophyllus carthaginensis ISA3
PN 9
2008_5
2011_1
2007_12
2008_1
2011_4
2008_36
PN 56
Ref 292
9_3
2007_4
C. albidus 1
C. albidus 3
C. albidus 2
C. albidus 4
C. monspelinesis 1
C. monspelinesis 2

Fig.7

Table 1 Genetic distances (*p-distance*) within and between groups of samples. Specimens identified as hybrids by Structure were removed. The groups from Murcia (natural population, reference collection, plants from seeds collected in 2007, in 2008, and 2011) are all very similar (*p-distance* ≤ 0.000502). The distances of the groups from Murcia with the plants from Valencia are higher (*p-distance* ≥ 0.001489). The within-group diversity in Murcia (0.000208 ≤ *p-distance* ≤ 0.000417) is in the range of the mean distance found in *Cistus albidus* (0.000415).

	Cistus albidus	**Morocco and Valencia**	**Natural population**	**Reference collection**	**2007**	**2008**	**2011**
Cistus albidus	0.000415						
Morocco and Valencia	0.005360	0.000208					
Natural population	0.004084	0.001496	0.000278				
Reference collection	0.004259	0.001489	0.000313	0.000341			
2007	0.004350	0.001705	0.000311	0.000414	0.000307		
2008	0.004428	0.001595	0.000328	0.000370	0.000429	0.000417	
2011	0.004788	0.001584	0.000368	0.000372	0.000502	0.000426	0.000365

