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Abstract
Jurinea pinnata (Lag.) DC. is one of the three species of the genus that grows in the Iberian Peninsula. This species grows on gypsum and dolomite, substrates rich in endemic taxa. Actually, the genus *Jurinea* Cass. is particularly rich in endemic taxa associated with special substrates. The island-like distribution of *J. pinnata* makes it an interesting case for the study of genetic differentiation processes. The geographical proximity of *J. pinnata* and *J. humilis* makes this evolutionary puzzle even more difficult to solve. These facts could affect the genetic attributes of the species as far as diversity and differentiation are concerned. Our field research involved the sampling of individuals from eight populations of *J. pinnata.* Of them, six were located in the Guadix-Baza Basin. We used sequences of ribosomal and plastid DNA to perform the genetic analyses. Results revealed no differences between individuals occurring on the two kinds of substrates and evidences of hybridization between *J. pinnata* and *J. humilis*. Furthermore, the phylogenetic analysis revealed a possible polyphyletic origin for the adaptation to special substrates within the *Jurinea* genus, while monophyly was observed in species from the Iberian Peninsula. This could indicate a more general adaptation to arid or saline environments.

Keywords: *cpDNA, dolomitophily, gypsophile, gypsophyly, nrDNA, serpentines*

Introduction

The genus *Jurinea* Cass. (Compositae) comprises about 200 species (Pujadas-Salvà 2013) that occur throughout central and southern Europe, NW Africa and central Asia (Bremer 1994). Some of them grow on gypsum steppes, limestone or other special substrates (Iljin 1997; Stevanović et al. 2010). *J. elegantisima* Iljin and *J. lasiopoda* Trautv. (Iljin 1997), for example, can be found on gypsum. *J. micevskii* Stevanović, Matevski & Kit Tan, *J. taygetea* Halcsy (Stevanović et al. 2010) and *J. turcica* Dogan & Duran (Dogan et al. 2010) grow on limestone. Other species grow on two kinds of substrates. This is the case of *Jurinea stoechadifolia* (Bieb.) DC. that occurs on gypsums in Turkey (Duman & Aytaç 1999) and on limestone in neighbouring areas of Europe (Kozuharov 1976; Iljin 1997). *Jurinea pinnata* (Lag.) DC. (Compositae) is an Iberian endemic plant that grows on gypsum (Martínez-Hernández et al. 2009, 2011) and dolomite soils (Salmerón-Sánchez et al. 2011).

In addition to *J. pinnata*, there are two other species of the *Jurinea* genus in the Iberian Peninsula. The widely distributed *Jurinea humilis* DC. occurs in the western Mediterranean on high mountain limestone and siliceous soils, and *Jurinea fontqueri* Cuatrec., exclusive to Sierra Mágina, occurs on dolomitic screes. *J. pinnata* has been considered both as a dolomitophyte (Mota et al. 2008) and as a gypsophyte (Mota et al. 2009). The fact that *J. pinnata* grows on these two very different edaphic substrates that harbour different species compositions (Mota 2007) offers an excellent opportunity for investigating the effect of soil profile on the

genetic features of this species, both in terms of diversity and genetic differentiation.

Recent studies with Amplified Fragment Length Polymorphism (AFLP) markers found no genetic differentiation between the species populations growing on dolomite and those growing on gypsum (Salmerón-Sánchez et al. 2014). Were it not for the geographical proximity between some of the populations growing on gypsum and dolomite, this fact could be striking given the huge differences in soil profile. Accordingly, the absence of genetic differentiation is probably due to the existence of gene flow between close populations. This scenario is particularly apparent in the Guadix-Baza Basin.

The occurrence of hybridization events [as extensively documented in the genus by Iljin (1997)] is another fact to be considered, because it could have an impact on the genetic differentiation. Dr Mota (personal communication) has found individuals with clearly intermediate morphologies between *J. pinnata* and *J. humilis*. On the other hand, the different edaphic affinities of the species belonging to the *Jurinea* genus provide a suitable opportunity to investigate into the evolutionary origin of gypsophily. This fact has been already noted by some authors (Levin 2000; Moore & Jansen 2007).

As this research covers the three Iberian species of the *Jurinea* genus, the molecular evidence for the inclusion of *J. fontqueri* within the genus *Aegopordon* Boiss. (Tscherneva 2008) has also been contemplated as a third issue. In addition to *J. fontqueri*, this genus would include *J. berardioides*, a species that occurs in Iran and nearby territories.

To answer all these questions, we used plastidial and ribosomal sequences. Numerous studies have recorded the geographical distribution of the intraspecific variation of the cpDNA (Mengoni et al. 2003; DeChaine & Martin 2005). Of particular interest are the internal spacers, widely used both in phylogenetic and phylogeographical studies (Zhang et al. 2005; Shaw et al. 2007; Borzatti Von Loewenstern et al. 2013). Plastid DNA has also been very useful in the study of special edaphic substrates (Kurata et al. 2008) and in the differentiation of ancestral lineages where AFLP markers proved useless for this purpose (Ikeda et al. 2006, 2008).

