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Genetic diversity and phylogeographical assessment of *Helianthemum caput-felis* Boiss. (Cistaceae) based on AFLP markers.

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Abstract

Analyses of AFLP markers were carried out for three populations of *Helianthemum caput-felis*. The selected populations corresponded to well-fragmented areas from Spanish territories: Iberian Peninsula (Alicante), Balearic Islands (Majorca) and North Africa (Melilla). Analyses of molecular data (PCO, Fst values and Fst dendrograms) revealed a separation between the North African population and the Iberian-Balearic populations. The importance of the geological events within the Mediterranean basin, especially changes of the water level after the Messinian salinity crisis, is discussed. Multivariate analyses yielded close relationships between populations from the Balearic Islands and the Iberian Peninsula, though some uncertain data were detected. Balearic subpopulations with different ecological habitats appeared to be genetically similar. Finally, the obtained genetic data are also relevant for conservation purposes.

Keywords: AFLP, Cistaceae, Gene flow, Mediterranean Sea, Population isolation, Spain.

Riassunto

Diversità genetica e valutazione filogeografica *Helianthemum caput-felis* Boiss. (Cistaceae) basata sui marcatori AFLP.
Le analisi dei marcatori AFLP sono state condotte per tre popolazioni di *Helianthemum caput-felis*. Le popolazioni selezionate corrispondono ad aree ben frammentate del territorio spagnolo: la penisola Iberica (Alicante), le isole Baleari (Majorca) e il Nord Africa (Melilla). Le analisi dei dati molecolari (PCO, valori Fst e dendogrammi Fst) rivelano una separazione tra la popolazione del Nord Africa e le popolazioni Iberiche-Baleariche. Viene discussa l'importanza degli eventi geologici nel bacino del Mediterraneo, con speciale riferimento ai cambiamenti del livello dell'acqua che provocarono la crisi di salinatà nel Messinano. Le analisi multivariate hanno evidenziato relazioni strette tra le popolazioni delle Isole Baleari e della Penisola Iberica, anche se sono stati evidenziati alcuni dati incerti. La subpopolazione balearica con differenti habitats ecologici, appare essere geneticamente simile. Infine, i dati genetici ottenuti sono considerati come rilevanti per avanzare proposte di conservazione della specie.

Parole chiave: AFLP, cistaceae, flusso geni, mar Mediterraneo, isolamento della popolazione, Spagna.

Introduction

Helianthemum caput-felis Boiss. (Cistaceae) is the only taxon belonging to *H.* sect. *Caput-felis* G. López. This section is characterized by two features exclusive in the genus (López-González, 1992; 1993): seeds with a cerebriform external surface and chromosome number 2*n*=24. Tébar *et al.* (1997) and Rodríguez-Pérez (2005) reported the alogamous character of this species, being a generalist entomogamous plant. Within the genus *Helianthemum*, this species has been considered as the unique component of an antique evolutive line (Arrigoni, 1971; Valdés-Bermejo, 1980; López-González, 1992). From an ecological point of view, *H. caput-felis* typically grows on coastal environments, under the direct influence of the sea, mostly on horizontal and vertical calcareous rocky cliffs (0-200 m). As other Cistaceae species, *H. caput-felis* participates in xeric perennial shrubs that have been catalogued as fragile and endangered habitats (cf. Directive of Habitats 96/62/CEE) due to their proximity to anthropized areas. The main threats of these populations are the disturbance and/or total destruction of their habitats, mainly as a result of urbanization and tourist activities (Pujol, 2001). Other relevant threat is derived from habitat competition with aggressive aloctonous plants such as *Carpobrotus* sp. (Sáez & Roselló, 2001).