Figures

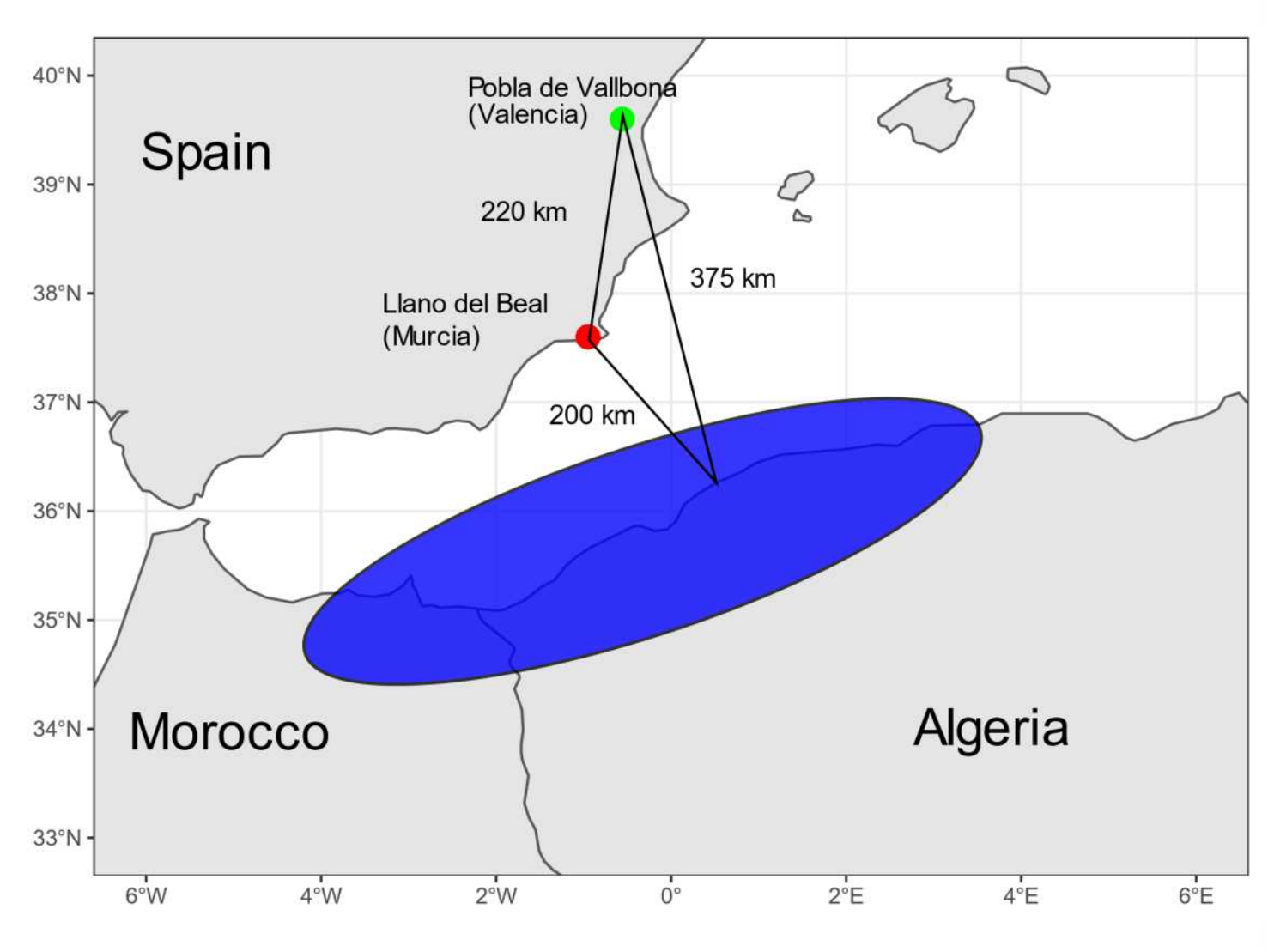

Figure 1

Distribution of Cistus heterophyllus. The species is frequent in the NE of Morocco and the NW of Algeria. In Spain, there are only two known localities, one in Llano del Beal (Cartagena municipality, Autonomous Community of the Region of Murcia) and the other with only one plant in La Pobla de Vallbona (Valencian Community). The distance between the Spanish localities and between these and the nearest point in North Africa are indicated

