PHYLOGENY, BIOGEOGRAPHY, AND CHARACTER EVOLUTION IN MEDITERRANEAN, ASIATIC, AND MACARONESIAN *HELICHRYSUM* (ASTERACEAE, GNAPHALIEAE) INFERRED FROM NUCLEAR PHYLOGENETIC ANALYSES

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The genus *Helichrysum* has 500–600 species in Africa, Madagascar, the Mediterranean basin, Macaronesia, western and central Asia, and India. The aims of this study are to confirm the exclusion of *Castroviejoa* from *Helichrysum*, to elucidate the origin of the Macaronesian endemics, to confirm the monophyly of the Mediterranean-Asiatic group, and, within this group, to contrast molecular data with sectional classification and to provide insight into trends in character evolution and biogeography. To address these issues, ITS and ETS sequences were used in phylogenetic analyses. Results show that *Castroviejoa* is excluded from *Helichrysum*. The phylogeny obtained suggests a southern African origin for *Helichrysum* and several subsequent migrations throughout the African continent. The species from the Canary Islands do not form a monophyletic clade, suggesting that at least two independent colonization events occurred. *Helichrysum makranicum*, from Oman and southern Iran, is the closest relative of a clade consisting of two endemics of the Canary Islands, providing new evidence of the phytogeographical links between Macaronesia and eastern Africa–southern Arabia. *Helichrysum gossypinum*, the third endemic of the Canary Islands, is the sister species of a monophyletic Mediterranean and Asiatic group, with the four endemics of Madeira sister to all of them. Within the Mediterranean-Asiatic clade, sect. *Stoechadina* is monophyletic, but sect. *Helichrysum* and sect. *Virginea* are not. Once in the Mediterranean region, the genus is hypothesized to have diversified and expanded to the east up to western and central Asia and to have suffered a reduction in woodiness.

Keywords: *Castroviejoa*, Canary Islands, Madeira, ETS, ITS, hybridization.

Online enhancement: appendix table.

Introduction

The genus *Helichrysum* Mill. (Gnaphalieae) is distributed throughout the African continent, Madagascar, the Mediterranean basin, Macaronesia, western and central Asia, and India (Anderberg 1991) and comprises ~500 (Hilliard 1983) to ~600 (Anderberg 1991; Bayer et al. 2007) species, making it one of the largest genera in the Asteraceae. It includes a great diversity of life forms, from annual plants to trees, although most of the species are subshrubs or shrubs. The genus is characterized as having homogamous or heterogamous capitula, generally with hermaphrodite florets outnumbering female florets; phyllaries with a fenestrated stereome; a flat, generally smooth or toothed receptacle; cypselae glabrous or covered with duplex or twin hairs; and a pappus that is monomorphic and usually uniseriate, consists of several scabrid to plumose bristles, and is free at the base (Hilliard and Burtt 1981).

Helichrysum is a large and taxonomically difficult genus, and its circumscription has undergone notable changes, because the traditional concept of the genus included an assemblage

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of numerous heterogeneous taxa. It has been repeatedly stated that Australian and New Zealand species traditionally known as *Helichrysum* certainly belong to other taxonomic groups, mainly to genera from either the subtribe Angianthinae or the subtribe Cassiniinae (Anderberg 1991; Bayer et al. 2002). These suggestions have since been supported by a molecular phylogenetic work based on ITS sequences (Galbany-Casals et al. 2004a).

A considerable number of changes, based on morphology, have been made concerning the generic delimitation of African *Helichrysum* species and several related genera. Many species have been placed in other genera (e.g., *Syncarpha* DC. [Anderberg 1991; Nordenstam 2003], *Edmondia* Cass., *Dolichothrix* Hilliard & B. L. Burtt, *Atrichantha* Hilliard & B. L. Burtt, and *Plecostachys* Hilliard & B. L. Burtt [Hilliard and Burtt 1981]), while others have been transferred into *Helichrysum* from related genera (e.g., *Leontonyx* Cass. [Hilliard and Burtt 1981]). Finally, some genera closely related to *Helichrysum*, such as *Achyrocline* (Less.) DC. and *Pseudognaphalium* Kirp., cannot be segregated from *Helichrysum* by any qualitative morphological character, and some authors suggest that their circumscription should be revised (Hilliard and Burtt 1981; Anderberg 1991). Despite all these rearrangements of generic

circumscription based on morphology, recent molecular phylogenetic works that included African *Helichrysum* species have confirmed that *Helichrysum* is still polyphyletic (Bayer et al. 2000; Galbany-Casals et al. 2004*a*). For example, *H. dasyanthum* (Willd.) Sweet, from southern Africa, is closely related to *Anaxeton laeve* (Harv.) Lundgren, from the Cassiniinae, rather than to other *Helichrysum* species.

Finally, still in relation to generic delimitation, in a phylogenetic work based on ITS sequences, *H. frigidum* (Labill.) Willd. and *H. montelinasanum* Schmid, two Mediterranean species, were excluded from the *Helichrysum* clade and placed with other genera coded as outgroup taxa (Galbany-Casals et al. 2004*a*). After a detailed study based on morphological traits, these two species were transferred to a new genus, *Castroviejoa* Galbany, L. Sáez & Benedí (Galbany-Casals et al. 2004*b*, 2004*c*).

The only exhaustive treatment of *Helichrysum* is that of de Candolle (1838). However, de Candolle's classification is very incomplete (including 215 of the 500–600 species currently recognized) and does not always reflect natural phylogenetic relationships within species, which makes his classification unreliable. Main recent treatments are those of floristic works and therefore are partial. Hilliard (1983) classified the 245 southern African species into 30 informal groups based on differences in habit; receptacle, stereome, and pappus morphology; and ratio of pistillate and hermaphrodite florets, among other characters. Humbert (1962) also proposed 11 informal groups to classify the 115 species from Madagascar, mainly using differences in habit, leaf morphology, capitula shape and size, and receptacle morphology. Finally, Tadesse and Reilly (1995), in their treatment of the species from northeastern tropical Africa (Ethiopia, Sudan, and Somalia), did not propose any infrageneric classification for the 29 species included, nor did Beentje (2002) in his proposal for the 46 species from eastern tropical Africa (Tanzania, Uganda, and Kenya). This lack of a complete and satisfactory infrageneric classification for the African species reflects the taxonomic difficulty of the genus and the scarcity of morphological characters that could allow the establishment of infrageneric categories.

The genus *Helichrysum* also has some representatives in Macaronesia. Three species are endemic to the Canary Islands: *H. gossypinum* Webb, from Lanzarote, *H. monogynum* B. L. Burtt & Sunding, also from Lanzarote, and *H. alucense* J. García-Casanova, S. Scholz & E. Hernández, from La Gomera; and four species are endemic to Madeira: *H. devium* J. Y. Johnson, *H. melaleucum* Rchb. ex Holl, *H. monizii* Lowe, and *H. obconicum* DC. The geographic origins of the Macaronesian flora have been examined in both traditional and molecular studies. Using the former approach, Bramwell (1976) examined patterns of geographic distribution, endemism, chromosome data, and morphology and stated that part of the flora of the Canary Islands is relict in origin and derived from Mediterranean relatives present during the Tertiary, since a continuous flora was present in northern Africa and the Mediterranean region in the late Miocene (Axelrod 1975; Quézel 1978; Sunding 1979). Other data on distribution patterns of many genera indicate that most of the modern flora of the Canary Islands is of African origin and that the closest relatives of many taxa on these islands are in eastern Africa and southern Arabia. A general "track" unifying southern Africa, eastern Africa, and, through the Sahara, the Mediterranean region and Macaronesia has been proposed (Bramwell 1985). This general African migration track mostly agrees with African migration routes for the Pliocene and Pleistocene flora suggested by Quézel (1978).

Molecular studies hold that several elements of Macaronesian flora derive from a range of areas including Eurosiberia (e.g., *Saxifraga*: Vargas et al. 1999*b*), northwestern Africa (e.g., *Lotus*: Allan et al. 2004; *Reseda*: Martín-Bravo et al. 2007), eastern Africa (several examples, reviewed by Andrus et al. [2004]), southern Africa (e.g., *Phyllis* and *Anthospermum*: Bremer 1996; Andersson and Rova 1999), and the Mediterranean region (e.g., *Argyranthemum*, the *Asteriscus* alliance: Francisco-Ortega et al. 1997, 1999; *Ixanthus*: Thiv et al. 1999; *Bencomia*: Helfgott et al. 2000; *Sideritis*: Barber et al. 2002; *Descurainia*: Goodson et al. 2006). A number of these studies show monophyletic groups of Macaronesian taxa, which indicates a single origin and colonization of the islands followed by diversification within the islands, including interisland colonization (Francisco-Ortega et al. 1997, 1999, 2001; Helfgott et al. 2000; Barber et al. 2002; Allan et al. 2004). In contrast, other studies support the hypothesis of multiple colonization events of Macaronesia from continental sources (*Hedera*: Vargas et al. 1999*a*; *Ilex*: Cuénoud et al. 2000; *Olea*: Hess et al. 2000; *Tolpis*: Park et al. 2001; *Lavatera*: Fuertes-Aguilar et al. 2002; *Teline*: Percy and Cronk 2002). In a previous phylogenetic work, *Helichrysum alucense*, endemic to La Gomera and the only species from Macaronesia included in the analysis, was grouped with the African *Helichrysum*, which is more closely related to African representatives than to Mediterranean ones (Galbany-Casals et al. 2004*a*). The origin of the other Macaronesian species is still uncertain, as is the matter of whether all the Macaronesian species constitute a monophyletic group derived from a single colonization event, on the one hand, or from several events, on the other.

