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A MOLECULAR PHYLOGENY OF *ARCEUTHOBIUM* (VISCACEAE) BASED ON NUCLEAR RIBOSOMAL DNA INTERNAL TRANSCRIBED SPACER SEQUENCES[1]

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Ribosomal DNA (rDNA) internal transcribed spacer (ITS) and 5.8S rDNA sequences were obtained from 22 species of dwarf mistletoes (*Arceuthobium*—Viscaceae) to test phylogenetic relationships. Interspecific distances ranged from 0 to 21.4% between New World species, values two to five times higher than those measures for the ITS region in other plants. One Old World species (*A. oxycedri*) and one New World species (*A. abietis-religiosae*) were remarkably similar to each other but exhibited up to 41% sequence divergence from the remaining species. Minimum length trees support the concept of a verticillately branched subgenus *Arceuthobium*; however, interspecific distances indicate this group is extremely heterogeneous. Subgenus *Vaginata*, Section *Vaginata*, is centered in Mexico and encompasses all the taxa previously placed in this group but is expanded to include several species previously classified in Section *Campylopoda* (e.g., *A. divaricatum*, *A. rubrum*, and *A. strictum*). The sister group relationship between *A. divaricatum* and *A. douglasii*, first seen following isozyme analysis, is supported by ITS sequence data. Section *Campylopoda* s. s. is now composed of 13 mainly U.S. species that show a high degree of morphological and genetic similarity. The eastern dwarf mistletoe, *A. pusillum*, is not closely related to *A. douglasii* but rather with *A. bicarinatum* from Hispaniola, which suggests that these taxa represent highly modified relicts that shared an ancestor in the early Tertiary. Two endemic species from Mexico and Central America (*A. guatemalense* and *A. pendens*) formed a sister group and have been placed in a new Section (*Penda*). Rapid molecular evolution in *Arceuthobium* may be associated with the adaptive radiation of this genus on numerous conifer hosts.

Arceuthobium M. Bieb. (dwarf mistletoes, Viscaceae) includes 41 species of mistletoes parasitic on Pinaceae (New and Old World) and Cupressaceae (Old World). These plants have long presented a challenge to systematists interested in elucidating their taxonomic and host relationships. The monograph by Hawksworth and Wiens (1972) included information on morphological characters, flavonoids, flowering and fruiting phenology, and host relationships. Their classification was based largely on results of a numerical phenetic analysis that utilized these features. Classifications of this genus have been constructed with the knowledge that implied relationships among taxa could be the result of convergence based upon their extremely reduced leaves (scales) and floral organs, lack of roots, and chromosome number uniformity ($x = 14$).

The classifications of Hawkworth and Wiens (1972, 1984) divided the genus into two subgenera: *Arceuthobium* and *Vaginata* (Table 1). The former is composed of three New World and eight Old World species characterized by verticillate secondary branching (Mark and Hawksworth, 1981). Subgenus *Vaginata*, which includes 30 species of mistletoes restricted to the New World, is marked by the presence of flabellate secondary branching, if secondary branching is present at all. Included in this subgenus was Section *Minuta*, composed of *A. pusillum* and *A. douglasii*, characterized by diminutive aerial shoots that develop from extensive systemic host infections called witches' brooms. The dwarf mistletoes of Section *Campylopoda* form nonsystemic infections on their host trees, have larger aerial shoots, and flower in the summer or fall (Wiens, 1968). This group is especially speciose in the western United States where an apparent adaptive radiation resulted in parasites specific to *Abies*, *Larix*, *Picea*, *Pinus*, and *Tsuga*. Finally, Section *Vaginata*, found in the southwestern United States, Mexico, and Central America, is composed of mistletoes that also form nonsystemic brooms but flower in winter or spring. Some species in this section are relatively large plants, such as *A. vaginatum* ssp. *vaginatum* and *A. durangense*, the latter having shoots up to 50 cm in length. The greatest host latitudes in *Arceuthobium* are seen in parasites of this section such as *A. globosum*, which has been recorded from over 15 species of pines.