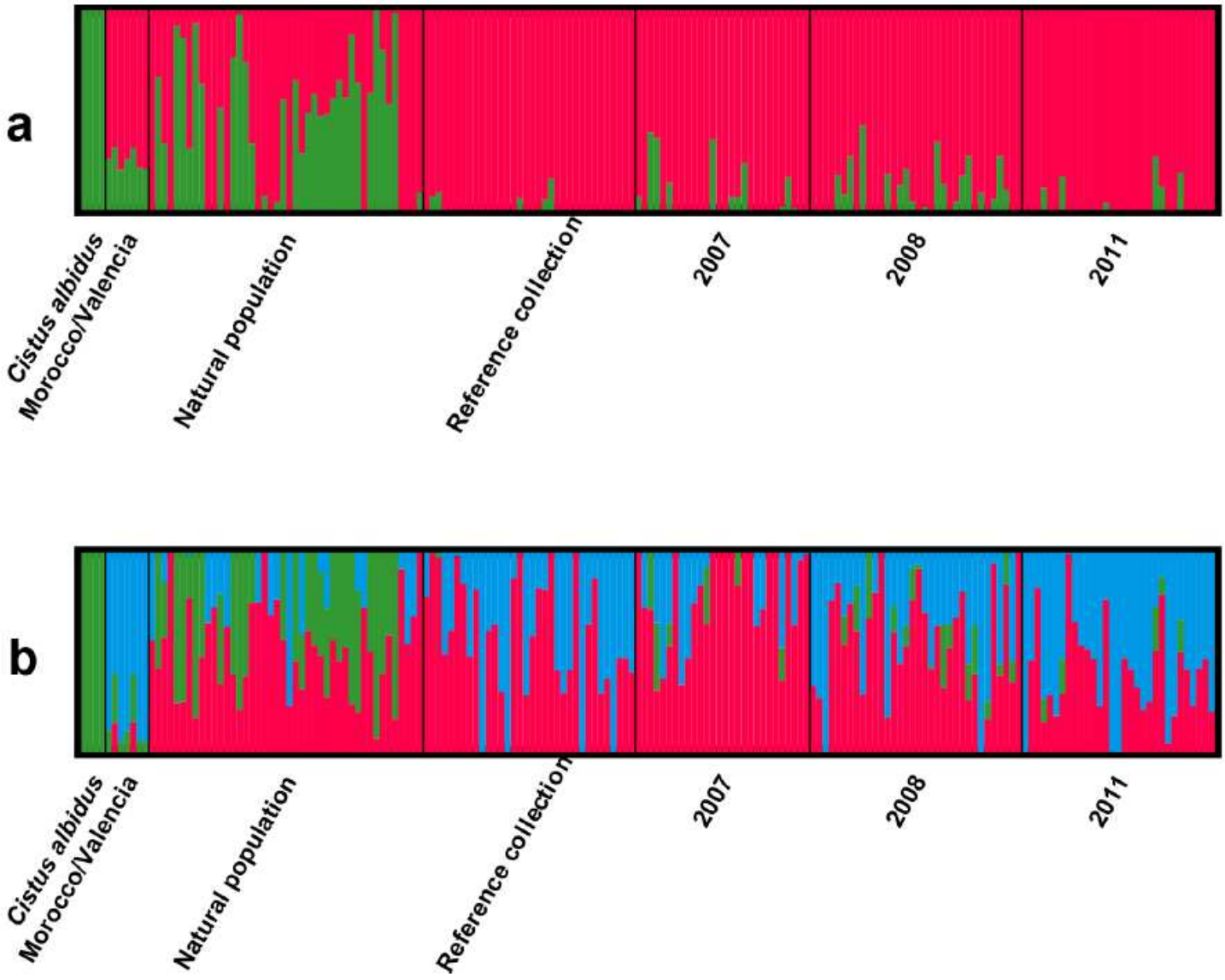

Figure 2

Structure results for K = 2 (A) and K = 3 (B). The genetic component of Cistus albidus is of green color. The natural population is heavily influenced by hybridization, but also the plants from Morocco and Valencia show a minor influence of C. albidus. The samples proceeding of seeds collected in 2007, 2008, and 2011 and the reference collection show a much lower signal of introgression than the present natural population. The values for each individual are given in the Online Resource

