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Evolution of Mexican *Bursera* (Burseraceae) inferred from ITS, ETS, and 5S nuclear ribosomal DNA sequences

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Abstract

I reconstructed a phylogeny of 66 species and varieties of *Bursera* and 9 outgroup species using sequences of the internal transcribed spacer region (ITS), the 5S non-transcribed region (5S-NTS), and the external transcribed region (ETS) of nuclear ribosomal DNA. This study extends a previously proposed parsimony-based phylogenetic study that used the ITS sequences of 57 *Bursera* species and five outgroups. Parsimony and maximum likelihood methods were used to infer the phylogeny in this new study. Analyses of the combined data sets largely confirmed the phylogenetic relationships proposed by the previous molecular study but generated a considerably more robust topology. The new phylogenies corroborate the monophyly of the genus, and its division into the two monophyletic subgenera or sections, *Bursera* and *Bullockia*. The current analyses also identify four main groups of species in section *Bursera*, and two in section *Bullockia*, confirming some of the previously proposed groups based on fruit, flower, and leaf morphology. One previously problematic species *B. sarcopoda*, which has sometimes been placed in *Commiphora*, is shown to belong in *Bursera*. Another controversial species, *Commiphora leptophloeos*, which was thought to belong to *Bursera*, falls within *Commiphora*. © 2002 Elsevier Science (USA). All rights reserved.

1. Introduction

Bursera, the past and current source of copal and incense of Aztec and Maya Indians comprises between 90 and 100 tree and shrub species distributed from Peru to the South of United States. Its main center of diversification is Mexico, with about 85 species, of which 75 are endemic. There, the genus is one of the most abundant and diversified components of the tropical dry forests and it is also conspicuous in the central and northwest deserts (Miranda, 1947; Rzedowski, 1978).

Bursera species are highly resinous and release a characteristic pine-lemony odor when a leaf or branch is broken. The smell is due to highly volatile terpene compounds such as α -pinene, β -phelandrene, limonene, carene, etc. (Becerra et al., 2001; Evans et al., 2000). Many species have succulent trunks and some display a spectacular, brightly colored bark that exfoliates in colorful papery flakes or sheets. This last trait is responsible for the Aztec name “cuajote” or leprous tree that is still applied to some of these plants (Rzedowski

and Kruse, 1979). The genus is relatively well known taxonomically, and it has been divided into two sections that are distinguished by the characteristics of the bark, among other traits. Section *Bursera* includes species with colorful trunks and peeling bark, while species in section *Bullockia* have rough, non-peeling bark (Bullock, 1936; McVaugh and Rzedowski, 1965; Rzedowski and Kruse, 1979; Toledo, 1982; Daly, 1993).

Bursera has also been the subject of several ecological, chemical, and evolutionary studies; (Becerra, 1994, 1997; Becerra et al., 2001; Becerra and Venable, 1999a,b; Evans et al., 2000). These plants have a long history of interaction with the herbivorous beetle genus *Blepharida* and their phylogenetic relationships, as well as the insects' ones have been central for testing coevolutionary ideas (Becerra, 1997; Hillis, 1997; Mitchell-Olds and Bergelson, 2000).

Several hypotheses have been advanced to identify the phylogenetic relationships among the individual species (Becerra and Venable, 1999a; McVaugh and Rzedowski, 1965; Rzedowski and Kruse, 1979). The most recent was reconstructed under the assumption of maximum parsimony using the nucleotide sites of the

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internal transcribed spacer regions (ITS1 and ITS2) of 18S–26S and the 5.8S coding region of nuclear ribosomal DNA (Becerra and Venable, 1999a) and included 54 species and five outgroups.

This first molecular phylogeny shed light on the relationships of the main lineages, and also on the relationships of some previously problematic species. It suggested that *Bursera* is monophyletic and closely related to the genus *Commiphora*. Previously, it had been suggested that the genus was diphyletic with one section related to *Commiphora* and the other related to the allied genus *Boswellia*. This molecular investigation also supported the traditional division of the genus into two subgenera or sections, and distinguished some of the subclades that had been hypothesized earlier. Also, the distinctive morphology of some of the species, such as *B. paradoxa*, *B. mirandae*, *B. tecomaca*, and *B. schlechtendalli* had made it previously difficult to determine their affinities. The molecular analyses provided insight as to their phylogenetic relationships. One drawback of this molecular analysis was low support (estimated by nonparametric bootstrapping) for many individual clades. The level of DNA divergence was particularly low among species of the *Bullockia* section, resulting in poorly supported groupings.

Lack of sufficient nucleotide variation has been a common problem for discerning phylogenetic relationships among species for many plant groups (Baldwin and Markos, 1998). The most widely used source of molecular data for plant phylogenetic studies at the species level is the ITS region (Baldwin, 1992). But, as was the case for *Bursera* (Becerra and Venable, 1999a,b), these two spacers often have too few variable nucleotide sites to provide robust inferences at all nodes. The external transcribed spacer (ETS) has been shown to be useful to supplement ITS data for plant phylogenetic studies (Baldwin and Markos, 1998; Bena et al., 1998; Linder et al., 2000). However, is not as easy to sequence as the ITS. It requires the initial amplification of the whole intergenic spacer, often longer than 4 Kb, from at least two divergent taxa. These sequences are then used to design internal primers for amplification of a selected region of ETS. Recently, it has also been reported that sequences from the 5S non-transcribed region (5S-NTS) provide further useful nucleotide variation (Persson, 2000).

