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Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trnL-trnF*, and *matK* sequences

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Abstract

This paper presents the first phylogenetic hypotheses for the 20 species of *Cistus* based on plastid (*trnL-trnF*, *matK*) and nuclear (ITS) DNA sequence data. Phylogenetic relationships reveal that: (1) *Halimium* and *Cistus* form a cohesive, natural group; (2) two major lineages of purple-flowered and white-flowered species are defined, except for the purple-flowered *C. parviflorus*; (3) monophyly of conspecific populations is congruent with the circumscription of species. Topological congruence between nuclear and plastid phylogenies does not support a predominant reticulate system of evolution in *Cistus*. Reconstruction of character evolution suggests an increment of number of fruit valves in the Cistaceae from 3 to 12 in a unidirectional manner. In contrast, reproductive characters, such as sepal number, petal color, and style length, evolved multiple times in the course of evolution. A single colonization of *Cistus* into the Canary Islands appears to be responsible for a lineage of four species sharing a most recent common ancestor with five sepals, purple flowers, styles as long as stamens, and five fruit valves. Species diversity in *Cistus* (14) and *Halimium* (8), coupled with sister-group relationships and molecular divergence, lead us to suggest the western Mediterranean as a major center of present-day differentiation, but paleobotanical data indicate an earlier formation of the *Cistus–Halimium* assemblage in different areas.

Keywords: Canary Islands; Character evolution; Cistaceae; *Cistus*; ITS; *matK*; Mediterranean; Systematics; *trnL-trnF*

1. Introduction

Cistus (Cistaceae) is one of the most characteristic genera of the Mediterranean flora. Shrubby species primarily occur as woodland understory and others (*C. ladanifer*, *C. laurifolius*, and *C. monspeliensis*) are dominant in evergreen scrub. The adaptation of the genus to Mediterranean environments is evident from ecological characteristics such as fire-dependent seed germination (Roy and Sonié, 1992; Trabaud and Renard, 1999), insect-dependent pollination (Talavera et al., 1993), flower-dependent reproduction (Herrera, 1987), and spring-dependent phenology (Herrera, 1986). A long history of human activities has favored distribution and abundance of *Cistus* species in the Mediterranean (Thompson, 2005). Impenetrable masses of *Cistus* plants are formed as early successional stages following woodland disturbances such as fire and soil overturning. Co-occurring species of *Cistus* are frequent, particularly in mountain ranges composed by both acidic and basic soils. Environmental specificity referring to substrate confers additional value to acidiphilous and basiphilous species as predictable indicators of woodland disturbances. In marked contrast to the detailed knowledge of ecological characteristics of *Cistus*, understanding of the evolution of morphological characters and phylogenetic relationships within the genus is extremely limited.

Cistaceae comprises about 180 species, typically displaying loculicidal capsules of three valves, except in *Cistus* that is characterized by capsules with five or more valves. Circumscription of species in the eight genera of the Cistaceae is still problematic, particularly in genera

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such as *Helianthemum* and *Halimium*. This has resulted in the publication of multiple combinations for the same taxon under different generic names (Arrington and Kubitzki, 2003). The taxonomy of *Cistus* has traditionally been based on vegetative (nerve number, shape, and hairiness of leaves) and reproductive characters (sepal number, petal color, style length, and number of fruit valves). Worldwide monographs of *Cistus* have recognized between 16 species (Grosser, 1903) and 28 species (Dunal, 1824) (Table 1). Following Grosser's (1903) treatment with additional species described more recently, the genus is currently thought to comprise approximately 20 species, of which 16 occur in Europe (Warburg, 1968), 11 in Spain (Martín and Guinea, 1949), 12 in Iberia (Demoly and Montserrat, 1993), and 12 in Morocco (Soriano, 2002) (Fig. 1). The highest species diversity therefore occurs in the western Mediterranean, where 14 species are distributed in the Iberian Peninsula and northwestern Africa.

Disparate infrageneric classifications of *Cistus* have been proposed (Table 1). In the last taxonomic treatment three subgenera, namely *Cistus*, *Leucocistus*, and *Halimioides*, are described based on morphological characters (Demoly and Montserrat, 1993). The subgenus *Halimioides* (three species) is distributed exclusively in the western Mediterranean, whilst the subgenera *Leucocistus* (eight species) and *Cistus* (nine species) occur in the Mediterranean basin and the Canary Islands. This widespread distribution of *Cistus* subgenera and species clearly indicates the successful mobility of seeds and colonization in Mediterranean habitats.

Evolutionary mechanisms responsible for the morphological diversity within *Cistus* remain poorly understood. Plants are predominantly self-incompatible (Bosch, 1992) promoting crossing between individuals of the same and different species. Identification in the field of individuals as hybrids is relatively easy because they display characteristics that are intermediate between those of nearby, putative progenitors. Crossing between two plants of any species potentially generates offspring with intermediate traits, particularly when they are closely related congeners (Demoly, 1996). Hybrid polyploidy (allopolyploidy) has not played an important role in speciation of *Cistus*, as all species display a chromosome number of $2n = 18$. In fact, variation of DNA content is not significant among species (Ellul et al., 2002).

A proper phylogeny of *Cistus* has not been proposed to date. Dansereau (1939) outlined a phyletic diagram based on morphological features. Examination of 13 isozyme loci indicates high values of genetic divergence among four Canarian species (Batista et al., 2001), although evolutionary relationships of island endemics with respect to continental species remain unknown. Phylogenetic relationships among Cistaceae genera indicate that *Cistus* is closely related to *Halimium* and *Helianthemum* (Arrington and Kubitzki, 2003; Savolainen et al., 2000). In addition, angiosperm phylogenies reveal that the family forms a lineage coupled with Dipterocarpaceae and Sarcolaenaceae (Soltis et al., 2000). A larger sample is needed, however, to determine sister-group relationships of Cistaceae and *Cistus*.

Four basic objectives are addressed in the present study: (1) to evaluate congruence between nuclear (ITS) and plastid (*trnL-trnF*, *matK*) sequences; (2) to identify major lineages and test the monophyly of infrageneric groupings recognized in existing classifications of *Cistus*; (3) to interpret evolution of key morphological characters; and (4) to describe biogeographic patterns in the Mediterranean basin and in the colonization of the Canary Islands.

2. Materials and methods

2.1. DNA extraction, gene amplification, and sequencing

A total of 47 individuals representing the 20 species of *Cistus*, one of *Fumana*, two of *Halimium*, two of *Helianthemum*, and one of *Tuberaria* were sampled for ITS, *trnL-F*, and *matK* sequencing (Supplementary Table S1). Total genomic DNA was extracted from material collected in the field, material in the living collections of R.G. Page, O. Filippi, and the Royal Botanic Garden of Madrid, and from two herbarium specimens (MA). Field collections were dried in silica gel. DNA was extracted using Kneasy Plant Mini Kit (Qiagen, California) and amplified using the polymerase chain reaction (PCR) on a Perkin-Elmer PCR System 9700 (California) or an MJ Research (Massachusetts) thermal cycler. After 1–4 min pretreatment at 94 °C, PCR conditions were: 24–35 cycles of 1 min at 94 °C, 30 s–1 min at 50–52 °C, and 1–2 min at 72 °C. Standard primers were used for amplification of the *trnL*(UAA)-*trnF*(GAA) spacer (Taberlet et al., 1991), the *matK* intron (Johnson and Soltis, 1994), and the ITS region (Sun et al., 1994 for 17SE; White et al., 1990 for ITS4). A volume of 1 μL of dimethyl sulfoxide (DMSO) was included in each 25 μL reaction. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism Model 3700 automated sequencer. PCR primers were used for cycle sequencing of the *trnL-F* spacer and the *matK* intron, while the ITS5 and ITS4 (Sun et al., 1994) primers were used for cycle sequencing the ITS region. Sequenced data were assembled and edited using the program Seqed (Applied Biosystems, California). The limits of the

Table 1
Comparison of historical taxonomic treatments of *Cistus* using taxa names as published in original publications