The dwarf mistletoe monograph by Hawksworth and Wiens (1972) provided the first comprehensive classification of *Arceuthobium*; however, a number of questions remained. Relatively little systematic or phylogenetic work focused on intergeneric or interspecific relationships has been conducted on the genus since that time. Isozyme electrophoresis has proven especially valuable in providing data, independent of morphology, useful for examining species relationships in *Arceuthobium*. Nickrent, Guttman, and Eshbaugh (1984) and Nickrent (1986) first examined the isozymes of 19 mainly U.S. taxa in the genus. This study showed that the genus had remarkably high levels of genetic diversity: 67% of the loci were polymorphic with an average of 2.23 alleles per locus. This result was surprising given the relative morphological homogeneity characteristic of the genus and the previous flavonoid survey (Crawford and Hawksworth, 1979) which

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identified few species-specific patterns. Many results of this isozyme study were consistent with the classification by Hawksworth and Wiens (1972) such as the recognition of two subgenera (*Arceuthobium* and *Vaginata*), the close relationship between ten species of Section *Campylopoda*, and the clustering of *A. gillii* s. lat. and *A. vaginatum* s. lat. The isozyme analysis did not, however, support a relationship between *A. douglasii* and *A. pusillum* (i.e., Section *Minuta*). An unexpected result was the grouping of *A. douglasii* with *A. divaricatum*, the latter a parasite of pinyon pines. The isozyme study clearly raised as many questions as it resolved, hence further work was called for, especially on Mexican and Central American taxa.

Additional isozyme data have since been obtained for seven Mexican taxa that were not included in the previous study (Nickrent, in press). Subgenus *Arceuthobium* showed greater within-group heterogeneity than any of the other sections. Cluster analysis grouped *A. americanum* and *A. verticilliflorum* followed by *A. abietis-religiosae*. This grouping joined the remainder of the genus at a genetic distance of 0.82. These data thus suggest that verticillate secondary branching is a distinguishing character for this subgenus. *Arceuthobium gillii* and *A. nigrum* (the latter previously classified as *A. gillii* ssp. *nigrum*) clustered at a distance value of 0.553. Although clearly related, this distance supports the recognition of two species. The relatives of *A. vaginatum* had high levels of genetic diversity, and cluster analysis indicated that significant genetic differentiation had occurred between populations. Despite their host preference of pinyon pines, *A. divaricatum* and *A. pendens* were not shown to be closely related. The isozyme data were supported by markedly different flavonoid chemistry, systemic broom formation in the latter species, and different principal host species (Hawksworth and Wiens, 1980). *Arceuthobium strictum* appeared distantly related to the majority of Section *Campylopoda* species and clustered with *A. rubrum*. Both of these species were placed in their own Series (*Stricta* and *Rubra*, respectively) by Hawksworth and Wiens (1972). It appears that the majority of Section *Campylopoda* species reside in the United States, whereas species of Section *Vaginata* occur predominately in Mexico.

Until recently, few studies have used DNA sequences to examine intrageneric relationships among plants because a gene or segment of DNA of adequate size and (fast) evolutionary rate needed to be identified. The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) have been shown to evolve at rates appropriate for examining recently diverging lineages (Baldwin, 1993; Wojciechowski et al., 1993). The above two studies focused on intrageneric relationships; however, evolutionary rates (i.e., the number of nucleotide substitutions per site per year) for the ITS appears to vary widely depending on the plant group. For example, Suh et al. (1993) showed that rates for ITS in Winteraceae are about ten times lower than rates observed for protein coding genes. This allowed the authors to use ITS data to examine intergeneric relationships. In addition, they demonstrated that two types of ITS sequences exist within individuals of one major lineage.

Interspecific relationships among 15 New World dwarf mistletoe species using ITS rDNA sequences were first determined by Schuette (1992). In the present study, both ITS regions as well as 5.8S rDNA were sequenced and analyzed in 22 Old and New World species of *Arceuthobium*. These results were compared with previously proposed phylogenetic relationships and classifications in the genus.

TABLE 1. Classification (according to Hawksworth and Wiens, 1972, 1984) of *Arceuthobium* taxa used in this study.

Subgenus *Arceuthobium* Hawksw. & Wiens
- *A. abietis-religiosae* Heil
- *A. americanum* Nutt. ex Engelm.
- *A. oxycedri* (DC) M. Bieb.
- *A. verticilliflorum* Engelm.

Subgenus *Vaginata* Hawksw. & Wiens
Section *Vaginata* Hawksw. & Wiens
- *A. gillii* Hawksw. & Wiens
- *A. gillii* subsp. *nigrum* Hawksw. & Wiens (= *A. nigrum*)
- *A. globosum* subsp. *globosum* Hawksw. & Wiens
- *A. vaginatum* (Wild.) Presl. subsp. *vaginatum*
- *A. vaginatum* subsp. *durangense* Hawksw. & Wiens (= *A. durangense*)
- *A. vaginatum* subsp. *cryptopodum* (Engelm.) H. & Wiens

Section *Minuta* Hawksw. & Wiens
- *A. douglasii* Engelm.
- *A. pusillum* Peck.