In the present study, I extend the ITS phylogenetic analysis of Becerra and Venable (1999a) to include 66 *Bursera* species and varieties and 9 outgroup species. I also supplement the nuclear ribosomal DNA database of species of section *Bursera* and outgroups to include partial sequences from the ETS region and, for the species in the *Bullockia* section, sequences of the 5S non-transcribed region. One of the goals was to assess the robustness of some of the previously defined clades that had relatively weak bootstrap support. I also wanted to determine the relationships of some species, such as

B. sarcopoda and *Commiphora leptophloeos*, whose affiliation to *Bursera* or *Commiphora* has been controversial. The results, which are analyzed both with maximum parsimony and maximum likelihood, largely corroborate the phylogenetic relationships described in Becerra and Venable (1999a) but with greater support for most clades.

2. Materials and methods

2.1. Plant samples and DNA extraction

The samples used by Becerra and Venable (1999a,b) were also used in the present study. The additional species included in this investigation were collected by J. Becerra and D.L. Venable directly from natural field populations, excepting *C. leptophloeos* and *Boswellia sacra* whose samples were donated by private collectors (Table 1). I am including *B. ruticola*, a recently described species (Pérez-Navarro, 2001), and a variety of *B. epinata* from El Tecolote Beach, Baja CA, which is very distinctive morphologically and has sometimes been confused with *B. cerasifolia* (Jose Luis León de la Luz and José Juan Pérez-Navarro, pers. comm.). DNA of fresh or silica-gel dried samples was extracted using the Dneasy Plant Mini Kit (Qiagen).

2.2. Amplification, sequencing, and sequence alignment

ETS. The whole intergenic spacer was amplified using the 18S-IGS and 26S-IGS primers and protocol described by Baldwin and Markos (1998) for *B. simaruba*, and *B. penicillata*. This procedure yielded a product of between 4 and 5 Kb. Approximately 650 bases from this product were sequenced using the 18S-IGS primer. A conserved region located about 500 bases away from the ETS/18S boundary was selected to design a primer, BUR1 (GGG CGT GTG AGT GGT GTT). This region was then amplified for all of the species using BUR1 and 18S-IGS as primers. PCR conditions for these amplifications followed the short-distance PCR protocol of Baldwin and Markos (1998), with an annealing temperature of 52° and Platinum Taq (Life Technologies).

5S-NTS. I used the primers designed by Cox et al. (1992). PCR conditions also followed short-distance protocol for ETS amplifications of Baldwin and Markos (1998) using Platinum Taq and 52° for primer annealing.

ITS. The ITS1, ITS2 regions, and the 5.8S gene were amplified using the primers C26A and N-nc18s10 (Wen and Zimmer, 1996). PCRs were the same as in Becerra and Venable (1999a) with the implementation of a “touchdown” procedure with an initial annealing temperature of 56 °C, then a decrease of 2 °C every two cycles until 48 °C, at which 30 amplification cycles were conducted.

Table 1
Species of *Bursera* and related taxa added in the present analysis

Species	Collection site	Distribution	Voucher specimen*
<i>Bursera</i>			
Section <i>Bursera</i>			
Mulatos group			
<i>B. cinerea</i> Engl.	Sierra de Huautla, Morelos, Mex.	Morelos, Veracruz	648
Red cuajotes group			
<i>B. medranoana</i> Rzedowski and Ortiz	Barranca Tolantongo, Hidalgo, Mex.	Narrow endemic, Barranca de Tolantongo	654
<i>B. rzedowski</i> Toledo	Chilpancingo, Acapulco, Guerrero, Mex.	Narrow endemic, East side of the Balsas depression	671
Yellow cuajotes group			
<i>B. bolivarii</i> Rzedowski	Xochipala, Guerrero, Mex.	Narrow endemic, Zopilote Canyon	649
<i>B. odorata</i> Brandege	La Paz, Baja California, Mex.	Narrow endemic, Cape region	701
Section <i>Bullockia</i>			
Copales with fruit partially covered by the pseudoaril			
<i>B. cerasifolia</i> Brandege	Sierra de La Laguna, Baja California, Mex.	Narrow endemic, Cape region	734
<i>B. citronella</i> McVaugh and Rzedowski	Colima, Colima, Mex	Pacific Coast, Colima to Guerrero	642
<i>B. epinnata</i> (Rose) Engler	La Paz, Baja California, Mex.	Baja California Sur.	745
<i>B. epinnata</i> var <i>El tecolote</i> (Rose) Engler	Playa de El tecolote, Baja California, Mex	Narrow endemic, Cape region	712
<i>B. sarcopoda</i> P.G. Wilson	Colima, Colima, Mex	Pacific Coast, Colima to Guerrero	635
<i>B. ruticola</i> Leon and Perez-Navarro	Rancho Leon, La Paz, Baja California, Mex	Narrow endemic, Sierra Cacachilas	707
<i>Commiphora</i>			
<i>C. africana</i> (Rich) Engl.	Messina, Northern Province, South Africa	Southeast Africa	1003
<i>C. leptophloeos</i> (Mart) Gillett	Private collection, Boris Vrskovy	Brazil, Bolivia	1010
<i>C. mollis</i> (Oliver) Engl.	Messina, Northern Province, South Africa	Southeast Africa	1023
<i>Boswellia</i>			
<i>B. sacra</i> Flueck	Private collection, Jason Eslamieh	Arabia	