Dunal (1824)	Spach (1836)	Willkomm (1856)
Sect. I. *Erythrocistus* Dunal	Genus *Ladanium* Spach	Subgen. I. *Erythrocistus* Dunal
C. albidus L.	*L. officinarum* Spach (*C. ladanifer* L.)	Sect. I. *Macrostylia* Willk.
C. candidissimus Dunal (*C. ochreatus* C. Sm. ex Buch)	*L. laurifolium* Spach (*C. laurifolius* L.)	*C. vaginatus* Aiton (*C. symphytifolius* Lam.)
C. complicatus Lam. (*C. parviflorus* Lam.)	*L. cyprium* Spach (*C. ladanifer* x *C. laurifolius*)	*C. candidissimus* Dunal (*C. ochreatus* C. Sm. ex Buch)
C. creticus L.		Sect. II. *Brachystylia* Willk.
C. crispus L.	Genus *Rhodocistus* Spach	*C. albidus* L.
C. cymosus Dunal (*C. parviflorus* x *C. creticus*)	*R. berthelotianus* Spach (*C. symphytifolius* Lam.)	*C. polymorphus* Willk. (*C. creticus* L.)
C. heterophyllus Desf.		*C. creticus* L.
C. hybridus Vahl (?)	Genus *Cistus* (Tourn.) Spach	*C. crispus* L.
C. incanus L. (*C. albidus* x *C. crispus*)	Sect. I. *Rhodopsis* Spach	*C. heterophyllus* Desf.
C. parviflorus Lam.	*C. purpureus* Lam. (*C. ladanifer* x *C. creticus*)	*C. purpureus* Lam. (*C. ladanifer* x *C. creticus*)
C. purpureus Lam. (*C. ladanifer* x *C. creticus*)	Sect. II. *Eucistus* Spach	Sect. III. *Astylia* Willk.
C. sericeus Vahl (*C. albidus* ?)	*C. vulgaris* Spach (*C. creticus* L.)	*C. parviflorus* Lam.
C. undulatus Dunal (*C. creticus* L.)	Sect. III. *Ledonella* Spach	
C. vaginatus Dryand. (*C. symphytifolius* Lam.)	*C. parviflorus* Spach (*C. parviflorus* Lam.)	Subgen. II. *Leucocistus* Willk.
C. villosus Lam. (*C. creticus* L.)		Sect. IV. *Stephanocarpus* Spach
	Genus *Stephanocarpus* Spach	*C. monspeliensis* L.
Sect. II. *Ledonia* Dunal	*S. monspeliensis* Spach (*C. monspeliensis* L.)	*C. pouzolzii* Delile
C. clusii Dunal		*C. florentinus* Lam. (*C. monspeliensis* x *C. salviifolius*)
C. corbariensis Pourr. (*C. populifolius* x *C. salviifolius*)	Genus *Ledonia* Spach	Sect. V. *Ledonia* Spach
C. cyprius Lam. (*C. ladanifer* x *C. laurifolius*)	*L. heterophylla* Spach (*C. monspeliensis* x *C. populifolius*)	*C. ledon* Lam. (*C. laurifolius* x *C. monspeliensis*)
C. florentinus Lam. (*C. monspeliensis* x *C. salviifolius*)	*L. populifolia* Spach (*C. populifolius* L.)	*C. populifolius* L.
C. hirsutus Lam. (*C. psilosepalus* Sweet)	*L. hirsuta* Spach (*C. psilosepalus* Sweet)	*C. longifolius* Lam. (*C. monspeliensis* x *C. populifolius*)
C. ladaniferus L. (*C. ladanifer* L.)	*L. peduncularis* Spach (*C. salviifolius* L.)	*C. obtusifolius* Sweet (*C. psilosepalus* x *C. salviifolius*)
C. laurifolius L.		*C. hirsutus* Lam. (*C. psilosepalus* Sweet)
C. laxus Aiton (*C. populifolius* x *C. psilosepalus* ?)		*C. salviifolius* L.
C. ledon Lam. (*C. laurifolius* x *C. monspeliensis*)		Sect. VI. *Ladanium* Spach
C. longifolius Lam. (*C. monspeliensis* x *C. populifolius*)		*C. cyprius* Lam. (*C. ladanifer* x *C. laurifolius*)
C. monspeliensis L.		*C. ladaniferus* L. (*C. ladanifer* L.)
C. populifolius L.		*C. laurifolius* L.
C. salviifolius L.		Sect. VII. *Halimioides* Willk.
		C. clusii Dunal
		C. bourgaeanus Coss. (*C. libanotis* L.)
		C. sericeus Munbyi (*C. munbyi* Pomel)

Grosser (1903)	Dansereau (1939)	Demoly and Montserrat (1993) (Iberian species)
Group A.	Subgen. I. *Erythrocistus* (Dunal) Willk.	Subgen. I. *Cistus* L.
Sect. I. *Rhodocistus* (Spach) Grosser	Sect. I. *Macrostylia* Willk.	*C. albidus* L.
C. ochreatus C. Sm. ex Buch	*C. osbeckiaefolius* Webb ex Christ (*C. osbeckiifolius* Webb ex Christ)	*C. creticus* L.
C. symphytifolius Lam.	*C. symphytifolius* Lam.	*C. crispus* L.
Sect. II. *Eucistus* Spach		*C. heterophyllus* Desf.
C. albidus L.	Sect. II. *Erythrocistus* Dunal	
C. villosus L. (*C. creticus* L.)	*C. albidus* L.	Subgen. II. *Leucocistus* Willk.
C. crispus L.	*C. villosus* L. (*C. creticus* L.)	Sect. 1. *Ledonia* Dunal
C. heterophyllus Desf.	*C. crispus* L.	*C. monspeliensis* L.
Sect. III. *Ledonella* Spach	*C. heterophyllus* Desf.	*C. populifolius* L.
C. parviflorus Lam.x	Sect. III. *Ledonella* Spach	*C. psilosepalus* Sweet
	C. parviflorus Lam.	*C. salviifolius* L.
Group B.		Sect. 2. *Ladanium* (Spach) Gren.
Sect. IV. *Stephanocarpus* (Spach) Willk.	Subgen. II. *Leucocistus* Willk.	*C. ladanifer* L.
C. monspeliensis L.	Sect. IV. Stephanocarpoidea Rouy et Foucaud	*C. laurifolius* L.
Sect. V. *Ledonia* Dunal	*C. varius* Pourr. (*C. pouzolzii* Del.)	
C. populifolius L.	Sect. V. *Stephanocarpus* (Spach) Gren.	Subgen. III. *Halimioides* (Willk.) Demoly & P. Monts.
C. hirsutus Lam. (*C. psilosepalus* Sweet)	*C. monspeliensis* L.	*C. clusii* Dunal
C. salviifolius L.	Sect. VI. *Ledonia* Dunal	*C. libanotis* L.
	C. populifolius L.	
Group C.	*C. hirsutus* Lam. (*C. psilosepalus* Sweet)	
Sect. VI. *Ladanium* (Spach) Willk.	*C. salviifolius* L.	
C. ladaniferus L. (*C. ladanifer* L.)	Sect. VII. *Ladanium* (Spach) Gren. et Godr.	
C. laurifolius L.	*C. ladaniferus* L. (*C. ladanifer* L.)	
Sect. VII. *Halimioides* Willk.	*C. laurifolius* L.	
C. rosmarinifolius Pourr. (*C. clusii* Dunal)	Sect. VIII. *Halimioides* Willk.	
C. bourgaeanus Coss. (*C. libanotis* L.)	*C. libanotis* L. (*C. clusii* Dunal)	
C. sericeus Munby (*C. munbyi* Pomel)	*C. bourgeanus* Coss. (*C. libanotis* L.)	
	C. munbyi Pomel	

Taxa in brackets as interpreted.

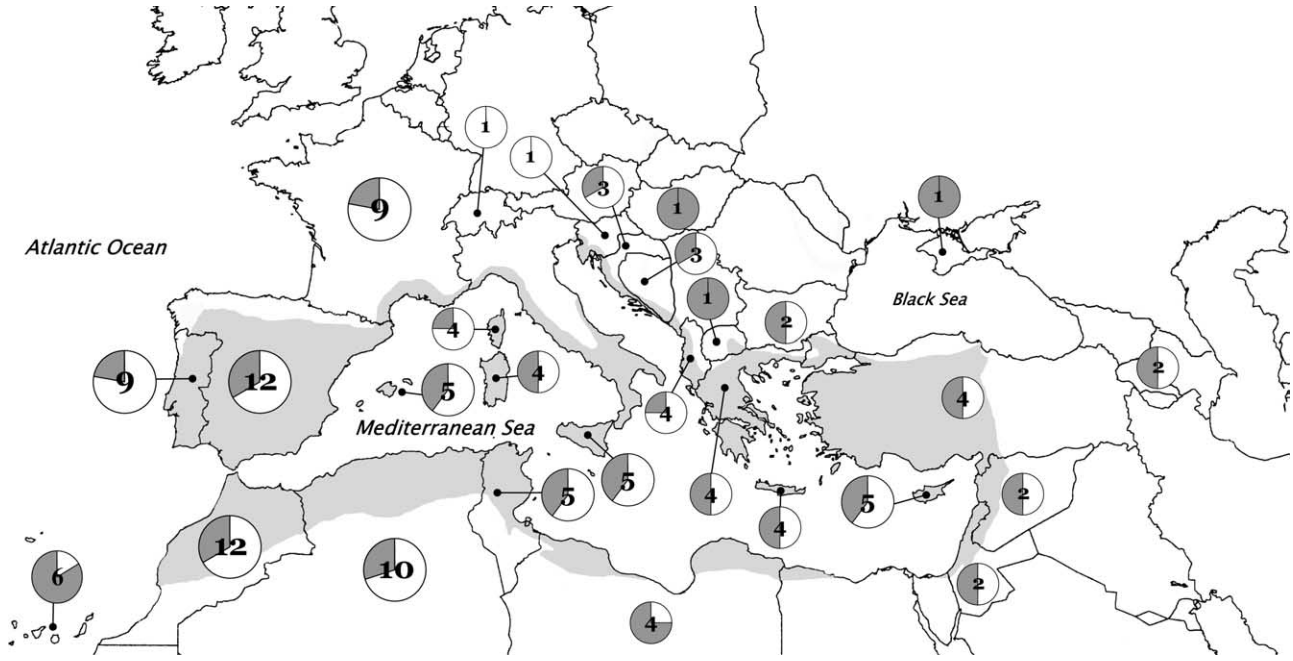

Fig. 1. Distribution map and number of *Cistus* species. Pie diagrams include proportion of white-flowered (white) and purple-flowered (grey) species in every country. Notice the highest species diversity in the western Mediterranean. The Mediterranean region shown in grey.

regions were determined by position of flanking primers. IUPAC symbols were used to represent nucleotide ambiguities.

