

**PHYLOGENY OF THE MACARONESIAN ENDEMIC *CRAMBE*
SECTION *DENDROCRAMBE* (BRASSICACEAE) BASED ON
INTERNAL TRANSCRIBED SPACER SEQUENCES OF
NUCLEAR RIBOSOMAL DNA¹**

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The 14 species of *Crambe* L. sect. *Dendrocrambe* DC. (Brassicaceae) form a monophyletic group endemic to the Canary and Madeira archipelagos. Both parsimony and maximum likelihood analyses of sequence data from the two internal transcribed spacer regions of nuclear ribosomal DNA were used to estimate phylogenetic relationships within this section. These analyses support the monophyly of three major clades. No clade is restricted to a single island, and therefore it appears that inter-island colonization has been the main avenue for speciation in these two archipelagos. The two species endemic to Fuerteventura (*C. sventenii*) and Madeira (*C. fruticosa*) comprise a clade, providing the first evidence for a floristic link between the Eastern Canary Islands and the archipelago of Madeira. Both maximum likelihood and weighted parsimony analyses show that this clade is sister to the two other clades, although bootstrap support for this relationship is weak. Parsimony optimizations of ecological zones and island distribution suggest a colonization route from the low-altitude areas of the lowland scrub toward the high-elevation areas of the laurel and pine forests. In addition, Tenerife is likely the ancestral island for species endemic to the five westernmost islands of Gran Canaria, La Gomera, El Hierro, La Palma, and Tenerife.

Key words: adaptive radiation; angiosperms; biodiversity; biogeography; *Crambe*; evolution; Macaronesia; molecular systematics; oceanic islands; speciation.

With approximately 40 species, *Crambe* L. (Brassicaceae) is the only large (>10 species) monophyletic genus of the tribe Brassiceae (Francisco-Ortega et al., 1999). A phylogenetic analysis of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA) showed that *Crambe* comprises three major monophyletic groups (Francisco-Ortega et al., 1999). One of these groups, *Crambe* sect. *Dendrocrambe* DC., has 14 species (Santos-Guerra, 1996; Prina, 2000) and is endemic to the Macaronesian archipelagos of the Canary Islands (13 species) and Madeira (1 species). Each of the Canary Islands has at least one endemic species from this section, with the exception of Lanzarote where no species of *Crambe* occur (Fig. 1). The only Madeiran species, *C. fruticosa*, also occurs on the islands of Desertas and Porto Santo. Another species that occurs on more than one island is *C. strigosa*, an endemic

of Tenerife and La Gomera. Reports of *C. santosii* on La Gomera and Tenerife and of *C. strigosa* on La Palma (Carquero-Alamo et al., 1997; Prina, 2000) need further investigation.

Three of the endemic species are restricted to the laurel forest (*Pruno-Lauretea azoricae*) (Rodríguez-Delgado et al., 1998; Fig. 1). This vegetation zone is found on northern slopes of the islands, which are under the direct influence of the humid and cool northeastern trade winds, and receive up to 1000 mm of rainfall per year (Fernández-Palacios, 1999). Traditionally, the laurel forest in Macaronesia has been considered a relict of an ecological zone that existed in the Mediterranean basin during the Miocene (Quézel, 1995; Nakamura et al., 2000). Fossil data from southern Europe support this hypothesis (reviewed by Sunding, 1979). However, recent phylogenetic studies indicate that some endemics in the laurel forest have a post-Tertiary origin and cannot be considered directly linked to the Miocene vegetation of southern Europe (Carvalho and Culham, 1998; Thiv, Struwe, and Kadereit, 1999). A post-Tertiary origin for species of the laurel forest has been reported primarily for plants in the understory; few studies have included canopy trees and most of the canopy species do not show major radiations in Macaronesia.