Section *Campylopoda* Hawksw. & Wiens
Series *Rubra* Hawksw. & Wiens
- *A. rubrum* Hawksw. & Wiens
- *A. bicarinatum* Urban.

Series *Stricta* Hawksw. & Wiens
- *A. strictum* Hawksw. & Wiens

Series *Campylopoda* Hawksw. & Wiens
- *A. abietinum* (Engelm.) Hawksw. & Wiens f. sp. *magnificae*
- *A. apachecum* Hawksw. & Wiens
- *A. campylopodum* Engelm.
- *A. divaricatum* Engelm.
- *A. guatemalense* Hawksw. & Wiens
- *A. microcarpum* (Engelm.) Hawksw. & Wiens
- *A. pendens* Hawksw. & Wiens

MATERIALS AND METHODS

The collection information for the 22 species sampled for sequence analyses are shown in Table 2. Both shoot and seed material was used as a source for DNA. Genomic DNA was obtained from shoots by grinding on liquid nitrogen and extracting in 2× cetyltrimethylammonium bromide (Nickrent, 1994). Crude homogenates of seeds (embryo plus endosperm) were made and used to directly amplify the rDNA ITS region using the polymerase chain reaction (PCR; Mullis and Faloona, 1987). Individual seeds were homogenized using sterile glass microhomogenizers (Radnoti Glass Technology, Inc., Arcadia, CA) in 30–50 μl of buffered protease (10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 9% w/vol. Tween 20, 0.5 units/μl protease). The extract was transferred to a microcentrifuge tube and centrifuged for 1 min to sediment the debris. Five microliters of the supernatant were transferred to a new tube and diluted with 45 μl of buffered protease. The extract was incubated at 60 C for 20 min after which the protease was deactivated by heating to 95 C for 5 min. This extract was diluted 1:30 for PCR amplification.

In addition to seed extracts, genomic DNA was obtained from some species and used for PCR. A number of conserved sites on both the 18S and 26S rDNA allow

TABLE 2. Dwarf mistletoe taxa used for ITS sequencing.