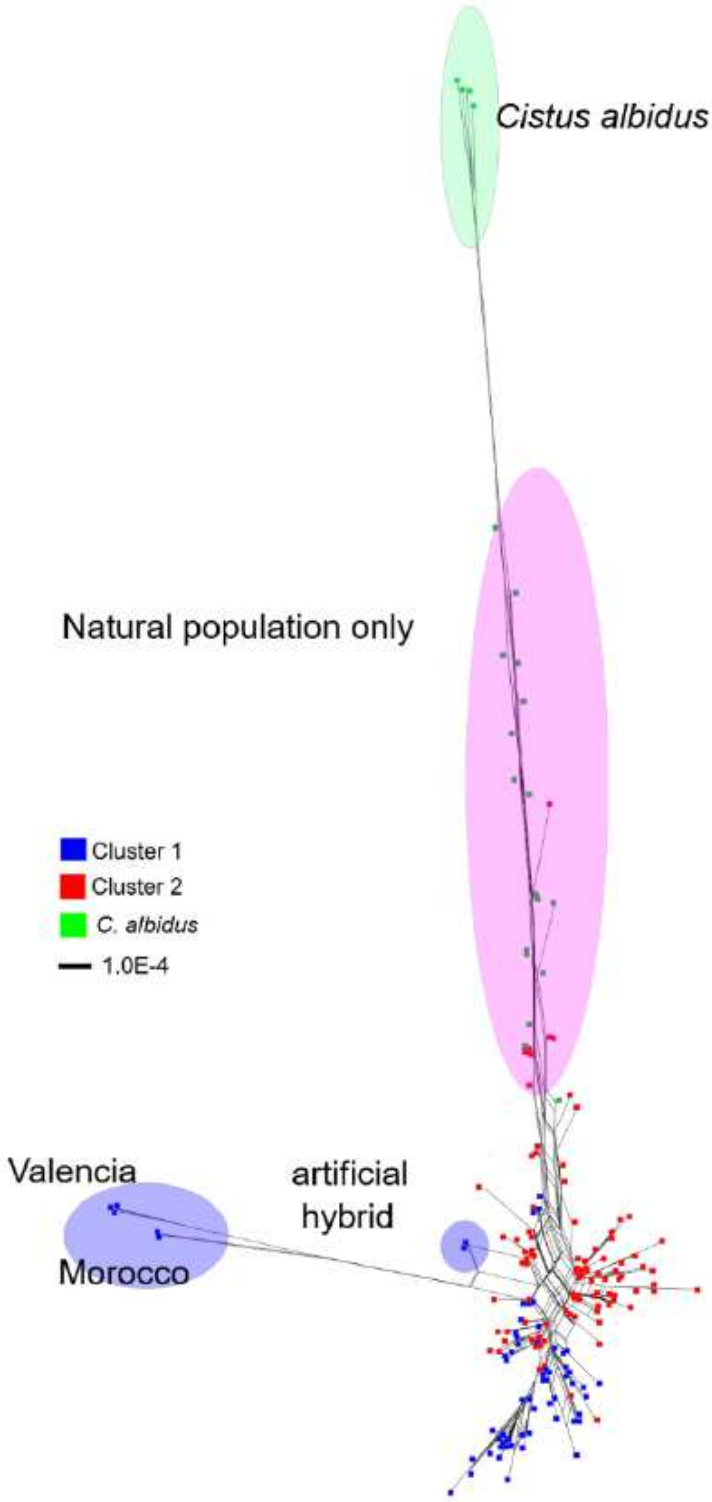

Figure 3

Neighbor-net obtained with SplitsTree4 based on the sequence data. The samples from Morocco and Valencia (to the left) are situated away from the samples from Murcia (center). Several samples from Murcia occupy positions in the upper direction where Cistus albidus is situated. The color code indicates the major genetic component of the samples according to K = 3 in the Structure analysis: green C. albidus, blue C. heterophyllus cluster 1, red: C. heterophyllus cluster 2 (compare with Fig. 2)