2.2. Molecular analysis

Phylogenetic analyses were performed on three molecular data sets (*trnL-F*, *matK*, and ITS) using the same methodology and a similar number of sequences from each of the 20 species of *Cistus* and six of Cistaceae (Supplementary Table S1). In addition, an analysis of *trnL-F* sequences from Old World Cistaceae (*Fumana*, *Helianthemum*, *Tuberaria*, *Halimium*, and *Cistus*) and Dipterocarpaceae was performed to investigate relationships of *Cistus* with respect to another Cistaceae. In this analysis, four Dipterocarpaceae (*Dipterocarpus*, *Parashorea*, *Shorea*, and *Hopea*) from GenBank (Li et al., unpublished) were used as outgroup taxa on the basis of an earlier *rbcL* phylogeny (Ducousso et al., 2004).

Sequences were aligned using Clustal X 1.62b (Thompson et al., 1997), with further adjustments by visual inspection. Insertion/deletion mutations (indels) were manually coded for parsimony analyses as appended characters following the logic of Kelchner (2000) and Simmons and Ochoterena (2000). Maximum Parsimony (MP) and Bayesian Inference (BI) analyses were then performed on each data set. All parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford, 1999) with equal weighting of all characters and of transitions/transversions. Heuristic searches were replicated 100 times with random taxon–addition sequences, tree–bisection–reconnection (TBR) branch swapping, and the options MulTrees and Steepest Descent in effect. Additionally, as a result of memory limitation in completing the analysis of *trnL-F* sequences of the Dipterocarpaceae–Cistaceae matrix, 10 trees only were saved from each of the 1000 replicates to minimize time searching thousands of trees. All trees thus collected were combined and used as starting trees, with MulTrees on and no tree limit (these trees were then swapped to completion) and Subtree–Pruning-Regrafting (SPR) (Salamin et al., 2003). Internal support was assessed using 1000 replicates with simple taxon addition and SPR branch swapping, but permitting only 10 trees per replicate to be held (Chase et al., 2003).

To determine the simplest model of sequence evolution that best fits the sequence data, the Hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC) were implemented using MrModeltest 1.1b (Posada and Crandall, 1998; Nylander, 2002). A Bayesian Inference analysis was conducted on each data set using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) and sampling for one million generations (four MCMC, chain temperature = 0.2; sample frequency = 100; and burn–in <500). A 50% majority–rule consensus tree was calculated for each matrix from the pooled sample using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as alternative estimate of robustness (Alfaro et al., 2003).

To assess whether data provide significantly less support for a specified alternative topology, we used the Significantly Less Parsimonious test of Templeton (SLP_T)

(Templeton, 1983) and compared matrices and most parsimonious topologies recovered by analyzing ITS, *trnL-F*, and *matK* sequences. SLP_T was implemented in PAUP* using the strict consensus tree obtained from parsimony analyses of the data (Johnson and Soltis, 1998).

2.3. Morphological characters

The distribution of 10 morphological characters, upon which classification of *Cistus* has been traditionally based, is indicated in Supplementary Table S2. Information on some characters is missing for some species and we consequently performed reconstructions of two vegetative (shape and base of leaves) and four reproductive (sepal and fruit-valve number, petal color, style length) characters. Patterns of evolution were explored using the character-state optimization function of MacClade 4.06 (Maddison and Maddison, 1992), assuming Fitch parsimony. Both ACCTRAN (maximizing the proportion of the homoplasy that is accounted by parallelism) and DELTRAN (maximizing the proportion accounted by reversal) optimizations were considered and analyzed. Characters were traced initially onto the strict consensus of shortest trees obtained. To gain further insights into morphological character evolution, the MP tree displaying most congruence with the BI tree, under the simplest model of sequence evolution, was chosen (see below).

3. Results

3.1. Characteristics of trnL-F, ITS, and matK sequences

The characteristics of the three data sets are summarized in Table 2. Within *Cistus*, *trnL-F* sequence divergence ranges from 0.0% (between the 17 conspecific accessions and between *C. clusii–C. munbyi*, *C. symphytifolius–C. chinamadensis*, and *C. albidus–C. creticus*) to 3.15% (between *C. parviflorus* 1–*C. monspeliensis* 1) using the K-2-p model of evolution; *matK* sequence divergence ranges from 0.0% (between 12 conspecific accessions and between *C. albidus–C. creticus*, *C. albidus–C. heterophyllus*, and *C. creticus–C. heterophyllus*) and 1.78% (between *C. salviifolius–C. osbeckiifolius*); and ITS sequence divergence ranges from 0.0% (between eight conspecific accessions) to 4.86% (between *C. crispus–C. parviflorus*). Nucleotide additivity for direct ITS sequencing was clearly observed in direct and reverse chromatograms at 15 positions of 10 accessions (see Supplementary table S2). More than one ITS copy with different sequence length was also detected in two accessions (*C. psilosepalus* 1, *C. parviflorus* 1). A single gap allowed re-establishing nucleotide chromatogram matching, and the resulting sequence was used in the phylogenetic analyses.

3.2. Phylogenetic analyses

Availability (Li et al., in GenBank) and alignability (clustal X, Thompson et al., 1997) of *trnL-F* sequences using four Dipterocarpaceae genera (*Dipterocarpus*, *Parashorea*, *Shorea*, and *Hopea*) allowed performing suitable phylogenetic analysis of Cistaceae–Dipterocarpaceae accessions. MP and BI analyses recognize Cistaceae as monophyletic, with 100% bootstrap value (BS) and 100 posterior probability (PP). The strict consensus tree of 362,200 MP trees is shown in Fig. 2. Within Cistaceae, a successive branching is depicted in the strict consensus tree, in which *Fumana* comes out first (92% BS) followed by *Helianthemum* (100% BS), and then *Tuberaria*. Accessions of *Halimium* and *Cistus* form a largely

Table 2
Summary of phylogenetic characteristics obtained from the analyses of ITS, *trnL-trnF*, and *matK* sequences of the Cistaceae and *Cistus*

	ITS				*trnL-trnF*	*matK*
	ITS region	ITS-1	5.8 S	ITS-2		
Cistaceae						
Length range (bp)	585–671	201–268	167	199–248	377–461	1302–1357
Aligned length (bp)	698	274	168	256	505	1403
Number of variables/informative characters	203/104	108/62	4/3	91/39	127/66	265/143
Maximum sequence divergence (K-2-p)	20.03%	29.70%	2.47%	33.86%	20.07%	13.75%
Informative indels (no. bp)	19 (1–41)	9 (1–41)	0	10 (1–29)	15 (1–26)	17 (1–48)
CI′ (CI)	0.64 (0.78)	—	—	—	0.84 (0.9)	0.87 (0.92)
RI	0.82	—	—	—	0.93	0.95
Mean G + C content	65%	69%	49%	68%	33%	33%
Cistus						
Number of variables/informative characters	92/73	58/45	1/0	33/28	48/42	56/46
Maximum sequence divergence (K-2-p)	4.86%	9.33%	0.60%	5.26%	3.15%	1.78%
Informative indels (no. bp)	7 (1–2)	4 (1–2)	0	3 (1–2)	10 (1–26)	3 (1–48)
Number of nucleotide additivities	15	10	0	5	0	0
Number of accessions with nucleotide additivities	10	7	0	7	0	0