Helianthemum caput-felis is distributed along the Western Mediterranean basin, in several fragmented populations located in the Iberian Peninsula, the Balearic Islands, North Africa and Sardinia (López-González, 1993). Among them, the widest distribution and the largest populations are situated in Spain, in three different areas: Iberian Peninsula (Alicante province), Balearic Islands (Majorca) and Melilla (in North Africa) (Guinea, 1954; López-González, 1993; González-García *et al.*, 2003). Furthermore, those populations are rare and punctual within the cited areas, and are completely fragmented without any current terrestrial connection. The presence of *H. caput-felis* in other localities (e.g. Sardinia or Morocco) is restricted to unique or reduced places (Arrigoni, 1971; Quézel & Santa, 1963; Pignatti, 1982; Valdés *et al.*, 2002; Amini *et al.*, 2008; Fenu & Bachetta, 2008) and no differentiated populations could be assigned within them on the basis of large geographical distances or remarkable barriers. In addition, a wider variety of ecologies could be achieved among the Spanish territories. Apart from the typical ecology (coastal cliffs), some particular populations also grow on different habitats such as sand dunes (Majorca) or rocky slopes bordering inland ravines (Melilla).

Conversely to other *Helianthemum* taxa, the populations of *H. caput-felis* are quite sparse and rare throughout its Spanish distribution. In addition, one of the most remarkable features is the presence of a natural barrier, the Mediterranean Sea, which origins the current fragmentation and isolation among populations (figure 1). The natural history of certain Mediterranean plant populations would be usually connected to the continuous geological changes happened in the Mediterranean basin (e.g. Juan *et al*., 2004; Ortiz *et al*., 2007), especially those related to changes of the sea level. One of the most important phenomena is focused on the Messinian salinity crisis, c. 5.9-5.3 Mya ago (Duggen *et al*., 2003). During this time, the sea level drop down dramatically, and many of the Western Mediterranean territories were connected without any water barrier; consequently, many plant populations could have maintained an active gene flow among them (Juan *et al*., 2004). However, these populations might have suffered a dramatic isolation after sudden and rapid refill of the Mediterranean basin c. 5.3 Mya ago (Duggen *et al*., 2003; García-Castellanos *et al*., 2009).

With regard to plant conservation, *H. caput-felis* has been included in several Red Lists in Spain (Laguna *et al*., 1998; Sáez & Roselló, 2001; Moreno, 2008) and Italy (Pignatti *et al*., 2001), but no information is available from Morocco and Algeria. According to IUCN categories, this species has been considered as endangered (EN) for Spanish and Italian populations (Pignatti *et al*., 2001; Moreno, 2008). However, the category was considered as vulnerable (VU) in certain Spanish local Red Lists such as the Valencian Community (Laguna *et al*., 1998) or the Balearic Islands (Sáez & Roselló, 2001). In addition, this plant was also included in the Directive of Habitats 97/62/CEE as a species of Community interest to be conserved. This Directive recommends the establishment of special conservation areas (appendix II) and a strict protection (appendix IV). However, this normative is effective only in European territories.

In the present study, amplified fragment length polymorphism (AFLP) marker was used to i) characterize the genetic structure of *H. caput-felis* over the Spanish populations (Iberian Peninsula, Balearic Islands and Melilla-North Africa), ii) to clarify their genetic relationships, and iii) to detect eventual gene flow among populations. The identification of these genetic features assesses the importance of the existence of natural barriers as the Mediterranean Sea. Moreover, the identity of inter- and intrapopulation genetic variation should provide relevant information for conservation purposes for fragmented populations, as it happens to *H. caput-felis*.

Material and methods

Young leaf samples were collected from three fragmented areas, which comprises the current Spanish plant distribution (figure 1; table 1): (i) Alicante (Iberian Peninsula); (ii) Majorca (Balearic Islands); and (iii) Melilla (North Africa). Moreover, two subpopulations were differentiated in Alicante (PMO, GLE) due to a large geographical distance between them (more than 100 km), as well as in Majorca (BLA, TRE) where different ecologies were detected (rocky cliffs vs. sand dunes, respectively). In the case of Melilla, one population was only considered since the distance between the recollection areas was less than 1 km.