In addition, nrDNA sequences can provide relevant information at generic, species (Nieto-Feliner & Rosselló 2007; de la Fuente et al. 2013) and infraspecific levels (Álvarez & Wendel 2003). nrDNA sequences are useful in the detection of hybridization events (Emshwiller & Doyle 1998; Widmer & Baltisberger 1999). In this case, nucleotide additive patterns, which combine parental ribotypes differentially inherited in the hybrid genome as a result of a recent hybridization event, can emerge (Fuertes-Aguilar et al. 1999a; Plume et al. 2013). nrDNA sequences have also been used in the study of plants adapted to different types of substrates (Cecchi et al. 2011) and ecotypes (Schechter & Bruns 2008). Furthermore, they could be helpful in opening up new lines of research not only in the study of the evolutionary dynamics involved in the adaptation of the genus *Jurinea* to dolomite and gypsum, but also in the study of the origin of species that have developed tolerance to these selective substrates.

As already mentioned, the *Jurinea* genus represents a complex evolutionary puzzle in the Iberian Peninsula. A combination of ancestral lineages associated with different substrates and favoured by palaeoclimatic events and the orographical and geological complexity of the territory cannot be dismissed.

The main objectives of this investigation have been to determine the possible monophyly of the Iberian species belonging to the *Jurinea* genus, to search for possible hybridization events and to compare the geographical differentiation with the ecological differentiation (geological profile of the substrate).

More precisely, our objectives were as follows:

(1) To determine possible genetic differences between the populations of *J. pinnata* growing on gypsum and dolomite.
(2) To detect hybridization events between the species of the *Jurinea* genus in the Iberian Peninsula.
(3) To establish phylogenetic relationships among them and in relation with *J. berardioides*.
(4) To further investigate the evolutionary origin of the adaptation to gypsum substrates.

Material and methods

Plant material

The study concentrates on *J. pinnata* populations within the Guadician–Bacensean Sector (Rivas-Martínez 2007) where the plant can be found both on gypsum and on dolomite outcrops at a short distance from one another (Salmerón-Sánchez et al. 2014). Dolomite populations are found in the foothills of the Sierra de Baza, whereas gypsum populations are found in the sedimentary depression known as the Guadix-Baza Basin. In this territory, we sampled material from three populations on gypsum and three others on dolomite. For analytical purposes, we also made use of material taken from isolated areas where only gypsum outcrops (Los Yesares) or dolomite outcrops (Sierra de las Nieves) can be found. Figure 1 shows the distribution of the populations under study.

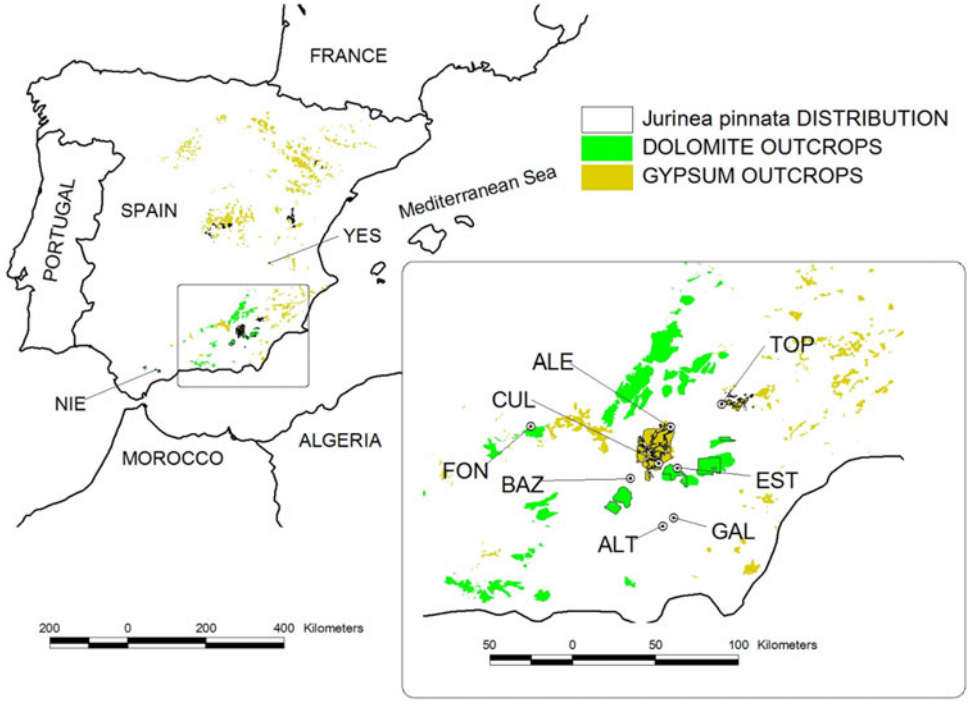

Figure 1. Distribution of gypsum and dolomite outcrops and location of sampled *J. pinnata*, *J. humilis* (ALT) and *J. fontqueri* (FON) populations. Correspondence of codes is shown in Table I.

Leaf material was taken from five individuals in each population at minimum distances of 15 m from one another. The plant material was dried in silica gel and stored at room temperature. Three individuals of two other species, namely, *J. humilis* and *J. fontqueri*, were also used to test the monophyly of the genus in the Iberian Peninsula and to check possible hybridization processes. Two individuals with morphologi-

Table I. Population, population code, substrate affinity (G, gypsum; D, dolomite; L, limestone), coordinates, number of cloned sequences, number of individuals analysed (nrDNA/cpDNA), ribotypes and haplotypes detected for each of the populations under study, haplotype diversity (H) and nucleotide diversity ($\Pi \times 10^{-3}$).