In the Mediterranean region and western and central Asia, 41 taxa of *Helichrysum*, including subspecies, are found, 19 of which were included in a previous phylogeny of the genus and that showed them to be a highly supported monophyletic group derived from African ancestors (Galbany-Casals et al. 2004*a*). The most recent treatments of this group recognize three sections that do not overlap and have no correspondence with the African informal groups: sect. *Stoechadina* (DC.) Gren. & Godr., sect. *Helichrysum*, and sect. *Virginea* (DC.) Gren. & Godr. (Clapham 1976; Galbany-Casals et al. 2006). Species of sect. *Stoechadina* generally have a western-central Mediterranean distribution and consist of shrubs or subshrubs (fig. 1*A*). They never have conspicuous overwintering buds, stolons, or rhizomes, and only *H. crassifolium* (L.) D. Don has basal leaf rosettes. Representatives of sect. *Stoechadina* were grouped in a moderately supported monophyletic clade in a previous study, but internal relationships within this group were unresolved (Galbany-Casals et al. 2004*a*). Species from sect. *Helichrysum* and sect. *Virginea* are suffruticose and generally have leaf rosettes that often bear conspicuous overwintering buds (fig. 1*B*). Some species have homogamous capitula and others heterogamous ones. The morphological differences between sect. *Helichrysum* and sect. *Virginea* are as follows (Clapham 1976): Species in sect. *Helichrysum* have capitula with yellow, rarely white, nonradiating phyllaries that nearly
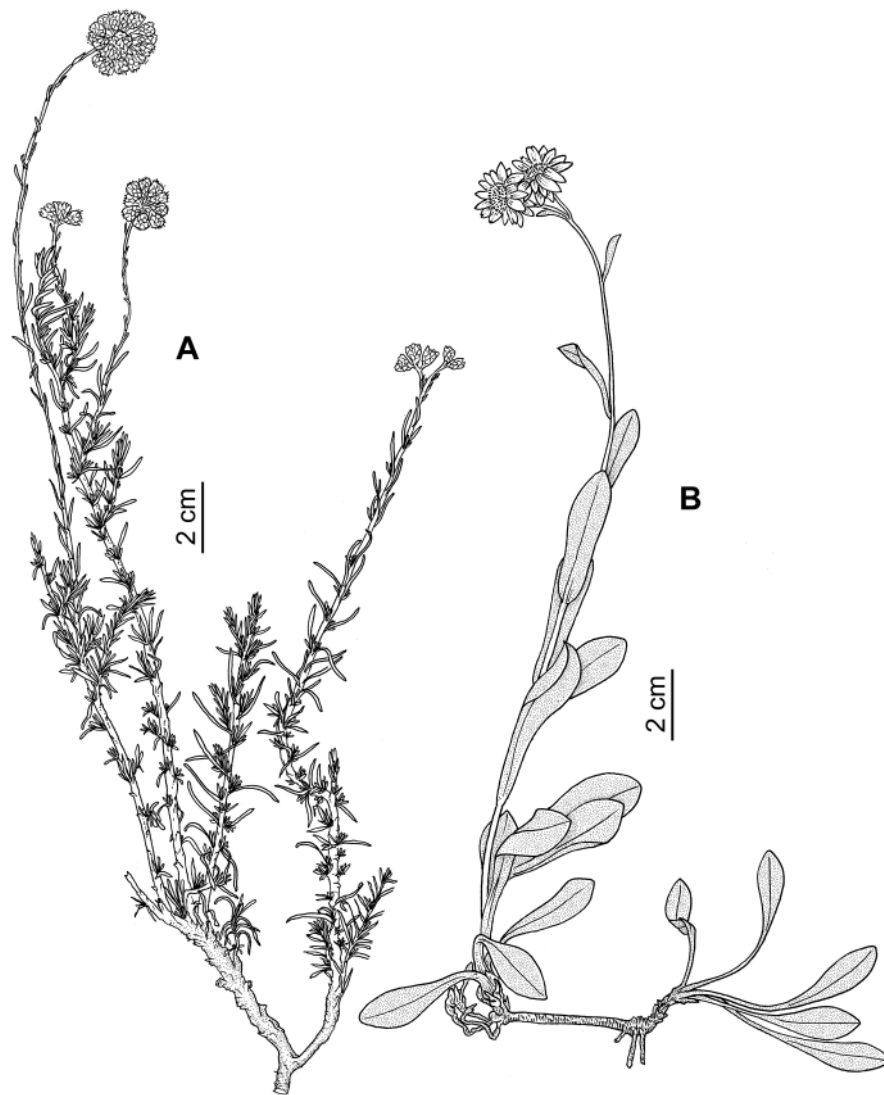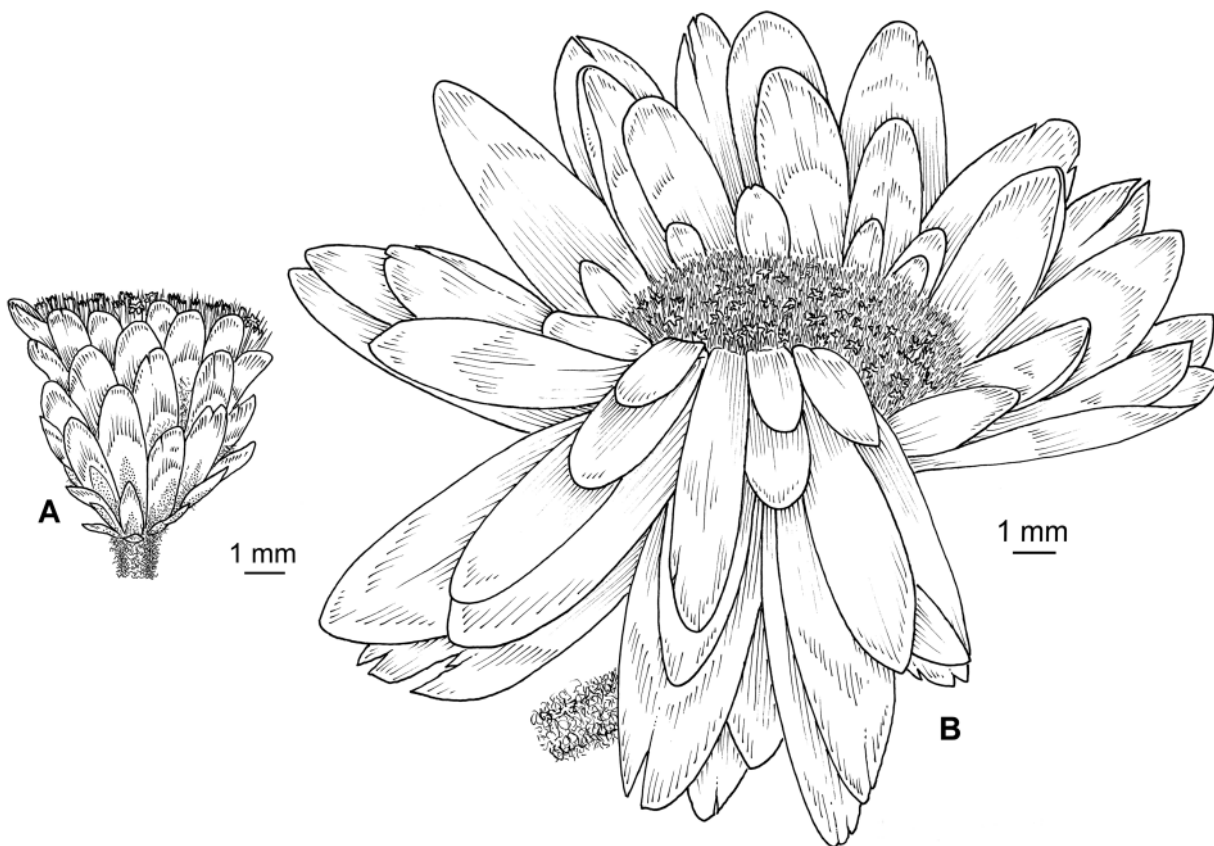
Fig. 1   Two types of habit in the Mediterranean and Asiatic *Helichrysum*: *A*, Shrubby plants, with vegetative stems well developed and without leaf rosettes; *B*, suffruticose plants, with leaf rosettes and often with overwintering buds conspicuously swollen.

equal the florets in length, and the innermost phyllaries are longer than the middle ones (fig. 2*A*). In contrast, species in sect. *Virginea* have capitula with white, radiating phyllaries that extend beyond the florets, and the middle phyllaries are longer than the innermost ones (fig. 2*B*). The species included in sect. *Virginea* are distributed in the central and eastern Mediterranean regions, whereas sect. *Helichrysum* includes taxa with eastern Mediterranean and western and central Asiatic distributions. In a previous ITS sequence-based study, sect. *Virginea* was not supported, because the two representatives of the section, *H. sibthorpii* Rouy and *H. taenari* Rothm., were not grouped in a monophyletic clade (Galbany-Casals et al. 2004*a*).