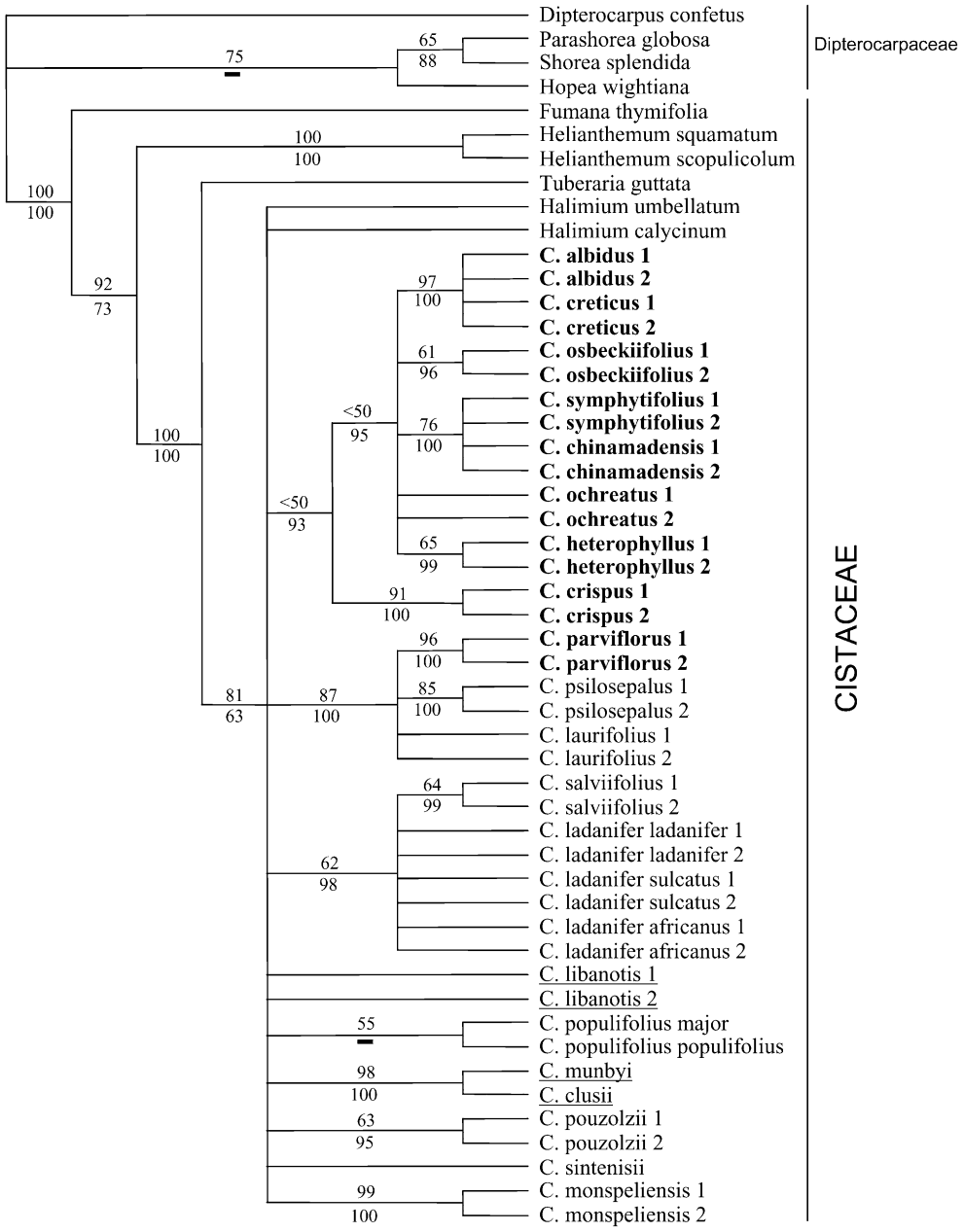

Fig. 2. Strict consensus tree of 362,200 shortest trees of 220 steps (CI = 0.90; CI′ = 0.83, excluding uninformative characters; RI = 0.94) from the analysis of *trnL-F* sequences. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR + *G* as the simplest model of DNA substitution selected by Modeltest 3.06 (Posada and Crandall, 1998). BI resolution incongruent with MP clades as indicated with a hyphen (-) below branches. Taxa circumscription in subgenera is coded as follows: Cistus (in bold); Leucocistus (in roman), and Halimioides (underlined).

unresolved, monophyletic group (81% BS). Bayesian inference, using GTR + *G* as the simplest model of sequence evolution, reached equilibrium after 350,000 generations. The BI reconstruction is mostly consistent with the strict consensus tree, but more resolved: (i) *Halimium calycinum* is sister to *Cistus* (76 PP), whilst *Halimium umbellatum* is nested within a group of white-flowered *Cistus* species (67 PP); (ii) within this group, a subgroup of six species (*C. laurifolius*, *C. parviflorus*, *C. psilosepalus*, *C. pouzolzii*, *C. populifolius*, and *C. monspeliensis*) is also retrieved (80 PP); and (iii) *C. monspeliensis* and *C. populifolius* are sister species (95 PP) (results not shown). In the MP and BI analyses, accessions of the same species either formed monophyletic groups or were placed in unresolved polytomies. We used hereafter *Fumana thymifolia* as the outgroup taxon based on its sister-group relationship to the rest of Cistaceae in the *trnL-F* (Fig. 2) and *rbcL* (Guzmán et al., unpublished) analyses. The analysis of Cistaceae-only accessions using *Fumana* as the outgroup taxon resulted in a

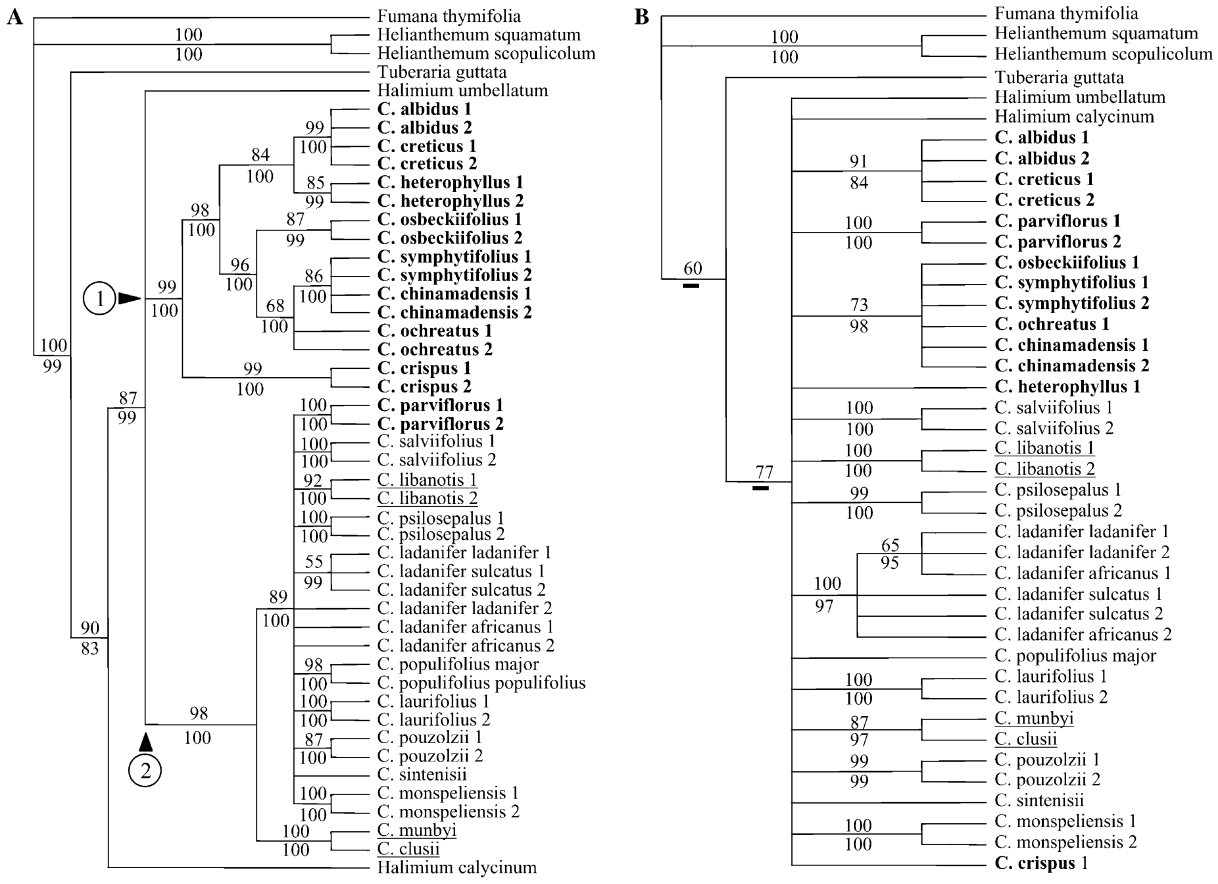

Fig. 3. Strict consensus trees of MP analyses. *F. thymifolia* served as the outgroup taxon. Insertions/deletions (indels) recorded as additional characters. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR + *G* model of DNA substitution selected by Modeltest 3.06 (Posada and Crandall, 1998). BI resolution incongruent with MP clades as indicated with a hyphen below branches (-). Two major clades indicated in circled numbers. (A) Strict consensus tree of 224 shortest trees of 512 steps (CI = 0.90; CI′ = 0.85 excluding uninformative characters; RI = 0.94) from the combined analysis of *trnL-F* and *matK* sequences. (B) Strict consensus tree of 69,486 most parsimonious trees of 933 steps (CI = 0.78; CI′ = 0.64; RI = 0.82) from the analysis of ITS-region sequences. Taxa circumscription in subgenera is coded as follows: Cistus (in bold); Leucocistus (in roman), and Halimioides (underlined).

similar resolution, support, and consistency indices (see Table 2).