Populations	Code	Subs.	Coor. (N/O)	Cloning	No. of ind.	Ribotype	Haplotype	H	$\Pi \times 10^{-3}$
Jurinea pinnata									
Baza	BAZ	D	37°28′/2°48′		3/4	R1	H2	0	0
S. Estancias	EST	D	37°31′/2°29′		3/4	R1	H2	0	0
Calar del Gallinero	GAL	D	37°15′/2°31′		3/4	R1/R2	H2/H4	0.500 ± 0.265	1.413 ± 1.138
S. Nieves	NIE	D	36°42′/4°59′	8	3/4	R6–R13[a]	H2/H4	0.667 ± 0.204	1.884 ± 1.451
Total dolomite					12/16		H2/H4	0.325 ± 0.125	0.919 ± 0.641
Galera	ALE	G	37°45′/2°31′		3/4	R1	H2/H3/H4	0.833 ± 0.222	2.167 ± 1.637
Cúllar	CUL	G	37°33′/2°37′		3/4	R1/R2	H2	0	0
Topares	TOP	G	37°52′/2°11′		3/4	R1	H2	0.667 ± 0.204	1.884 ± 1.451
Yesares	YES	G	39°08′/1°44′	8	3/4	R1/R3/R4/R5	H1	0	0
Total gypsum					12/16		H1/H2/H3/H4	0.725 ± 0.069	1.427 ± 0.908
Total					24/32			0.563 ± 0.081	1.160 ± 0.743
J. pinnata × *J. humilis*									
Baza	BAZ	D	37°28′/2°48′	8	2	R1/R14–R18[b]	H4		
Jurinea humilis									
Calar Alto	ALT	L	37°13′/3°32′	4	3	RH1/RH2/ RH3/RH4	H4		
Jurinea fontqueri									
Cerro Cárceles	FON	D	37°44′/3°28′		3	RF1/RF2/ RF3/RF4	H4		

[a] Ribotypes: R6, R7, R8, R9, R10, R11, R12, R13. [b] Ribotypes: R14, R15, R16, R17, R18.

cally intermediate features between *J. humilis* and *J. pinnata* (a-priori interpreted as putative hybrids) were also included in the analyses. These two individuals were collected near the population of Baza (BAZ). More details about the studied populations and voucher information of the material used are shown in Table I and Appendix 1, respectively.

DNA extraction

Dry material (0.05 g per leaf) was pulverized with the aid of liquid nitrogen. Total DNA was extracted by means of cetyltrimethylammonium bromide method (Doyle & Doyle 1987) with minor modifications, and resuspended in 100 µl of buffer TE (10 mM Tris; 0.1 mM EDTA; pH 8). Average DNA concentration was estimated by means of a spectrofluorometer (Synergy Mx Microplate Reader, Biotek, Winooski, VT, USA) and the quality determined in agarose gel at 1% (Tremetsberger et al. 2004).

Extraction of ribosomal sequences from data bases

To determine the phylogenetic relationships between the different Iberian species of the *Jurinea* genus, we used ribosomal internal transcribed spacers region (ITS; ITS1–5.8S–ITS2) available in the GenBank. We also used sequences belonging to the species of the subtribe Carduinae (Cass.) Dumort, taking into account those that have shown signs of gypsophily. Consequently, our analyses included species such as *J. mollis* (L.) Reichnb. and *J. stoechaedifolia* within the *Jurinea* genus, and some others such as *Cousiniopsis atractryloides* (C. Winkl.) Nevski, *Plagiobasis centauroides* Schrenk or *Lamyropappus scharaptaricus* (B. Fedtsch.) Knorring & Tamamsch (Iljin 1997). Appendix 2 shows details on the sequences used and their edaphic affinity.

Amplification of plastid DNA (trnT–trnL and trnQ–rps16) and nuclear DNA (ITS) and sequencing

Both the nuclear ITS sequences (ITS1–5.8S–ITS2) and the plastid DNA (*trnT–trnL* and *trnQ–rps16*) were obtained from three and four individuals, respectively, from each population. In the case of the ITS sequences, the polymerase chain reaction (PCR) was performed with 20 µl volume containing ca. 10 ng of genomic DNA, 1 × Gotaq buffer (Promega, Madison, WI, USA), 1 µM of each primer (ITS1 and ITS4, White et al. 1990), 1.25 U of Gotaq polymerase (Promega), 1.875 mM of $MgCl_2$ and 0.4 mM of each dNTP. Conditions for the PCR were as follows: 5 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C. After another step of 10 min at 72°C, samples were stored at 4°C. Primers, reaction mixtures and conditions initially used for the amplification of plastid *trnT–trnL* [Tab D and Tab A from Taberlet et al. (1991)] and *trnQ–rps16* [$trnQ_{UUG}$ and rps 16x1 from Shaw et al. (2007)] sequences were those suggested by Shaw et al. (2007) with minor modifications.

PCRs were carried out in agarose gel and purified in the Genelute PCR cleanup kit (Sigma-Aldrich®, St Louis, MO, USA). Sequencing reactions were carried out by means of the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's recommendations, with the same primers as in the amplification reaction. Sequences were electrophoresed in an automated sequencer ABI 3100 Avant (Applied Biosystems). Forward and reverse sequenced chromatograms were com-pared, assembled and corrected where necessary using the Geneious software (v4.8.5; Biomatters Ltd, Auckland, New Zealand) to establish the consensus sequence of the sample. International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes were used to codify polymorphic positions. Following the recommendations of Fuertes-Aguilar et al. (1999a), sites were considered polymorphic (additive polymorphic sites (APSs)). When it was possible, identification of matching ITS ribotypes was inferred by subtraction (Clark 1990). All sequences were stored in GenBank under the codes showed in Appendix 3.