In this article, we add new material that contributes to a more complete sampling of the Mediterranean-Asiatic group, including all the Macaronesian endemic species, within a wider survey of the genus, including many species from tropical and southern Africa. Moreover, in view of the low resolution of the ITS within the Mediterranean-Asiatic group, we add a new marker, the ETS (external transcribed spacer), which has been used in the Asteraceae (e.g., Baldwin and Markos 1998; Clevinger and Panero 2000; Urbatsch et al. 2003; Suárez-Santiago et al. 2007) and the Gnaphalieae (Bayer et al. 2002).

Our aims are (1) to test the hypothesized monophyly of the Mediterranean-Asiatic *Helichrysum*, using a more complete sampling; (2) to contrast molecular data with the traditional sectional classification and morphology in this group; (3) to provide additional insight into character evolution in this group; (4) to examine the origin and affinities of *Helichrysum* species from the Canary Islands and Madeira and infer the colonization pattern of these islands; and (5) to provide additional support, based on broader taxonomic sampling, for the exclusion of *Castroviejoa* from *Helichrysum*.

Fig. 2 Two types of capitula in the Mediterranean and Asiatic *Helichrysum*: *A, Stoechadina* and *Helichrysum* type, with nonradiating phyllaries that nearly equal the florets in length and innermost phyllaries that are longer than the middle ones; *B, Virginea* type, with radiating phyllaries that extend beyond the florets and middle phyllaries that are longer than the innermost ones.

Material and Methods

Plants

We have included 36 representatives of the 41 Mediterranean and Asiatic *Helichrysum* and 47 representatives of African *Helichrysum*. The African species were selected to widen the representation of the genus and comprise 15 species from tropical Africa and 32 from southern Africa, representing 28 of the 30 informal groups recognized by Hilliard (1983). In addition, *H. makranicum* (Rech. f. & Esfand.) Rech. f., from Oman and southern Iran, and *H. arwae* J. R. I. Wood, from Yemen, have been included. Our sampling of the genus also comprises the three endemic species of the Canary Islands and the four endemic species of the Madeiran Archipelago. We have also included *Anaxeton laeve*, *Syncarpha mucronata* (P. J. Bergius) B. Nord., *Helichrysum dasyanthum*, and *Plecostachys serpyllifolia* (P. J. Bergius) Hilliard & B. L. Burtt as outgroup taxa, according to previous sequence analyses (Bayer et al. 2000; Galbany-Casals et al. 2004*a*). Finally, *Castroviejoa frigida* (Labill.) Galbany, L. Sáez & Benedí and *C. montelinasana* (Schmid) Galbany, L. Sáez & Benedí have been included. In total, we have included in the analyses 96 ITS sequences, of which 58 are new, and 46 ETS sequences, all of which are new. Voucher data, the origin of the material, and GenBank sequence accession numbers are given in table A1 in the online edition of the *International Journal of Plant Sciences*.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted with the CTAB method of Doyle and Doyle (1987), as modified by Cullings (1992), from silica-gel-dried leaves collected in the field, fresh plants cultivated at the Botanic Institute of Barcelona, or herbarium material. For difficult material, the commercial kits NucleoSpin Plant (Macherey-Nagel, Düren, Germany) and DNeasy Extraction Kit (Qiagen, Hilden, Germany) were used, following the manufacturers' instructions.

Nuclear Ribosomal DNA (nrDNA) ITS Region Strategies

The ITS DNA region was amplified using the 17SE forward and 26SE reverse primers (Sun et al. 1994). The profile used for amplification was as described in Galbany-Casals et al. (2004*a*). Double-stranded PCR products were purified with either a QIAquick Purification Kit (Qiagen, Valencia, CA) or DNA Clean and Concentrator-5 (Zymo Research, Orange, CA) and sequenced with the same primers. Direct sequencing of the amplified DNA segments was performed with a Big Dye Terminator v3.1 kit (Applied Biosystems, Foster City, CA), following the protocol recommended by the manufacturer.

Nucleotide sequencing was carried out at the Serveis Científico-Tècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (Applied Biosystems). In all cases, only one PCR product was obtained, and direct sequencing produced generally unambiguous sequences. For that reason, no species was cloned for ITS. However, a few double peaks appeared in some chromatograms, and these were coded with ambiguous bases, although we cannot be sure whether they are noise or genuine variation, given that we did not clone them.

nrDNA ETS Region Strategies

The ETS DNA region was amplified using the forward primer ETS1f (Linder et al. 2000) and the reverse primer 18S-2L (Linder et al. 2000) or 18S-ETS (Markos and Baldwin 2001). In some cases, Ast-1 and Ast-2 were also used as internal primers (Markos and Baldwin 2001). The profile used for amplification included a 5-min denaturing at 95°C, followed by 30 cycles of 94°C denaturing for 45 s, 48°C annealing for 45 s, and 72°C extension for 40 s, with an additional 7 min at 72°C. Purification and sequencing was performed as for the ITS region, but with the corresponding primers. In all cases, only one PCR product was obtained, and all but five ETS sequences could be obtained by direct sequencing. When a possible polymorphism was observed in some positions of the ETS sequences obtained by direct sequencing, nucleotides were replaced with ambiguous bases, although they are interpreted as noise rather than as genuine variation because these ambiguous positions were mainly found at the end of the sequence or in the internal primers' annealing zone.

Direct sequences could not be obtained for five species: *H. oligocephalum* DC., *H. crassifolium* (L.) D. Don, *H. pallasii* (Spreng.) Ledeb., *H. thianschanicum* Regel, and *H. graveolens* (M. Bieb.) Sweet. In these cases, the ETS PCR product was cloned by using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, except that only half-reactions were used. When available, eight positive colonies from each reaction were screened with direct PCR and the T7 and M13R universal primers, according to the amplification profile described in Vilatersana et al. (2007). All PCR products obtained were the same size. Finally, four PCR products for each species, when possible, were selected randomly for sequencing in both directions with the same primers.

All sequences obtained for each cloned species were included in a first analysis. When multiple sequences of the same species formed a highly supported monophyletic group and sequence dissimilarity was low (*H. pallasii*, *H. thianschanicum*, and *H. graveolens*), a consensus sequence was generated for subsequent analyses. This was done by replacing nucleotides at polymorphic positions with ambiguous bases in the consensus sequence. This strategy was chosen to include all available data potentially contributing to the phylogeny construction. Also, this reduced the size of the matrices as well as the effect of potential PCR artifacts (chimerical sequences and Taq errors; Cline et al. 1996; Popp and Oxelman 2001). When multiple sequences of the same species did not form a highly supported monophyletic group, all sequences were used in final analyses.

Analyses

Nucleotide sequences were edited with Chromas 2.0 (Technelysium, Tewantin, Australia) and aligned visually by sequential pairwise comparison (Swofford and Olsen 1990). Data matrices are available on request from the corresponding author.

The evolutionary relationships in *Helichrysum* were examined for four data sets. Data set 1 consisted of ITS sequences of 95 species, including 90 *Helichrysum* species and five species from other genera. In analyses using data set 1, *Anaxeton laeve*, *Syncarpha mucronata*, *Helichrysum dasyanthum*, and *Plecostachys serpyllifolia* were coded as outgroup taxa. A subset of data set 1 was used to construct data sets 2–4. This subset, which was the main objective of our study, contained the Asiatic, Macaronesian, and Mediterranean *Helichrysum* species that constituted a derived monophyletic clade in the analyses from data set 1. *Helichrysum milfordiae* Killick and *H. populifolium* DC., two African species, were also included and were designated as outgroup taxa in the analyses of data sets 2–4. Data set 2 contained 43 ITS sequences of the same number of species; data set 3 contained 46 ETS sequences of the 43 species, because for some of the species several clones were included; and data set 4 was a combined ITS and ETS data set and contained 46 sequences corresponding to the 43 species. In this combined data set, for each cloned species, the same ITS sequence was repeated for each of the ETS clones.

A partition homogeneity test (incongruence length difference [ILD]; Farris et al. 1994) was carried out to test the heterogeneity of phylogenetic signal across the ITS and ETS data sets (data sets 2 and 3, respectively). This test (implemented in PAUP*, ver. 4.0b10; Swofford 2002) was accomplished by excluding uninformative characters and using heuristic search and a simple addition of taxa for 300 random partitions of the data.

Parsimony analyses of all data sets involved heuristic searches conducted with PAUP*, version 4.0b10 (Swofford 2002), using tree bisection reconnection (TBR) branch swapping with character states specified as unordered and unweighted. The indels were not excluded from analyses and were coded as missing data. All most parsimonious trees (MPTs) were saved, except in the case of the data set 1 analysis, in which only 500 trees were held at each step to minimize the time of search. To locate other potential islands of MPTs (Maddison 1991), we performed 1000 replications with random taxon addition, also with TBR branch swapping. Bootstrap (BS) analyses (Felsenstein 1985) were performed with 100 replications and heuristic search with the default options, except for data set 1 analysis, which followed the approach of Lidén et al. (1997), using 1000 replicates, random taxon addition with 20 replicates, and no branch swapping. For the parsimony analyses, consistency index (CI), retention index (RI), and homoplasy index (HI) are given (table 1), excluding uninformative characters.