The GTR + *G* model was also selected for the *matK* data set. MP and BI analyses yielded similar topology, although with less resolution and lower support values in the MP analysis (results not shown). The strict consensus tree of the combined *matK* and *trnL-F* sequences depicts *Halimium* and *Cistus* as monophyletic (90% BS), and a basal polytomy is formed by *H. umbellatum* and two *Cistus* clades (Fig. 3A). A well-resolved *Cistus* clade (clade 1) comprises all purple-flowered species (99% BS) except for *C. parviflorus*. A second *Cistus* clade (clade 2) consists of all white-flowered species plus *C. parviflorus* (98% BS). The BI analysis of the combined *matK* and *trnL-F* matrix using the common, simplest model of sequence evolution for both data sets (GTR + *G*) reached equilibrium after 40,000 generations. Again, the BI analysis displays better resolution and higher support values than those of the MP analysis, including the sister relationship of *H. calycinum* to a group of all *Cistus* species and *H. umbellatum* and of this *Halimium* species to the group of white-flowered *Cistus* species (clade 2). High BS and PP values (over 85 support values) were retrieved for 11 groups of conspecific accessions (Fig. 3A).

The analysis of ITS sequences yielded limited resolution (Fig. 3B). Eight conspecific accessions are resolved into well-defined monophyletic groups, mostly in agreement with those in the plastid DNA tree (Fig. 3A). In addition, other supported clades are: the four accessions of *C. albidus–C. creticus* (91% BS); the six Canarian accessions (73% BS); and the two accessions of *C. clusii–C. munbyi* (87% BS). Bayesian inference using the selected GTR + *G* + *I* model reached equilibrium after 50,000 generations. The BI analysis retrieved similar relationships at clade tips to those in Fig. 3B, plus a group of *C. psilosepalus* sister to *C. ladanifer* accessions (88 PP) and *C. heterophyllus* sister to the Canarian group (82 PP). Visual inspection of ITS chromatograms revealed 15 positions containing nucleotide double

peaks (Table 2). Although it was not possible to determine whether, in some cases, double-peak patterns may be the result of sequencing artifacts, equimolar proportions of alternative nucleotide peaks in many accessions suggested the presence of more than one ITS copy. This view is supported by the facts that forward and reverse chromatograms displayed double peaks of the same nucleotide proportions and that five affected matrix positions turned to be parsimony-informative characters.

In the analysis of the Cistaceae, resolution and support at clade tips and deep nodes is higher in plastid than in nuclear trees. Consensus-tree topologies display polytomies primarily as a result of insufficient number of informative characters and character incongruence across accessions. In *Cistus*, the number of parsimony-informative characters is higher in the ITS (73) than in the *trnL-F* (42) and *matK* (46) sequences, indicating that the ITS analysis had a sufficient number of informative characters for better resolution. A search for the causes behind low levels of resolution revealed higher measure of fit for the *trnL-F* and *matK* analyses ($CI' = 0.84$ and $CI' = 0.87$, respectively) than that for the ITS analysis (0.64). These values fall into the CI and RI range provided by Álvarez and Wendel (2003), who also reported that ITS data sets have higher levels of homoplasy in angiosperms than plastid data sets. Additionally, the occurrence of more than one nucleotide (additivity) at the same seven informative sites in some ITS accessions contributed to a low resolution, as a result of multiple searches using alternative character states.

3.3. Data congruence and combined phylogeny

The plastid genome is generally considered free from recombination and the *trnL-F* and *matK* sequences consequently share a hypothetical common phylogenetic history. This provides a strong argument for inferring character evolution by combining a *priori* both data sets. Additionally, a significance test for heterogeneity between nuclear and plastid data sets was implemented. Characters in each of the three data sets statistically support alternative topologies found in the set of the shortest trees recovered for those data sets (Table 3). As statistical sub-optimality exists in at least one direction in each comparison, the SLP_T supports data homogeneity (Johnson and Soltis, 1998). The combined plastid and nuclear data matrix of 42 samples consisted of 2606 characters, of which the number of variable/parsimony-informative characters was 595/313 in the Cistaceae (Table 2). The strict consensus tree reveals, once again, a well-defined assemblage of all the *Halimium* and *Cistus* accessions (99% BS), in which *H. calycinum* is sister (71% BS) to a group formed by *H. umbellatum* and two clades of *Cistus* (Fig. 4). Clade 1 contains exclusively purple-flowered, 5-sepaled, mid-to-long styled species (subgenus *Cistus*) (100% BS). Within clade 1, *C. crispus* is sister (96% BS) to the remaining members; they, in turn, form three subclades. The first is a well-defined (100% BS) group of *C. albidus* and *C. creticus* accessions. The second forms a well-supported group (98% BS) of Canarian species, and includes a subgroup of *C. symphytifolius* and *C. chinamadensis* accessions (95% BS). *C. heterophyllus* constitutes the third, unresolved subclade. Clade 2 contains all white-flowered species of subgenera Leucocistus and Halimioides, plus the purple-flowered *C. parviflorus* (93% BS). The three species of subgenus Halimioides do not form a monophyletic group. Whilst *C. munbyi* and *C. clusii* constitute a clade (100% BS) that is resolved as sister to the rest of species in clade 2, *C. libanotis* is unresolved in the large polytomy of white-flowered species. The results from the BI analysis, implementing partitions with the respective simplest models of evolution, were consistent with the strict consensus of the MP trees, but with better resolution and similar or higher support values in most cases. Interestingly, a group of white-flowered *Cistus–Halimium* species is resolved in the BI tree (96 PP), displaying a pectinate topology and high support values (results not shown). In this BI tree, *H. umbellatum* is sister to the white-flowered species of *Cistus* (100 PP), which are also arranged in a pectinate fashion with *C. munbyi–C. clusii* (99 PP) as the earliest diverging group, followed by *C. libanotis* (86 PP), *C. sintenisii* (56 PP), and then a biphyletic group consisting of *C. salviifolius* sister to *C. ladanifer* (97 PP) and *C. monspeliensis* sister to the remaining five species (99 PP) (results not shown). Multiple conspecific accessions within clade 2 form well-supported monophyletic groups (100% BS, 100 PP) in eight cases (*C. parviflorus*, *C. salviifolius*, *C. libanotis*, *C. psilosepalus*, *C. ladanifer*, *C. laurifolius*, *C. pouzolzii*, and *C. monspeliensis*).

Table 3
Results of the Templeton's test (SLP_T) on alternative topologies of strict consensus trees

Data set	Alternative topology	Increase	Decrease	Net	Probability
ITS	*trn*L-F	28	8	48	0.028*
*trn*L-F	ITS	34	2	36	0.0001*
ITS	*mat*K	27	4	50	0.825
*mat*K	ITS	58	49	67	0.0001*
*trn*L-F	*mat*K	18	13	23	0.24
*mat*K	*trn*L-F	19	1	28	0.0021*

Probability values greater than 0.05 indicate that the alternative topology is not significantly less parsimonious than at least one shortest tree.

3.4. Character-state reconstruction

A summary of significant character states obtained from the literature and from our own observations is shown in Fig. 4. Exploration of character changes and ancestral-state reconstruction was undertaken using the total-evidence analysis of nuclear and plastid sequences.