Nine species of *Crambe* sect. *Dendrocrambe* are restricted to the lowland scrub (*Oleo-Rhamnetalia crenulatae*) (Fig. 1). This vegetation zone is situated above the coastal xerophytic belt (*Kleinio-Euphorbietalia canariensis*) and receives up to 550 mm of rainfall per year (Fernández-Palacios, 1999). The two species in the pine forest (*Chamaecytiso-Pinetalia can-*

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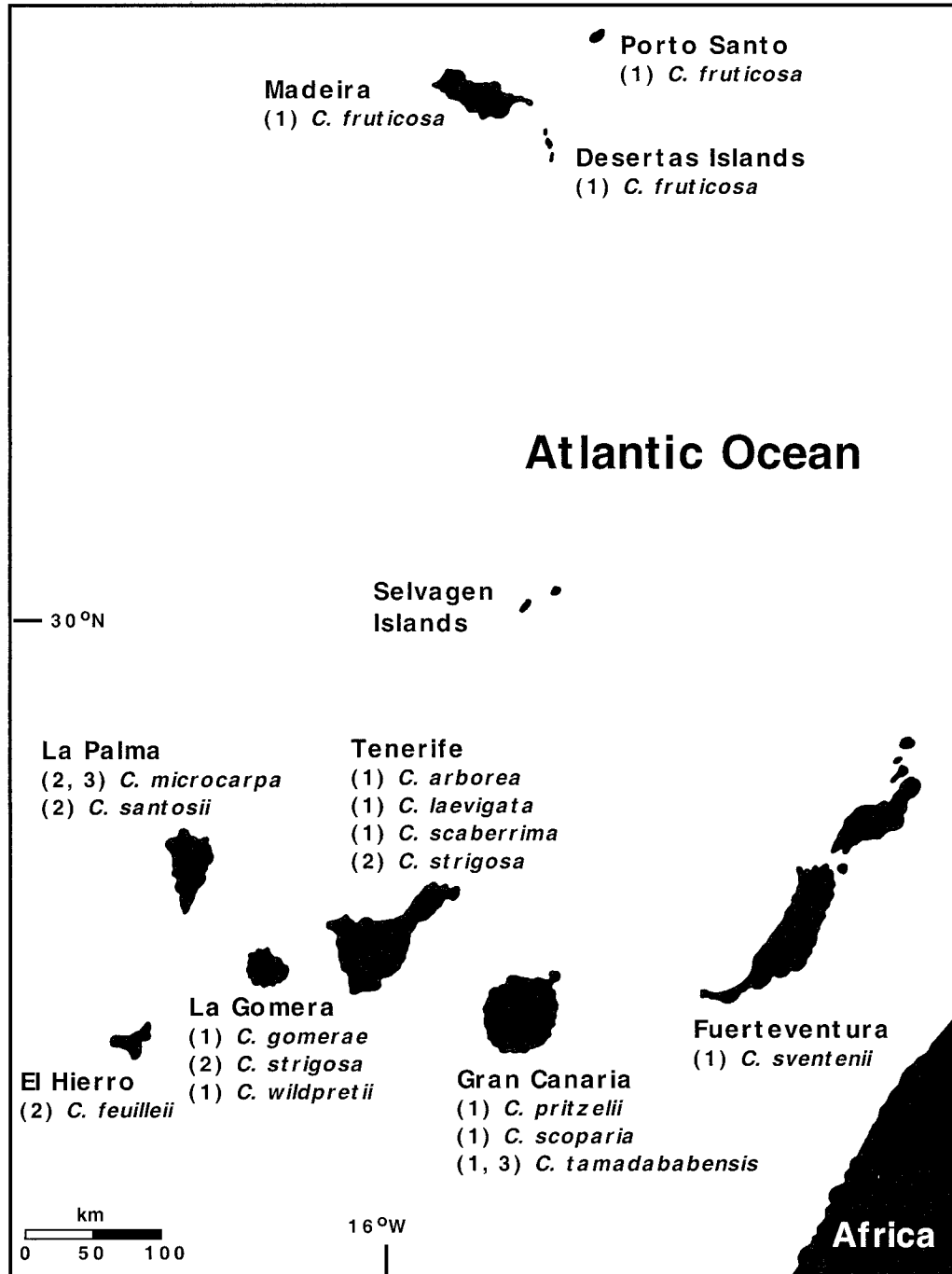


Fig. 1. The Canary, Selvagen, and Madeira archipelagos and *Crambe* species found on each island. The habitats of the species are also indicated and are coded as follows: (1) lowland scrub, (2) laurel forest, (3) pine forest.

ariensis) are also found in other ecological zones. This type of pine forest is restricted to the Canary Islands and is situated above the laurel forest (on northern slopes of the islands) or above the lowland scrub (on southern slopes). The only islands without pine forest are Fuerteventura and Lanzarote. Rainfall in this forest ranges between 400 and 800 mm per year.