* Specimens are deposited at the University Herbarium (ARIZ). Accession numbers are Becerra and Venable.

PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced in both directions. Sequencing reactions used the same primers as for amplification and were conducted on an ABI automated sequencer at the DNA sequencing facility of the Biotechnology Division of the University of Arizona.

Sequences were edited and assembled using the program Sequencher 4.1 for Macintosh (Gene Codes). ITS sequences were aligned with the PILEUP program of GCG version (University of Wisconsin Group) using the default options. ETS and 5S sequences were aligned using Sequencher 4.1. Correction of the last small misalignments was performed with MacClade 4 (Maddison and Maddison, 2000). Indels of equal length that occurred in more than one sequence were added to the sequence data matrix as binary characters with three-fourths the weight of the nucleotide characters.

2.3. Phylogenetic analyses

Phylogenetic analyses were done on using PAUP 4.08 (Altivec; Swofford, 2000) and PAUP PPS (Swofford, 2000) on two G3 and two G4 Power Macintosh com-

puters. I chose maximum parsimony and maximum likelihood methods to infer the phylogenetic relationships among species. Parsimony analyses are fast and often consistent, but may yield the wrong phylogeny if long branches are separated by short branches (Felsenstein, 1978; Maddison et al., 1999). Maximum likelihood methods tend to be consistent and robust when their assumptions are met, but calculations are often time-consuming.

Parsimony analysis. The data were analyzed as tree separate data sets (ITS, ETS, and 5S) and also as a single, combined data set. Before combining, a partition homogeneity test was implemented to determine whether the ITS and the ETS data sets (for species of section *Bursera* and outgroups) were congruent. Another partition homogeneity test was used to test for the congruency between the ITS and 5S data sets (for species of section *Bullockia*). For both tests, 100 replicates were performed, each with 10 random addition sequence replicates and TBR branch swapping. These tests did not detect significant incongruence between the ITS and ETS data sets ($P = 0.48$) nor between the ITS and the 5S data sets ($P = 0.34$). For each of the separate data

Table 2

–ln Likelihoods of the most parsimonious tree under 16 different evolution models, relative to the most parameter model for (A) the ITS and ETS data matrices of species of the *Bursera* section and (B) the ITS and 5S data matrices of species of the *Bullockia* section

Site-to-site rate variation model	ITS				ETS			
	JC69	F81	HKY85	GTR	JC69	F81	HKY85	GTR
(A) <i>Bursera</i> section								
Equal rates	269	185	143	142	137	106	63	59
%I	89	59	21	16	78	47	7	5
Gamma	65	33	6	5	75	44	7	2
%I + gamma	55	32	5	0	73	42	3	0
(B) <i>Bullockia</i> section								
Equal rates	112	95	87	77	27	25	15	10
%I	39	24	14	8	21	19	4	1
Gamma	41	29	17	14	22	20	6	2
%I + gamma	36	13	6	0	20	19	3	0

Values shown are the increase in –ln L with respect to the GTR + %I + gamma model.

matrices as well as the combined data set, heuristic searches were conducted using 100 random-taxon-addition replicates, tree bisection reconnection (TBR) branch swapping, and the MULPARS option in effect. To estimate the support of individual clades, I implemented bootstrap analysis, which involved 250 bootstrap searches with 20 replicates of random addition, TBR branch swapping and MULPARS in effect.

Analyses excluding putative hybrid species. Several *Bursera* are believed to have originated by hybridization. These include: *B. medranoana*, believed to be a hybrid between *B. morelensis* and *B. schlechtendalii* (Rzedowski and Ortiz, 1988); *B. fagaroides* var. *elongata*, a putative hybrid between *B. fagaroides* var. *fagaroides* (McVaugh and Rzedowski, 1965) and *B. microphylla*; and *B. diversifolia*, which in the case of my sample is presumed hybrid between *B. copallifera* and *B. bipinnata* (Toledo, 1982). Because of a concern that they may affect the general phylogenetic arrangement of groups that involve their parental species, another heuristic search based on parsimony was performed excluding these three putative hybrid species.

