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TWO LINEAGES OF *ARCTOSTAPHYLOS* (ERICACEAE) IDENTIFIED USING THE INTERNAL TRANSCRIBED SPACER (ITS) REGION OF THE NUCLEAR GENOME

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ABSTRACT

The understanding of evolutionary relationships in *Arctostaphylos* has been hampered by taxonomic difficulties in this large and complex genus. A phylogenetic analysis of sequences from the ITS region for 38 species was used to provide a phylogenetic perspective for interpreting evolutionary patterns and relationships in *Arctostaphylos.* Phylogenetic relationships were estimated using maximum likelihood and Bayesian inference. ITS sequence data do not support a previously published subgeneric classification based on morphological characteristics, but do support the two lineages of *Arctostaphylos* described by a previous molecular phylogeny based on RFLP data. Topology tests indicate morphological characters are not useful in defining monophyletic clades.

Key Words: *Arctostaphylos,* California, Ericaceae, Arbutoideae, manzanita, polyploidy, hybridization, life history evolution.

One of six genera included in subfamily Arbutoideae (Hileman et al. 2001), *Arctostaphylos* (Ericaceae) is a taxonomically complex genus with over 100 taxa of evergreen shrubs and trees. The center of *Arctostaphylos* diversity is in the California Floristic Province (Raven and Axelrod 1978); only 8 taxa are found outside this province (Wells 2000). Over half of these species are considered rare, threatened or endangered by the California Native Plant Society. Species diversity is highest (over 30 species) along the coast of California from Mendocino County to San Luis Obispo County. Many of the species have limited distributions and are restricted to specific substrates such as serpentine and shale (Wells 1962; Gankin and Major 1964). In contrast, one widespread taxon, *A. uva-ursi,* has a circumboreal distribution and is common in Canada and the northern United States with a continuous distribution across Asia and Europe, occurring in coastal or high mountain areas farther south (e.g., the Alps and Caucasus Mountains in Eurasia or the Rocky Mountains in North America) (Packer and Denford 1974).

Diversification of *Arctostaphylos* has been attributed to life history changes in the context of a complex and changing ecological environment, especially the exposure of a diversity of soil types and the increase in fire frequencies in the last 1.5 MY (Stebbins and Major 1965; Stebbins 1974; Axelrod 1981). Two different life history patterns are found within the genus. In the first, plants survive wildfire and resprout (facultative sprouters). In the second, plants are killed by fire (obligate seeders). In both cases, populations recover from persistent dormant seed banks that are stimulated by fire (Keeley and Zedler 1978; Parker and Kelly 1989). Obligate seeding has been proposed as facilitating the radiation of *Arctostaphylos* into different soil and habitat types (Wells 1969; Stebbins 1974; Raven and Axelrod 1978).

Polyploidy and diploid hybridization (Gottlieb 1968; Stebbins 1974; Kruckeberg 1977; Roof 1978; Schierenbeck et al. 1992) are considered to be major evolutionary processes involved in the rapid speciation in the genus (Stebbins and Major 1980). The base chromosome number for *Arctostaphylos* is $x = 13$ (Wells 1968) and the genus contains both diploids ($n = 13$) and tetraploids ($n = 26$); (Wells 1992). Evidence for allopolyploidy has been found for the origin of at least two taxa (Schierenbeck et al. 1992). Interspecific hybridization has been documented for *Arctostaphylos,* where two compatible species have overlapping distributions and come into close enough contact for cross-pollination (Dobzhansky 1953; Gottlieb 1968; Schmid et al. 1968); diploid hybridization has been suggested as the origin of several species (Gankin 1967; Parker and Vasey 2004). Although hybridization exists, it is not pervasive enough to result in the breakdown of species boundaries (Dobzhansky 1953; Gottlieb 1968; Keeley 1976; Kruckeberg 1977).

A combination of vegetative diversification in *Arctostaphylos* with little divergence in floral characters has led to varying taxonomic interpretations (Jepson 1922, 1939; Eastwood 1934, 1937; McMinn 1939; Adams 1940; Wells 1987, 1992, 2000). Recently, Wells (1992, 2000) provided the most comprehensive and hierarchical treatment. Wells (1992, 2000) divided the genus into two subgenera

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based on fruit characteristics: *Micrococcus* (pulpless, cylindroid, somewhat achene-like drupe with 2–5 separable nutlets) and *Arctostaphylos* (subglobose, berry-like drupes with mealy, granular mesocarp and/or thick leathery pericap, nutlets 8–10, separable, or partially or fully fused). Subgenus *Micrococcus* was divided into three sections, *Myrtifolia, Nissenana,* and *Micrococcus* based on several morphological features of the flower, floral bracts, leaves and bark. Using features of inflorescence bracts, Wells also further divided subgenus *Arctostaphylos* into three sections: *Arctostaphylos, Foliobracteata,* and *Pictobracteata.* The sections were further subdivided into subsections based on presence of basal burl, stomatal distribution and other morphological characters.