Cloning of ribosomal sequences

The presence of APSs in sequences of *J. pinnata* and *J. humilis*, together with the putative hybrids, led us to isolate the contained ribotypes by cloning techniques. Two products of PCR belonging to the populations S. Nieves (NIE) and Jumilla (JUM) were selected, one in the case of *J. humilis* and two more in the case of the putative hybrids (one from each individual). Ligations and transformations were prepared by means of pGEM-T Easy Vector System (Promega) following the manufacturer's instructions. Sequencing was performed as described earlier.

Analysis of APSs

Variation patterns in nrDNA sequences were investigated by means of SPLITSTREE V4 (Huson & Bryant 2006) generating a network using the NeighbourNet algorithm (Bryant & Moulton 2003) on a matrix of uncorrected *p*-distances with average scores in ambiguous sites. The network generated included both the sequences derived by direct sequencing and those obtained by cloning (Figure 2(b)).

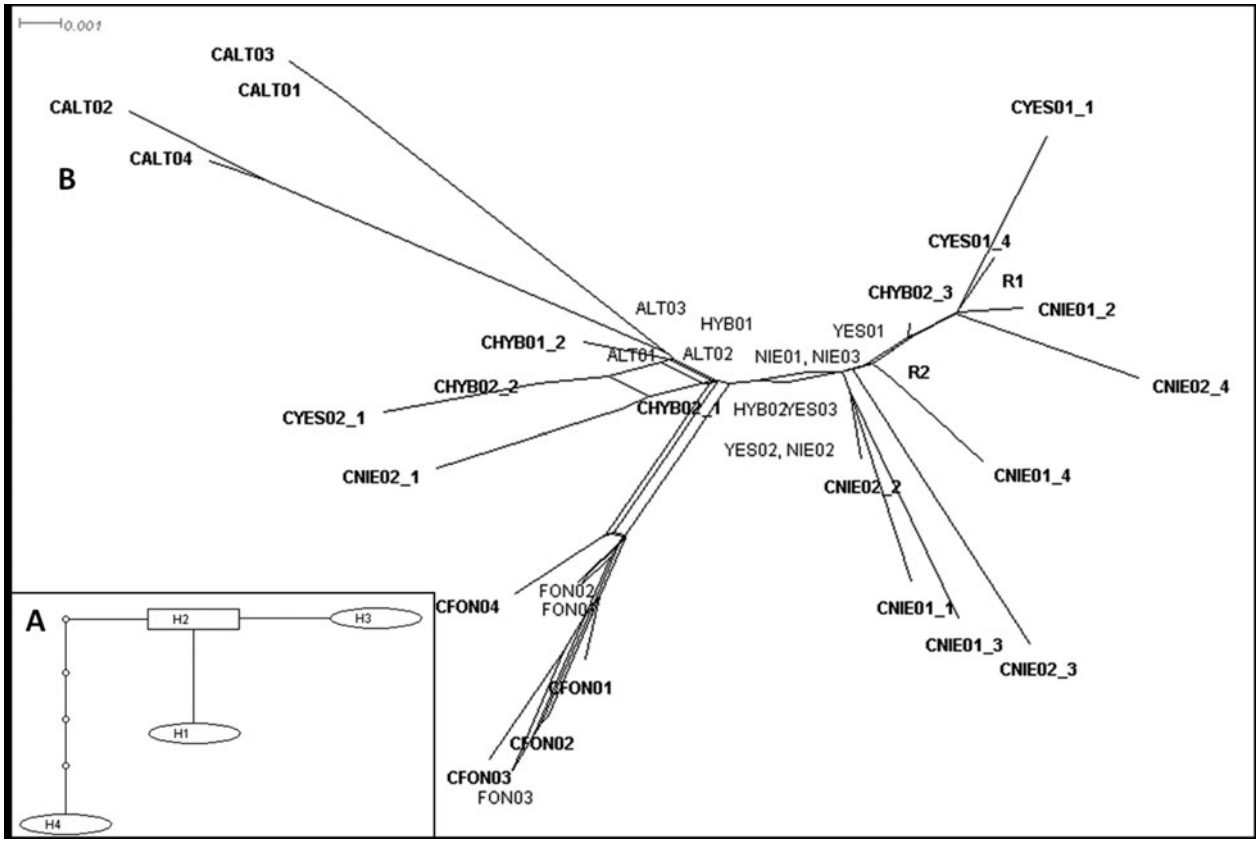

Figure 2. (a) Relationships of haplotypes of *J. humilis*, *J. fontqueri* and *J. pinnata* (indicated by their population number given by Table I) shown as a haplotype network. (b) NeighbourNet network of ITS ribotypes derived from *J. pinnata*, *J. humilis*, *J. fontqueri* and putative hybrids (from both direct-sequencing and cloned PCR). Individual accession names were omitted in the case of those that corresponded to ribotypes R1 and R2.

Analysis of plastid DNA sequences

The sequences of *J. pinnata*, *J. humilis*, *J. fontqueri* and the putative hybrids corresponding to amplified plastid fragments were combined and aligned by eye assuming that the plastid genome forms one single ligament group. The network of haplotypes was generated using TCS 1.21 (Clement et al. 2000), a method of statistical parsimony for constructing phylogenetic networks of haplotypes by treating gaps as a fifth state. Haplotype diversity (*H*) and nucleotide diversity (Π) were estimated for each population, edaphic group and species levels using ARLEQUIN V 3.5.1.2 (Excoffier et al. 2005).