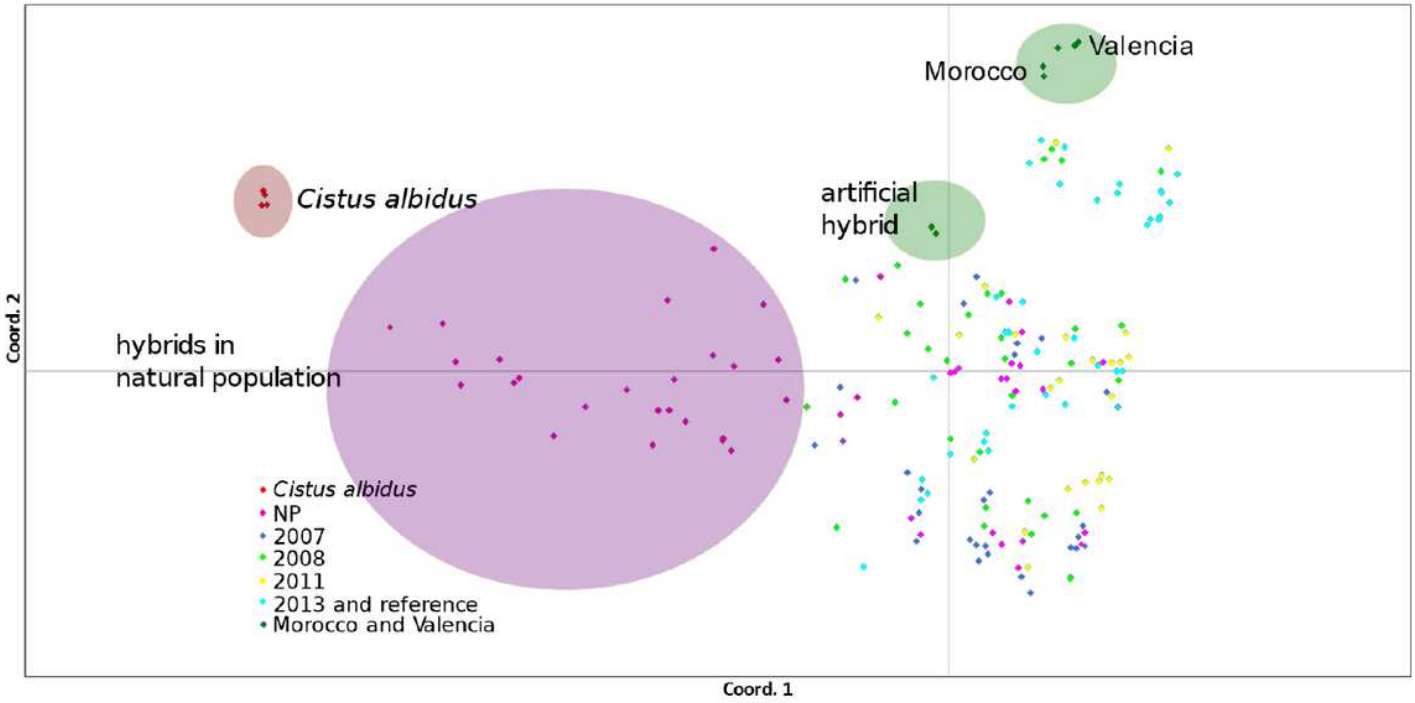

Figure 4

PCA analysis of the sequence data. The colors codify the groups indicated in the legend the samples belong to. The general outcome confirms the results obtained with the neighbor-net. The samples from Morocco and Valencia are close to each other and separated from the samples from Murcia. The natural population of Murcia harbors several specimens that are near Cistus albidus

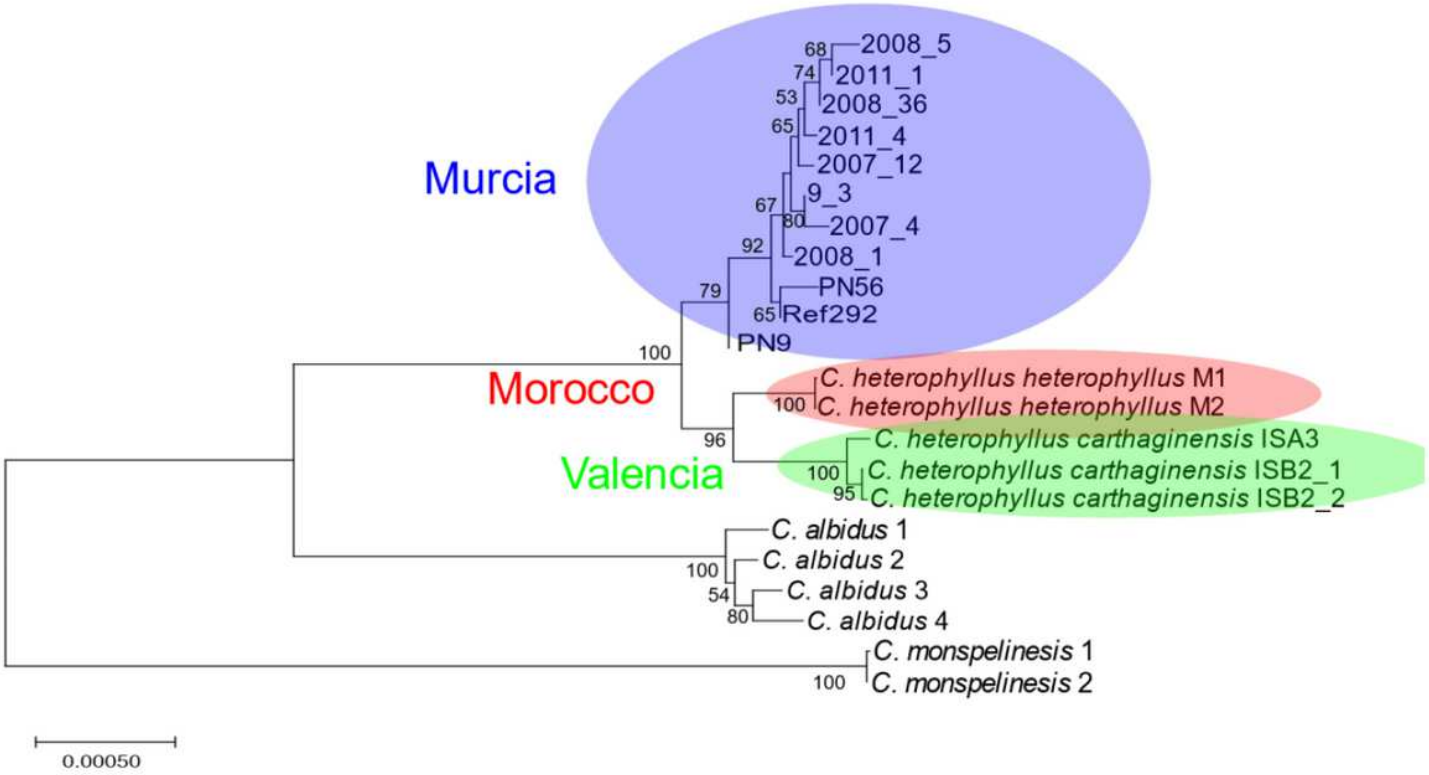

Figure 5

NJ tree with selected samples. Cistus monspeliensis was chosen as an outgroup. The samples from Morocco and Valencia share a clade sister to the samples from Murcia. The two samples of C. heterophyllus subsp. heterophyllus M, C. heterophyllus subsp. carthaginensis ISB2 and C. monspeliensis are replicates included in different GBS library preparations. The very similar sequences show the high reproducibility of our analyses