Maximum likelihood. To infer whether the modes of evolution of the 5S, ITS, and ETS regions were similar, I examined several models of nucleotide substitution for each of the data sets. These models which differ in complexity and number of parameters were the JC69 (“Jukes–Cantor 1969”), F81 (“Felsenstein 1981”), HKY85 (“Hasegawa–Kishino–Yano 1985”), and GTR (general time reversible). The first two models include one parameter in the rate matrix, while the third model uses two, and the fourth model involves six (Swofford et al., 1996). To decrease calculation times, the ITS data set was divided into two corresponding to the species in the two sections and only *C. africana* was included as outgroup. This seemed reasonable since the division of the genus into these two sections has never been controversial (McVaugh and Rzedowski, 1965), and also because this division was strongly supported with pre-

vious ITS analysis. Since estimation of model parameters is fairly insensitive to tree topology (Yang et al., 1995), estimations were performed using the most parsimonious tree.

A likelihood-ratio test (Sullivan and Swofford, 1997; Yang et al., 1995) determined that the HKY85 + gamma was the model that fit the data best (considering its number of parameters) for both the ITS and ETS sequences of species in section *Bursera*. For the ITS and 5S sequences of section *Bullockia* the HKY85 + %I fit the data best, followed very closely by the HKY85 + gamma model. The difference in log likelihood between these two models for section *Bullockia* is insignificant. (Table 2). Therefore, the HKY85 + gamma model was chosen to search for maximum likelihood trees for the whole genus. This was done by invoking this model in PAUP with the three data sets combined, with parameters estimated on the most parsimonious tree (ti/tv ratio = 0.99; empirical nucleotide frequencies $A = 0.2326$, $C = 0.2774$, $G = 0.2747$, $T = 0.2152$; gamma distribution of rates at variable sites; α -shape parameter = 0.396; rate categories = 4). The heuristic search was performed using SIMPLE addition, TBR branch swapping, and MULPARS option in effect.

Analyses excluding putative hybrid species. Another series of maximum likelihood analyses using the same model and parameters as the previous heuristic search was done in which *B. diversifolia*, *B. medranoana*, and *B. fagaroides* var. *elongata* were excluded one at a time.

3. Results

Alignment of the ETS sequences of section *Bursera* and outgroups resulted in a matrix of 449 characters, of which 11 were gaps and 124 (28%) were phylogenetically informative under maximum parsimony. The 5S matrix (for species in section *Bullockia*) included 257 characters. Of these, four of them were gaps and 64 (25%) were

informative. The ITS matrix (for species in both sections and outgroups) included 814 nucleotide sites plus 63 gaps. Of these sites, 268 (33%) were phylogenetically informative, but most of them were in section *Bursera* and outgroup species (235, 29%). Only 60 informative sites (7%) were for species of section *Bullockia* (GenBank accession numbers for ETS: AF445804 throw AF445882; for ITS AF445883 throw: AF445958; for 5S: AF445959 throw AF445992).

Parsimony. Analysis of the ITS data matrix resulted in 12 equally parsimonious trees of 1184 steps (Fig. 1A, CI=0.46, RI=0.73), while the 5S analysis yielded 9 trees of 215 steps (Fig. 1B, CI=0.75, RI=0.78). Analysis of the ETS data set generated 288 trees of 461 steps (Fig. 1C, CI=0.70, RI=0.77). As in previous analysis (Becerra and Venable, 1999a), the ITS bootstrap values suggested more support for clades in section *Bursera* and outgroups, than clades of section