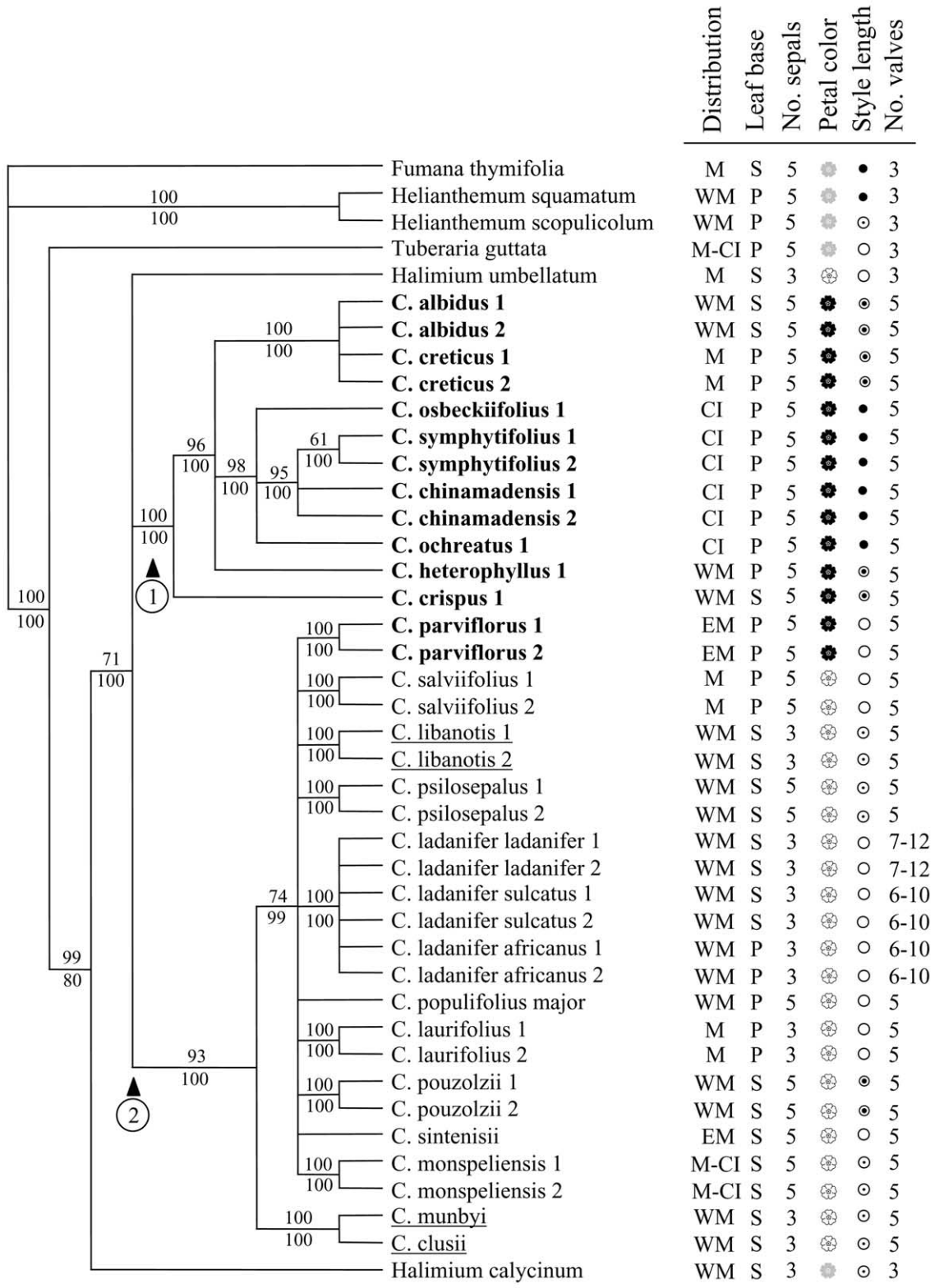

Fig. 4. Strict consensus tree of 328 shortest trees of 895 steps (CI = 0.81; CI′ = 0.70; RI = 0.85) from the combined analysis of *trnL-F*, *matK*, and ITS sequences (total-evidence tree). Insertions/deletions (indels) recorded as additional characters. *F. thymifolia* served as the outgroup taxon. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities. Species distribution (M, Mediterranean; WM, western Mediterranean; EM, eastern Mediterranean; and CI, Canary Islands) and five relevant morphological characters are plotted on the right side of the tree: leaf base (P, petiolate; S, sessile), number of sepals (3, 5); petal color (❁, yellow; ❀, white; and ❁, purple); style length (○, sessile; ⊙, shorter than stamens; ⦿, as long as stamens; and ●, longer than stamens); and number of fruit valves (3, 5, 6 or more). Taxa circumscription in subgenera is coded as follows: Cistus (in bold); Leucocistus (in roman), and Halimioides (underlined).

ACCTRAN and DELTRAN optimizations gave extremely similar results, and only results using ACCTRAN are presented. The most likely of the shortest trees was chosen based on congruence with the BI analysis under the simplest model of sequence evolution (Fig. 5). MacClade reconstructions of character states indicate that leaf shape, sepal number, petal color, and style length are homoplastic in the *Cistus–Halimium* assemblage. For example, purple petals seem to have occurred twice, being the only flower color maintained in the eight species of clade 1. Trace of the four states for style length (sessile, shorter, similar, and longer than stamens) appears to be extremely complex, arising many times not only in this assemblage, but also in the Cistaceae (Fig. 5A). The number of fruit valves is not a synapomorphy supporting the monophyly of *Cistus* (which has five or more valves), in contrast to the three valves found in the remaining seven genera of Cistaceae. Remarkably, the three subspecies of *C. ladanifer* do not display five valves. Rather, between 6 and 12 fruit divisions are observed within this species, representing a unique increment in fruit segmentation from a 3-valved ancestor (Fig. 5B).

4. Discussion

4.1. Systematic implications

Analysis of *trnL-F* sequences supports the monophyly of the Cistaceae genera using *Fumana*, *Helianthemum*, *Tuberaria*, *Halimium*, and *Cistus* (Fig. 2). All phylogenetic analyses are congruent with the monophyly of the *Cistus–Halimium* assemblage. A close relationship between these two genera was suggested in a phyletic diagram by Dansereau (1939). The two representatives of *Halimium* section Halimium (*H. umbellatum*) and section Commutata (*H. calycinum*) appear to have arisen from the same lineage involved in the formation of *Cistus* (Fig. 4). Although we obtained limited phylogenetic support, a sister-group relationship between *H. umbellatum* and the white-flowered species of *Cistus* is observed in some reconstructions (Fig. 5). In fact, the three species (*C. clusii*, *C. munbyi*, and *C. libanotis*) of *Cistus* subgenus *Halimioides* are morphologically similar to *Halimium* in terms of leaf shape (linear), sepal number (3), and seed production (oligosperm placenta). *Cistus* subgenus *Halimioides* was recognized by some authors (Demoly and Montserrat, 1993) but not by others (Dansereau, 1939; Dunal, 1824; Willkomm, 1856). None of these taxonomic treatments of *Cistus* (Table 1) is fully congruent with the strict consensus tree of the combined analysis of *trnL-F*, *matK*, and ITS sequences (Fig. 5). The division of *Cistus* into two more subgenera formed by species with purple (subgenus *Cistus*) and white (subgenera Leucocistus) flowers is mostly supported, as *C. parviflorus* appears in all analyses as the only purple-flowered species placed in an otherwise white-flowered lineage. Its distinctiveness has been historically recognized by creating a monotypic, supraspecific taxon (section) for this species as *Ledonella*. The recognition of a group of Canarian species, as a supraspecific taxon (usually called Rhodocistus) within *Cistus* subgenus *Cistus*, accords with the well-diagnosed natural group of long-styled species exclusive to the Canary Islands (Fig. 4). One more supraspecific taxon (section Erythrocistus), consisting of *C. albidus*, *C. creticus*, *C. heterophyllus*, and *C. crispus*, is paraphyletic because the Canarian lineage originated from a most recent common ancestor to only three of them. At a finer level of taxonomic resolution, this study supports present-day delimitation of some species (*C. parviflorus*, *C. salviifolius*, *C. libanotis*, *C. psilosepalus*, *C. ladanifer*, *C. laurifolius*, *C. pouzolzii*, and *C. monspeliensis*). Our population sample, although limited, indicates that neither paraphyly nor polyphyly affect species formation in *Cistus*. One more lineage consisting of the three subspecies of *C. ladanifer*, as circumscribed by Demoly and Montserrat (1993), receives strong support (Fig. 4). However, our phylogenetic hypothesis does not resolve relationships among these subspecies and our data are unable to determine whether populations from southern Portugal should be recognized as *C. ladanifer* subsp. *sulcatus* or as a distinct species (*C. palinhae* Ingram).

4.2. Evolution of morphological characters

Ten morphological characters have been traditionally considered for circumscription of *Cistus*. Data for some of these characters were not available for all species included in the study (Supplementary Table S2), but five are shown mapped on the total-evidence phylogeny: petal color, leaf base, sepal number, style length, and number of fruit valves (Fig. 4). MacClade reconstructions indicate a dynamic course of evolution of morphological characters (Fig. 5). Our phylogenetic hypothesis suggests that purple petals appear to originate twice in the *Halimium–Cistus* assemblage, result partly in agreement with taxonomic treatments since petal color serves to define a natural group of all purple-flowered species, except for *C. parviflorus*. Leaf bases experienced multiple changes not only in *Cistus* lineages, but also in the Cistaceae and potentially within a single species (*C. ladanifer*). Three and five sepals are found across the Cistaceae reflecting multiple shifts in many groups. Five sepals are, however, maintained within the lineage of purple-flowered species. Our data suggest that the four states of style length (sessile, shorter, similar, and longer) did not evolve in a unidirectional manner in either Cistaceae or the *Cistus–Halimium* assemblage (Fig. 5A). Stigmas exceeding stamens occur only in the Canarian species of *Cistus* and appear to have evolved from an ancestor in