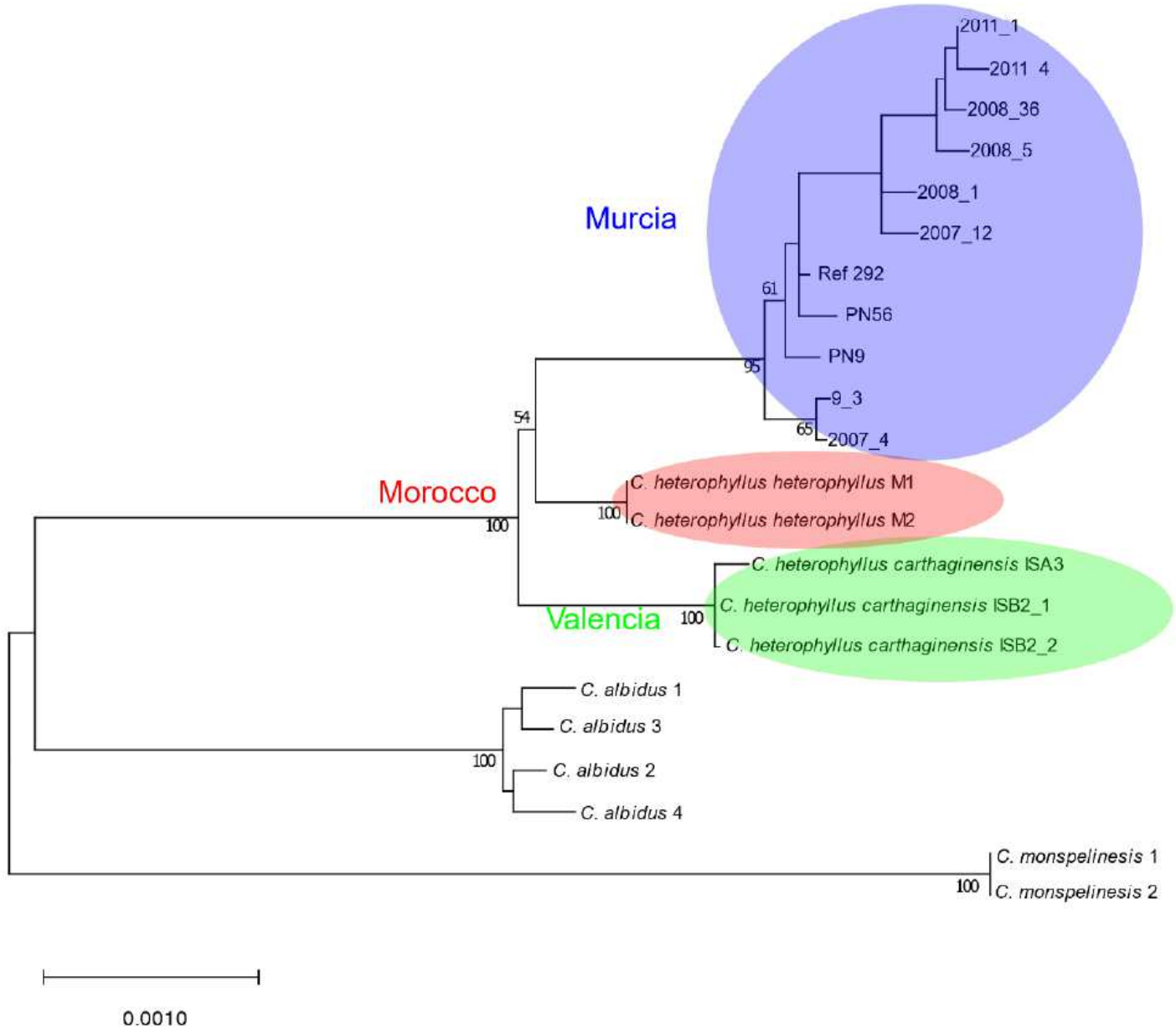

Figure 6

ML tree with selected samples. In this case, the samples of Valencia occupy the basal position within Cistus heterophyllus and the samples of Morocco and Murcia share a common clade, although with very low bootstrap support. Two replicates were used for C. heterophyllus subsp. heterophyllus M, C. heterophyllus subsp. carthaginensis ISB2 and C. monspeliensis as in Fig. 5