Recent systematic research on the Arbutoideae (Hileman et al. 2001) and *Arctostaphylos* (Markos et al. 1998) has underscored the value of using molecular data to evaluate phylogenetic relationships and to test the previous morphology-based classifications. Hileman et al. (2001) used nuclear ribosomal sequence data to study the phylogenetic relationships of the six genera of Arbutoideae: *Arbutus, Arctostaphylos, Arctous, Comarostaphylis, Ornithostaphylos,* and *Xylococcus. Arctostaphylos* was determined to be one of the more recently derived genera in this group. Markos et al. (1998) were the first to use molecular data in phylogenetic research within *Arctostaphylos.* The nuclear ribosomal Internal Transcribed Spacer (ITS) region was sequenced for a small group of nine taxa (five of which were subspecies of *A. hookeri*) that indicated two clades in the genus. This was tested with sequences from a portion of the 26S nuclear ribosomal DNA of 17 species. These data indicated two clusters of species that conflicted with Wells' classification; while there seemed to be a deep split between the two clusters, there was no further resolution. Restriction fragment data derived from the nuclear ribosomal ITS region were then used to test Wells' 1992 subgeneric classification of *Arctostaphylos,* examining 34 species in the genus. The recognition of Wells' two subgenera in the genus was not supported although Markos et al. (1998) did consistently find two different but distinctly supported groups (''Group one'' and ''Group two'') in each analysis.

The goal of the present study is to assess phylogenetic relationships within *Arctostaphylos* using ITS sequence data. We have extended the taxa sequenced for ITS in the genus beyond that of Markos et al. (1998) from 9 to 38 taxa. The phylogenetic hypotheses generated using the molecular data are used to address these specific questions:

1. Does the ITS phylogeny support the infrageneric classification proposed by Wells (1992) or the two groups recognized by Markos et al. (1998)?
2. Will traditionally used morphological characters, such as shreddy bark, unequal stomata, bract type, and chromosome number support monophyletic clades found in the molecular phylogeny?

Materials and Methods

Taxa and Regions Sampled

Thirty-eight species of *Arctostaphylos* were included in this study (Table 1). Sequence data were deposited in TreeBASE (accession number SN1430) and Genbank (Table 1). Markos et al. (1998) indicated that the lack of intraspecific variation in the ITS region precluded the need for inclusion of more than one individual per species in the study. This was supported in another study of wide-ranging diploid species of *Arctostaphylos*; no variation in ITS sequence was found from geographically widespread individuals in three species, while in another species, one individual differed by 1 base pair from 4 others (Parker et al. unpublished data). For this reason, only one individual per taxon was sampled in this study. Sampling was designed to include taxa from the two subgenera and three sections described by Wells (1992). *Arbutus* was used as the outgroup based on the results of Hileman et al. (2001).

DNA Extraction, Amplification, and Sequencing

Total DNA was isolated from dried leaves of individual plants. DNA extraction followed a modified Doyle and Doyle (1987) CTAB extraction (Cullings and Bruns 1992). Double-stranded PCR products were amplified using the universal primers ITS4 and ITS5 (White et al. 1990). The 50-μl PCR reactions were heated at 94°C for three minutes. The reactions underwent 35 cycles in a Perkin-Elmer 480 thermocycler. Each cycle consisted of 35 sec at 97°C denaturation, 45 sec at 50°C annealing and 1 min 15 sec at 72°C extension. Prior to sequencing, the amplified products were cleaned using a PEG precipitation method (Kusukawa 1990).

Amplification primers were used for sequencing. All sequencing was done using dye primer sequencing on a Catalyst 800 Molecular Biology Lab Station following the protocol specified by the ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit (Revision B, August 1995, Perkin-Elmer). Sequence fragments were assembled with Sequencher® version 3 (Gene Code Corporation, Ann Arbor, MI) and then visually inspected.

Molecular Data Analyses

Skewness of distribution of tree length was tested using the methods described by Huelsenbeck (1991). Evaluating 20,000 random trees using PAUP* (Swofford 2003) generated a g_1 skewness statistic that assessed the non-randomness of the data set. Base frequencies were also calculated using PAUP*. Base composition bias was calculated according to Irwin et al. (1991).

TABLE 1. COLLECTIONS EXAMINED IN THE PHYLOGENETIC STUDY OF *ARCTOSTAPHYLOS* AND RELATIVES. VTP = V. Thomas Parker and MV = Michael Vasey. Data in table are listed in the following order: taxa, voucher, herbarium, location (state, county), Genebank accession numbers (ITS1, ITS2).

Subgenus *Micrococcus*

A. mendocinoensis Wells. McCabe & Shierenbeck 0037. SFSU. CA, Mendocino. AF297750, AF297795
A. myrtifolia Parry. VTP & MV 0497. SFSU. CA, Amador. AF297760, AF297805
A. nissenana Merriam. VTP & MV 0490. SFSU. CA, El Dorado. AF297782, AF297727
A. nummularia A. Gray. Dunne 0040. SFSU. CA, Marin. AF297755, AF297800

Subgenus *Arctostaphylos*
Section *Arctostaphylos*

A. bakeri subsp. *sublaevis* Wells. VTP & MV 0547. SFSU. CA, Sonoma. AF297774, AF297819
A. canescens subsp. *canescens* Eastw. MV 0179. SFSU. CA, Santa Cruz. AF297781, AF297826
A. catalinae Wells. VTP & MV 155. SFSU. Santa Catalina Island. AF297787, AF297832
A. colombiana Piper. VTP 0299. SFSU. CA, Mendocino. AF297765, AF297810
A. cruzensis Roof. VTP & Schierenbeck 0012. SFSU. CA, San Luis Obispo. AF297750, AF297795
A. glauca Lindley. VTP 0235. SFSU. CA, San Luis Obispo. AF297778, AF29723
A. hispidula Howell. MV 0360, SFSU. CA, Del Norte. AF297752, AF297797
A. hookeri subsp. *hookeri* G. Don. Strybing Arboretum. CA, San Francisco. AF297756, AF297801
A. mewukka subsp. *truei* Knight. MV 0029. SFSU. CA, Butte. AF297759, AF297804
A. parryana Lemmon. J. Keeley 22,291. LOC. CA, San Bernardino. AF297757, AF297802
A. patula Greene. VTP 0313. SFSU. CA, Sierra. AF297754, AF297799
A. pechoensis Abrams. Markos 0264. SFSU. CA, San Luis Obispo. AF297767, AF297812
A. peninsularis Wells. MV 0804, SFSU. Mexico, Baja California. AF297785, AF297830
A. stanfordiana subsp. *stanfordiana* Parry. MV 0468. SFSU. CA, Sonoma. AF297751, AF297796