Bayesian inference (BI) estimation was calculated with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The best available model of molecular evolution, required for Bayesian estimations of phylogeny, was selected using hierarchical likelihood ratio tests (hLRT) and the Akaike Information Criteria (AIC; Akaike 1973), as

Table 1

Main ETS and ITS Sequence Characteristics and Analysis Results for the Different Data Sets

	Data set 1	Data set 2	Data set 3	Data set 4
No. of taxa	95	43	46	46
Aligned length	503	479	1151	1630
No. of gaps	40	12	15	27
Gap length range (bp)	1–45	1	1–291	1–291
No. of variable sites	248	88	264	352
No. of sites with missing data	0	0	12	12
No. of ambiguous/polymorphic sites	15	5	57	62
Parsimony analyses:				
Parsimony-informative characters	143	32	120	153
No. of most parsimonious trees	390,500	160	18,892	660
No. of steps	497	56	231	295
Consistency index	.4708	.6429	.6623	.6475
Retention index	.7882	.8540	.8770	.8670
Homoplasy index	.5292	.3571	.3377	.3525
Bayesian inference:				
Model of molecular evolution	GTR + Γ	GTR + Γ	GTR + Γ	GTR + Γ

Note.   Data set 1 includes ITS sequences of all species studied. Data set 2 contains ITS sequences of the Asiatic, Macaronesian, and Mediterranean *Helichrysum* species that constituted a derived monophyletic clade in the analyses from data set 1, with *Helichrysum milfordiae* and *H. populifolium* as outgroup taxa. Data set 3 contains ETS sequences for the species in data set 2. Data set 4 combines data sets 2 and 3. The consistency, retention, and homoplasy indices are calculated excluding uninformative characters.

implemented in MrModeltest 2.2 (Nylander 2004), which considers only nucleotide substitution models that are currently implemented in PAUP and MrBayes 3.1.2. For all data sets, the General Time-Reversible model (Rodríguez et al. 1990) with variable sites assumed to follow a discrete gamma distribution (GTR+Γ; Yang 1996) was identified as the best-fitting symmetrical model for the sequence data by both the hLTR and the AIC statistics, so GTR+Γ was the model used in all analyses. Phylogenetic reconstruction using BI was performed with MrBayes 3.1.2. Two simultaneous and independent analyses were performed; for each analysis, four Markov Monte Carlo chains were run simultaneously, starting from random trees. Each analysis was run for 1,000,000 generations and sampled one of every 100 generations, which resulted in 20,000 sample trees. A critical aspect of the Bayesian analysis is to ensure that the Markov chain has reached stationarity. With this aim, the first 1000 trees (burn-in) were excluded before the majority-rule consensus tree was computed, to avoid trees that might have been sampled before convergence of the Markov chains. Posterior probability support (PP) was estimated to be significant for nodes with PP > 0.95.

Results

Numeric results of main sequence characteristics for each data set are summarized in table 1.

Size and Composition of ITS

In data set 1, the length of the ITS1 region in the studied taxa ranged from 213 bp in *Helichrysum lambertianum* DC. to 260 bp in *H. simillinum* DC. The ITS2 region ranged from 217 bp in *H. acutatum* DC. to 228 bp in *Anaxeton laeve*. Within *Helichrysum*, *H. confertum* N. E. Br. had the longest ITS2 region, with 223 bp. In data set 2, the length of the ITS1

region in the studied taxa ranged from 253 bp in *H. sanguineum* (L.) Kostel. to 256 bp in *H. milfordiae* and *H. populifolium*. The ITS2 region was 218 bp long in *H. leucocephalum* Boiss. and 219 bp long in the other species.

Sequence divergence was calculated separately for the ITS1 and ITS2 regions with the distance matrix option available in PAUP. ITS1 sequence divergence within data set 1 varied from 0% (between 53 pairs of species of *Helichrysum*) to 16.05% (pairwise distance between *H. monogynum* and *Syncarpha mucronata*). ITS1 sequence divergence within data set 2 varied from 0% (between 53 pairs of species of *Helichrysum*) to 6.67% (pairwise distance between *H. thianschanicum* and *H. populifolium*). ITS2 sequence divergence within data set 1 varied from 0% (between 75 pairs of species of *Helichrysum*) to 14.87% (pairwise distance between *H. retortum* [L.] Willd. and *Castroviejoa montelinasana*). ITS2 sequence divergence within data set 2 varied from 0% (between 74 pairs of species of *Helichrysum*) to 11.01% (pairwise distance between *H. pamphylicum* Davis & Kupicha and *H. populifolium*).

Ambiguous positions in ITS sequences were very scarce (table 1). Since no ITS sequences were cloned, we cannot be sure whether they are due to noise or to genuine variation.

Size and Composition of ETS

The length of the ETS portion used (data set 3) ranged from 837 bp in *H. arenarium* (L.) Moench ssp. *arenarium*, *H. arenarium* ssp. *aucheri* (Boiss.) P. H. Davis & Kupicha, *H. lacteum* Coss. & Durieu, *H. graveolens*, and *H. artemisioides* Boiss. & Hausskn. to 1112 bp in *H. milfordiae*. ETS sequence divergence was calculated with the distance matrix option available in PAUP. It varied from 0.12% (pairwise distance between *H. doerfleri* Rech. f. and *H. amorginum* Boiss. & Orph.) to 9.77% (pairwise distance between one of the clones of *H. oligocephalum* and *H. milfordiae*).

Direct ETS sequences could not be obtained for five species: *H. oligocephalum*, *H. crassifolium*, *H. pallasii*, *H. thianschanicum*, and *H. graveolens*, and these were cloned. The different clones obtained for each of the last three species formed highly supported monophyletic clades because of high sequence similarity: the clones of *H. pallasii* presented only five polymorphic positions, those of *H. thianschanicum* only three, and those of *H. graveolens* only one. Therefore, they are represented by a single consensus sequence in the final phylogenetic trees (figs. 4, 5). The cases of *H. crassifolium* and *H. oligocephalum* are different: the ETS clones obtained for both of these species did not form monophyletic groups and presented notable levels of polymorphism: the clones of *H. oligocephalum* presented 27 polymorphic positions and those of *H. crassifolium* 13.

Phylogenetic Analyses

The numerical results of the analyses with all data sets are given in table 1. Both parsimony and BI analyses showed highly congruent topologies for each data set. Therefore, for each data set, we discuss only one of the analyses, either the BI or the parsimony strict consensus tree. However, BS values from the parsimony analysis and Bayesian PP are shown in all figures.

In the analyses of data set 1 (fig. 3), the species of *Helichrysum* coded as ingroup are monophyletic (BS = 100%, PP = 1). *Castroviejoa frigida* and *C. montelinasana* constitute a clade (BS = 100%, PP = 1; fig. 3) that is excluded from the *Helichrysum* group and placed in the outgroup. Within the *Helichrysum* clade, the southern African *H. retortum*, *H. lambertianum*, *H. asperum* (Thunb.) Hilliard & B. L. Burtt, and *H. spiralepis* Hilliard & B. L. Burtt form a basal polytomy. Many African *Helichrysum* appear in a general polytomy, and the few clades moderately supported include mixed tropical and southern African species. However, some results are worthy of comment. *Helichrysum monogynum* and *H. alucense*, from the Canary Islands, are grouped together (BS = 99%, PP = 1; fig. 3) with *H. makranicum*, from Oman and southern Iran, as sister taxa (BS = 100%, PP = 1), with *H. populifolium*, from South Africa, as sister to this three-species clade (BS = 55%, PP = 0.97). Within the general *Helichrysum* clade, another highly supported clade is observed that includes all the Mediterranean and western and central Asiatic species and most of the Macaronesian ones (excluding *H. monogynum* and *H. alucense*; BS = 98%, PP = 1).

The analyses from data sets 2 and 3 provided congruent phylogenies (fig. 4). Low general resolution and the absence of any supported incongruence justified combination of the ITS and ETS data sets. The result of the ILD test was $P = 0.3$, which meant that both data sets contained highly congruent phylogenetic signals and supported the possibility of combining the two regions.