The total DNA was extracted from silica gel-dried material using a modified 2x CTAB protocol (Csiba & Powell, 2006). After precipitation in double volumen of frozen absolut ethanol, DNA was resuspended in 1000 μl 0.1xTE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Extracted DNA was purified using MOBIO purification kits, according to the manufacturer's protocols.

AFLP was performed according to AFLP^{TM} Plant Mapping Protocol (Applied Biosystem Inc.), for plants with normal genome size. A primer trial was done with 29 different primers combinations, but the primers EcoRI-ACA + MseI-CTG (blue-labelled), EcoRI-ACA + MseI-CAT (blue-labelled) and EcoRI-AGG + MseI-CAC (green-labelled) only showed clearly distinguised bands.

Fluorescently labelled fragments from the selective amplification were separated using an ABI 3100 automated sequencer. Gel analysis was carried out using Genescan 3.1. and Genotyper 2.0 (Applied Biosystems, Inc.). Only amplified fragments with sizes ranging from 50 to 500 bases were scored because bands beyond this size range cannot be accurately sized.

Genetic diversity was evaluated as the mean effective number of alleles (A), the percentage of polymorphic loci (P), gene diversity (Nei 1973) and Shannon diversity (Shannon & Weaver 1949). All these values were obtained with Popgene 1.31 (Yeh *et al*., 1997). A Principal Coordinate Analysis (PCOA) was also