Data analysis for phylogenies

The phylogenetic analysis of nrDNA sequences was performed using the criteria of maximum parsimony as implemented in PAUP 4.0b10 (Swofford 2002) and Bayesian inference as implemented in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). In parsimony analysis, gaps were considered as missing data. Heuristic searches were performed using 1000 random additions of sequences and Tree Bisection Reconection (TBR) branch swapping in which a maximum of 500 trees were saved per replicate. Robustness of each node obtained was checked via a non-parametric bootstrap analysis with 500 pseudo-replicates, each of them with 10 random additions of sequences, and TBR branch swapping, with 100 trees saved per replicate.

In the case of the Bayesian analysis, the election of the best suited model of nucleotide substitution was made taking into account Akaike and Bayesian Information Criteria (AIC and BIC), implemented in JMODELTEST 0.01 (Posada 2008). We produced 5 $\times 10^6$ generations with four Markov chains and samples every 100 generations. The first 10% of the sampled trees were discarded as burn-in. With the remaining trees, we obtained a consensus tree by majority rule (50%) using the posterior probability as a measure for the support of the clade (Alfaro et al. 2003).

Results

Plastid sequences (trnT–trnL and trnQ–rps16)

The primers that amplified *trnT–trnL* fragment generated a region of 785 nucleotides in all the

samples under study. The variation in the sequence remained restricted to one single variable site producing two different haplotypes. The intergenic spacer *trnQ–rps16* produced an amplicon of 960–984 bp in *J. pinnata* and 960 bp in *J. humilis* and *J. fontqueri*. The aligned length of the region was of 984 bp. Five variable sites and one indel (2.95% or 0.51% of the total, depending on whether or not the indel was included) were identified in the 40 sequences analysed. All variable positions and haplotypes (H1, H2, H3 and H4) detected as a result of the concatenation of the two fragments of plastid DNA are described in Table II. Haplotypes H1, H2 and H3 only appeared in *J. pinnata*, whereas H1 was found in all the individuals from Yesares (YES). H2 was present in both gypsum and dolomite populations and was the most frequent. H3 was found only in Galera (ALE). Finally, H4 was present in *J. pinnata* (in both substrates), *J. humilis*, *J. fontqueri* and the two putative hybrids studied (Table I).

The haplotype network (Figure 2(a)) revealed that H4, present in all the species, was the haplotype most differentiated from the rest and that the most frequent in *J. pinnata*, H2, was the haplotype from which the other haplotypes present only in *J. pinnata* derived. As far as sites are concerned, four populations exhibited one single haplotype (YES H1; BAZ, Cullar (CUL) and S. Estancias (EST) H2), whereas ALE exhibited three (H2, H3 and H4). Interestingly, when haplotypes are brought into relation with the corresponding substrate, H2 takes place much more frequently in dolomite (0.813) than in gypsym (0.438). On the other hand, the populations of gypsum had higher average levels of haplotype (*H*) and nucleotide diversity ($\Pi \times 10^{-3}$) than those of dolomite (0.725 vs. 0.325 and 1.43×10^{-3} vs. 0.92 $\times 10^{-3}$). Results are shown in detail in Table I.

Sequences of ribosomal DNA (ITS)

ITS primers generated a fragment of 638–639 bp in *J. pinnata*, *J. humilis*, *J. fontqueri* and in the samples of the two putative hybrids. *J. fontqueri* revealed four additive polymorphisms, all of them of autapomorphic origin. Accordingly, that sequences were excluded from further analysis. Including the indel, 28 polymorphic sites (17, if *J. humilis* is excluded) were found (4.38% and 2.66% of the total, respectively). Of these, 12 or 5 (1.9% and 0.8%) corresponded to ITS1, and 12 or 9 (1.9% and 1.4%) were located in ITS2, respectively (Table II). In the region 5.8S, we found up to three APSs in the samples belonging to YES and, NIE (positions 380, 385 and 388) and two in Calar Alto (ALT) (385 and 388), with one of the nucleotides being coincidental with the rest of the samples.

By direct sequencing, two ribotypes present in *J. pinnata* were distinguished: R1 in Topares (TOP), ALE, BAZ, EST, CUL and Calar del Gallinero (GAL), and R2 in CUL and GAL. In the rest of the populations, as well as in putative hybrids and *J. humilis*, cloning was necessary to identify new ribotypes. Further details on polymorphic sites are shown in Appendix 4. The addition of the 32 cloned ITS sequences derived from the two accessions belonging to *J. pinnata* (16), one belonging to *J. humilis* (4) and two more belonging to the putative hybrid of *J. pinnata* and *J. humilis* (8) raised to 66 variable sites, i.e. 51 non-informative and 15 informative. These sequences allowed the identifi-cation of 23 new ribotypes. The increased levels of variation recorded in the cloned sequences suggest that at infra-individual level there are more ITS polymorphisms than what the direct sequencing revealed. Accordingly, 4 ribotypes in *J. fontqueri*, 4 ribotypes in *J. humilis*, 13 ribotypes in *J. pinnata* and 4 more in the putative hybrid individuals were distinguished (the hybrids also exhibited ribotype R1, present in all *J. pinnata* populations). Distribution of ribotypes is shown in Table I.