Arceuthobium species	Taxon abbrev.	Host	Collection no.[a]	Genbank acc. no.	Locality
A. abietinum f. sp. *magnificae* Hawksw. & Wiens.	ABM	*Abies magnifica*	1916	L25786	F.H. 7N09 at jnc. with S.H. 4, Calaveras Co., CA
A. abietis-religiosae Hiel.	ABR	*Abies religiosa*	2010	L25787	Km. 17 E of Amacameca on rd. to Popacatepetl and Ixtaccihuatl, Estatado Mexico, Mexico.
A. americanum Nutt. ex Engelm.	AME	*Pinus contorta*	1911	L25788	SW side of Upper Sky Ranch Rd., just W of turnoff from Beasore Rd., Madera Co., CA
A. americanum Nutt. ex Engelm.	AME	*Pinus contorta*	1925	—	S.H. 89, 4.8 km N of entrance to D. L. Bliss State Park, El Dorado Co., CA
A. apachecum Hawksw. & Wiens.	APA	*Pinus strobiformis*	1945	L25789	Madera Canyon on trail to Josephine Saddle and Mt. Wrightson, Santa Rita Mts., Santa Cruz Co., AZ
A. bicarinatum Urban.	BIC	*Pinus occidentalis*	2750	L25684	Dominican Republic. Coll. S. Thompson No. 7634.
A. campylopodum Engelm.	CAM	*Pinus jeffreyi*	2161	L25685	5.3 km N of Greenhorn Summit on F.H. 90, Kern Co., CA
A. divaricatum Engelm.	DIV	*Pinus edulis*	1953	L25686	F.H. 567, 9.6 km E of U.S. 666/180, Greenlee Co., AZ
A. divaricatum Engelm.	DIV	*Pinus* monophylla	2160	—	8.5 km E of Horseshoe Meadow on Horseshoe Mdw. Rd. from Lone Pine, Inyo Co., CA
A. douglasii Engelm.	DOU	*Pseudotsuga menziesii*	1955	L25687	Mt. Withington, 22 km S of U.S. 60 along S.H. 52, Socoro Co., NM
A. durangense Hawksw. & Wiens.	DUR	*Pinus* sp.	2178	L25688	Hwy. 40 W of El Palmito, 1.1 km W of km marker 215. Elev. 5,850 ft., Estado Sinaloa, Mexico.
A. gillii Hawksw. & Wiens.	GIG	*Pinus leiophylla* var. *chihuahuana*	1663	L25689	Bear Canyon, Santa Catalina Mts., Pima Co., AZ
A. globosum ssp. *globosum* Hawksw. & Wiens.	GLO	*Pinus durangensis*	2053	L25690	Km. 155 along Hwy. 40 ca. 2.8 km E of Buenos Aires and La Ermita turnoff, Estado Durango, Mexico.
A. guatemalense Hawksw. & Wiens.	GUA	*Pinus ayacahuite*	2039	L25691	Km 129 on Hwy. 175, ca. 46 km N of Ixtlan, Estado Oaxaca, Mexico.
A. microcarpum (Engelm.) Hawksw. & Wiens.	MIC	*Picea pungens*	1950	L25692	Three Forks Creek and F.H. 249, ca. 14 air km W of Alpine, Apache Co., AZ
A. nigrum Hawksw. & Wiens.	NIG	*Pinus lawsonii*	2041	L25693	Km. 121 on Hwy. 175, ca. 40 km N of Ixtlan, Estado Oaxaca, Mexico.
A. oxycedri (DC) Bieb.	OXY	*Juniperus oxycedrus*	2832	L25694	Near Madrid, Spain in the Parke Regional Alta Manzanares. Coll. R. Scharpf (S.N.)
A. pendens Hawksw. & Wiens.	PEN	*Pinus cembroides* var. *orizabensis*	2014	L25695	Mountains 3 km NW of El Frijol Colorado, ca. 16 air km NW of Perote, Estado Veracruz, Mexico.
A. pusillum Peck.	PUS	*Picea mariana*	1969	L25696	Pine Island State Forest near Big Falls, Minnesota, Koochiching Co., MN. Col. K. Zuzek (S.N.)
A. rubrum Hawksw. & Wiens.	RUB	*Pinus durangensis*	2071	L25697	Hwy. 40, 1.6 km W of El Salto, Estado Durango, Mexico.
A. strictum Hawksw. & Wiens.	STR	*Pinus teocote*	2061	L25698	N side of Hwy. 40, 35 km W of Durango just W of the Ojo de Agua turnoff, Estado Durango, Mexico.
A. vaginatum spp. *cryptopodum* (Engelm.) Hawksw. & Wiens.	VAC	*Pinus ponderosa* var. *scopulorum*	1978	L25699	Just N of jnc. of S.H. 4 and F.H. 776, Sandoval Co., NM
A. vaginatum ssp. *vaginatum* (Willd.) Presl.	VAV	*Pinus teocote*	2018	L25806	4.0 km S of Sierra de Aqua (off of Hwy. 140), Estado Veracruz, Mexico.
A. verticilliflorum Engelm.	VER	*Pinus engelmannii*	2065	L25700	Hwy. 40, ca. 42 km W of Durango, just before the Los Mimbres canyon, Estado Durango, Mexico.

[a] Voucher specimens deposited at ILL and SIU.

the construction of forward and reverse primers that bracket ITS-1, 5.8S rDNA, and ITS-2. A common primer combination employed the 18S 1830 forward and 26S 25 reverse (5'-AACAAGGTTTCCGTAGGTGA-3' and 5'-TATGCTTAAAYTCAGCGGGT-3', respectively), which, upon symmetrical amplification, yielded a 0.64-kb fragment in dwarf mistletoes. The PCR reaction mixture contained (final concentrations in a 100-μl reaction): 50 mM KCl, 10 mM Tris pH 8.8, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1.25 mM of each dNTP, 1 μl of each primer at 125 μg/ml (ca. 20 pmoles), 2.5 units of *Taq* polymerase (Promega, M166), and ca. 10–30 ng of genomic DNA. The mixture was overlaid with mineral oil, centrifuged briefly, and placed in the thermal cycler. After an initial incubation (time delay) at 94 C for 3 min, 35 step cycles were performed, each of which consisted of 1 min at 94 C, 1 min at 50 C, and 2.5 min at 72 C with 2 sec added to each subsequent polymerization step. The resulting fragment was used either as a template for an asymmetrical PCR reaction (Gyllensten and Erlich, 1988) that produces single-stranded DNA, or the double-stranded template was sequenced directly (Nickrent, 1994). *Arceuthobium oxycedri* and *A. abietis-religiosae* apparently had mutations at one or both of the above two priming sites, hence the ITS region was encompassed through the use of alternate internal 18S (forward) and 26S (reverse) primers. The PCR product was then gel purified using DEAE (diethylaminoethyl) membranes (Nickrent, 1994). Sequencing reactions were carried out using the terminal amplification primers or forward and reverse primers constructed for the conserved sites on the 5.8S rDNA (sites 32–48). Chain-termination sequencing reactions using dideoxynucleotides (Sanger, Nicklen and Coulson, 1977) were conducted using Sequenase® (U.S. Biochemical Corp., Cleveland, Ohio).