Subgenus *Arctostaphylos*
Section *Foliobracteata*

A. andersonii A. Gray. MV 0089. SFSU. CA, Santa Cruz. AF297780, AF297825
A. auriculata Eastw. MV 0170. SFSU. CA, Contra Costa. AF297779, AF297824
A. densiflora Baker. MV 0069. SFSU. CA, Sonoma. AF297753, AF297799
A. glandulosa subsp. *glandulosa* Eastw. VTP & MV 0157. SFSU. CA, Santa Barbara. AF297775, AF297820
A. hooveri Wells. MV 0667. SFSU. CA, Monterey. AF297773, AF297818
A. morroensis Wiesl. & Schreiber. VTP & MV 0149. SFSU. CA, San Luis Obispo. AF297763, AF297808
A. montereyensis Hoover. VTP 0581. SFSU. CA, Monterey. AF297770, AF297815
A. obispoensis Eastw. VTP & MV 0236. SFSU. CA, San Luis Obispo. AF297764, AF297809
A. pajaroensis Adams. VTP & MV 0459. SFSU. CA, Monterey. AF297772, AF297817
A. pallida Eastw. VTP & MV 0565. SFSU. CA, Contra Costa. AF297771, AF297816
A. pilosula Jepson & Wiesl. VTP 0233. SFSU. CA, San Luis Obispo. AF297766, AF297811
A. purissima Wells. VTP 0238. SFSU. CA, Santa Barbara. AF297769, AF297816
A. refugioensis Gankin. MV 0156. SFSU. CA, Santa Barbara. AF297776, AF297821
A. silvicola Jepson & Wiesl. MV 0082. SFSU. CA, Santa Cruz. AF297768, AF297813
A. tomentosa subsp. *tomentosa* (Pursh) Lindley. MV 0243. SFSU. CA, Monterey. AF297786, AF297831
A. uva-ursi (L.) Sprengel. MV 0019. SFSU. CA, San Mateo. AF297761, AF297806
A. viridissima (Eastw.) McMinn. MV 0875. SFSU. CA, Santa Barbara. AF297777, AF297802
A. viscida subsp. *mariposa* (Dudley) Wells. VTP & MV 0569. SFSU. CA, Tuoleme. AF297783, AF297828

Subgenus *Arctostaphylos*
Section *Pictobracteata*

A. pringlei subsp. *Drupacea* (C. Parry) Wells. MV 0232. SFSU. AZ, Pima. AF297784, AF297829

Outgroup

Arbutus andrachne L. UC Botanical Garden. Isreal. AF297789, AF297834
Arbutus menziesii Pursh. UC Botanical Garden. CA, Alameda. AF086828, AF086828

Phylogenetic Analysis of Molecular Data

Molecular data were evaluated using maximum likelihood (ML) and Bayesian methods. ML analyses were performed using PAUP* (Swofford 2003). Bayesian analyses were done using MrBayes version 2.01 (Huelsenbeck and Ronquist 2001). Prior to likelihood or Bayesian analysis of the best-fit model of evolution was determined using Modeltest (Posada and Crandall, 1998).

Tree searches. Heuristic searches with ten random addition sequence replicates and TBR branch swapping were performed for all ML estimates. Maximum likelihood estimates of the ITS phylogeny were obtained using the TrNef+G model,

which has equal base frequencies and varying transition rates including gamma distribution for rate heterogeneity (Posada and Crandall 1998).

Branch support. Maximum likelihood bootstrap analyses of 100 replicates were performed using a heuristic search with 10 random addition sequence replicates and TBR branch swapping. A Bayesian approach for inferring phylogenies was also used because of its easy interpretation of results, its ability to incorporate prior information (Huelsenbeck and Ronquist 2001), and some computational advantages (Larget and Simon 1999). The analysis used MrBayes (Huelsenbeck and Ronquist 2001), which employs Markov Chain Monte Carlo (MCMC) to approximate the posterior probabilities of phylogenies (Metropolis et al. 1953; Hastings 1970; Green 1995). The model of evolution used for all runs was TrNef+G (determined by ModelTest). MrBayes was run with four chains from 10 different starting points. Five of the 10 runs were 100,000 generations and trees were sampled every 10 generations. The remaining five runs were 1,000,000 generations and trees were sampled every 50 or 100 generations. All 10 runs reached a plateau in likelihood. Trees that were suboptimal at the beginning of the runs were discarded (burn-in phase). All trees saved from all 10 runs were summarized in PAUP* (See MrBayes manual). Posterior probabilities for nodes of interests were recorded in Table 2.