The NeighbourNet analysis of ribotypes revealed three very distinctive groups and a slightly conflicting signal (caused by the ribosomal sequences of NIE, YES and the putative hybrids). These groups correspond to the ribosomal sequences of

Table II. Summary of the nucleotide variation of the sites in the chloroplast regions trnT–trnL and trnQ–rps16 in *J. pinnata*, *J. humilis* and *J. fontqueri* defining four haplotypes.

Plastidial region		trnT–trnL			trnQ–rps16			
Taxon/nucleotide position	Haplotype	200	197	501	631	731	844	865–888
J. pinnata	H1	C	G	A	G	C	C	–[a]
	H2	C	G	A	G	C	A	–[a]
	H3	C	G	A	A	C	A	–[a]
	H4	T	A	C	G	A	A	–
J. humilis × *J. pinnata*	H4	T	A	C	G	A	A	–
J. humilis	H4	T	A	C	G	A	A	–
J. fontqueri	H4	T	A	C	G	A	A	–

[a] Insertion of the sequence TTTATTATCTATGTAGATAAGAAA takes place.

J. humilis, *J. pinnata* and *J. fontqueri*. In addition, analysis revealed that there are no differences between the ribosomal sequences of *J. pinnata* on account of the substrate. The addition of cloned ITS sequences (Figure 2(b), cloned sequences in bold) in the network revealed their resolution outside the groups of ribotypes of *J. pinnata* and *J. humilis*. This could be attributed to their chimaeric nature. These ITS types were a mixture between *J. pinnata* and *J. humilis* ribotypes. The nucleotides present in the different polymorphic positions are shown in Table III.

Phylogenetic analysis by ribosomal sequences

The nucleotide substitution pattern that best fits our data and could be used in MrBayes was General Time Reversed (GTR) with Gamma distributed rates (G) mixed with invariables sites (I). The topology of the trees obtained by Bayesian analysis (Figure 3) and parsimony analysis (not shown) diverged in the grouping of the sequences of *J. humilis* with the rest of the species from the Iberian Peninsula. In all other respects, the trees coincided entirely. The 50% majority rule tree revealed that the ribosomal sequences belonging to the *Jurinea* genus from the Iberian Peninsula group well in the case of *J. pinnata* and *J. fontqueri* (1/97), but with lower support of the node when including *J. humilis* (0.67/-). On the other hand, *J. berardioides* remained outside the group formed by the Iberian species. This species was included in the genus *Jurinea* (a monophyletic group); therefore, the existence of an independent genus (*Aegopordon*) including *J. fontqueri* and *J. berardioides*, as Tscherneva (2008) suggested, is hardly defensible. In the case of the species growing on gypsum, no evidence of monophyly was found, neither in the genus *Jurinea* nor in the Carduinae subtribe.

Discussion

Absence of genetic differentiation on account of the substrate in J. pinnata

As far as the analyses by ITS sequences and cpDNA sequences are concerned, the absence of genetic differentiation between the two edaphic typologies is in line with the results obtained in *J. pinnata* (Salmerón-Sánchez et al. 2014) and *Onosma echioides* (L.) L. (Mengoni et al. 2006) using AFLP markers. These authors found close genetic relationships between geographically close populations regardless of the features of the soil they were growing on. Similar evidence was found in *Heteropappus hispidus* ssp. *Leptocladus* (Thunb.) Less by Yokoo et al. (2009) using microsatellites, or in the gypsophyte *Tiquilia gossypina* (Wooton & Standl.) A.T. Richardson (Moore & Jansen 2007) using plastidial and nuclear sequences.

According to Salmerón-Sánchez et al. (2014), there is no molecular indication of locally adapted populations. Instead, these authors suggest that the adaptation to these kinds of substrates is probably due to shared physiological mechanisms. In other words, plant tolerance to these substrates can be

Table III. Summary of the variation of nucleotide sites of the ITS1, ITS2 and 5.8S gene in *J. pinnata*, *J. humilis*, potentially hybrid individuals (HYB01 and HYB02) cloned sequences.

POS sample	Ribotype	86	206	255	388	438	446	459	544	566	575
CALT01	RH1	T	C	–	A	T	T	T	C	C	C
CALT03	RH2	T	C	–	A	T	T	T	C	C	C
CALT02	RH3	C	C	A	A	G	A	G	C	T	C
CALT04	RH4	T	C	A	A	G	A	G	C	T	C
CYES01_1	R3	C	T	–	G	G	T	T	T	C	C
CYES01_4	R4	C	T	–	G	G	T	T	T	C	C
CYES02_1	R5	**T**	**C**	–	**A**	G	**A**	T	**C**	C	C
CNIE01_1	R6	C	T	**A**	**A**	G	T	T	T	C	C
CNIE01_2	R7	C	T	–	G	G	T	T	T	C	C
CNIE01_3	R8	C	T	–	**A**	G	T	T	T	C	C
CNIE01_4	R9	C	T	–	G	T	T	T	T	C	C
CNIE02_1	R10	C	T	–	**A**	G	**A**	T	T	C	T[a]
CNIE02_2	R11	C	T	**A**	**A**	G	T	T	T	C	C
CNIE02_3	R12	C	T	–	G	G	**A**	**G**	**C**	**T**	C
CNIE02_4	R13	C	T	–	G	G	T	T	T	C	C
CHYB01_2	R14	C	T	**A**	**A**	G	**A**	T	**C**	**T**	C
CHYB02_1	R15	C	T	–	**A**	G	**A**	T	T	C	C
CHYB02_2	R16	**T**	T	–	**A**	G	**A**	T	**C**	C	C
CHYB02_3	R18	C	T	–	**A**	G	T	T	T	C	C
R2	R2	C	T	–	G	G	T	T	T	C	C
R1	R1	C	T	–	G	G	T	T	T	C	C

Note: Recombinant positions are in bold. [a] Position 575 polymorphic sites *Y* (C/T) were detected in *J. humilis* individuals.