The majority of mutations were base substitutions, thus allowing manual alignment using "Eyeball Sequence Editor" (Cabot and Beckenbach, 1989). Sequence divergence in *A. oxycedri* and *A. abietis-religiosae* ITS-2 prevented alignment beyond position ca. 470. Deletions and alignment gaps were coded as missing data. Minimum length Fitch trees were constructed using PAUP version 3.1 (Swofford, 1993) using the heuristic search algorithm. Character state changes were given equal weight in all analyses. The heuristic searches employed tree bisection-reconnection branch swapping with MULPARS. Bootstrap analysis, conducted to test the statistical confidence of the resulting clades, used 200 replicates of the heuristic search algorithm with tree bisection-reconnection branch swapping and MULPARS in effect. Pairwise nucleotide differences were calculated using PAUP. Variable and informative sites were determined using MacClade version 3.0 (Maddison and Maddison, 1992).

For use as potential outgroups to *Arceuthobium*, ITS sequences from *Phoradendron serotinum, Korthalsella lindsayi, Viscum album,* and *Notothixos subaureus* were determined (Nickrent, unpublished data). All sequences were extremely divergent from those obtained from *Arceuthobium*, thus precluding multiple sequence alignment and their use as outgroups. Complete 18S rDNA sequences have been obtained from at least one species representing all seven genera of Viscaceae, including three species of *Arceuthobium*: *A. pendens, A. verticilliflorum* and *A. oxycedri* (Nickrent, in press; Genbank accession numbers L24082, L24042, and L24081, respectively). Bootstrap analysis of 13 aligned 18S rDNA sequences produced a partially resolved tree containing four genera and/or generic pairs that could be considered for use as an outgroup to *Arceuthobium*: *Phoradendron/Dendrophthora, Korthalsella/Ginalloa, Viscum* and *Notothixos*. The genus *Arceuthobium* was monophyletic (98% bootstrap confidence) and showed *A. verticilliflorum* and *A. pendens* as sister taxa. Since *A. oxycedri* was sister (and basal) to that clade, this species and *A. abietis-religiosae* were used to root the trees obtained from ITS sequences.

RESULTS

Boundaries and size of ITS regions—The boundaries of ITS-1, 5.8S rDNA, and ITS-2 were determined by inspection and comparison with other published angiosperm sequences. The beginning of ITS-1 is clearly defined given the conserved sequence of CATTG at the 3' end of the 18S rDNA. Similarly, the 5' end of the 5.8S rDNA is recognized by the common occurrence of YAMA, although some variation exists (see *A. oxycedri, A. abietis-religiosae, A. bicarinatum,* and other angiosperms). For most dwarf mistletoes, the 5.8S rDNAs were 167 bp in length, although *A. oxycedri, A. abietis-religiosae,* and *A. divaricatum* had a 5.8S rDNA of length 166. These sequences are slightly larger than the average length seen in other angiosperms (164 bp). The 3' ends of most dicot 5.8S rDNAs are variable; however, they generally show CACRY followed by ATCG that marks the beginning of ITS-2. In most dwarf mistletoes, the 5.8S rDNA ends as CGTAT followed by AYRA for the start of ITS-2. The boundary between the 3' end of ITS-2 and the 5' end of the 26S rDNA can be difficult to recognize. Many asterids (including *Arceuthobium*) have RACC at the end of ITS-2 followed by GCGA for the start of the 26S rDNA. In *Arceuthobium,* the 26S starts as TTTTGACC, which is similar to *Daucus* which has TTGTGACC (Yokota et al., 1989). These boundaries were determined from multiple sequence alignments; however, their exact positions would require verification using S1 nuclease mapping. The average length of dwarf mistletoe ITS-1 sequences was 208 bp whereas ITS-2 averaged 226 bp. Other plants that show a shorter ITS-1 than ITS-2 include *Cucumis, Daucus, Mimulus, Oryza,* and *Vigna,* but apparently the more common situation is for ITS-1 to be larger than ITS-2 as in *Astragalus, Calycadenia, Canella, Fragaria, Nicotiana, Populus, Vicia,* and Winteraceae.