Morphological constraint trees. To test the monophyly of species with shreddy bark, unequal stomata, scale-like bracts and chromosome number all species in our data set with a particular character were constrained to a monophyletic group. These characters were chosen because they have been used for distinguishing species or higher taxa in previous treatments (Adams 1940; McMinn 1939; Wells 1969, 1992). All character states were extracted and defined by Wells (1992) and confirmed by the authors both on herbarium sheets and in the field. Each species set was tested using both the Kishino-Hasegawa (1989) and the Shimodaira-Hasegawa (1999) tests in a likelihood context to compare competing tree topologies. For tests, settings were set to full-optimization with 1000 bootstrap replicates. All analyses were performed using PAUP*.

Results

Missing data represented zero percent of the data set. There were 120 phylogenetically informative sites out of 531 base pairs for ITS 1 & 2 including all taxa listed in Table 1. Uncorrected P values among species range from 0.0 to 0.09. Base frequencies were: A = 0.24147, C = 0.24423, G = 0.24133, T = 0.27297. Base composition bias was calculated as 0.03, showing minimal bias. A g_1 statistic of −1.356 (SD = 13.18) was calculated indicating significant structure in the data set.

Table 2. Parameters Used in MrBayes Runs. Nodes A, B, C and D are labeled on Fig. 1. Probabilities are expressed as posterior probabilities of all trees (omitting burn-in trees).

Run	Number of generations	Tree sample frequency	Suboptimal trees (burn-in)	Number of trees	Likelihood (final tree)	Probability clade A	Probability clade B	Probability clade C	Probability clade D
1	100,000	10	1–3,187	6,813	−2618.26	65	64	73	51
2	100,000	10	1–2,325	7,675	−2615.09	68	56	60	59
3	100,000	10	1–1,885	8,115	−2622.73	71	69	76	58
4	100,000	10	1–2,597	7,403	−2613.45	69	78	87	79
5	1,000,000	100	1–306	9,694	−2630.15	51	65	67	59
6	1,000,000	100	1–288	9,712	−2621.01	56	69	71	64
7	1,000,000	100	1–268	9,732	−2625.63	55	75	76	71
8	1,000,000	100	1–218	9,782	−2628.85	59	67	68	62
9	1,000,000	50	1–255	19,745	−2627.74	59	67	68	62
10	1,000,000	50	1–646	19,354	−2618.80	53	64	64	60

Phylogenetic Analyses

A ML search resulted in one most likely tree (Fig. 1, −ln = 2025.40). There is relatively strong support along the backbone of the *Arctostaphylos* phylogeny (Fig. 1) and weaker branch support on the tips. There is a deep split (nodes labeled A and B in Fig. 1) in the phylogeny for *Arctostaphylos* (Fig. 1). We have labeled the two clades that result in the splits at both ''A'' and ''B'' Clade 1 and Clade 2 respectively. The deep split into two lineages is marginally supported by both a ML bootstrap (60 and 70) and posterior probabilities from Bayesian analyses (Table 2). There are two other nodes that are well supported along the backbone of the phylogeny, nodes C and D. Other relationships supported by both ML bootstrap and Bayesian runs are: *A. pechoensis* and *A. purissima* (100/98, posterior probability/ML bootstrap), *A. viridissima* and *A. cruzensis* (89/86), *A. nissenana* and *A. viscida* (100/98), and *A. pringlei* and *A. peninsularis* (100/99).

All 10 runs of MrBayes differed slightly in number of trees included in final summary tree, number of burn-in trees, and posterior probabilities (Table 2). Nodes A, B, C, and D (Fig. 1) are of interest because they are the nodes that define clades along the backbone of the phylogeny. There are six other clades in the phylogeny supported by posterior probability and not a ML bootstrap value (Fig. 1).

Morphology Constraint Trees

All constrained ML searches of morphological characters (i.e., shreddy bark, unequal stomata, type of bracts and chromosome number) resulted in significantly less likely trees (Table 3). Constraining the species with shreddy bark resulted in the biggest difference in likelihood (54.796) while chromosome number resulted in the smallest difference (27.277).

Discussion

Phylogenetic Analyses and Relationships

The two clades in the *Arctostaphylos* ITS phylogeny (Fig. 1) present a different model of evolution than have relationships based on morphology (e.g., Wells 1992, 2000). One clade consists of *A. mendocinoensis, A. stanfordiana, A. hispidula, A. densiflora, A. hookeri* subsp. *hookeri, A. nummularia, A. parryana, A. patula, A. mewukka* and *A. myrtifolia.* The second clade contains the remaining 28 taxa sampled in this study. The results found here agree with those of Markos et al. (1998) who also found two groups within a 26S sequence tree of *Arctostaphylos.*

The ML phylogeny and the Bayesian analyses for the ITS data do not support Wells' (1992, 2000) classification. In these analyses, subgenus *Micrococcus* is not monophyletic, and two sections of subgenus *Arctostaphylos* proposed by Wells (1992, 2000), section *Foliobracteata* and section *Arctostaphylos,* are clearly not monophyletic (Fig. 1, Tables 2 and 3). The strongly supported clades along the backbone of the ITS phylogeny show this. The incongruence between the morphologically based classification and Fig. 1 suggests a different interpretation of the evolution of the genus. For example, common to both trees are species with elliptic, simple green leaves like *A. pungens* and *A. pringlei* in one clade, *A. hookeri* ssp. *hookeri* and *A. densiflora* in the other, similar in shape to ancestral fossil leaves of *Arctostaphylos* from the Miocene and Pliocene (Chaney and Mason 1934; Mason 1934; Axelrod 1950; Wolfe 1964). Other processes that would influence a morphological approach are hybridization, allopolyploidy or convergence.