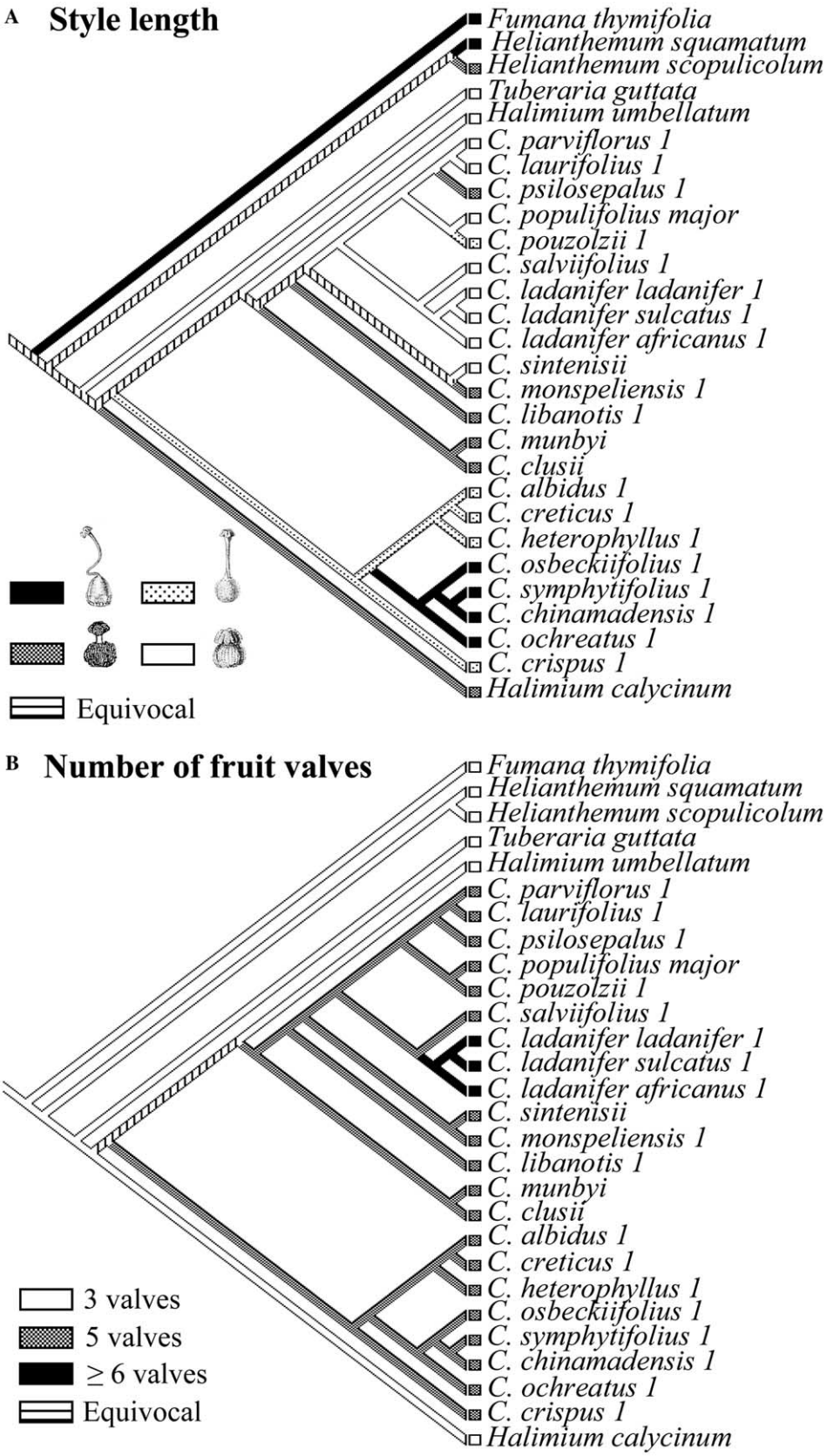

Fig. 5. Hypothesis of character evolution for style length (A) and number of fruit valves (B) using sequences from one individual per taxon. This MP tree of the combined analysis of *trnL-F*, *matK*, and ITS sequences chosen for character reconstruction, onto which the two characters have been mapped, is congruent with the BI tree (see text) and shows "all parsimonius states" as implemented in MacClade (Maddison and Maddison, 1992).

the lineage of purple-flowered species with styles equal in length to the stamens. The optimization of long styles is consistent with an early acquisition of this unique character state, which was then maintained during the course of speciation in the Canary Islands. It is intriguing to interpret prominent stigmas in the Canarian *Cistus* as evolution of a trait related to particular environment conditions of oceanic islands. The occurrence of long styles in other continental Cistaceae indicates recurrent acquisition of this character (Fig. 5A).

Historical reconstruction of the evolution of the number of fruit valves provides evidence of equivocal transition from three valves, consistently displayed in over 180 species of the Cistaceae, to five valves in *Cistus* (Fig. 5B). A further step resulted in the increment of fruit valves to 6–12 exclusively in a single species (*C. ladanifer*). A consistent number of fruit valves is not only exhibited within species of the Cistaceae but also within genera of closely related families (Nandi, 1998). *C. ladanifer* constitutes then a remarkable species model to explore multiplication of fruit valves during the development of the ovary wall. Maximum ITS sequence divergence (0.93% K-2-p distance) of extant subspecies, in comparison to another angiosperms (Richardson et al., 2001), suggests that the multi-valved fruit of *C. ladanifer* evolved following establishment of the Mediterranean climate 2.8 Ma (Suc, 1984) and after plants with 5-valved fruits had been in existence for million of years (see below). Although this character may be subject to phylogenetic processes, variation between 6 and 12 valves should be studied in a broader sense because the highest number of valves appears to be environment dependent in some populations of *C. ladanifer* subsp. *ladanifer* (Narbona, Guzmán, and Vargas, unpublished data).

4.3. Hybridization and evolution in Cistus

Reproductive mechanisms that act to prevent mating occur at the individual level (self-incompatibility) in many species of *Cistus* and *Halimium* (Dansereau, 1940; Herrera, 1992). Consequently, outcrossing favors both inter-individual and inter-specific production of hybrids. Artificial hybridization experiments carried out between 1860 and 1868 by Bornet (Gard, 1910, 1913, 1914) illustrate the facility in which species of *Cistus* generate F_1, F_2, F_3, and F_4 offspring. Bornet successfully undertook crosses between six species of *Cistus* that gave rise to fertile progeny (Gard, 1910). Formation of an intergeneric hybrid between *Cistus* and *Halimium* (x *Halimiocistus*) demonstrates even a wider range of genetic compatibility. Natural hybridization is common as species pairs of *Cistus* occur in sympatry. For instance, 38 natural hybrids between varieties and species were recorded in the field by Dansereau (1940) and 20 inter-specific hybrids of *Cistus* have been described from the Iberian Peninsula (Demoly and Montserrat, 1993; Martín and Guinea, 1949). In light of these results, hybridization was hypothesized as the major mode of evolution in *Cistus* since the early 20th century (Dansereau, 1940; Demoly, 1996). Molecular evidence for hybridization is manifested by (1) nucleotide double-peak patterns (additivity) in 10 ITS sequences, (2) more than two ITS copies of different length size from PCR amplifications of the same DNA samples, and (3) five of the 15 nucleotide additivity sites are found at parsimony-informative positions. This, together with the biparental inheritance of the ITS region, is interpreted as an argument of alternative ITS copies inherited by two or more parental donors (Fuertes et al., 1999), followed by failure to fully homogenize (concerted evolution) multiple ITS copies in the nuclear genome over generations (Rauscher et al., 2002). Apart from success in obtaining artificial inter-species crossings and detecting a molecular pattern of sequence additivity in *Cistus*, the occurrence of hybrid swarms in certain locations of Morocco and Spain (unpublished data) supports the viability of hybridization as an evolutionary mechanism in *Cistus*.

Conflicting signals observed from phylogenies of recombinant, biparental nuclear ribosomal ITS vs non-recombinant, uniparental organelles have typically been interpreted as evidence for extensive reticulation processes at the species level (Wendel and Doyle, 1998). Unfortunately, the limited resolution obtained in the ITS phylogeny precludes detecting fundamental discordance with the plastid phylogeny (Fig. 3). A potential case of speciation by hybridization should be further explored in *C. parviflorus*, as suggested by the detection of sequence additivity (ITS additivity of nucleotides in two sites and different-length sequences in one accession) and the combination of two morphological characters within this species that otherwise define exclusively the two *Cistus* lineages, i.e., purple petals and sessile stigmas. While incongruence between nuclear and organelle genomes may reveal evidence for reticulation, the converse is not necessarily true based only on ITS sequences (Chase et al., 2003). The use of alternative nuclear markers, such as single-copy genes (already in progress), may shed further light on whether balanced concerted evolution of ITS sequences impedes obtaining full-resolved phylogenies (Nieto Feliner et al., 2001).