The ETS alone (data set 3) provided most of the resolution and support of the internal nodes in the Mediterranean-Asiatic-Macaronesian group (fig. 4), although the combined ITS-ETS analyses (data set 4) showed the highest overall support values, for which we discuss the following results (see fig. 5). Within the Mediterranean-Asiatic-Macaronesian clade, the four Madeiran endemic species constitute a monophyletic group (BS = 100%, PP = 1; fig. 5, clade E) sister to the rest of the species, which also constitute a monophyletic group (BS = 100%, PP = 1). *Helichrysum gossypinum*, from Lanzarote, is sister to the whole Mediterranean-Asiatic group (BS = 87%, PP = 1), according to our sampling. Internal relationships within the Mediterranean-Asiatic group are much better resolved than those in the previous study using ITS alone (Galbany-Casals et al. 2004a). In this clade, two main groups sister to each other are highly supported: a clade that includes all the species of sect. *Stoechadina* (BS = 100%; PP = 1; fig. 5, clade C) and another that comprises all the representatives of sect. *Helichrysum* and sect. *Virginea* (BS = 99%, PP = 1; fig. 5, clades A, B). Sections *Virginea* and *Helichrysum* do not form monophyletic groups, because representatives of sect. *Virginea* are scattered among representatives of sect. *Helichrysum*.
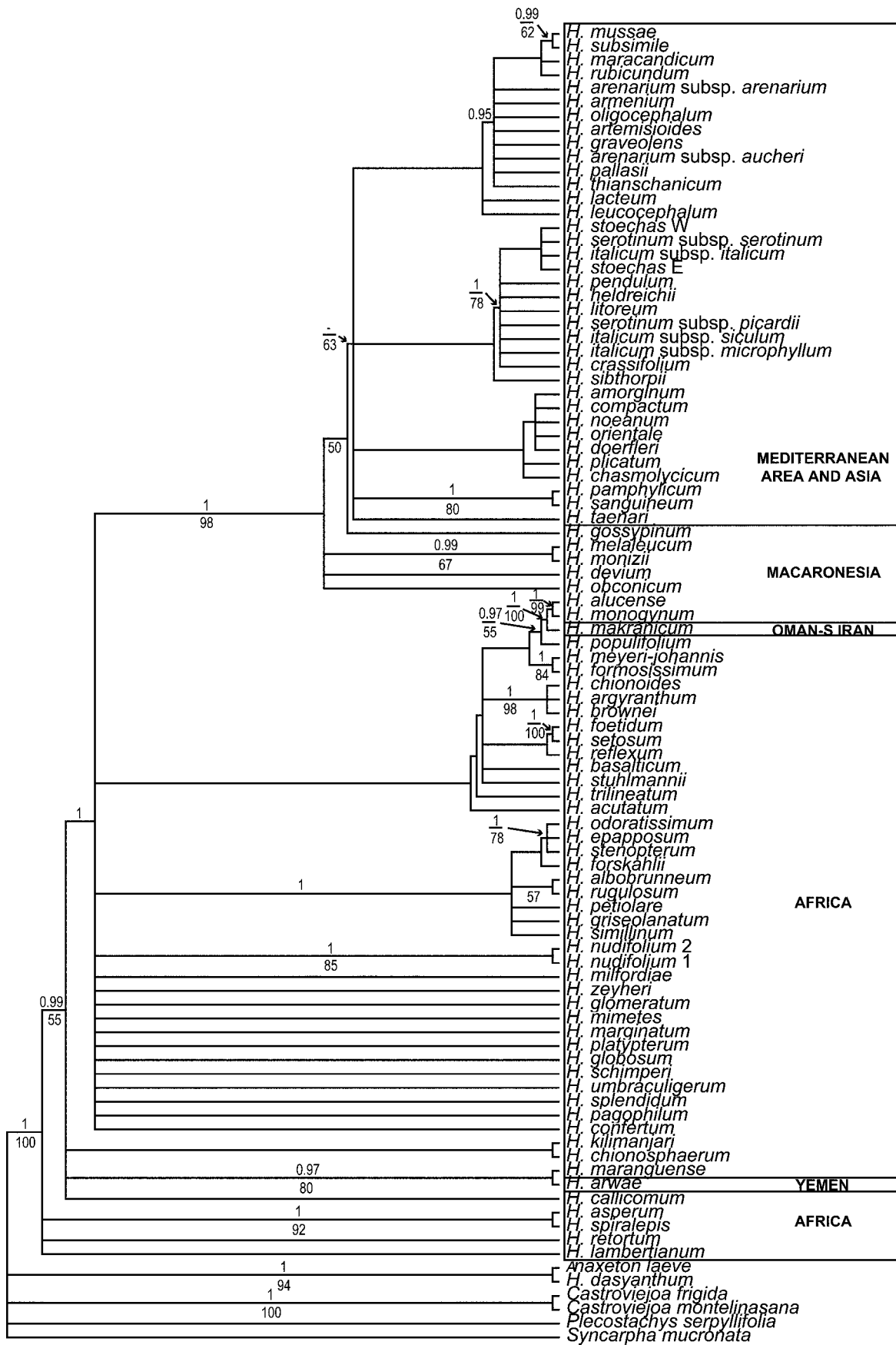
Discussion

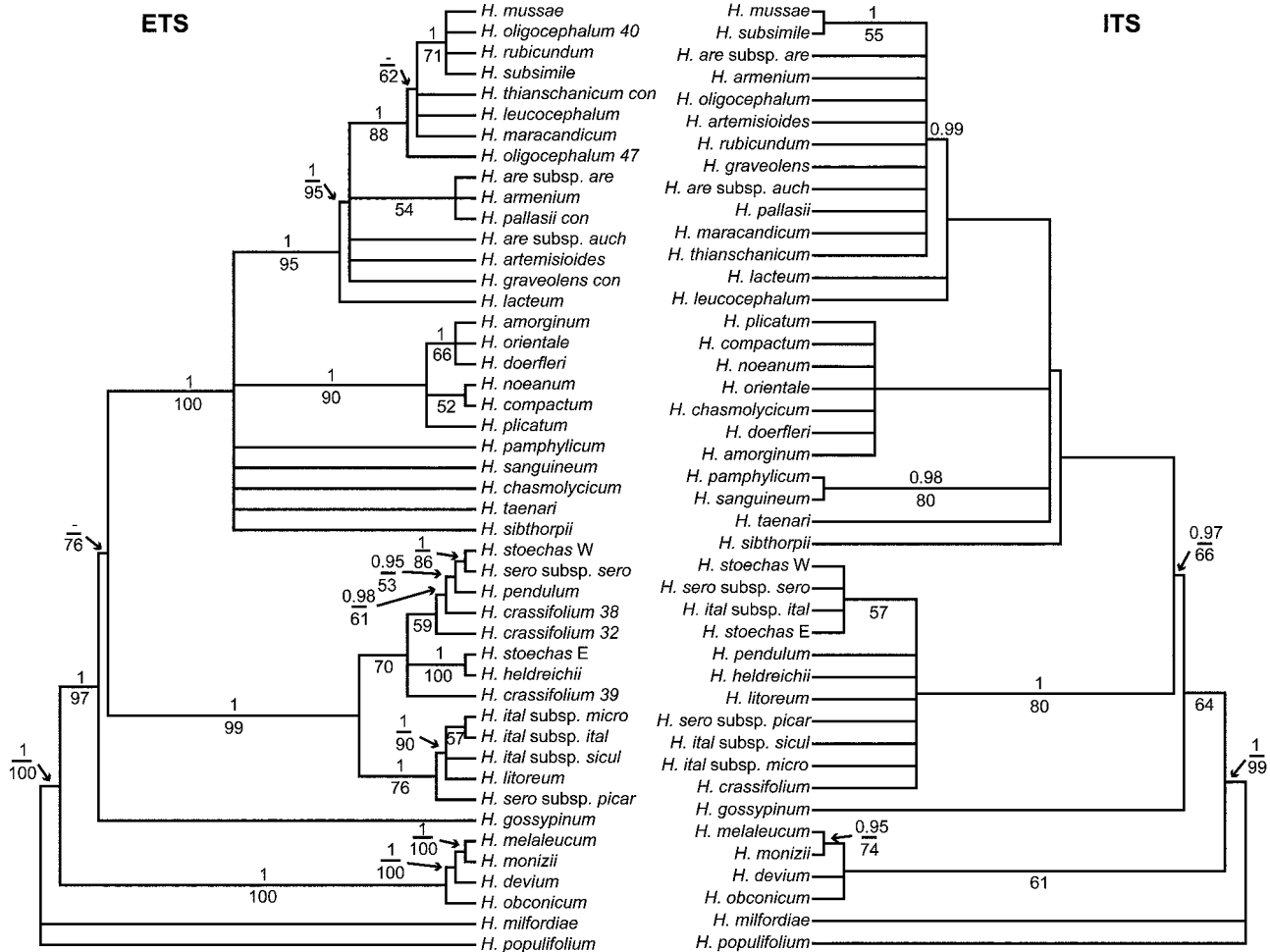#### Utility of ETS and Congruence between ITS and ETS Phylogenies

The ETS is longer than ITS1 and ITS2 together, and it provides at least as many variable and potentially informative sites than the ITS in this and other studies (Baldwin and Markos 1998; Bena et al. 1998; Linder et al. 2000; Andreasen and Baldwin 2003). In our case, in the ITS 6.68% of the characters are informative, while in the ETS the percentage is 10.43%, which represents a ratio of 1.5. However, we used nearly the entire ETS region, including the 5′ portion, which is generally more variable than the 3′ portion, and in our case it was easily aligned because of high sequence similarity. In ETS alignment, most of gaps were 1–5 bp, and only one large gap (291 bp) was needed in the alignment of *H. populifolium* and *H. milfordiae* with the Mediterranean-Asiatic-Macaronesian group. In contrast, nearly all studies use only the 3′ portion because of the general difficulties in aligning the too-variable 5′ end.

As they are parts of the same transcriptional unit, the ITS and ETS regions are said to be evolving under similar functional constraints and at comparable rates (Baldwin and Markos 1998). ITS and ETS phylogenies are usually congruent and combinable, so that the ETS becomes a source of additional characters for increasing resolution and support in nrDNA–based phylogenies (Baldwin and Markos 1998). However, Okuyama et al. (2005) caution that sometimes the ITS and the ETS can provide incongruent phylogenies because of uneven rates of concerted evolution after introgressive hybridization. In our case, the ITS and ETS data are highly congruent (fig. 4).