Sequence alignment—Secondary structural features of rRNA provide essential information for determination of homologous nucleotide positions. Attempts to align *Arceuthobium* ITS sequences with other published dicot sequences were mostly unsuccessful. Despite the claim that "sufficient sequence homology is retained in the higher plants to examine the evolutionary changes which make these regions diverse" (Venkateswarlu and Nazar, 1991), only one region in ITS-1 appears to be significantly conserved. The region is toward the 3' end of ITS-1 (beginning at bp 131 in *A. americanum,* 177 in *Calycadenia*) and can be identified as CAAGGAA. Venkateswarlu and Nazar (1991) have proposed that a conserved secondary

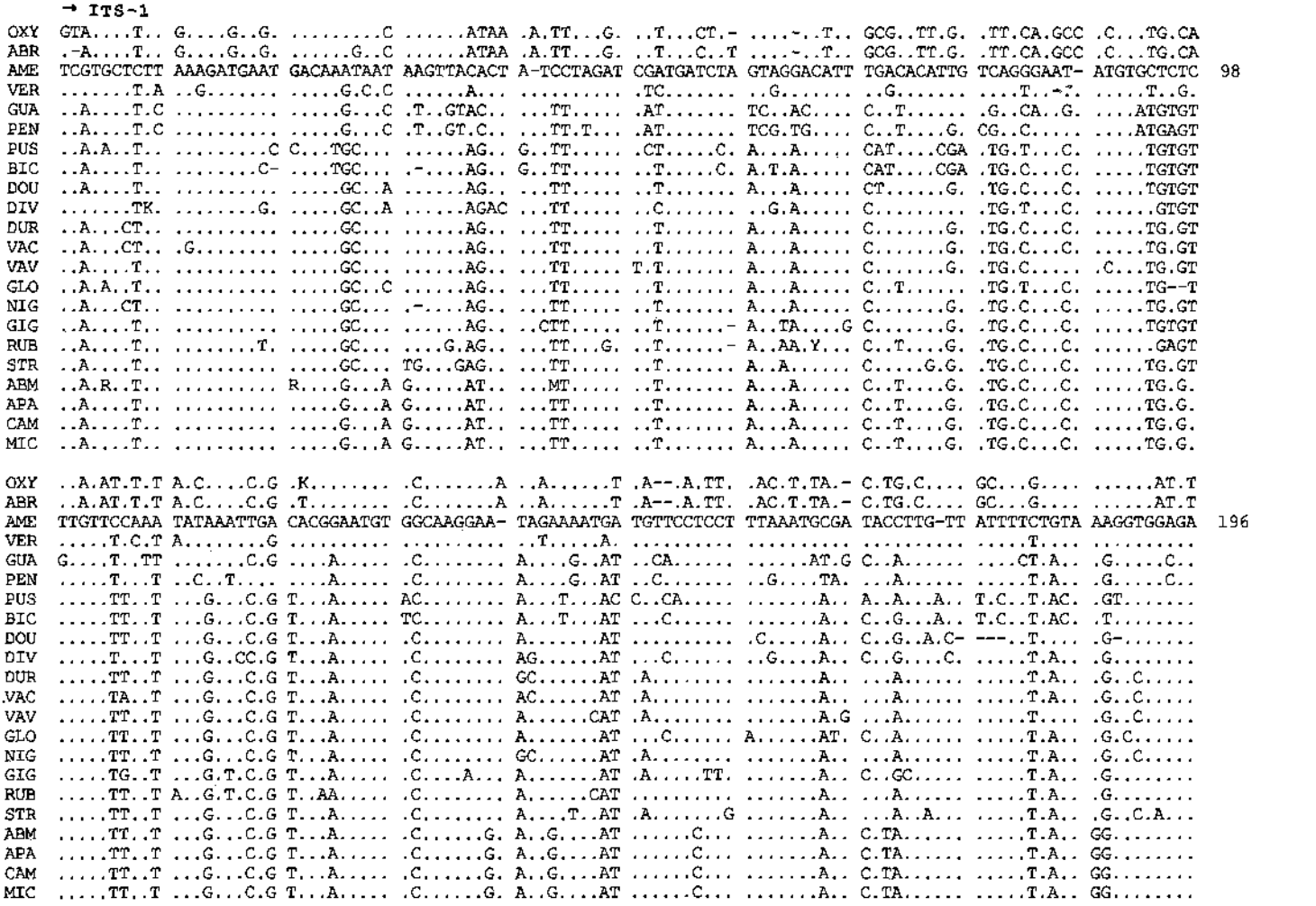

Fig. 1. An alignment of ITS-1, 5.8S, and ITS-2 sequences (5' to 3') for 22 *Arceuthobium* species. See Table 2 for species name abbreviations. A dot in a column indicates the same nucleotide as the reference (*A. americanum*). Ambiguous nucleotides (designations follow IUPAC recommendations) represent either ambiguous sequence data or a true polymorphism at that site. Sequence divergence in *A. oxycedri* and *A. abietis-religiosae* ITS-2 prevented alignment beyond position ca. 470. See text for a discussion of the boundaries of the ITS regions.