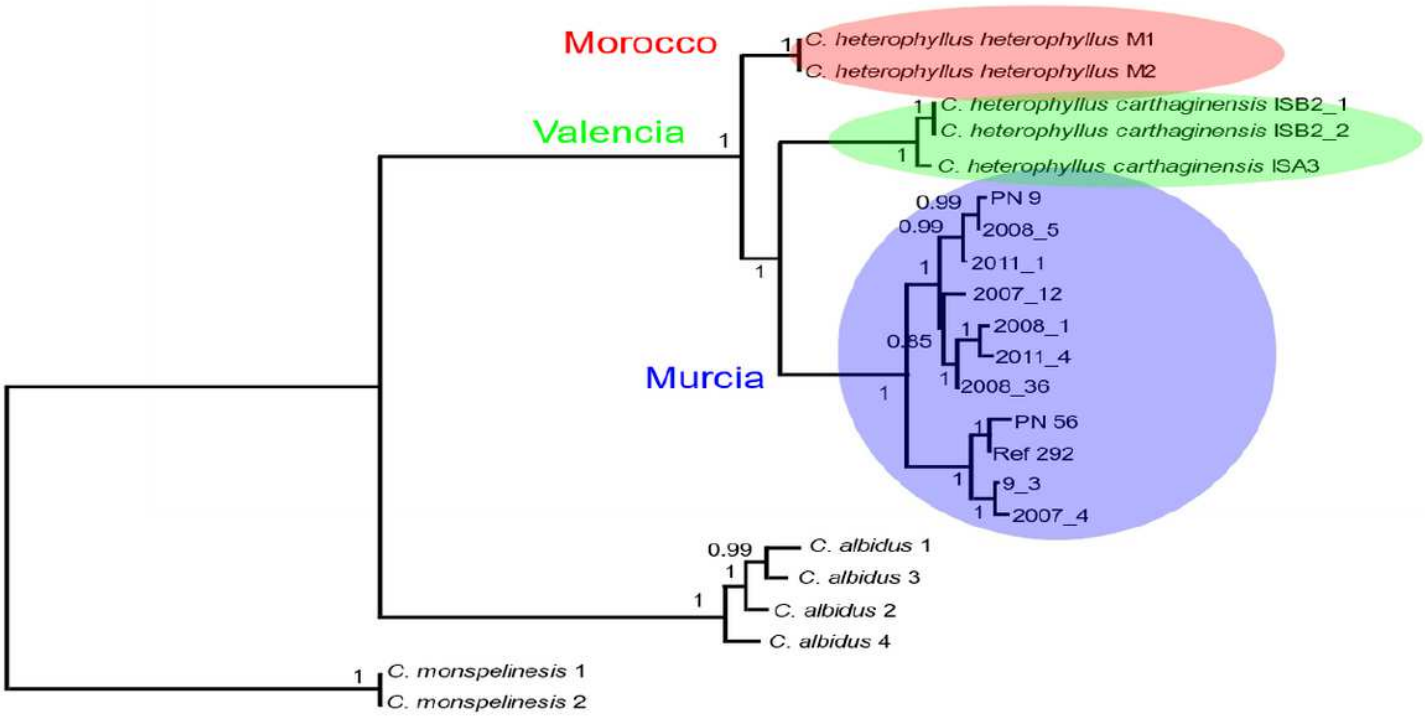

Figure 7

Bayesian analysis of the sequence data. In this case, the sample from Morocco occupies the basal position, with the samples from Valencia and Murcia on sister clades, although with some distance in between. Two replicates were used for C. heterophyllus subsp. heterophyllus M, C. heterophyllus subsp. carthaginensis ISB2 and C. monspeliensis as in Fig. 5

Supplementary Files

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- SupplementaryTable.pdf