Okuyama et al. (2005) stated that sequence heterogeneity within an individual is a good indicator of the extent to which concerted evolution has progressed. In *Helichrysum*, a few species contained different ETS sequences, suggesting that concerted evolution is not complete in these cases. Several factors could be the cause for sequence polymorphism, such as incomplete lineage sorting, a high mutation rate relative to the rate of concerted evolution, and hybridization (Andreasen and Baldwin 2003). We can discuss only the polymorphism observed in the cloned species, because the cause of ambiguous bases in sequences not cloned may be either genuine variation or noise. The five species cloned for this study

H. mussae
H. subsimile
H. maracandicum
H. rubicundum
H. arenarium subsp. *arenarium*
H. armenium
H. oligocephalum
H. artemisioides
H. graveolens
H. arenarium subsp. *aucheri*
H. pallasii
H. thianschanicum
H. lacteum
H. leucocephalum
H. stoechas W
H. serotinum subsp. *serotinum*
H. italicum subsp. *italicum*
H. stoechas E
H. pendulum
H. heldreichii
H. litoreum
H. serotinum subsp. *picardii*
H. italicum subsp. *siculum*
H. italicum subsp. *microphyllum*
H. crassifolium
H. sibthorpii
H. amorginum
H. compactum
H. noeanum
H. orientale
H. doerfleri
H. plicatum
H. chasmolycicum
H. pamphylicum
H. sanguineum
H. taenari

MEDITERRANEAN AREA AND ASIA

H. gossypinum
H. melaleucum
H. monizii
H. devium
H. obconicum
H. alucense

MACARONESIA

H. monogynum
H. makranicum

OMAN-S IRAN

H. populifolium
H. meyeri-johannis
H. formosissimum
H. chionoides
H. argyranthum
H. brownei
H. foetidum
H. setosum
H. reflexum
H. basalticum
H. stuhlmannii
H. trilineatum
H. acutatum
H. odoratissimum
H. epapposum
H. stenopterum
H. forskahlii
H. albobrunneum
H. rugulosum
H. petiolare
H. griseolanatum
H. similinum
H. nudifolium 2
H. nudifolium 1
H. milfordiae
H. zeyheri
H. glomeratum
H. mimetes
H. marginatum
H. platypterum
H. globosum
H. schimperi
H. umbraculigerum
H. splendidum
H. pagophilum
H. confertum
H. kilimanjari
H. chionosphaerum
H. maranguense

AFRICA

H. arwae

YEMEN

H. callicomum
H. asperum
H. spiralepis
H. retortum
H. lambertianum

AFRICA

Anaxeton laeve
H. dasyanthum
Castroviejoa frigida
Castroviejoa montelinasana
Plecostachys serpyllifolia
Syncarpha mucronata

Fig. 4 *Left*, strict consensus tree of the 18,892 equally most parsimonious trees obtained in the parsimony analysis of ETS sequences for the Mediterranean-Asiatic-Macaronesian group (data set 3). *Right*, strict consensus tree of the 160 equally most parsimonious trees obtained in the parsimony analysis of ITS sequences for the same group (data set 2). Bayesian posterior probabilities ≥0.95 are shown above branches, and bootstrap values >50% from the parsimony analyses are shown below branches. Taxon abbreviations: *are = arenarium; auch = aucheri; con = consensus sequence; ital = italicum; micro = microphyllum; picar = picardii; sero = serotinum; sicul = siculum.*

presented some degree of polymorphism, but the cases of *H. crassifolium* and *H. oligocephalum* are the most interesting because both presented many more polymorphic positions than the other three species and because the ETS clones obtained for each species did not form monophyletic groups. We believe that, in both cases, besides the lack of complete concerted evolution, these results probably show hybridization and introgression with other species. In particular, the sequenced specimen of *H. crassifolium* was collected by two of the authors near populations of *H. pendulum* (C. Presl) C. Presl, and putative hybrid specimens between these two species have been reported on the basis of morphology (Galbany-Casals et al. 2006). The comparison of sequences obtained for both species also seems to support our hybridization hypoth-

esis: although the three clones of *H. crassifolium* are invariable in several positions where *H. pendulum* differs, in the positions where the clones are polymorphic there is always one of the multiple nucleotides that coincides with the one that *H. pendulum* has (table 2). The case of *H. oligocephalum* deserves some comments as well. This species overlaps in distribution area with *H. rubicundum* (K. Koch) Bornm. and *H. leucocephalum* in Iran, and the three specimens of these species that we have sequenced came from Iran. The variable sites of *H. rubicundum, H. leucocephalum,* and the two clones of *H. oligocephalum* are compared in table 3. Although definite conclusions cannot be drawn about hybridization, we can appreciate that the clone *H. oligocephalum* 40 is notably similar to *H. rubicundum,* while the clone *H. oligocephalum* 47

Fig. 3 Consensus tree obtained from the Bayesian analysis of ITS sequences (data set 1). Bayesian posterior probabilities ≥0.95 are shown above branches, and bootstrap values >50% from the parsimony analyses are shown below branches.
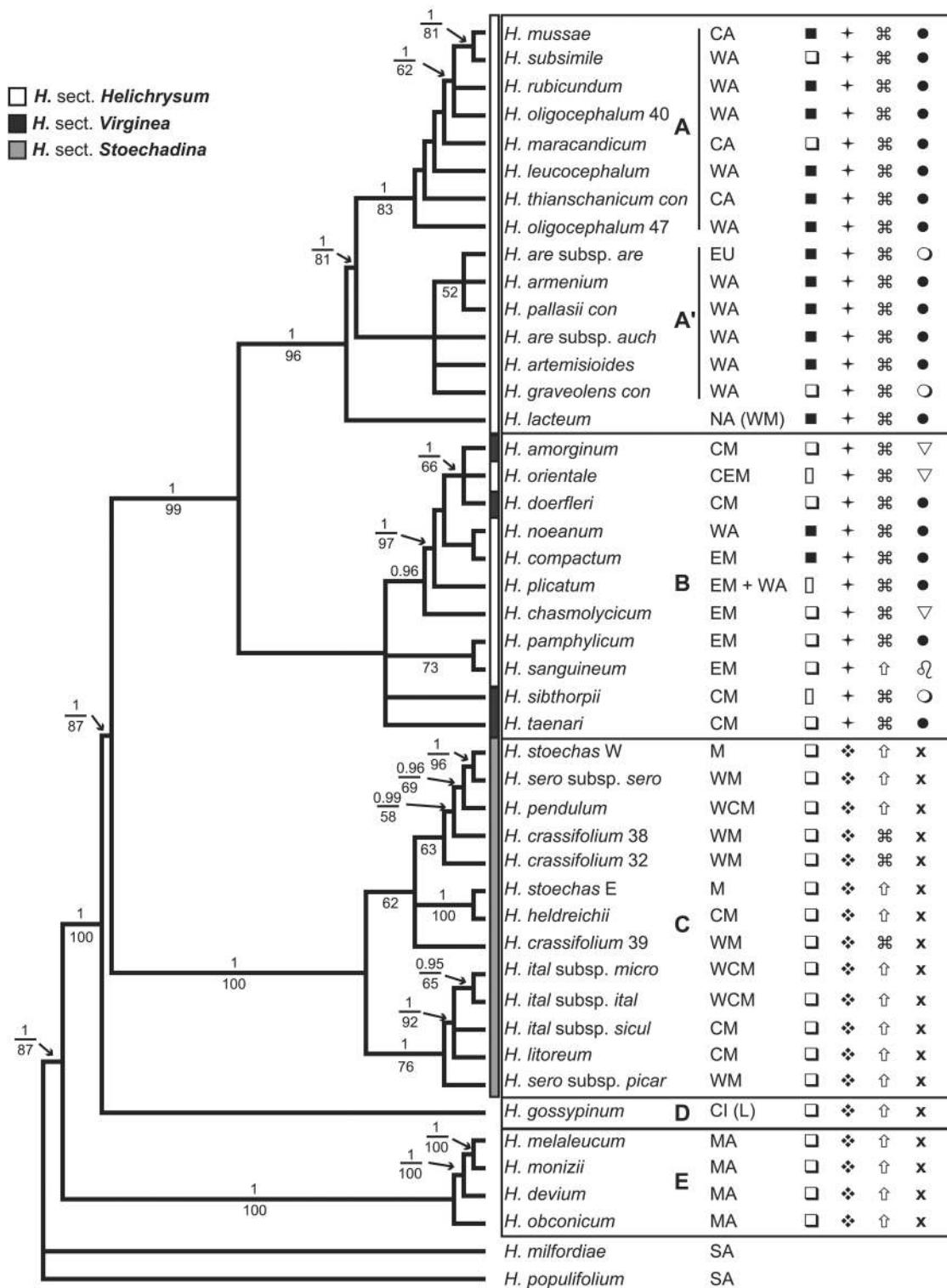
Fig. 5 Consensus tree obtained from the Bayesian analysis of the combined ITS-ETS sequences data set (data set 4). Bayesian posterior probabilities ≥0.95 are shown above branches, and bootstrap values >50% from the parsimony analyses are shown below branches. Region acronyms: *CA* = central Asia; *CI* = Canary Islands; *CEM* = central and eastern Mediterranean; *CM* = central Mediterranean; *EM* = eastern Mediterranean; *EU* = Eurasia; *L* = Lanzarote; *M* = Mediterranean; *MA* = Madeira Islands; *NA* = North Africa; *SA* = southern Africa; *WA* = western Asia; *WCM* = western and central Mediterranean; *WM* = western Mediterranean. Taxon abbreviations: *are* = *arenarium*; *auch* = *aucheri*; *con* = consensus sequence; *ital* = *italicum*; *micro* = *microphyllum*; *picar* = *picardii*; *sero* = *serotinum*; *sicul* = *siculum*. Morphological characters: open square = capitula heterogamous; filled square = capitula homogamous; rectangle = capitula heterogamous or homogamous; diamonds = shrubs or

Table 2

Summary of the Nucleotide Site Variation in ETS Sequences among *Helichrysum pendulum* and the Clones of *H. crassifolium*

	H. pendulum	*H. crassifolium* 39	*H. crassifolium* 38	*H. crassifolium* 32
16	G	G	G	A
42	A	G	A	A
53	G	T	T	T
125	C	C	C	G
167	A	G	A	A
202	C	T	T	T
603	…	T	…	…
661	A	T	A	T
663	C	T	T	T
687	T	T	T	C
765	C	C	T	C
769	G	A	G	G
771	T	C	T	T
783	A	A	A	G
918	A	T	A	A
1106	T	C	T	T

presents some exclusive nucleotides and is more similar to *H. leucocephalum* than to *H. rubicundum*.