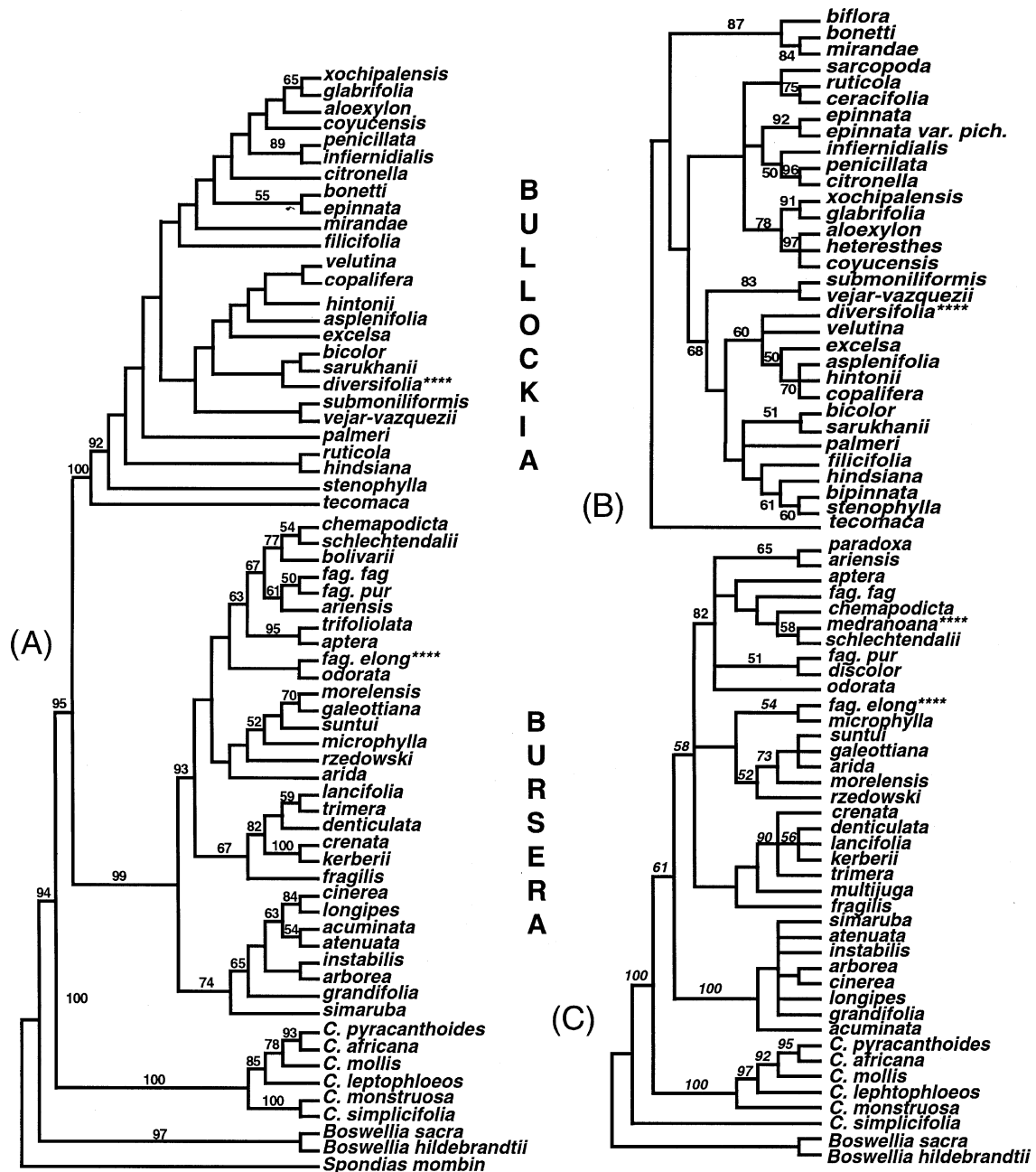


Fig. 1. Results of parsimony analyses of uncombined data sets. (A) Consensus tree of 12 equally most parsimonious trees generated by analysis of the ITS sequences. (B) Consensus tree of nine most parsimonious trees resulting from analysis of 5S sequences. (C) Consensus tree of 288 most parsimonious trees yielded by the ETS sequences. Numbers above branches are bootstrap percentage values for clades supported above a 50% bootstrap value. Putative hybrid species are indicated with ****.