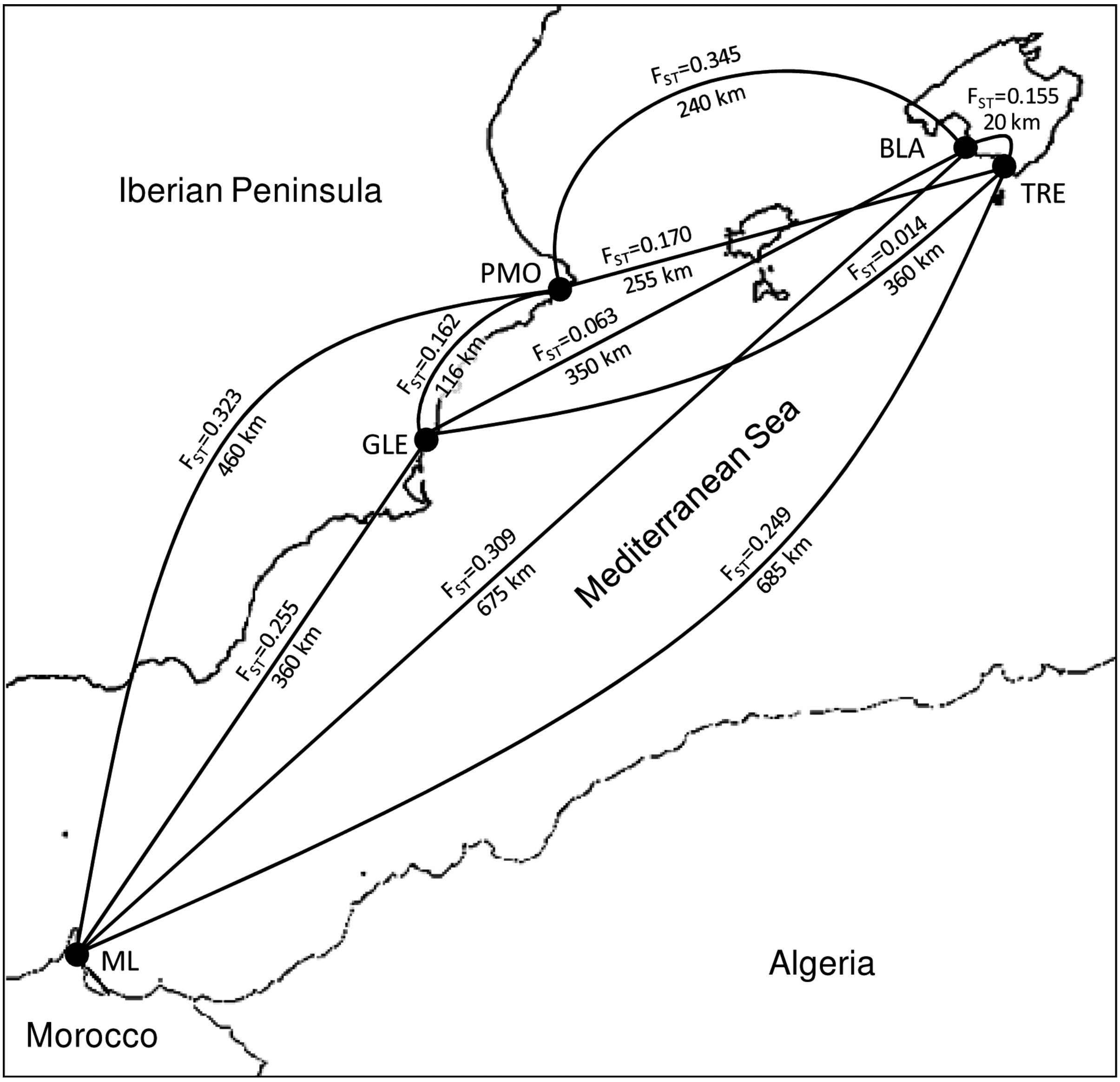

Fig. 1 - Map of the studied populations of *Helianthemum caput-felis*, with the indication of geographical distance and Fst values among them.

performed based on gene diversity (Nei 1978) using Genalex 6.2 (Peakall & Smouse, 2006). A neighbour joining (NJ) dendrogram was done based on Fst pairwise values using MEGA4 (Tamura *et al*., 2007). The genetic differentiation coefficient (Fst), Mantel test and an analysis of Molecular Variance (AMOVA; Excoffier *et al*., 1992) were done using Arlequin 3.0 (Excoffier *et al*., 2005). AMOVA was estimated with the variance components for (i) within populations, (ii) among populations within regions, and (iii) among regions.

Results

The AFLP was carried out on 70 individuals, which cover the range of the species in Spain. However, some of them showed weak electropherograms or did not amplify at all, therefore the number of studied samples was diminished (5 for BLA, 11 for TRE, 4 for PMO, 5 for GLE, and 7 for ML). The final matrix contained 149 characters, of which 70 bands (47%) were polymorphic (table 1). The population with the lowest polymorphic value corresponds to Alicante,

Population	Subpopulation	Abbr.	MGRS	Ind.
Balearic Islands	Cap Blanc	BLA	31SDD8158	15
	Es Trenc	TRE	31SDD5944	15
Alicante	Portet Moraira	PMO	31SBC5185	15
	Punta Glea	GLE	30SXG9898	15
Melilla	Barranco del Nano	ML	30SWE0307	5
	Barranco del Quemado	ML	30SWE0408	5

Tab. 1 - Studied localities of *Helianthemum caput-felis*. Names of populations, subpopulations, abbreviatures (Abbr.), geographic coordenates (MGRS), and number of sampled individuals (Ind.), are provided.

whereas those from the Balearic Islands and Melilla showed equivalent data. Similar relationships were detected for the genetic diversity values (table 1).

The global Fst obtained for the Spanish populations was 0.221. The pairwise values between subpopulations varied between 0.014 (GLE-TRE) and 0.345 (PMO-BLA) (figure 1 map of Fst and geographic distances). AMOVA analysis pointed out to a greater genetic variability within populations (77.9%) whilst among populations of Alicante, Balearic Islands and Melilla the percentage shown the lowest value (9.4%).