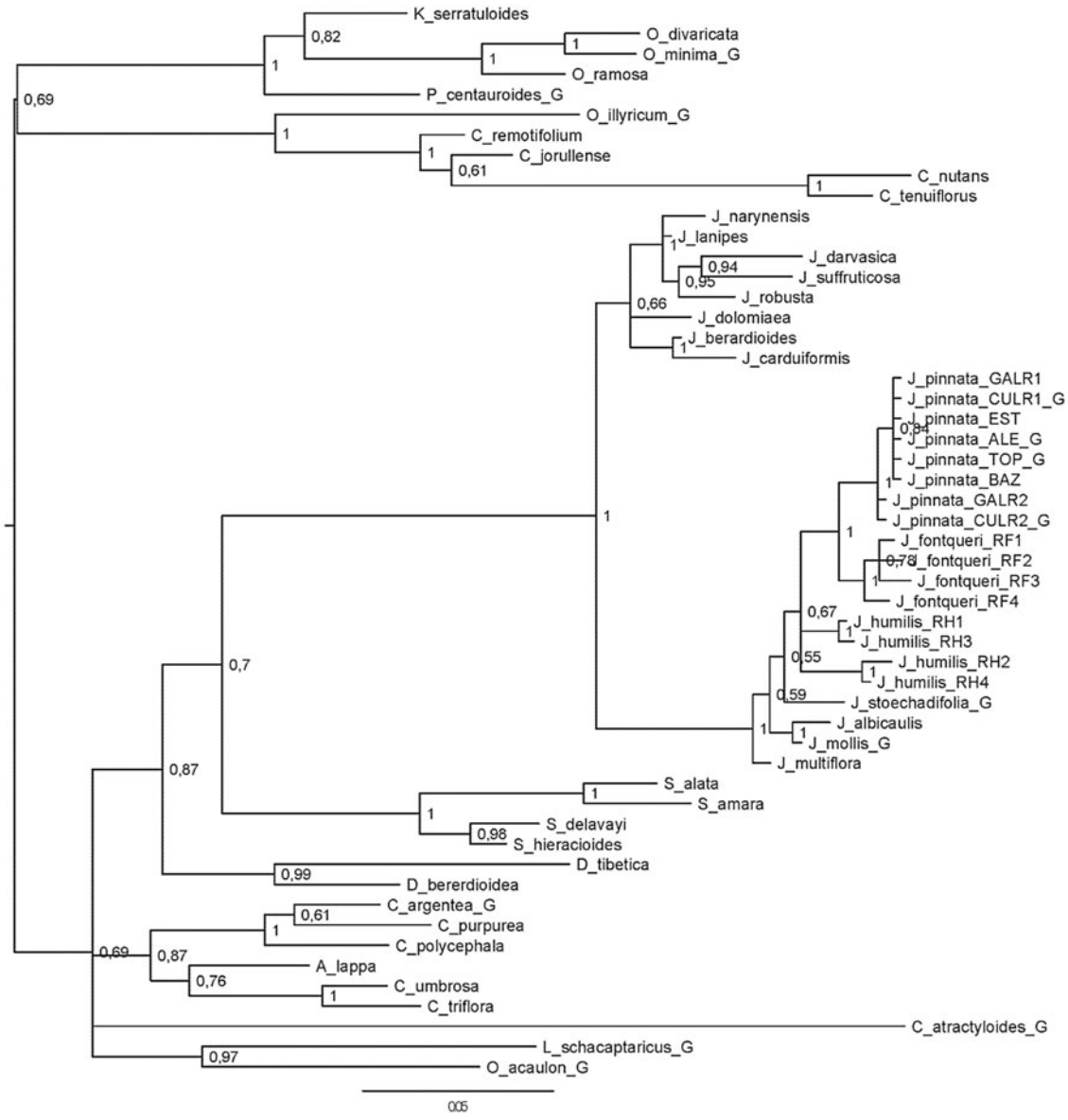

Figure 3. Phylogenetic relationships obtained by MrBayes from nrDNA sequences of *J. pinnata*, *J. humilis*, *J. fontqueri* and from other species belonging to the Carduinae subtribe (with special emphasis on *Jurinea* genus) available in GenBank. Results are shown in a consensus tree obtained by majority rule. The numbers on the branches are posterior probabilities. The scale bar represents substitutions per site.

simply interpreted as a wide-spectrum adaptation to substrates with an altered ionic balance (high Mg contents were recorded in *J. pinnata growing* both on gypsum and dolomite) or to arid environment (Mota & Valle 1992). All these together with the existence of gene flow between populations on account of their proximity and possible hybridization events in the *J. humilis* could explain why the species exhibits such a low substrate-related genetic differentiation.