The recently described *C. tamadababensis* from Gran Canaria is found both in the pine forest and in the lowland scrub (Prina and Marrero-Rodríguez, 2000). Likewise *C. microcarpa* from La Palma can be found in both the pine forest and the

laurel forest zones of this island. Endemic species of *Crambe* do not occur in the coastal xerophytic belt or in the high altitude scrub (*Spartocytision supranubii*). The direction of evolutionary change in habitat preference in sect. *Dendrocrambe* is ambiguous because species are present in three of the major ecological zones of Macaronesia.

In this paper, we present a phylogenetic study of the species of sect. *Dendrocrambe* based on nucleotide sequences of the ITS regions of nuclear rDNA. Previous studies have shown that in many cases the ITS can provide enough phylogenetic

signal to estimate phylogenies of island groups (Baldwin et al., 1998). The main objectives of this research were to clarify the phylogenetic relationships of the Macaronesian species of *Crambe* and to use the molecular phylogeny to understand the biogeographical, ecological, and morphological patterns of variation in this group.

MATERIALS AND METHODS

Taxon sampling, DNA isolation, polymerase chain reaction procedures, and DNA sequencing—All species of *Crambe* sect. *Dendrocrambe* were included in our analysis. For nine species, multiple accessions were included to sample the entire geographical range of the section (<http://ajbsupp.botany.org/v89/>). The outgroup included three continental species of sections *Leptocrambe* DC. (*C. kralikii* and *C. kilimandscharica*) and *Orientecrambe* I. Khalilov (*C. orientalis*). These were chosen based on a previous ITS phylogeny of the entire genus and related genera in the Brassicaceae (Francisco-Ortega et al., 1999).

DNA isolation, polymerase chain reaction (PCR) amplification, and direct sequencing of the ITS region for most species followed protocols used in Francisco-Ortega et al. (1999). Sequences of *C. tamadababensis* and *C. wildpretii* were obtained following the protocols of Fuertes-Aguilar, Rossello, and Nieto-Feliner (1999). Accession data are given in <http://ajbsupp.botany.org/v89/>.

Phylogenetic analyses—Sequences were easily aligned manually. All phylogenetic analyses were performed using version 4.0b5 of PAUP* (Swofford, 1999). Maximum parsimony (MP) and maximum likelihood (ML) methods were used to reconstruct phylogenies. Due to computer memory limitations, the ML analyses only included one sequence from each of the ingroup species (the reduced data set of 17 species included in this analysis are marked with an asterisk in the table found at <http://ajbsupp.botany.org/v89/>). In contrast, maximum parsimony analyses included sequences from all 37 samples, although the reduced data set of the 17 species was used for parsimony optimization of biogeographical ecological features (see below).

In maximum parsimony analyses, nucleotide changes were weighted. Transversions were weighted over transitions by a 2 : 1 ratio based on the ML analysis (see below). Heuristic searches for most parsimonious trees were performed with 100 random entries using the ACCTRAN, MULTREES, and TBR options. Informative gaps were coded as binary characters (present or absent) and added to the original data matrix using the "simple indel coding" method for recoding of gaps (Simmons and Ochoterena, 2000). The consistency index (CI; Kluge and Farris, 1969) and the retention index (RI; Farris, 1989) were also calculated. One hundred bootstrap replicates (Felsenstein, 1985) were performed using a heuristic search with one random taxon entry.

The HKY85 model of DNA sequence evolution (Hasegawa, Kishino, and Yano, 1985) with among-site variation approximating a gamma distribution was used in the ML analyses, and gaps were treated as missing data. The ML search involved estimation of parameters (i.e., transition/transversion ratios, proportion of invariant sites, base frequencies, and shape of the gamma distribution). Support for monophyletic groups was evaluated with 500 bootstrap replicates (Felsenstein, 1985) using a heuristic search with ten random entries. Parameter values obtained from the initial ML search (see above) were used for the bootstrap analysis. Due to memory limitations, the number of trees saved in each bootstrap replicate was limited to a maximum of 500.