Hybridization has been postulated as an important factor in the evolution of *Arctostaphylos* (Stebbins and Major 1965; Shapin 1966; Gottlieb 1968; Raven and Axelrod 1978; Roof 1978; Kruckberg 1977; Schierenbeck et al. 1992). Further directions might include looking at known hybrids and their parental origin in a molecular phylogenetic framework. McDade (1992, 1997) has employed parsimony techniques to show how hybrids affect phylogenies; this technique, along with maximum likelihood and a Bayesian analysis might shed light on hybridization of *Arctostaphylos* taxa and how they affect phylogenies. Hardig et al. (2000, 2002) have used sequence data to examine species of putative hybrid origin in *Ceanothus* although they could not rule out allopatric origins.

Constraint Trees

The morphological characters that were included in this study were type of bracts, type of bark, stomatal distribution, and ploidy level. Of these characters, only bract type has been suggested to be monophyletic, while the others have been used to segregate smaller sets of species or subspecies. The sections of subgenus *Arctostaphylos* described by Wells (1992), for example, are defined based in part on bract type. Nineteen species of the leafy bracted group (section *Foliobracteata*) and sixteen species with a scale-like bract (section *Arctostaphylos*) were included in our study. These two sections of subgenus *Arctostaphylos* do not form monophyletic groups in Fig. 1 and when the constraint of bract type was carried out (Table 3) the resulting tree was significantly different than the unconstrained phylogeny. Based on our study, bract shape in *Arctostaphylos* is not a phylogenetically useful character at the sectional level in the genus.

In Wells' (1992) classification, bark characteristics generally are given minor significance in the taxonomy of the group. Species with rough or shreddy bark fall into both subgenera and sections. Although not recognized by Wells (1992), northern populations (Mendocino Co.) of *A. nummularia* have shreddy bark in contrast to southern popula-