structure, consisting of a crucifix or tRNA-like core, exists for eukaryotic ITS rRNA. Attempts to fold *Arceuthobium* (and other angiosperm) ITS sequences into this conformation were not successful, hence sufficient compensatory changes have not been identified to justify the acceptance of such secondary structures.

The alignment of ITS-1, 5.8S rDNA and ITS-2 produced a matrix of 22 *Arceuthobium* species by 619 sites (Fig. 1). Overall, most mutations were base substitutions as opposed to insertion/deletion events (indels). The ITS-2 sequences of *A. oxycedri* and *A. abietis-religiosae* are extremely divergent when compared with the other 20 dwarf mistletoes. No attempt was made to align these sequences beyond ca. position 470. These two mistletoes are, however, remarkably similar to each other (see Discussion).

ITS inter- and intraspecific variability—When all 22 *Arceuthobium* taxa were aligned, 388 of the 619 sites (62.6%) were variable. Given the inability to align *A. oxycedri* and *A. abietis-religiosae* with other dwarf mistletoe sequences, these taxa were excluded from further variability calculations. With 20 taxa, a total of 304 variable sites were identified. Of these sites 174 are phylogenetically informative (exclusive of ambiguous nucleotides). Many of the informative sites differentiated *A. verticilliflorum* and *A. americanum* from the remaining species (i.e., Section *Arceuthobium* from *Vaginata*). Of the 174 sites, 71 occurred in ITS-1 (40.8% of total), 15 in the 5.8S (8.6%), and 88 in ITS-2 (50.6%). When the number of phylogenetically informative sites was taken as a percentage of the number of variable positions for each of the three cistronic regions, the following resulted: 53.7% (71/132) for ITS-1, 46.8% (15/32) for 5.8S, and 63.7% (88/138) for ITS-2. Interspecific distance values between *A. oxycedri/A. abietis-religiosae* and the remaining taxa were high (36%–41%), mainly owing to extreme divergence of ITS-2. Excluding these two taxa, interspecific distances ranged from 0 (*A. apachecum* and *A. microcarpum*) to 21.4% (*A. americanum* and *A. bicarinatum*).

To test for the possibility of significant intraspecific variation, partial ITS sequences (100 bp) were determined