Character optimizations were performed using parsimony for three sets of characters using MacClade (Maddison and Maddison, 2000): (1) ecology (with three states, laurel forest, lowland scrub, and pine forest); (2) insular distribution (with seven states, Fuerteventura, La Gomera, Gran Canaria, El Hierro, Madeira/Porto Santo, La Palma, and Tenerife); and (3) archipelago distribution (with two states, Canaries and Madeira). Character optimizations were mapped onto the two most parsimonious trees obtained after weighted parsimony analysis with the reduced data set of 17 species. Character states for ecological and island data of the outgroup taxa were coded as "0," and they were different from the states assigned to the island endemics.

Nucleotide sequence divergence values based on the HKY85 model of

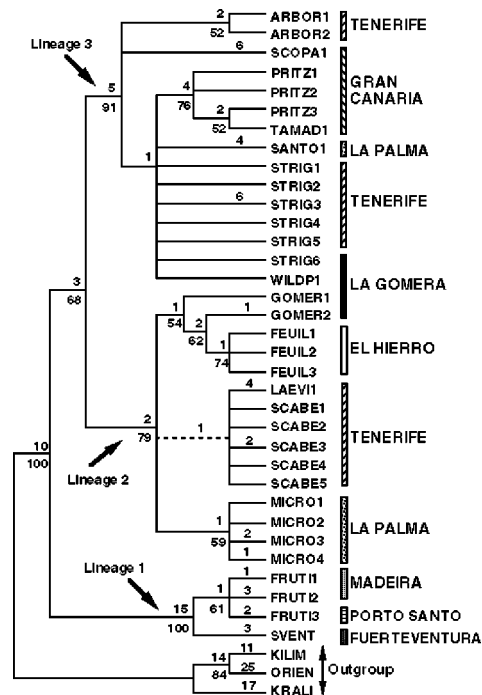


Fig. 2. One of the six most parsimonious ITS trees from the maximum parsimony analyses (transversions were weighted over transitions by a 2 : 1 ratio, number of steps = 112, CI = 0.835, excluding autapomorphies; RI = 0.940). The dashed line indicates the branch that collapses in the strict consensus tree. Numbers above and below each branch indicate the number of changes and bootstrap values (higher than 50%), respectively. Vertical bars at tips of branches indicate island distribution of Canarian endemics. The three major lineages are also indicated.

DNA sequence evolution were also estimated. A pairwise distance matrix was computed using the parameters estimated from the ML analysis (see below).

RESULTS

Length variation and sequence divergence—Aligned ITS sequences are 458 base pairs (bp) long (272 bp for ITS1 and 186 for ITS2). The longest and shortest sequences are 453 bp (SCAB5) and 448 bp (SANTO1) within sect. *Dendrocrambe*. There are 19 gaps after alignment, none of which is longer than 2 bp. Three of the gaps are parsimony informative, however only one of these occurs in sect. *Dendrocrambe*.

The two sequences of *C. arborea* are identical as are the three sequences of *C. feuilleii*. Other sets of identical sequences include two sequences of *C. microcarpa* (MICRO1 and MICRO2), four sequences of *C. scaberrima* (SCAB1, SCAB2, SCAB4, and SCAB5), and four sequences of *C. strigosa-C. wildpretii* (STRIG2, STRIG4, STRIG5, WILDP1). The two most divergent sequences of insular taxa are between the Madeira species *C. fruticosa* (FRUTI3) and the Gran Canarian endemic *C. scoparia* (6.50% nucleotide sequence divergence).