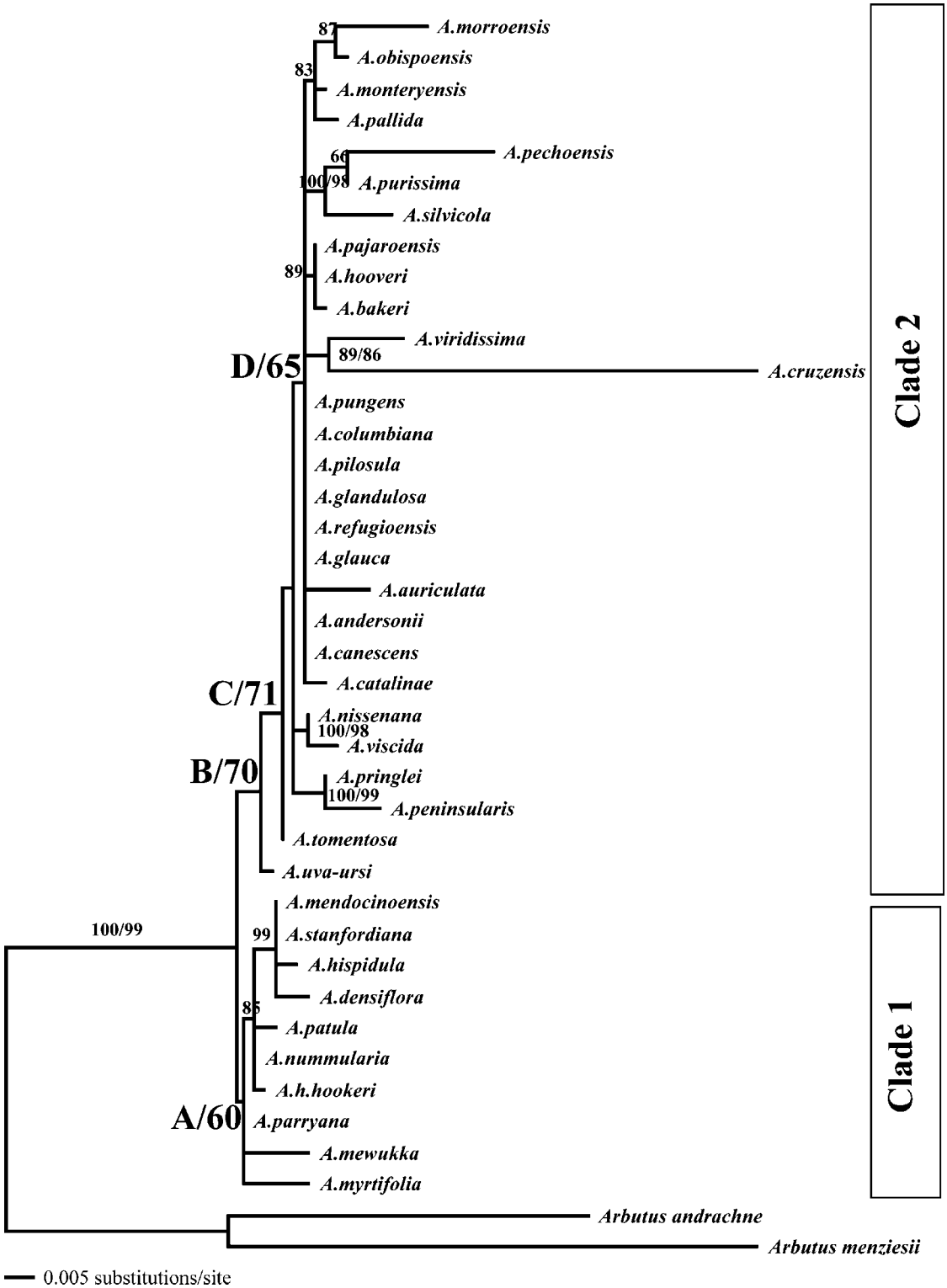

FIG. 1. Maximum likelihood tree generated using ITS data and the TrNef+G model of evolution (−ln = 2025.4027). A Bayesian analysis was run under the TrNef+G model (see Table 2 for parameters) to assess the posterior probabilities of the nodes. Numbers above the nodes are posterior probabilities/maximum likelihood bootstrap when two numbers are present and posterior probabilities when one number is shown. Letters above the nodes correspond to letters in Table 2.

TABLE 3. TOPOLOGY TEST STATISTICS. KH = Kishino-Hasegawa, SH = Shimodaira-Hasegawa. (Kishino and Hasegawa 1989; Shimodaira and Hasegawa 1999). Morphological character data were obtained from Wells (1992) and confirmed by the authors.

	LN	Difference	KH-test P-value	SH-test P-value	Significant
Shreddy bark					
Tree1	−2025.40271	best			
Tree2	−2080.19954	54.79682	0.000	0.006	YES
Stomata unequal					
Tree1	−2025.40271	best			
Tree2	−2070.89644	45.49373	0.000	0.008	YES
Scale-like bracts					
Tree1	−2025.40271	best			
Tree2	−2060.60708	35.20437	0.000	0.015	YES
Chromosome Number					
Tree1	−2025.40271	best			
Tree2	−2052.68067	27.27796	0.000	0.018	YES

tions of Marin, San Mateo and Santa Cruz Counties; in the same Wells' subgenus, *A. nissenana* also exhibits roughened grey bark. In subgenus *Arctostaphylos* (Wells 1992), *A. tomentosa, A. morroensis, A. rudis, A. pajaroensis* and several others have shreddy, gray bark. We found that a monophyletic group constrained on the ITS tree was significantly different from the unconstrained tree suggesting this character has arisen more than once. Hileman et al. (2001) found a similar result in *Arbutus* in which multiple species have either smooth reddish or roughened grayish bark but neither represents a monophyletic group.

Some members of *Arctostaphylos* have stomata on both sides of the leaf (isofacial), others have stomata on the underside of the leaf (bifacial), and some have more stomata on the bottom than on the top (heterofacial). Wells (1992) reports that there are 10 species of *Arctostaphylos* with bifacial/heterofacial leaves: *A. tomentosa, A. andersonii, A. pajaroensis, A. morroensis, A. uva-ursi, A. pumila, A. edmundsii, A. insularis, A. nummularia* and *A. mendocinoensis.* Ecologically, the bifacial/heterofacial species are restricted to the cooler, mild climate of the coastal fog belt (Wells 1992). We have included seven of the 10-bifacial/heterofacial species in our study: *A. mendocinoensis, A. nummularia, A. uva-ursi, A. pajaroensis, A. andersonii, A. tomentosa* and *A. morroensis.* When bifacial/heterofacial species are constrained to form a monophyletic group, the ML estimate is significantly different from the non-constraint tree (Table 3). Therefore, stomatal distribution likely has arisen independently in some of these species and is likely to have resulted from ecological convergence.

The majority of *Arctostaphylos* have a base chromosome number of $n = 13$ while others have $n = 26$ (Wells 1992). There are only two tetraploids in clade one (*A. parryana* and *A. mewukka*) and three in clade two (*A. bakeri, A.glandulosa* and *A. tomentosa*). When constraining all tetraploids to a monophyletic group a significant increase in likelihood resulted (Table 3) indicating the support that is present along the backbone of the phylogeny is significant. Figure 1 is also useful in looking at the hypothesis of Roof (1980). Roof (1980) hypothesizes that through the boreal species *A. uva-ursi,* a great gene pool of *Arctostaphylos* at the tetraploid level has entered California from the north while another vast gene pool, at the diploid level has come to California in *A. pungens,* a species derived from Mexico and the American southwest. We do not find support for Roofs' hypothesis based on the placement of *A. pungens,* which is embedded in ''Clade two''. If Roofs' hypotheses were supported we would expect to see *A. pungens* at the base of the two clades.

The results presented here suggest that a thorough reexamination of the current classification of *Arctostaphylos* is warranted. If hybridization and polyploidy are both important processes for evolution in this genus, then morphological characters may not consistently represent monophyletic groups. Introgression and hybridization can also influence the interpretation of molecular-based phylogenies. Because species within *Arctostaphylos* are known to hybridize (e.g., Gottlieb 1968) and some neutral characters spread across species boundaries (e.g., Ellstrand et al. 1987), molecular trees based on neutral molecular characters like the ITS region of the nuclear ribosome may be subject to similar problems. Hardig et al. (2000), for example, developed an ITS tree for *Ceanothus* that illustrated a few anomalies, such as a few examples of taxa with geographic proximity being clustered together, while taxa with morphologically unique characters (such as the two varieties of *C. jepsonii* with six-merous flowers) were separated. In another study, Schierenbeck et al. (1992) provided evidence for the hypothesized allopolyploid origin of *A. mewuk-*

ka from a hybrid cross between *A. viscida* and *A. patula.* In this study, these two latter species are in different clades; but this allopolyploid indicates a potential problem for interpretation of both morphological and molecular features. (*A. Mewukka* is found only near one of the parents.) Markos et al. (1998) found *A. pungens* to be ambiguous in its placement based on RFLPs while it fell clearly into the larger clade in this study. All of these results suggest caution with both morphological and molecular approaches in *Arctostaphylos.* This ITS phylogeny should be taken as an alternative to previous morphological models of the genus.