Possible events of ancestral hybridization between J. pinnata *and* J. humilis

Plastid capture. Convergent evolution, lineage sorting and recurrent hybridization are among the processes that could help to explain the discrepancies in plastid haplotypes. In the case of convergent evolution, the possibility that the same haplotype (H4) has emerged in two different species is low. Actually, there are at least four substitutions and one indel between this lineage and the rest. In the case of incomplete lineage sorting, we can say that only haplotype H4 is found in *J. humilis* and *J. fontqueri*. The absence of the other haplotypes present in *J. pinnata* is particularly striking. At all events, the existence of APSs and recombinant ribotypes and the occurrence of putative hybrids support the existence of hybridization phenomena.

Introgression of a plastid from one species into another (plastid capture) can result from recurrent

hybridization that involves unidirectional gene flow between species (Petit et al. 2003). This is the most reasonable explanation for the pattern observed of haplotype sharing between two species (Fehrer et al. 2007). Plastid capture usually takes place between species with sympatric distribution and reproductive compatibility.

Hybridization is likely to take place in populations where individuals of one species are in the minority and receive foreign pollen belonging to related taxa. In fact, the minority species will almost inevitably be the female parent of the hybrid. In our case, putative hybrids occur in dolomite outcrops, where some *J. humilis* also occur and *J. pinnata* is particularly abundant. Furthermore, although flowering time overlaps partially, the phenology in *J. humilis* is earlier than in *J. pinnata*; therefore, the existence of cross-pollination from the latter to the former is much more likely.

Ancestral interspecific hybridization and reticulate evolution. Although the presence of APSs could have different causes, such as evolutionary processes (incomplete lineage sorting, etc.), our data indicate that they are the consequence of hybridization processes, due to the existence of individuals with recombinant ribotypes, resulting from the combination of *J. pinnata* and *J. humilis* ribotypes.

The lack of ITS variation could be the result of crossings between formally differentiated genetic entities followed by ribotype homogenization (towards one of the parental genomes, peculiar to ancestral hybrids) during concerted evolution, a process not unusual in the *Armeria* Willd. genus (Fuertes-Aguilar et al. 1999b). In this genus, the compilospecies are widely distributed and polyphyletic taxa form a system in which free genetic exchange is possible (Fuertes-Aguilar et al. 1999b), blurring the phylogenetic signal of molecular markers. Something somewhat similar could have happened in the case of *J. pinnata* and *J. humilis*, although no complete convergence between species can be observed, probably due to the dissimilar substrate affinity with respect to *J. humilis*, which grows primarily on limestone soils.

Monophyly of the species in the Iberian Peninsula and polyphyly to gypsum adaptation. In the Iberian species of the *Jurinea* genus, monophyly is a clear phenomenon, particularly in the case of *J. pinnata* and *J. fontqueri*. In the case of *J. humilis*, a more cautious statement must be made because this species is more widely distributed in the western Mediterranean and the plant is not endemic to the Iberian Peninsula. The results obtained by the use of ribosomal sequences discredit the idea of including *J. fontqueri*, together with *J. berardioides*, in the *Aegopordon* genus. As a matter of fact, this latter species remains included in a clade together with the rest of the species belonging to the *Jurinea* genus, a fact that is supportive of the monophyly of the genus within the Carduinae subtribe. Consequently, there is no need to entertain the idea of the *Aegopordon* genus to reflect the phylogenetic relationships between the taxa under study.

As far as the gypsicolous species of the *Jurinea* genus and the rest of species within the Carduineae subtribe are concerned, our results do not support a monophyletic pattern conducive to an adaptation to gypsum soils. This could suggest that the endemic gypsum species belonging to the *Jurinea* genus have probably emerged in a polyphyletic process through independent speciation events [as Cecchi et al. (2010) suggested in the case of the genus *Alysum* L. or Patterson and Givnish (2003) suggested in the case of the *Calochortus* Pursh].

On the other hand, a deep analysis of the phylogeny of the Carduineae subtribe reveals a large number of species belonging to different genera (*Coussinia stricta* Tschern., *Cousinia iljinii* Takht, *Cousinia rotundifolia* C. Winkl., *Echinopus babatagensis* Tschrn., *Cirsium pseudolappaceum* Kharadze, *Saussarea weberi* Hulten, *Serratula suffruticosa* Trautv. and so on) capable of growing on gypsum soils. These results support the suggestion made by Salmerón-Sánchez et al. (2014) according to whom the plant's ability to live on special substrates is a more general trait, which led to the colonization of these habitats.

As in the case of *Onosma* L. (Mengoni et al., 2006), key pre-adaptive features are probably associated with drought tolerance and preference for basic substrates (most of them grow on calcareous substrates). In the case of ultramafic rocks, given their extremely xeric nature, drought tolerance is a crucial requirement (Brady et al. 2005). This has been proved experimentally in groups such as *Mimulus* L. (Hughes et al. 2001) and *Cerastium* L. (Nyberg-Berglund et al. 2004). Indeed, both dolomite and gypsum can be regarded as extremely xeric substrates (Mota et al. 1993). In addition, ultramafic rocks, such as serpentines, share other key features with dolomites, such as a low Ca/Mg ratio (Mota 2007; Merlo et al. 2011).

In accordance with presumptions made for other plant groups (de Kok 2002), in *Jurinea* the repeated colonization of gypsum (or calco-dolomitic) outcrops has probably been reinforced by a constitutive pre-adaptation that represents a symplesiomorphy when contemplated from an ecological point of view.

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