Phylogenetic analyses—There are 91 variable sites, with 53% (48) of these being parsimony informative. Weighted parsimony analysis (transversions were weighted over transitions by a 2 : 1 ratio) yields six trees with 112 steps, a CI of 0.835 (excluding autapomorphies), and an RI of 0.940. One of the six most parsimonious trees and the strict consensus tree from this analysis are shown in Fig. 2. Weighted parsimony analyses

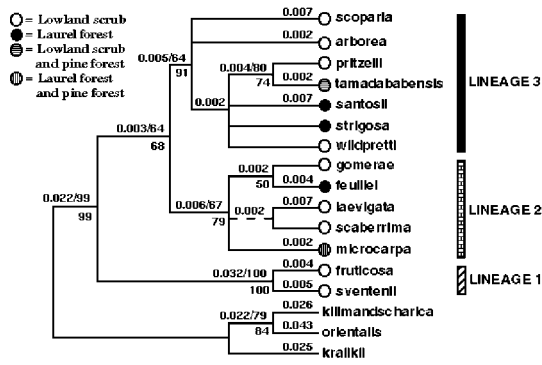


Fig. 3. Single tree obtained from the maximum likelihood analysis ($-\ln = 1217.51871$) and one of the two most parsimonious trees obtained after maximum parsimony analysis of the reduced data set of 17 species. Transversions were weighted over transitions by a 2 : 1 ratio for maximum parsimony analysis. Branch lengths followed by bootstrap values (higher than 50%) obtained after the maximum likelihood analysis are shown above the branches. Bootstrap support values (higher than 50%) obtained after the maximum parsimony analysis are shown below each branch.

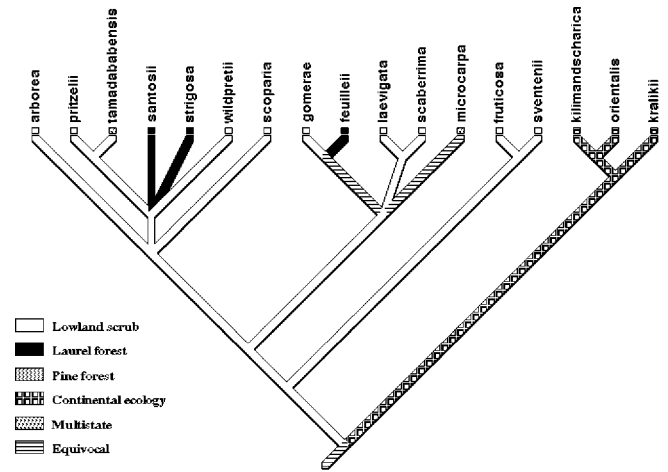


Fig. 4. Optimization of ancestral states for ecological data on one of the two most parsimonious trees obtained after maximum parsimony analysis of the reduced data set of 17 species.

with transversions weighted over transitions with weights of 1.1, 1.5, 2.1, and 2.5 also yield the same six tree topologies.

The strict consensus tree supports three major clades of *Crambe* in Macaronesia. The first (lineage 1, with 100% bootstrap support) includes the taxa from Madeira and Fuerteventura. Both weighted parsimony and ML (see below) analyses support this clade as sister to the rest of island species of *Crambe*. The second clade (lineage 2, with 79% bootstrap support) includes five species (*C. feuillei*, *C. gomerae*, *C. laevigata*, *C. microcarpa*, and *C. scaberrima*). This lineage includes three clades, two of which comprise two species. The third clade (lineage 3) has 91% bootstrap support and includes the seven remaining species. Five of these species (*C. pritzelii*, *C. santosii*, *C. strigosa*, *C. tamadababensis*, and *C. wildpretii*) form a monophyletic group. This clade of five species together with *C. arborea* and *C. scoparia* forms an unresolved trichotomy. All taxa from lineage 3 share a single base deletion in ITS2. Lineages 2 and 3 form a monophyletic group with 68% bootstrap support.

The ML analysis of 17 species yields one tree ($-\ln = 1217.51871$). Estimated base frequencies are A = 0.2427, C = 0.2511, G = 0.2456, T = 0.2606. Other estimated parameters are: transition/transversion ratio = 2.0 ($\kappa = 4.1110$); proportion of invariable sites = 0.1326; and value of gamma shape parameter = 0.649206. The ML tree is identical to one of the two trees generated by weighted parsimony analyses of 17 species (Fig. 3). The ML tree agrees with the weighted parsimony tree in placing the two taxa of lineage 1 (from Madeira and Fuerteventura) sister to the rest of the insular species. Bootstrap support for this relationship is weak in both the ML (64%) and the weighted parsimony analysis (68%).