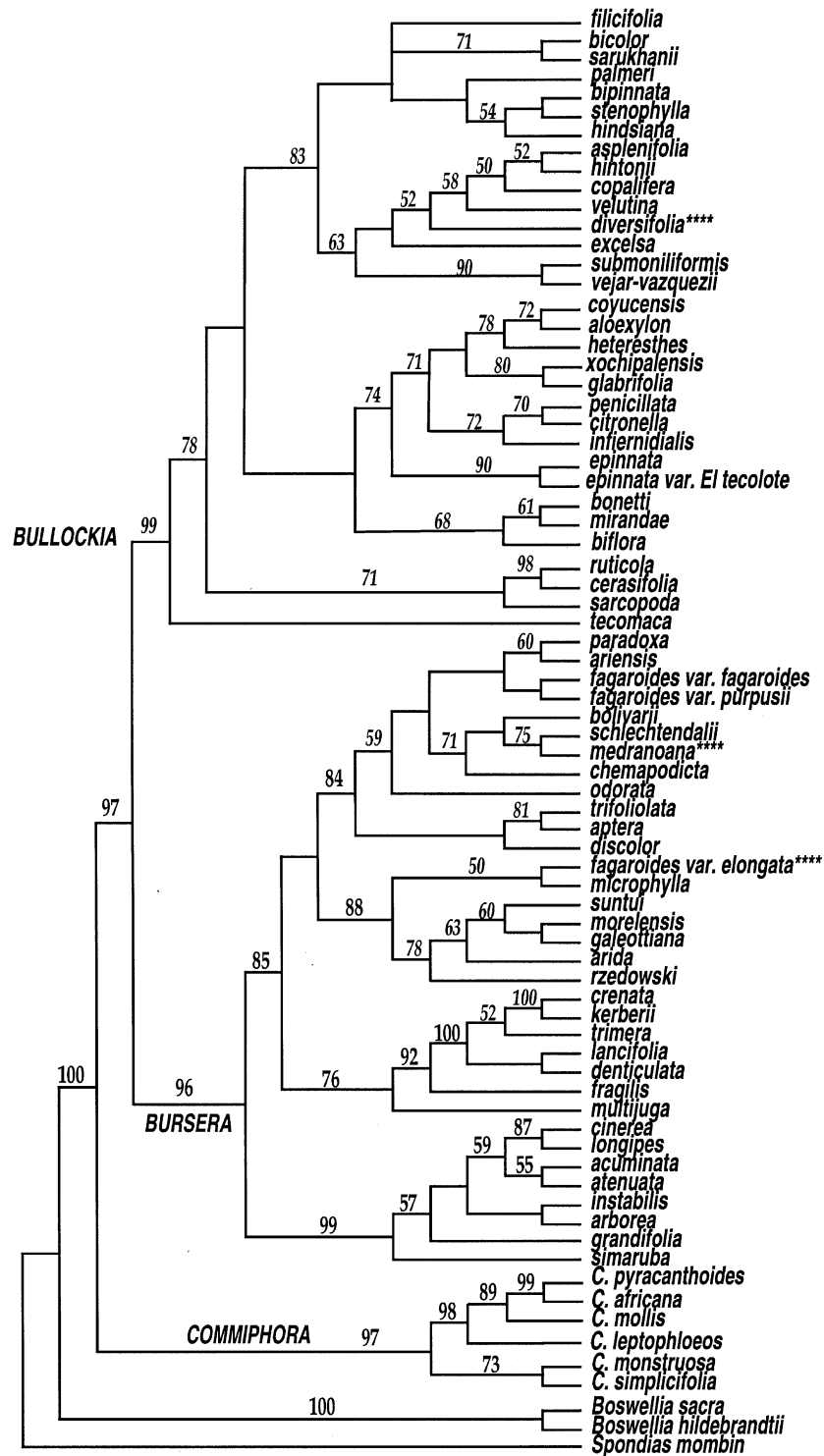


Fig. 2. Consensus tree of two most parsimonious trees resulting from analysis of the combined ITS, ETS, and 5S sequences. Numbers above branches are bootstrap percentage values for clades supported above a 50% bootstrap value. Numbers above branches are bootstrap percentage values for clades supported above a 50% bootstrap value. Putative hybrid species are indicated with ****.

Bullockia. Only 5 (18%) of the clades in section *Bullockia* had a bootstrap support of 50% or higher, while 64% of the clades in section *Bursera* did. The 5S bootstrap analyses yielded higher values for section

Bullockia and 56% of the individual clades had a bootstrap value of 50% or higher. Thirty-five percent of the individual clades analyzed with ETS had a support of 50% or higher.