While this molecular tree is not the only way to assess relationships in this group, research on hybridization in *Arctostaphylos* differentially supports it over previous models of evolution in this genus based on morphology. Dobzhansky (1953) concluded his study of hybridization in *Arctostaphylos* with the comment that there was little indication of the loss of species boundaries and just a small number of hybrids. He examined two diploid species (*A. viscida* and *A. patula*) in his study to assess whether there was some type of reproductive isolating mechanism. The co-occurrence of these and other pairs of diploid species that do not show extensive hybridization (e.g., *A. nummularia* and *A. silvicola* in the southern Santa Cruz Mts, *A. pechoensis* and *A. hookeri* near Prunedale, CA) all have one feature in common: these pairs of diploid species each combine a representative from both clades. This suggests the ITS molecular tree more accurately distributes species by their reproductive closeness, as studies in which hybridization was low (e.g., Dobzhanksy 1953) combine species from both clades, while studies in which hybridization was abundant (e.g., Gottlieg 1968, Schmid et al. 1968) examined species pairs from one of the clades indicated in the ITS tree.

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Literature Cited

Adams, J. E. 1940. A systematic study of the genus *Arctostaphylos.* Journal of the Elisha Mitchell Society 56:1–62.

Axelrod, D. I. 1950. Studies in late Tertiary paleobotany. Publication 590, Carnegie Institution of Washington, Washington, DC.

———. 1981. Holocene climatic changes in relation to vegetation disjunction and speciation. American Naturalist 117:847–870.

Chaney, R. W. and H. L. Mason. 1934. A Pleistocene flora from Santa Cruz Island, California. *In* Studies of the Pleistocene paleobotany of California. Carnegie Institution of Washington, U.S.A.

Cullings, K. W. and T. D. Bruns. 1992. Phylogenetic origin of the Montropoideae inferred from partial 28S Ribosomal RNA gene sequences. Canadian Journal of Botany 70:1703–1708.

Dobzhansky, T. 1953. Natural hybrids of two species of *Arctostaphylos* in the Yosemite region of California. Heredity 7:73–79.

Doyle, G. and E. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19:11–15.

Eastwood, A. 1934. A revision of *Arctostaphylos* with key descriptions. Leaflets of Western Botany 1:105–127.

———. 1937. Notes on *Schizococcus* with a key to the species. Leaflets of Western Botany 2:50.

Ellstrand, N. C., J. M. Lee, J. E. Keeley, and S. C. Keeley. 1987. Ecological isolation and introgression: biochemical confirmation of introgression in an *Arctostaphylos* (Ericaceae) population. Oecologia Plantarum 8:299–308.

Gankin, R. 1967. A new species of *Arctostaphylos* from Santa Barbara County, California. The Four Seasons 2:13.

——— and J. Major. 1964. *Arctostaphylos myrtifolia,* its biology and relationship to the problem of endemism. Ecology 45:792–808.

Gottlieb, L. 1968. Hybridization between *Arctostaphylos viscida* and *A. canescens* in Oregon. Brittonia 20:83–93.

Gene Codes Corporation, inc. 1994. Sequencher 2.1. Gene Codes Corporation, Inc. Madison, Wisconsin.

Green, P. J. 1995. Reversible jump Markov chain Monte Carlo computation and Bayesian model determinations. Biometrika 82:711–732.

Hastings, M. K. 1970. Monte Carlo sampling methods using Markov chains and their applications. Biometricka 57:97–109.

Hardig, T. M., P. S. Soltis, and D. E. Soltis. 2000. Diversification of the North American shrub genus *Ceanothus* (Rhamnaceae): conflicting phylogenies from nuclear ribosomal DNA and chloroplast DNA. American Journal of Botany 87:108–123.

———, P. S. Soltis, D. E. Soltis, and R. B. Hudson. 2002. Morphological and molecular analysis of putative hybrid speciation in *Ceanothus* (Rhamnaceae). Systematic Botany 27:734–746.

Hileman, L. C., V. T. Parker, and M. Vasey. 2001. Phylogeny and biogeography of the Arbutoideae (Ericaceae): implications for the Madrean-Tethyan hypothesis. Systematic Botany 26:131–143.

Huelsenbeck, J. P. 1991. Tree-length distribution skewness: an indicator of phylogenetic information. Systematic Zoology 40:257–270.

——— and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755.

Irwin, D. M., T. D. Kocher, and A. C. Wilson. 1991. Evolution of the Cytochrome *b* gene in mammals. Journal of Molecular Evolution 32:128–144.

Jepson, W. L. 1922. Revision of California *Arctostaphyli.* Madroño 1:78–86.

———. 1939. A Flora of California, Vol. III. Associated Student's Store, U.C. Berkeley pp. 745–750.

Keeley, J. E. 1976. Morphological evidence of hybridization between *Arctostaphylos glauca* and *A. pungens* (Ericaceae). Madroño 23:427–434.

——— AND P. H. ZEDLER. 1978. Reproduction of chaparral shrubs after fire: a comparison of sprouting and seeding strategies. American Midland Naturalist 99: 142–161.