Optimization of biogeographic and ecological data—The topologies from the two most parsimonious trees of the weighted parsimony analyses of the reduced data set of 17 species were used for the optimization of ecological and biogeographic data. Three characters—archipelago distribution, island distribution, and ecology—were optimized on these two trees using the parsimony method implemented in MacClade (Maddison and Maddison, 2000).

We show character optimizations for only one of the most

parsimonious trees (Figs. 4–5). The other most parsimonious tree showed identical optimization results for these ecological and biogeographic data. The most parsimonious character optimizations for archipelago distribution, island distribution, and ecology require two, ten, and four steps, respectively (Figs. 4–5). The character reconstructions suggest that ancestral lineages of *Crambe* originated in the lowland scrub of the Canary Islands, followed by colonization of the laurel forest and pine forests of both the Canary and Madeira archipelagos. Tenerife is the most likely ancestral area of the lineages 2 and 3, and the Canary Islands probably represent the ancestral archipelago.

Overall, there is a poor correlation between the topology of the ITS tree and island distribution/ecology (Figs. 2–3). Species endemic to the laurel forest occur in both lineages 2 and 3. Likewise, species from Tenerife, La Palma, and La Gomera are found in both of these clades (Fig. 2). The three species from Gran Canaria are part of lineage 3, however they do not form a monophyletic group. The only species endemic to El Hierro is sister to *C. gomerae* in lineage 2, although this relationship is only weakly supported (bootstrap value of 54% in Fig. 2).

DISCUSSION

Ecological shifts—There has been considerable debate concerning the origin of plants endemic to the Macaronesian laurel forest (e.g., Axelrod, 1975; Santos-Guerra, 1990; Thiv, Struwe, and Kadereit, 1999). Many phylogeographers consider most laurel forest endemics to be relicts of a flora that was prevalent in the Mediterranean in the Pliocene and early Pleistocene. The ITS phylogeny of *Crambe* provides two insights into the origin of laurel forest species: (1) there have been two independent colonization events into this forest; and (2) patterns of ecological diversification are from lower elevation zones (lowland scrub) to higher elevations zones (laurel forest and pine forest).

Multiple colonizations of the laurel forest by congeneric species have been reported from the *Aeonium* Webb & Berthel. alliance (Crassulaceae) (Mort et al., 2002), *Hedera* L. (Araliaceae) (Vargas et al., 1999), *Pericallis* D. Don (Asteraceae) (Panero et al., 1999), *Sideritis* L. (Lamiaceae) (Barber et al.,