The PCO analyses revealed a preliminary geographical pattern among some studied subpopulations. ML and PMO as appeared as separate groups, whereas the subpopulations BLA, TRE and GLE nested clearly intermingled (figure 2). No genetic separation was detected within Balearic subpopulations on the basis of their different ecology. The dendrogram based on Fst values yielded similar results; ML and PMO shown the largest genetic distances (figure 3).

Discussion

No previous reports on *H. caput-felis* exist about genetic variation of either the entire distribution range or any particular population. The present study should be taken as the first analysis relating the genetic identity of that species, though it is focused only on the Spanish populations. Nevertheless, these territories comprise the widest range for this species within its global distribution, due to the existence of three well fragmented and differentiated areas (figure 1), in which the greatest habitat variability is also found (e.g. coastal cliffs, dunes, ravines, etc.) (Agulló *et al.*, 2009).

According to our data (PCO analysis, Fst values and dendrogram), the current geographic fragmentation of *H. caput-felis* populations appears to be not directly correlated to a genetic isolation, as reported by Hamrick & Godt (1989, 1997). The three studied areas in Spain (Melilla, Alicante and Balearic Islands) did not group as three totally isolated genetic populations. However, according to recent data by Holmes *et al.* (2009), the global Fst value obtained for the Spanish populations of *H. caput-felis* (Fst = 0.221) would indicate a certain genetic isolation and low rates of gene flow among populations. Batista *et al.* (2001) reported similar or even higher Fst values (up to 0.6) for closer geographical populations of different *Cistus* taxa in the Canary Islands. Some features such as breeding systems, population and sample sizes, geology, ecology or also the used molecular technique might influence current genetic fragmentation of plant populations (Batista *et al.*, 2001). The Spanish populations of *H. caput-felis* showed a relatively high value of Fst, but lower than it could be expected for natural populations isolated by remarkable natural barriers such as the Mediterranean Sea.

The position of Melilla population, rather well differentiated from Alicante and Balearic populations (figures 2, 3), could be explained by geological events together with its well separated geographical position. Melilla is the most faraway population (geographical distances: from 360 to 685 km) and it also shows a total lack of terrestrial connection with the others. The genetic fragmentation obtained between Melilla and the rest of Spanish populations could depend on the convulsive geological history of the Mediterranean basin. North Africa became definitely disconnected