KISHINO, H., AND M. HASEGAWA. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in the Hominoidea. Journal of Molecular Evolution 29:170–179.

KRUCKEBERG, A. R. 1977. Manzanita (*Arctostaphylos*) hybrids in the Pacific Northwest: Effects of human and natural disturbance. Systematic Botany 2:233–250.

KUSUKAWA, N. 1990. Rapid, reliable protocol for direct sequencing of material amplified by the PCR. Biotechniques 9:66–72.

LARGET, B. AND D. SIMON. 1999. Markov chain Monte Carlo algorithms for the Bayesian analysis of phylogenetic trees. Molecular Biology and Evolution 16: 111–120.

MARKOS, S. M., L. C. HILEMAN, M. C. VASEY, AND V. T. PARKER. 1998. Phylogeny of the *Arctostaphylos hookeri* complex (Ericaceae) based on nrDNA data. Madroño 45:187–199.

MASON, H. L. 1934. Pleistocene flora of the Tomales Formation. Carnegie Institution of Washington Publication 415:81–179.

MCDADE, L. A. 1992. Hybrids and phylogenetic systematics II. The impact of hybrids on cladistic analysis. Evolution 46:1329–1346.

———. 1997. Hybrids and phylogenetic systematics III. Comparison with distance methods. Systematic Botany 22:669–683.

MCMINN, H. E. 1939. An illustrated manual of California shrubs. U.C. Press, Berkeley and Los Angeles, CA.

METROPOLIS, N., A. W. ROSENBLUTH, M. N. ROSENBLUTH, A. H. TELLER, AND E. TELLER. 1953. Equations of state calculations by fast computing machines. Journal of Chemical Physics 21:1087–1091.

PACKER, J. G. AND K. E. DENFORD. 1974. A contribution to the taxonomy of *Arctostaphylos uva-ursi*. Canadian Journal of Botany 52:743–753.

PARKER, V. T. AND V. R. KELLY. 1989. Seed banks of California chaparral and other Mediterranean climate shrublands. Pp. 231–255 *in* M. A. Leck, V. T. Parker, and R. L. Simpson (eds.), Ecology of Soil Seed Banks. Academic Press, San Diego, CA.

——— AND M. C. VASEY. 2004. *Arctostaphylos gabilanensis*, a newly described auriculate-leaved manzanita from the Gabilan Mountains, California. Madroño 51:322–325.

POSADA, D. AND K. A. CRANDALL. 1998. ModelTest: testing the model of DNA substitution. Bioinformatics 14:817–818.

RAVEN, P. H. AND D. I. AXELROD. 1978. Origin and relationships of the California flora. University of California Press, Berkeley, CA.

ROOF, J. B. 1978. Studies in *Arctostaphylos* (Ericaceae). The Four Seasons 5:3–15.

———. 1980. A fresh approach to the genus *Arctostaphylos* in California (continued). Changing Seasons 1(2):1–32.

SCHIERENBECK, K. A., G. L. STEBBINS, AND R. W. PATTERSON. 1992. Morphological and cytological evidence for polyphyletic allopolyploidy in *Arctostaphylos mewukka* (Ericaceae). Plant Systematics and Evolution 179:187–205.

SCHMID, R., T. E. MALLORY, AND J. M. TUCKER. 1968. Biosystematic evidence for hybridization between *Arctostaphylos nissenana* and *A. viscida*. Brittonia 20:34–43.

SHAPIN, S. 1966. Variation and hybridization in three Pacific Northwest taxa of *Arctostaphylos*. Senior thesis, Reed College, Portland, OR.

SHIMODAIRA, H. AND M. HASEGAWA. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Molecular Biology and Evolution* 16:1114–1116.

STEBBINS, G. L. 1974. Flowering plants: evolution above the species level. Harvard University Press, Cambridge, MA.

——— AND J. MAJOR. 1965. Endemism and speciation in the California flora. Ecological Monographs 35:1–35.

———. 1980. Polyploidy in plants: Unresolved problems and prospects. Pp. 495–520 *in* W. H. Lewis (ed.), Polyploidy: biological relevance. Plenum Press, New York, NY.

SWOFFORD, D. L. 2003. Phylogenetic Analysis using Parsimony (*and other methods) PAUP*. Sinauer and Associates, Sunderland, MA.

WELLS, P. V. 1962. Vegetation in relation to geological substratum and fire in the San Luis Obispo quadrangle, California. Ecological Monographs 32:79–103.

———. 1968. New taxa, combinations, and chromosome numbers in *Arctostaphylos* (Ericaceae). Madroño 19: 193–210.

———. 1969. The relation between mode of reproduction and the extent of speciation in woody genera of the California chaparral. Evolution 23:264–267.

———. 1987. The leafy-bracted, crown sprouting manzanitas, an ancestral group in *Arctostaphylos* (Ericaceae). The Four Seasons 7:5–27.

———. 1992. Subgenera and sections of *Arctostaphylos*. The Four Seasons 9:64–69.

———. 2000. The manzanitas of California, also of Mexico and the world. Published by the author, 151pp.

WHITE, T. J., S. L. BRUNS, AND J. TAYLOR. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Page 315 *in* M. Innis, D. Gelfand, J. Sninsky, and T. White (eds.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA.

WOLFE, J. A. 1964. Miocene floras from Fingerrock wash, southwestern Nevada. U.S. Geological Survey Professional Paper 454-N:1–31.