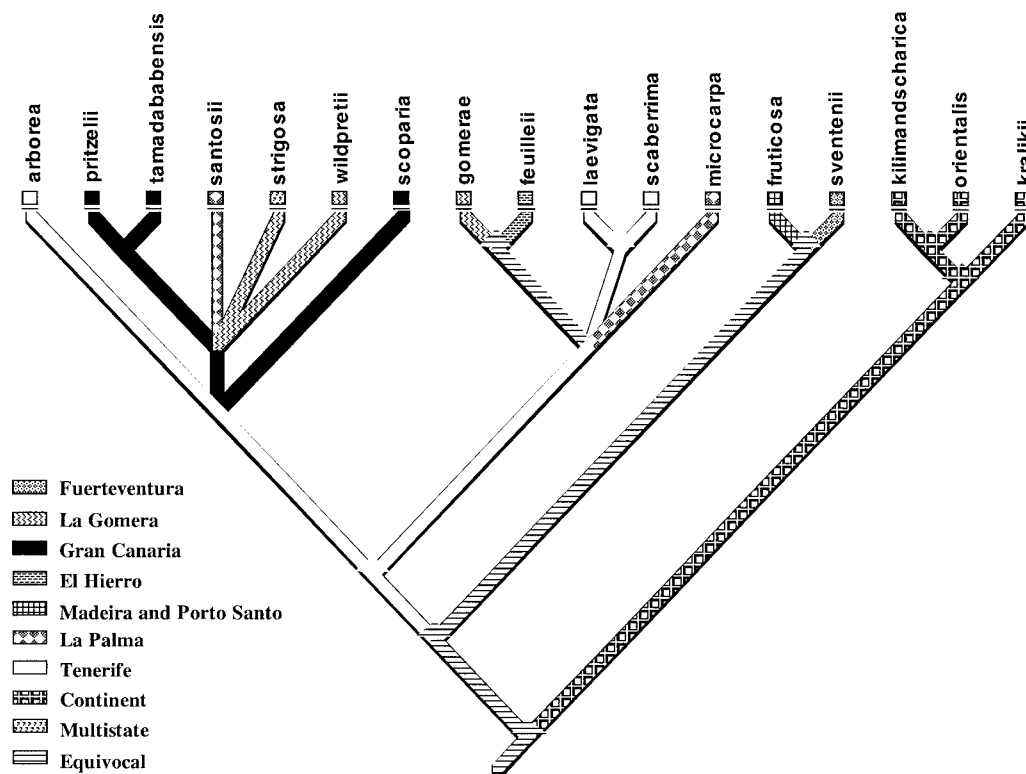


Fig. 5. Optimization of ancestral states for island distribution on one of the two most parsimonious trees obtained from maximum parsimony analysis of the reduced data set of 17 species.

2000), and *Tolpis* Adans. (Asteraceae) (Moore et al., 2002). In most of these examples, taxa from the laurel forest are in a derived position, which suggests a relatively recent origin. The ITS tree for *Crambe* also indicates a recent colonization of the laurel forest. The most obvious case of recent colonization involves *C. feuilleii*, an endemic from El Hierro in lineage 2 (Fig. 3). El Hierro is the youngest island of the Canary archipelago. The oldest subaerial rocks of this island are 1.12 million years old (Guillou et al., 1996), indicating a post-Tertiary origin for species of *Crambe* endemic to the laurel forest of this island. The two other species of *Crambe* restricted to the laurel forest (*C. santosii* and *C. strigosa*, both in lineage 3) also appear to be derived based on the ITS data (Figs. 2–3).

Biogeographic relationships—The ITS phylogeny supports a floristic connection between the eastern Canary Island of Fuerteventura and the Madeiran archipelago. A multivariate analysis of the Macaronesian flora suggests that the Canary and Madeira archipelagos form a distinct cluster (Nicolas et al., 1989; La-Roche and Rodríguez-Piñero, 1994). Previous phylogenetic studies of several plant genera suggest that the floras of the Canary Islands and Madeira are linked through the five western Canary Islands of El Hierro, Gran Canaria, La Gomera, La Palma, and Tenerife (e.g., Panero et al., 1999; Mort et al., 2002). The sister relationship between *C. sventenii* from Fuerteventura and *C. fruticososa* from Madeira provides the first phylogenetic evidence of a biogeographic connection between the eastern Canary Islands and Madeira. The two genera *Crepis* and *Helichrysum* in the Asteraceae may provide additional evidence of a connection between Madeira and the

eastern Canary Islands because they are restricted primarily to these islands (Hansen and Sunding, 1993; García-Casanova, Scholz, and Hernández, 1995).

The ITS tree supports Tenerife as the ancestral island for the common ancestor of all the species in the five westernmost islands of Gran Canaria, Tenerife, La Gomera, La Palma, and El Hierro. Tenerife is the largest and most ecologically diverse of the Macaronesian Islands. This island is approximately 12 million year old (Ancochea et al., 1990), and it is situated between the islands of Gran Canaria and Fuerteventura and the islands of La Gomera, El Hierro, and La Palma (Fig. 1). Previous phylogenetic studies of Macaronesian plants have not focused on identifying ancestral areas of dispersal. However, the age, large size, central location, and extraordinary ecological diversity of Tenerife suggest that this island may have played an important role as a center of dispersal in the Canary Islands.

Optimization of archipelago distribution on the ITS tree supports the Canary Islands as the ancestral archipelago. Thus, colonization in *Crambe* apparently occurred from the Canary Islands to Madeira. Phylogenetic evidence for the *Sonchus* L. alliance (Asteraceae) (Kim et al., 1996) and *Pericallis* (Panero et al., 1999) also support a dispersal route from the Canaries to Madeira.