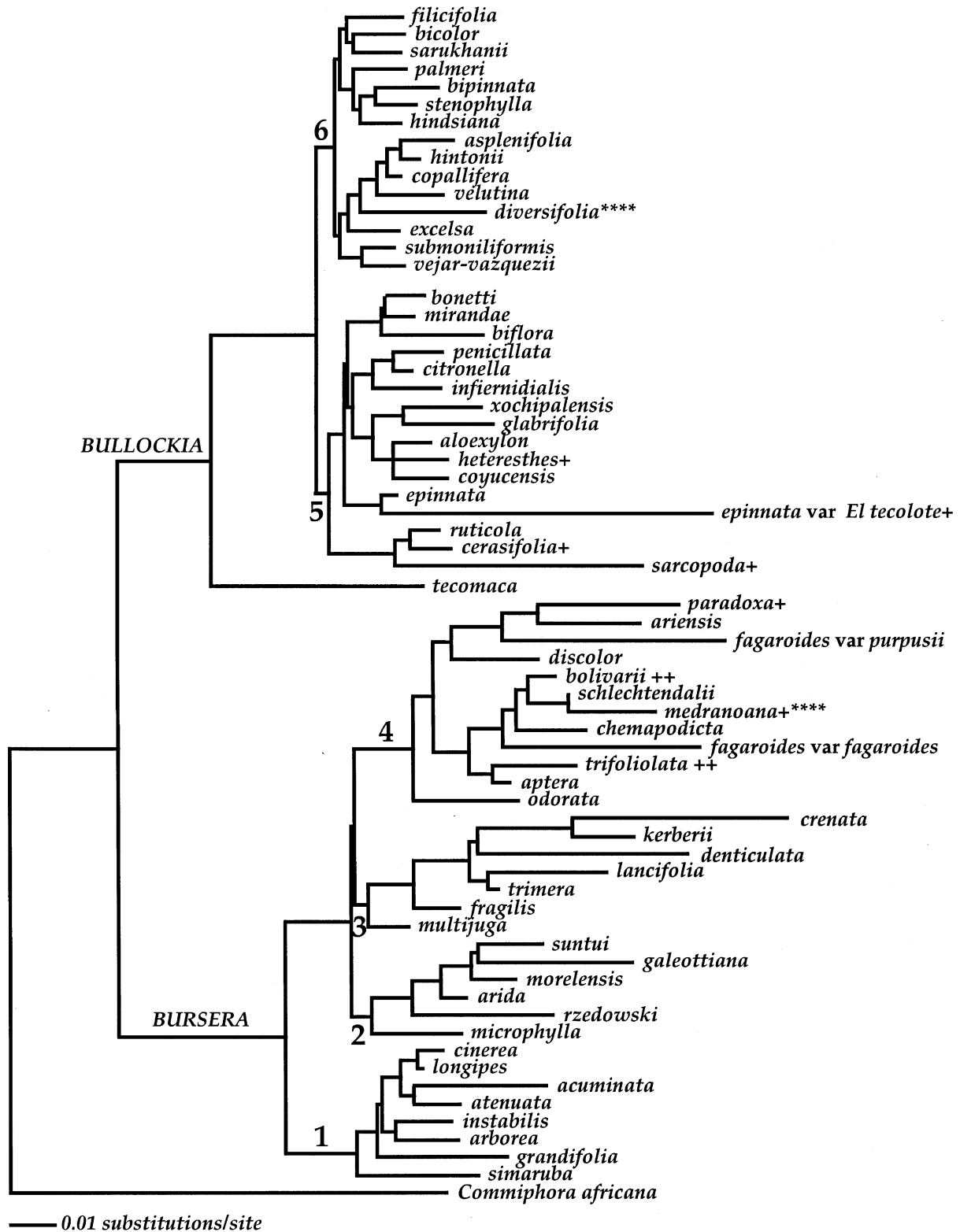


Fig. 3. Phylogeny of *Bursera* based on maximum likelihood. Tree of highest likelihood found under the HKY85+gamma model. Lengths of branches are proportional to the number of substitutions inferred but are comparable only among species from the same section. Also, some species were not analyzed with ITS (+), or with ETS (++) . Numbers indicate proposed subdivisions of section *Bursera*: (1) Simaruba group; (2) Microphylla group; (3) Fragilis group; (4) Fagaroides group; and subdivisions of section *Bullockia*: (5) Glabrifolia group; (6) Copallifera group (see text for details).

Phylogenetic analyses of the three regions combined yielded 2 most-parsimonious trees of 2235 steps (Fig. 2, CI = 0.57, RI = 0.73). When the putative hybrid species were excluded from the analyses, the searches generated more trees. Nevertheless, the consensus tree was identical to the consensus tree with the hybrid species included. Bootstrap support was overall higher when combining the three data sets. Seventy-nine percent of the individual clades had 50% or higher bootstrap support, and 54% of clades had 70% or higher bootstrap support.

Maximum likelihood. The heuristic search yielded one single tree with a score of $-\ln = 9784.5539$. Excluding the supposed hybrid species did not change the number of trees found, but it changed the topology of the tree lightly. When the analysis included *B. fagaroides* var. *elongata*, the clade containing *B. odorata* and the clade containing *B. microphylla* are sister groups. However, when *B. fagaroides* var. *elongata* was excluded, the clade of *B. multijuga* becomes the sister group to the clade of *B. odorata*. This tree had a score of $-\ln = 9734.089$ (Fig. 3). This reconstruction was very robust to changes in the values of parameters of the HKY85 model. The position of species and clades was the same either in separated analyses of species of section *Bullockia* and species of section *Bursera* or in joint analyses involving species of the two sections (see Fig. 3).

4. Discussion

In the current analyses, parsimony and maximum likelihood produced very similar results. The main differences were in the position of some clades in section *Bullockia* for which variation continues to be too low for parsimony analyses to give robust results, and in the placement of some species found at the terminus of long branches. Thus, differences in tree topology may be due to well-known weaknesses of the maximum parsimony method (Swofford et al., 1996). Maximum likelihood was robust to small model variations and generated identical tree topologies when section *Bullockia* and section *Bursera* were analyzed separately, each with its own parameter values for the HKY85 model, or in a combined analysis assuming a single set of parameters for the whole genus. This method also always produced the same groups as parsimony for clades that had 50% or greater bootstrap support. The positions of clades and species in the tree with highest likelihood are more consistent with previous suggestions based on morphology than groupings resulting from the parsimony analysis (see below).

Relationships among major lineages. The consensus tree of the maximally parsimonious reconstructions and the tree of highest likelihood of this study are, in general, very similar to the ITS phylogeny of Becerra and Venable (1999a). They all support the hypothesis that *Bursera*

is monophyletic and more closely related to *Commiphora* than to *Boswellia*. Controversy exists as to whether or not the South American species *C. leptophloeos* belongs in *Bursera* (Gillet, 1980). In this study this species clearly falls into *Commiphora* and favors the view that the distribution of *Bursera* does not go beyond the north of South America (Gillet, 1980). Also, because the pollen of *B. sarcopoda* and *B. tecomaca* are very different to that of other *Bursera* species, but share many similarities to the pollen of *Commiphora* species, it was previously proposed that these two species should be transferred to *Commiphora* (Rzedowski and Palacios, 1985). The present study confirms the suggestion by Becerra and Venable (1999a) that the transfer of *B. tecomaca* is not warranted and provides the first molecular evidence that *B. sarcopoda* also belongs in *Bursera*. My analyses also confirm the division of the genus into two groups corresponding to the two sections, *Bursera* and *Bullockia*, as well as the monophyly of these sections.

Subdivisions of section *Bursera*. The present analyses strongly support the division of section *Bursera* into four major subclades (Figs. 2 and 3). Becerra and Venable (1999a) found the same groups and suggested some of the following morphological differences:

1. *Simaruba* group. Often massive trees, trilobate cotyledons, red exfoliating bark, leaflets with entire margins and apices mostly acuminate, plants tend to produce less resin than plants in other groups of this section (Clade 1 in Fig. 3).
2. *Microphylla* group. Multilobate cotyledons, red exfoliating bark, leaflets with entire margins, small linear leaflets (except unifoliate *B. rzedowski*), medium-size trees and shrubs, highly resinous (Clade 2 in Fig. 3).
3. *Fragilis* group. Multilobate cotyledons, red exfoliating bark, leaflets with serrate or crenate leaflets that are mostly larger than in 2, often medium-size trees, highly resinous (Clade 3 in Fig. 3).
4. *Fagaroides* group. Multilobate cotyledons, red, or yellow exfoliating bark, leaflets usually larger than in 2 with entire margins, medium-size trees but often small shrubs, highly resinous (Clade 4 in Fig. 3).