Taxonomic Implications

Our ITS phylogeny clearly shows the independence of the two Mediterranean species *Castroviejoa frigida* and *Castroviejoa montelinasana*, traditionally classified under the genus *Helichrysum*, from the Mediterranean *Helichrysum* species and even from the entire genus *Helichrysum*, as they are placed with the outgroup taxa. In addition, our results demonstrate the close relationship between these two species, as they are grouped together with very high support.

Within the Mediterranean and Asiatic *Helichrysum*, a clear monophyletic group was resolved (fig. 5, clade C) that includes all the species recently considered, on the basis of morphological evidence, to constitute sect. *Stoechadina* (Galbany-Casals et al. 2006). They are all shrubs or subshrubs. They never have conspicuous resting buds, stolons, or rhizomes, and only *H. crassifolium* has leaf rosettes. All have heterogamous capitula and are tetraploid (2n=28). However, within this section, some results of the ETS phylogeny show incongruence with the morphology: some taxa of close geographic origin that are morphologically distinct are grouped together. First, the two specimens of *H. stoechas* (L.) Moench do not appear in a monophyletic clade. We think that the specimen *H. stoechas* E, from Crete, could have undergone recent hybridization with *H. heldreichii* Boiss., also from Crete, not detectable from the morphology, and that this would explain why they appear in a highly supported monophyletic clade (ETS: BS = 100%, PP = 1; ETS-ITS: BS = 100%, PP = 1; figs. 4 and 5, respec-

tively). Second, *H. serotinum* (DC.) Boiss. ssp. *serotinum* does not form a group with *H. serotinum* ssp. *picardii* (Boiss. & Reut.) Galbany, L. Sáez & Benedí but does form one with *H. stoechas* W, from Spain (ETS: BS = 86%, PP = 1; ETS-ITS: BS = 96%, PP = 1; figs. 4 and 5, respectively). Again, recent hybridization events may be the cause of our results, because potential hybrids between *H. stoechas* and *H. serotinum* have been reported on the basis of morphology (Galbany-Casals et al. 2006) and because the sequenced specimen of *H. serotinum* ssp. *serotinum* was growing close to populations of *H. stoechas*. Other cases of conflict among morphological and molecular data have been used by authors as a demonstration of reticulate evolution (Fuertes Aguilar et al. 1999; Comes and Abbott 2001; Andreasen and Baldwin 2003; Suárez-Santiago et al. 2007). Gene flow between sympatric species may allow geographical structuring (Fuertes Aguilar et al. 1999; Nieto Feliner et al. 2004), detectable from the similarity or sharing of their ITS and/or ETS sequences.

Species traditionally classified in *H.* sect. *Virginea* were not grouped together in the phylogeny but appeared scattered in the clade of eastern Mediterranean representatives of sect. *Helichrysum* (fig. 5, clade B). On the one hand, the positions of *H. taenari* and *H. sibthorpii* were not resolved in any of the analyses. On the other hand, *H. amorginum* and *H. doerfleri* constituted a moderately supported clade with *H. orientale* (L.) Gaertn. from sect. *Helichrysum* (BS = 66%, PP = 1; fig. 5).

Relationships observed within sect. *Helichrysum* and sect. *Virginea* are correlated with geographic distribution rather than with any infrageneric treatment. Although internal nodes were not strongly supported, we can recognize two main clades, the first comprising species mainly distributed in the eastern Mediterranean region (BS < 50%, PP = 0.82; fig. 5, clade B) and the second comprising species mainly distributed in Asia (BS = 96%, PP = 1; fig. 5, clade A). However, there are two exceptions to this pattern: *H. noeanum* Boiss. is endemic to inner Turkey but appears within the eastern Mediterranean clade, and *H. lacteum* is endemic to northern Africa but appears at the base of the western Asia–central Asia clade with high support (BS = 95%, PP = 1; fig. 5, clades A and A′, respectively). Within the Asiatic clade resolution is poor, and species from western and central Asia are intermixed. However, all the species endemic to central Asia—*H. mussae* Nevski, *H. maracandicum* Popov ex Kirp., and *H. thianschanicum*—are in the same clade (fig. 5, clade A).

Character Evolution

Regarding habit, the species of the Mediterranean sect. *Stoechadina* share with *H. gossypinum* and the four species endemic to Madeira, which are basal, a shrubby habit and the lack of rosettes and overwintering buds (fig. 1*A*). In contrast, species from the eastern Mediterranean basin and western and central Asia are predominantly caespitose-suffruticose, with rosettes and often with conspicuous overwintering buds (fig. 1*B*). Therefore, within the Mediterranean-Asiatic *Helichry-*

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subshrubs, vegetative stems well developed, plants in general without leaf rosettes; plus signs = suffruticose plants, vegetative stems usually short, plants in general with leaf rosettes; arrow = leaf rosettes absent; cloverleaf = leaf rosettes present; cross = resting buds absent; open circle = resting buds not conspicuous; filled circle = resting buds conspicuously swollen; triangle = resting buds not conspicuous or conspicuously swollen; "tadpole" = resting buds subterranean.

Table 3

Summary of the Nucleotide Site Variation in ETS Sequences among *Helichrysum rubicundum, H. leucocephalum,* and the Clones of *H. oligocephalum*

	H. leucocephalum	*H. rubicundum*	*H. oligocephalum* 40	*H. oligocephalum* 47
42	A	A	G	G
53	T	C	T	T
102	A	T	A	A
199	G	T	T	T
203	G	G	G	A
204	K	G	G	T
205	K	T	T	G
206	T	T	T	G
526	T	T	A	T
528	A	T	T	A
533	G	A	A	A
582	A	A	A	G
587	A	A	A	G
591	T	T	C	T
595	T	T	C	T
604	A	A	A	T
608	A	G	A	A
612	T	G	G	T
622	C	A	A	C
647	A	G	G	G
662	T	T	T	C
663	…	…	…	T
684	G	G	G	T
699	A	G	G	G
707	G	G	G	T
713	T	T	C	T
735	A	G	G	G
742	G	G	A	G
751	A	A	A	C
881	…	…	T	T
883	T	T	…	…
910	T	T	C	T
939	C	C	C	T
1032	C	C	C	T
1052	T	T	T	C
1086	G	A	A	G
1094	C	C	C	T
1116	C	G	G	G

Note. Underlining marks positions where *H. oligocephalum* 40 coincides with *H. rubicundum* and *H. oligocephalum* 47 coincides with *H. leucocephalum.*

sum clade, evolutionary trends may have led to a reduction in woodiness that is likely related to a change from a chamaephyte to a hemicryptophyte life form. The presence of rosettes and overwintering buds, conspicuous or not, seems to be related to the latter life form. Moreover, most of these caespitose-suffruticose species with overwintering buds grow in high mountain meadows or steppes in Turkey, the Transcaucasus, Iran, and central Asia, often at altitudes of more than 2000 m. Several authors have reported the predominance of hemicryptophytes in high mountain environments (Körner 1999; Illa et al. 2006). The acquisition of this hemicryptophyte growth form may have permitted adaptation to high-mountain climatic conditions, particularly very cold winters, and radiation and speciation within these habitats.

Another character worthy of comment is the capitulum gender ratio. Regarding this character, the Canary Island endemic *H. gossypinum*, the Madeiran species, and species of sect. *Stoechadina* group share the same state, i.e., heterogamous capitula. Within the eastern Mediterranean and Asiatic groups, species with heterogamous and homogamous capitula occur, although those with the latter predominate in the Asiatic group, while the former prevail in the eastern Mediterranean clade (fig. 5). On the basis of this observation, homogamous capitula may be derived within the Mediterranean-Asiatic *Helichrysum*.

Biogeographic Implications

Southern African origin and expansion throughout the African continent. Our results confirm that *Helichrysum* has a southern African origin, as four southern African species appear in a basal polytomy, sister to the rest of African species plus the Mediterranean-Asiatic clade: *H. retortum, H. lambertianum,* and the monophyletic pair *H. asperum* and *H. spiralepis* (fig. 3). Few other conclusions can be extracted from our results regarding African species, because many African *Helichrysum* appeared in a general polytomy. However, species from tropical Africa (*H. kilimanjari* Oliv., *H. maranguense* O. Hoffm., *H. forskahlii* (J. F. Gmel.) Hilliard & B. L. Burtt, *H. globosum, H. meyeri-johannis, H. schimperi, H. formosissimum, H. stuhlmannii* O. Hoffm., *H. argyranthum* O. Hoffm., *H. chionoides,* and *H. brownei* S. Moore) appear scattered in the phylogeny and do not constitute a monophyletic group, which may indicate that several migration events from southern Africa to tropical Africa have taken place.