The ITS phylogeny, unfortunately, does not resolve the issue of the direction of colonization between the eastern and the western Canaries and whether the island of Tenerife or Fuerteventura can be regarded as the ancestral island for the Macaronesian species of *Crambe*.

Taxonomy and morphology—A number of previous workers have discussed the morphology of this genus (Sventenius,

1953; Bramwell, 1969a, b, 1973; Prina, 2000). The ITS phylogeny has some implications for the taxonomy of the Macaronesian species and for our understanding of patterns of morphological variation.

Crambe scoparia is one of the most morphologically distinctive species of sect. *Dendrocrambe*. It is the only species that sheds its leaves in summer and has fruits with a relatively long distal beak. The leaves also have a unique combination of features, including a glabrous and chartaceous texture, a reddish color, and few lobes. Sventenius (1953) used these features to support the recognition of the distinct sect. *Rhipocrambe*. The ITS phylogeny does not support the segregation of *C. scoparia* at the sectional level because this species is nested within lineage 3 (Fig. 2).

There is a good correspondence between the monophyletic groups in the ITS tree and morphology. The Madeiran and Fuerteventuran species of lineage 1 share a combination of unique morphological features, including laterally compressed fruits and small, glabrous, and glaucous leaves with sinuate margins. These two species differ primarily in their fruit morphology with those of *C. sventenii* having two lateral wings, whereas the wings are either missing or greatly reduced in *C. fruticosa*.

Morphological similarities among species are also apparent in lineages 2 and 3. With the exception of *C. wildpretii* (Prina and Bramwell, 2000), all five species in the largest clade of lineage 3 share several morphological features, including large, wide, pubescent leaves and lax, highly branched inflorescences with long peduncles. The five species of lineage 2 are similar in having small, rhomboid leaves with a hard texture and compact inflorescences with short peduncles.

The strong concordance between patterns of morphological variation and the groups present in the ITS tree of *Crambe* is rarely seen in other Macaronesian groups. Previous molecular phylogenies of *Argyranthemum* Sch. Bip. (Asteraceae) (Francisco-Ortega, Jansen, and Santos-Guerra, 1996), the *Aeonium* alliance (Mort et al., 2002), the *Gonospermum* Less. alliance (Asteraceae) (Francisco-Ortega et al., 2001), *Pericallis* (Panero et al., 1999), and *Sideritis* (Barber et al., 2000) demonstrated that most of the morphologically defined sections in these genera are either paraphyletic or polyphyletic. The most extraordinary example of incongruence between morphology and molecular phylogenies is in *Pericallis*. It has been claimed that woodiness in this genus is a pleisomorphic character state (Nordenstam, 1978; Serrada et al., 1988). A recent molecular study indicates that woodiness is derived and that it originated multiple times (Panero et al., 1999).

Conclusions—Our ITS phylogeny demonstrates that the Macaronesian species of *Crambe* have experienced multiple inter-island colonization events and that radiation of this group has involved several ecological shifts. Similar ecogeographic patterns have been detected for the majority of the endemics of these islands (i.e., the *Aeonium* alliance [Mort et al., 2002], *Sideritis* [Barber et al., 2000], and the *Sonchus* alliance [Kim et al., 1996]), although in some cases, inter-island colonization among similar ecological zones has also been important (i.e., *Argyranthemum*; Francisco-Ortega, Jansen, and Santos-Guerra, 1996; Francisco-Ortega et al., 2001). Thus, *Crambe* follows the same phytogeographic patterns detected in many other Macaronesian endemics.

Crambe differs from other Macaronesian taxa because of the agreement between morphologically defined groups and

the molecular phylogeny. Our results seem to indicate that hybridization has not played a major role in the evolutionary history of the Macaronesian species of *Crambe*. The common occurrence of natural hybrids in *Argyranthemum* and *Sideritis* has been used as an argument to explain the poor correlation between molecular phylogenies and the morphological groupings in these two genera (Francisco-Ortega et al., 1996; Barber et al., 2000; Brochmann, Borgen, and Stabbetorp, 2000).

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