Some of these groups had already been recognized as natural, morphologically well-defined groups (Bullock, 1936; McVaugh and Rzedowski, 1965; Rzedowski and Kruse, 1979; Toledo, 1984). The closest division to the present one is the one proposed by McVaugh and Rzedowski (1965) who recognized two main groups, the mulatos and the cuajotes (Becerra and Venable, 1999a). The mulatos group is equivalent to the *Simaruba* group above, and the cuajotes include all of the remaining species in the section.

Further divisions of the cuajotes based on morphological differences had been difficult and not very consistent. (Becerra and Venable, 1999a; McVaugh and Rzedowski, 1965; Toledo, 1984). The present study

confirms the affinities of *B. bolivarii* and *B. odorata* with the species of the Fagaroides group. However, *B. bolivarii* appears closely related to *B. chemapodicta* and *B. schlechtendalii*, not to *B. discolor*, as it was previously suggested (Rzedowski, 1968). Also, *B. rzedowski* in the present analyses falls into the Microphylla group, not close to either *B. schlechtendalii* or *B. crenata* as suggested by Toledo (1982).

The parsimony-based phylogeny and the tree with highest likelihood differ in the positions of several species of the Fagaroides group. The more conspicuous differences are with respect to the positions of *B. fagaroides* var. *fagaroides*, *B. fagaroides* var. *pupusii*, *B. odorata*, and *B. discolor*. However, bootstrap support for the position of these species in the most parsimonious trees is low, and the reason why *B. fagaroides* var. *fagaroides* and *B. fagaroides* var. *pupusii* come together in the parsimony-base phylogeny maybe be the result of long branch attraction (Fig. 3). The tree with highest likelihood separates these two species, giving some support to the proposal of considering them different species (Becerra and Venable, 1999a). Having *B. discolor* closer to *B. ariensis* also makes more sense, since they are very similar except for leaf pubescence (absent in *B. discolor* and present in *B. ariensis*; McVaugh and Rzedowski, 1965).

Subdivision of section *Bullockia*. Toledo (1982) divided section *Bullockia* into two groups, distinguished by fruit and flower characteristics. In the present study, the tree with highest likelihood also has two main clades, and the species are divided according to Toledo (1982). The consensus tree based on maximum parsimony is different from the tree with highest likelihood mainly because it leaves the clade with *B. rutilica*, *B. cerasifolia*, and *B. sarcopoda* outside of these two groups. Leaving this clade out, however, has low bootstrap support, and might be caused by a long branch attraction between *B. sarcopoda* and *B. tecomaca* (Fig. 3). Also, some populations of *B. epinnata* are almost indistinguishable from *B. rutilica* (Jose Juan Perez-Navarro, pers. comm.).

Toledo (1982) originally proposed his division for *Bullockia* species from the Mexican state of Guerrero, but this study indicates that the traits he suggested are probably good, general synapomorphies for all *Bullockia* species. Following Toledo (1982) and Becerra and Venable (1999a), this study confirms two more natural groups in the genus:

5. *Glabrifolia* group. Seed partially covered (less than two-thirds) by the pseudoaril, flowers with united sepals, lobes smaller than 1.5 mm long (Clade 5 in Fig. 3).
6. *Copallifera* group. Seed covered completely or at least two-thirds flowers with the pseudoaril, flowers with free sepals, sepals bigger than 1.5 mm long (Clade 6 in Fig. 3).

This study suggests that the 5S nontranscribed spacer may be of high utility for inferring relationships among very closely related species. In the case of *Bursera*, it was

more variable than ITS, and because it comprised a short span of nucleotide bases, its amplification was even more robust than the one for ITS. Its utility for discerning basal relationships in the *Bullockia* section suggests that this marker may be variable in groups that have little variation to other available markers. This study also confirms the usefulness of the three markers ITS, ETS, and 5S together in generating robust phylogenies at the species level.

Several evolutionary studies were previously conducted on the basis of *Bursera*'s ITS phylogeny. For example, Becerra and Venable (1999a) used the ITS phylogeny as a basis for discussing evolutionary tendencies in *Bursera*'s bark, leaves, breeding systems, and fruits. The ITS phylogeny was also the foundation to explain the patterns of coevolution of *Bursera* and their insect herbivores (Becerra, 1997; Becerra and Venable, 1999b). The current parsimony and maximum likelihood analysis have yielded very similar topologies to the ITS phylogeny, but considerably more robust. This new ITS, ETS, and 5S phylogenetic analyses, thus, not only corroborates the previous molecular reconstruction of *Bursera*, but also strengthens previous claims concerning the evolutionary biology of the genus.

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