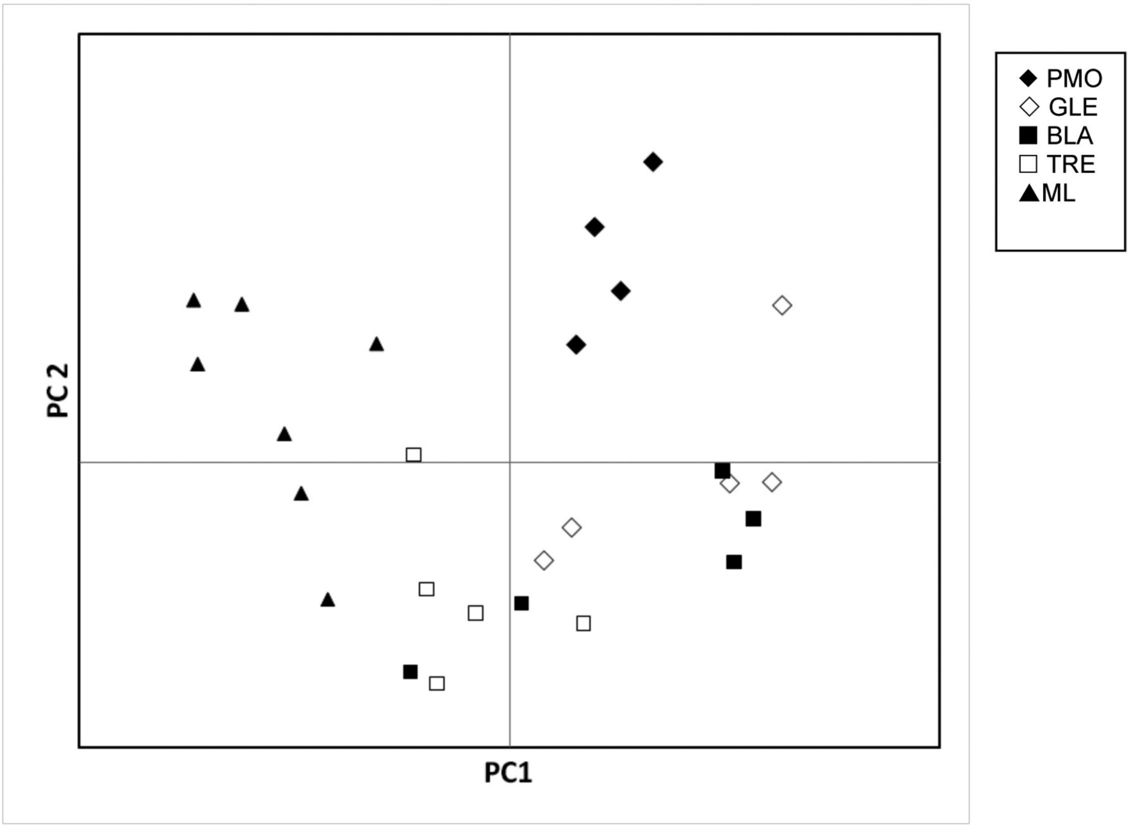

Fig. 2 - Principal Component Analysis of the studied Spanish populations of *Helianthemum caput-felis*. The principal component 1 (PC1) and PC2 explained 32.6% and 20% of the variance, respectively.

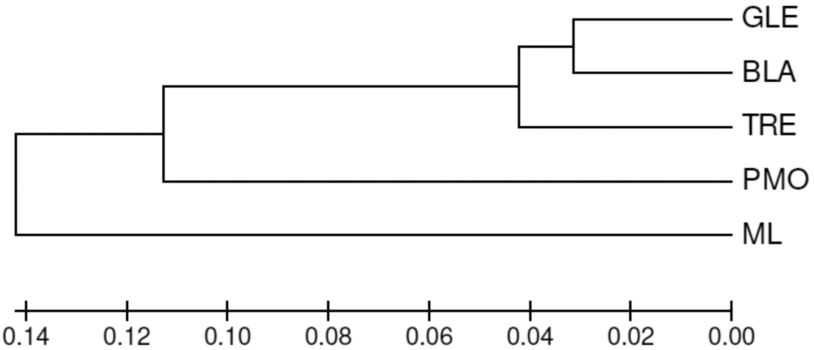

Fig. 3 - Neighbour-Joining dendrogram, based on Fst values between Spanish subpopulations of *Helianthemum caput-felis*.

from the Iberian Peninsula after the Messinian Event during the late Tertiary c. 5.3 Mya (Duggen *et al*., 2003). Most of similar previous studies have been focused on genetic differentiation among populations across the Strait of Gibraltar (Werner *et al*., 2002; Escudero *et al*., 2008; Guzmán & Vargas, 2009, among others) and also on the importance of this area as an active land bridge in the migration of different species (Quézel, 1978). However, neither current nor fossil populations of *H. caput-felis* are known around the Strait of Gibraltar; therefore this area does not seem to be relevant for genetic similarities or differences between populations of that species in both territories. Nonetheless, the fluctuant changes of the Mediterranean Sea level could have influenced directly on the gene flow among populations of the Iberian Peninsula and North Africa, far away from the Strait of Gibraltar. The refill of the Mediterranean basin after the Messinian salinity crisis (c. 5.3 Mya ago) followed a flood from the Atlantic Ocean in a

Populations	Subpopulations	ne	h	I	P
Balearic I.	BLA	1.134	0.077	0.114	20.7
	TRE	1.127	0.075	0.115	24.5
Alicante	GLE	1.106	0.061	0.091	16.4
	PMO	1.120	0.067	0.098	17.0
Melilla	ML	1.128	0.074	0.112	22.6
	Total	11.766	0.109	0.170	47