4.4. Differentiation in the Mediterranean

Cistus exhibits a dominant role in woodland understory and evergreen scrub of the Mediterranean region (Médail and Quézel, 1997). The major center of species diversity is in the western Mediterranean, particularly on both sides of the Strait of Gibraltar (14 of 20 in both Andalusia and northern Morocco (Fig. 1)). The same is true for *Halimium* (the closest genus to *Cistus*), with most species (8 of 10) distributed in this area. Early differentiation of present-day *Cistus* may have occurred

in the western Mediterranean based on the following molecular evidence: (1) the total-evidence phylogeny reveals that the six species of *Cistus* exclusively occurring in the eastern Mediterranean and the Canary Islands (*C. sintenisii* in Albania and Greece; *C. parviflorus* in Greece, Turkey, Italy, Cyprus, and Libya; and *C. chinamadensis*, *C. ochreatus*, *C. osbeckiifolius*, and *C. symphytifolius* in the Canary Islands) do not form basal-most sister groups (Fig. 4); (2) a western-Mediterranean species (*C. crispus*) is sister to the remaining species of the purple-flowered lineage (excluding *C. parviflorus*), as well as two western-Mediterranean species (*C. clusii*, *C. munbyi*) to the white-flowered lineage; (3) the 14 species distributed in the western Mediterranean reach levels of pairwise sequence divergence similar to those within the whole genus (1.62 vs 1.78% in *mat*K; 2.88 vs 3.15% in *trnL-F*; and 4.36 vs 4.86% in ITS (Table 2)). High morphological (taxonomy) and molecular (phylogenetics) divergence in the western Mediterranean and Macaronesia suggests a prime hotspot of diversity not only in *Cistus* but also in disparate angiosperms (Médail and Quézel, 1997). It has been suggested that regional diversity in mediterranean-climatic regions is the product of local diversity and differentiation diversity in relation to environmental heterogeneity (Cowling et al., 1996; Thompson, 2005). *Cistus* species do not fall into this diversity pattern in spite of absence of a long-distance dispersal syndrome (dry capsules and seeds) and environmental specificity referring to acidic and carbonate substrates. In fact, there are no endemics to particular Mediterranean countries and no pattern of geographic cohesion (Fig. 4). Dispersal and colonization of *Cistus* across areas in the Mediterranean basin is inferred to have taken placed successfully after divergence and species formation. Besides wide distribution of most species, the occurrence of circum-Mediterranean species (*C. creticus*, *C. monspeliensis*, and *C. salviifolius*) in the two major lineages supports this view.

The oldest pollen record for Cistaceae (*Cistacearumpollenites*) dates from the Lower Miocene from Czechia (Bohemia) (Konzalova, 1967). This identification should be taken cautiously as there are difficulties in identifying pollen samples at the genus level; identification of species is, however, most reliable once determining ascription to *Cistus* (Ukraintseva, 1991). In contrast, *Cistus* displays an unequivocal shape and number of fruit valves (Supplementary Table S1) in the Mediterranean flora. Fruits in the sedimentary rocks of Germany (Montbauer) and in the amber-bearing sands of the Baltic Sea (Zemland) from the Oligocene (Palibin, 1909) provide reliable evidence for a distribution of *Cistus* not restricted to the present-day Mediterranean region. Objections about inference of centers of origin have been extensively discussed in the past (Bremer, 1992). Despite present-day distribution and diversity of *Cistus* species, paleobotanical data strongly suggest centers of origin for *Cistus* out of the Mediterranean region (as nowadays outlined, Fig. 1), and a time of formation of at least 23 million years ago. We cannot consider calibrated divergence times for *Cistus* because no absolute substitution rate (molecular clock) has been estimated in the Cistaceae. Using both mean values of ITS divergence (4.37×10^{-9} nucleotide substitution/site/year) in angiosperms (Richardson et al., 2001) and the maximum ITS sequence divergence found within *Cistus* (4.86% K-2-p divergence), we infer that differentiation of extant species of *Cistus* might not predate 8–7 Ma. If this result is consistently obtained in future investigations, as increasing the sample and using alternative markers and molecular-clock methods, it would be plausible a hypothesis of that present-day species differentiation occurred much later than the formation of *Cistus*.

4.5. Historical biogeography of Canarian species

Floristic affinities, number of introductions, time of dispersal, and speciation patterns have been the major objectives inferred for Macaronesian plant groups by means of molecular phylogenetics (Carine et al., 2004). The biogeographic results presented in this paper support general patterns of plant colonization in the Canary Islands. The species of *Cistus* endemic to the Canary Islands are imbedded in the purple-flowered lineage in the total-evidence analysis (Fig. 4). Both plastid and nuclear phylogenies reveal a single colonization of *Cistus* in the Canary Islands to account for present-day differentiation into four species (*C. ochreatus*, *C. chinamadensis*, *C. osbeckiifolius*, and *C. symphytifolius*) (Fig. 3). Despite a significant number of phylogenies including Macaronesian taxa, few of them use molecular data from different cellular genomes and, where they have been used, few are congruent with the placement of Canarian lineages (Francisco-Ortega, 2004). We herein provide strong multigenome evidence for a single introduction of purple-flowered *Cistus* in the Canary Islands. Single introductions in Macaronesia appear to be the rule rather than the exception for plant groups consisting of numerous species (Carine et al., 2004; Silvertown, 2004; Vargas, 2005), although topological congruence between organullar and nuclear markers and a larger number of examples are needed. One question that remains to be resolved is whether the occurrence of the white-flowered species *C. monspeliensis* in the Canary Islands and Madeira is the result of natural or human-influenced introduction.

Phylogenetic reconstructions place the Canarian endemics of *Cistus* in a clade with three purple-flowered continental species (*C. albidus*, *C. heterophyllus*, and *C. creticus*). A set of morphological attributes, such as petiolate leaves and a high number of stamens, appear to relate *C. heterophyllus* to the Canarian species (Supplementary Table S2). This species is currently

distributed in the western Mediterranean supporting the close floristic relationship between the Canary Islands and the Mediterranean (Carine et al., 2004), and particularly with north-western Africa since *C. heterophyllus* occurs almost exclusively in Morocco and Algeria. Irrespective of the closest, extant relative of the Canarian lineage, character-state reconstruction using MacClade reveals that the four species endemic to the Canary Islands (*C. ochreatus*, *C. chinamadensis*, *C. osbeckiifolius*, and *C. symphytifolius*) originated from a 5-sepaled, purple-flowered, mid-styled, and 5 fruit-valved ancestor (Figs. 4 and 5). Once *Cistus* colonized and established in the archipelago, speciation took place in conjunction with maintenance of long styles exceeding stamens (Fig. 5A).

Previous allozyme diversity results (Batista et al., 2001) are in agreement with the levels of nucleotide divergence found in the present study, in which *C. symphytifolius* displays the highest levels of K-2-p pairwise divergence with respect to the other Canarian species: 0.32% for ITS (between *C. symphytifolius* 2 and *C. chinamadensis* 1); 0.00% for *matK*; and 0.71% for *trnL-F* (between *C. symphytifolius* and *C. osbeckiifolius*). Additionally, populations of *C. symphytifolius* from different islands display a polyphyletic pattern and the highest levels of nucleotide pairwise divergence for these three markers (unpublished data). Given that Tenerife and Gran Canaria harbor the four species of *Cistus* and the highest levels of molecular diversity, including isozyme and nucleotide divergence, we hypothesize that *Cistus* lineages from these two islands have spawned new lines of evolution via interisland dispersal. Two major evolutionary models have been described to explain speciation of angiosperms in the Canary archipelago: interisland dispersal followed by speciation and intraisland radiation followed by dispersal to similar habitats (Baldwin et al., 1998). The bulk of molecular evidence and present-day distributions suggest extensive interisland dispersal of *C. symphytifolius*, or a closely related ancestor, followed by differentiation of new taxa in some islands (Batista et al., 2001). These conclusions require further intraspecific sampling of *C. symphytifolius* from every island.

Accelerated morphological diversification has been hypothesized for insular plant groups commonly regarded as examples of explosive radiation in which sequence identity is maintained (Baldwin et al., 1998). We do not hypothesize explosive speciation for *Cistus* because of a low number of Canarian species (five including a recently described species by Demoly (2004)) separated by considerable tree branch length with respect to continental species. Levels of molecular divergence of Canarian and their closest continental relatives (0.94% for ITS; 0.47% for *matK*; and 0.71% for *trnL-F*) suggest a relatively old colonization, which contrasts with similar habit (shrubs) as that of their closest relatives in the continent. Differentiation of genera colonizing oceanic islands resulted in tree-like plants in a numerous number of plant groups and remarkable shifts from herbs to a woody condition (Baldwin et al., 1998). Given a considerable time for establishment of *Cistus* in the Canary Islands, it is intriguing to observe neither an increment in size (woodiness) nor occupation of new habitats. Competition-free environments characteristic as oceanic island formation were, however, likely to be rare by the relatively time of *Cistus* establishment, as inferred by low sequence divergence with regard to the origin of the oldest island (Fuerteventura, 20.7 Ma) (Silvertown, 2004). In addition, limited capability of *Cistus* to succeed in different habitats in the continent may be related to failure in exploitation of new, diverse habitats already occupied in oceanic islands. The four species of *Cistus* inhabit Canarian woodlands as understory, and form part of successional stages of Mediterranean and pine tree communities, therefore similar in ecology to their continental congeners (Ceballos and Ortuño, 1976). Lineages of two broom genera (*Adenocarpus*, *Teline*) also exhibit adaptation to woodland understory with no shift in woodiness, limited exploitation of new ecological niches, and similar levels of ITS sequence divergence related to species number (Percy and Cronk, 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.04.026.

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