Geographic origin and multiple colonization of Macaronesia. Our results show that *Helichrysum* has reached the Macaronesian islands more than once, because Macaronesian species appear in independent clades. As the Canary Islands are oceanic in origin (Carracedo et al. 1998), long-distance dispersal is a prerequisite. Multiple colonizations of volcanic archipelagos are rare because of geographical isolation from continental sources. However, as the Canary Islands are relatively close to the African mainland, an active dispersal route between part of Macaronesia and Africa may exist (Francisco-Ortega et al. 1999). There are several documented examples of multiple colonizations of the Macaronesian archipelago, particularly of the Canary Islands (Francisco-Ortega et al. 1999; Cuénoud et al. 2000; Hess et al. 2000; Park et al. 2001; Fuertes-Aguilar et al. 2002; Percy and Cronk 2002). In the case of *Helichrysum*, the different ancestors of the present species in Macaronesia probably reached the islands through wind dispersal of the tiny (~1 mm), light seeds.

With regard to origin, we have a clade, consisting of the four species endemic to Madeira (fig. 5), that is sister to *H. gossypinum*, endemic to Lanzarote, which in turn is sister to the whole Mediterranean-Asiatic group. These results suggest either (1) that one species colonized Madeira from Africa and originated the four present endemic species and that some founder species was dispersed from this region to the Canary Islands and the Mediterranean area, where it radiated from west to east; (2) that two independent radiations from a

common, probably extinct, ancestor in North Africa may have occurred to Madeira and the Mediterranean basin and that some founder species was dispersed from Madeira to the Canary Islands, where it originated *H. gossypinum*; or (3) that three independent radiations from a common, probably extinct, ancestor in North Africa may have occurred to Madeira, the Canary Islands, and the Mediterranean basin. In fact, several species of sect. *Stoechadina*, such as *H. stoechas*, *H. serotinum*, and *H. pendulum*, are present in Morocco. We think that the second and third scenarios are much more probable than the first one, although some cases of back-dispersal from Macaronesia to the mainland have been reported (*Aeonium*: Mes and Hart 1996; *Convolvulus*: Carine et al. 2004; *Tolpis*: Park et al. 2001); thus, this possibility cannot be ruled out. Some works indicate that the Madeiran endemics have their origin in the Canary Islands (*Pericallis*: Panero et al. 1999; *Crambe*: Francisco-Ortega et al. 2002; *Convolvulus*: Carine et al. 2004; *Genista tenera*: Percy and Cronk 2002; *Sonchus*: Lee et al. 2005; *Bystropogon*: Trusty et al. 2005), while others indicate a different origin (*Hedera*: Vargas et al. 1999a; *Olea*: Hess et al. 2000; *Tolpis*: Park et al. 2001). Regardless of possible evolutionary histories, the fact is that the sister taxon of the Macaronesian-Mediterranean-Asiatic *Helichrysum* remains unknown, although it is clear that it must be of African origin. In general, molecular phylogenies show that Macaronesian groups are derived rather than basal when compared with closely related continental taxa (see numerous examples in Francisco-Ortega et al. 1997; Goodson et al. 2006), although some other exceptions to these results have been reported, for example, the Tenerife endemic *Lavatera phoenicea* Vent., which appears in a basal position of its group (Fuertes-Aguilar et al. 2002), the genus *Argyranthemum*, which appears basal to three other Mediterranean genera (Francisco-Ortega et al. 1997), and *Tolpis azorica*, basal to a clade that includes two continental species and two other island endemics (Park et al. 2001).

Furthermore, our results place the other two species from the Canary Islands in a different lineage, indicating two different introductions into these islands. *Helichrysum alucense*, endemic to La Gomera, and *H. monogynum*, endemic to Lanzarote, form a monophyletic group sister to *H. makranicum*, which grows in Oman and southern Iran; *H. populifolium*, from South Africa, is sister to this three-species clade (fig. 3). This finding could support the phytogeographical links between eastern Africa–southern Arabia and the Macaronesian islands hypothesized by Quézel (1978). The close relationships between Macaronesian and eastern African–Arabian taxa were considered evidence of a continuous flora present in northern Africa and the Mediterranean region in the late Miocene (Axelrod 1975; Quézel 1978; Sunding 1979). Many elements of this hypothetical continuous flora disappeared from several areas of North Africa after major climatic changes during the late Tertiary and the Quaternary (Axelrod 1975; Quézel 1978). Thus, in the case of *Helichrysum*, a common, presumably extinct, ancestor of *H. makranicum*, *H. alucense*, and *H. monogynum* may have had a more widespread distribution and/or reached the western part of northern Africa from eastern Africa through the Sahara region before desertification; from there it probably colonized the Canary Islands. The length of phylogram branches (not shown) suggests that the divergence in these two

lineages must be quite ancient. Present-day *Helichrysum* species from the Canary Islands are not associated with sclerophyllous-lauroid flora but with open shrubby or subshrubby communities. Their ancestor may have had similar ecological preferences, in which case its area expansion through northwestern Africa would likely have been along open patches within the lauroid forests. Alternatively, colonizers of the Canary Islands could have evolved their present ecological preferences as an adaptation to the island environment. Whereas some close relationships between Macaronesian and eastern African–southern Arabian taxa have been demonstrated through molecular phylogenetic studies, such as the cases of *Aeonium* Webb & Berthel. (Mes and Hart 1996), *Euphorbia* (Molero et al. 2002; Bruyns et al. 2006), and *Solanum* (Bohs and Olmstead 2001), others have been rejected, such as *Adenocarpus* DC. (Percy and Cronk 2002), *Plocama* (Andersson and Rova 1999), and *Prenanthes* (Kim et al. 1996), among others (reviewed by Andrus et al. [2004]).

Interisland colonization and diversification in Macaronesia. A point of interest for studies of island biogeography is how species diversify within an insular environment. As explained above, *H. monogynum* is endemic to Lanzarote, and *H. alucense* is endemic to La Gomera; thus, the most parsimonious hypothesis is that a common ancestor of the two first reached Lanzarote, which is closer to the African coast, and later colonized La Gomera. These two species then evolved independently because of their isolation. Both *H. monogynum* and *H. alucense* grow on the lowland scrub of their respective islands. Similar patterns of allopatric speciation via interisland colonization across similar ecological zones have been reported for several groups: *Lotus* (Allan et al. 2004), *Pericallis* (Panero et al. 1999), *Sideritis* (Barber et al. 2002, 2007), *Sonchus* and *Gonospermum* (Francisco-Ortega et al. 2001), and *Bystropogon* (Trusty et al. 2005), among others. However, in all these genera, radiation into different ecological zones, allowing for differentiation within a single island, has also been a significant speciation mechanism in these insular environments. In the case of the four endemic Madeiran *Helichrysum*, which have a single origin, speciation events should be explained by diversification within a single island, which could have been favored by radiation into different, isolated geographic zones within the island rather than by ecological shifts, because the four species occupy similar ecological zones, mainly sea cliffs. However, at present the only species that is quite isolated is *H. monizii*, for which a unique and very small population is known in the south of Madeira. The other three species do overlap in distribution area in some localities. In particular, *H. melaleucum* is widely distributed, found from sea cliffs to the higher parts of the Madeira, Porto Santo, and Deserta islands (R. Jardim, Madeira Botanical Garden, Funchal, Madeira, Portugal, personal communication).

The origin and expansion of the Mediterranean-Asiatic group. All analyses showed that the Mediterranean-Asiatic *Helichrysum* is a strongly supported monophyletic group, and the ITS-ETS combined data showed *H. gossypinum* as its sister taxon with 87% BS value. It seems likely that an ancestor of the whole Mediterranean-Asiatic group reached the Mediterranean basin, where the group expanded and diversified, occupying the whole basin. Species of sect. *Stoechadina*, with a predominantly western-central Mediterranean distribution, share numerous

morphological characters with the Macaronesian species, which are sister to the Mediterranean-Asiatic group.

Our results suggest that the expansion through the Mediterranean was followed by colonization of the western and central Asiatic regions, likely related to the reduction in woodiness mentioned above, which may have permitted an adaptation to the high-mountain climate of these areas. Speciation appears to have been recent in the western and central Asiatic regions, evidenced by the scarce morphological and molecular divergence of the group, and it was probably affected by numerous hybridization events, as hybridization is still common within many of these species (M. Galbany-Casals and L. Sáez, personal observation). Moreover, the low levels of support for the internal nodes of a tree have sometimes been interpreted as a sign of reticulate evolution within a group of species (McDade 1992; Wendel and Doyle 1998; Font et al. 2002; Andreasen and Baldwin 2003; Fuertes Aguilar and Nieto Feliner 2003), because concerted evolution of the sequences in a hybrid results in their homogenization and therefore the loss of informative characters (Suárez-Santiago et al. 2007).

Helichrysum lacteum, which grows in the mountains of Morocco and Algeria, is a hemicryptophyte and appears as the basal species in the Asiatic clade (BS = 95%, PP = 1; fig. 5). This finding indicates a secondary colonization of the southwestern Mediterranean area from western Asia or the eastern Mediterranean region. The ancestor of *H. lacteum* could have reached the North African mountains during times of land connections in the Mediterranean, either during the Messinian crisis (Bocquet et al. 1978) or during Pleistocene glacial phases, when the sea level was also low (Quézel 1981), and then become isolated in several mountain areas. However, a long-distance dispersal event cannot be ruled out as a possible explanation for the presence of *H. lacteum* in the North African mountains. Further dispersion-vicariance and time estimation analyses would be useful to confirm one of these hypotheses.

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