Tab. 2 - The mean effective number of alleles (ne), the Nei genetic diversity (h), and Shannon genetic diversity (I), and proportion of polymorphic loci (P), for the studied Spanish populations and subpopulations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	p-value
Among regions	2	32.943	0.599	9.4	0.132
Among populations within regions	2	18.333	0.806	12.7	<0.010
Within populations	27	133.786	4.955	77.9	<0.001
Total	31	185.062	6.361	100.0	-

Tab. 3 - Analyses of molecular variance (AMOVA).

extremely short time, less than 2 years (cf. García-Castellanos *et al*., 2009), and as a direct consequence of this process plant populations suffered a very quick and dramatic isolation. Therefore, the Mediterranean Sea constituted an active barrier to gene flow and promoted genetic differentiation between natural plant populations, as recently reported for *Cistus ladanifer* L. populations (Guzmán & Vargas, 2009). The current genetic isolation of North African *H. caput-felis* populations might respond to a similar pattern, and the obtained genetic distance values between Melilla and the other Spanish populations would support this reasoning.

Populations from Alicante and the Balearic Islands show closer genetic similarities, appearing mixed in our genetic analyses (figures 2, 3). Similarly, the AMOVA analyses would also support this genetic structure since the highest value (78%) corresponds to intrapopulation variation. Similar genetic relationships were also achieved for other plant species with fragmented populations in similar distribution areas around Alicante and the Balearic Islands (e.g. *Silene hifacensis*, Prentice *et al*., 2003; *Juniperus oxycedrus* subsp. *macrocarpa*, Juan *et al*., in prep.). Both territories are next, and share a common geological history (Azañón *et al*., 2006). Therefore, different processes of active gene flow (without complete genetic isolation) might have occurred among populations prior to their final isolation c. 5.3 Mya, and they could also keep part of that common genetic information. Moreover, Wright (1965) suggested that Fst values above 0.20 reflect low rates of gene flow among populations. On this basis, some gene flow seems to be the rule among the studied Alicante and Balearic Islands subpopulations since that value varied between 0.014 and 0.170. However, the PMO subpopulation represented a notable exception since it showed the highest values of Fst (0.345) with the Balearic subpopulation BLA. This exceptional and unique result reveals a strong genetic isolation of the PMO subpopulation (figures 2, 3), conflicting with other data pointing to genetic connection between Alicante and Balearic subpopulations. However, these first molecular results should be considered as preliminary because of the existence of clear contradictory results within the same geographical area. No previous studies support similar data, as two different scenarios are reported for different plant groups: i) shared genetic information (Prentice *et al*., 2003; Juan *et al*., in prep.), or ii) clear fragmented genetic populations (Roselló *et al*., 2002; Juan *et al*., 2004; Rubio de Casas *et al*. 2006). Probably, the obtained results could be justified by the low number of studied samples, and hence more individuals should be added to obtain definitive conclusions about relationships of the Alicante and Balearic subpopulations.

As a conclusion, AFLP technique provides relevant data to establish the genetic relationships among *H. caput-felis* populations, as previously reported for other Cistaceae (Carlier *et al*., 2008; Jump *et al*., 2008). According to our data, the current geographical fragmentation of the North African population allows regarding it as a group genetically isolated from the other studied Mediterranean populations. That population should be treated as an Evolutionarily Significant Unit (ESU; Waples, 1991) and managed independently for conservation purposes. However, the genetic relationships between populations of the Balearic Islands and Alicante should be taken as preliminary, until a wider sampling in each population is achieved that allow a full understanding of the current uncertain genetic relationships. Moreover, further studies are needed to elucidate the genetic variation of *H. caput-felis* throughout its whole distribution range, including samples from Algeria, Morocco and Sardinia (Agulló *et al*. in prep.).

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