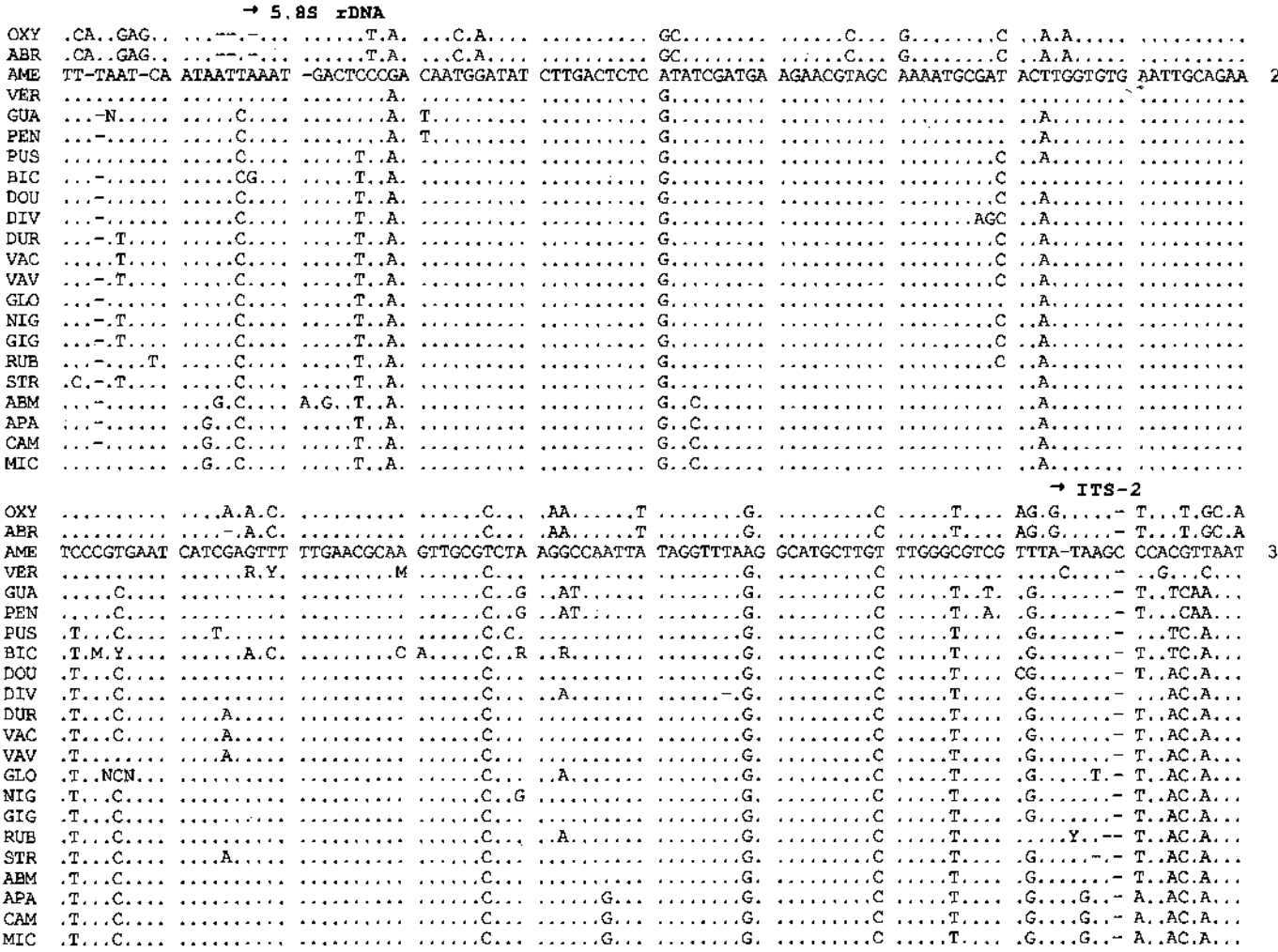

Fig. 1. Continued.

for individuals from three populations of *A. americanum* and two populations of *A. divaricatum*. One change was detected in *A. americanum* whereas no differences were seen in *A. divaricatum*. Several taxa have been sequenced twice from either the same genomic DNA sample or another crude seed extract. Only very rarely were true polymorphisms detected, and these were then coded in the matrix as ambiguous nucleotides.

Phylogenetic analyses—When the data matrix composed of 22 taxa was analyzed, six equally parsimonious trees of 738 steps resulted. The strict consensus cladogram of these trees is shown in Fig. 2. The same topology (for 20 taxa) was obtained when *A. oxycedri* and *A. abietis-religiosae* were excluded. In the latter analysis, *A. americanum* and *A. verticilliflorum* were designated the outgroup for rooting purposes. As expected from their amount of sequence divergence, *A. abietis-religiosae* and *A. oxycedri* form a clade well removed from the remainder of the genus. The clade composed of *A. guatemalense* and *A. pendens* is positioned intermediate between subgenus *Arceuthobium* and the remaining taxa. *A. divaricatum* and *A. globosum* form a clade in four of the six trees. Four clades of unresolved relationships to one another include: 1) the four Section *Campylopoda* species plus *A. douglasii*; 2) *A. pusillum* and *A. bicarinatum*; 3) *A. vaginatum* (both subspecies), *A. durangense*, and *A. strictum*; and 4) *A. gillii*, *A. nigrum*, and *A. rubrum*.

The bootstrap analysis showed strong support for several of the above clades (Fig. 3). For example, 100% bootstrap values were obtained for four of the above groups: 1) *A. americanum* and *A. verticilliflorum*; 2) *A. guatemalense* and *A. pendens*; 3) *A. pusillum* and *A. bicarinatum*; and 4) the four Section *Campylopoda* species. High bootstrap support was obtained for the *A. vaginatum/A. strictum* group (90%) and the *A. gillii/A. rubrum* group (75%). Three taxa arose from a polytomy which included groups 3 and 4 (above, *A. douglasii*, *A. divaricatum*, and *A. globosum*.

DISCUSSION

The ITS region of dwarf mistletoes is like that of other members of the Asteridae (such as *Daucus* and *Mimulus*) in having a shorter ITS-1 than ITS-2. In most classifications, Viscaceae have been placed in the Rosidae; how-

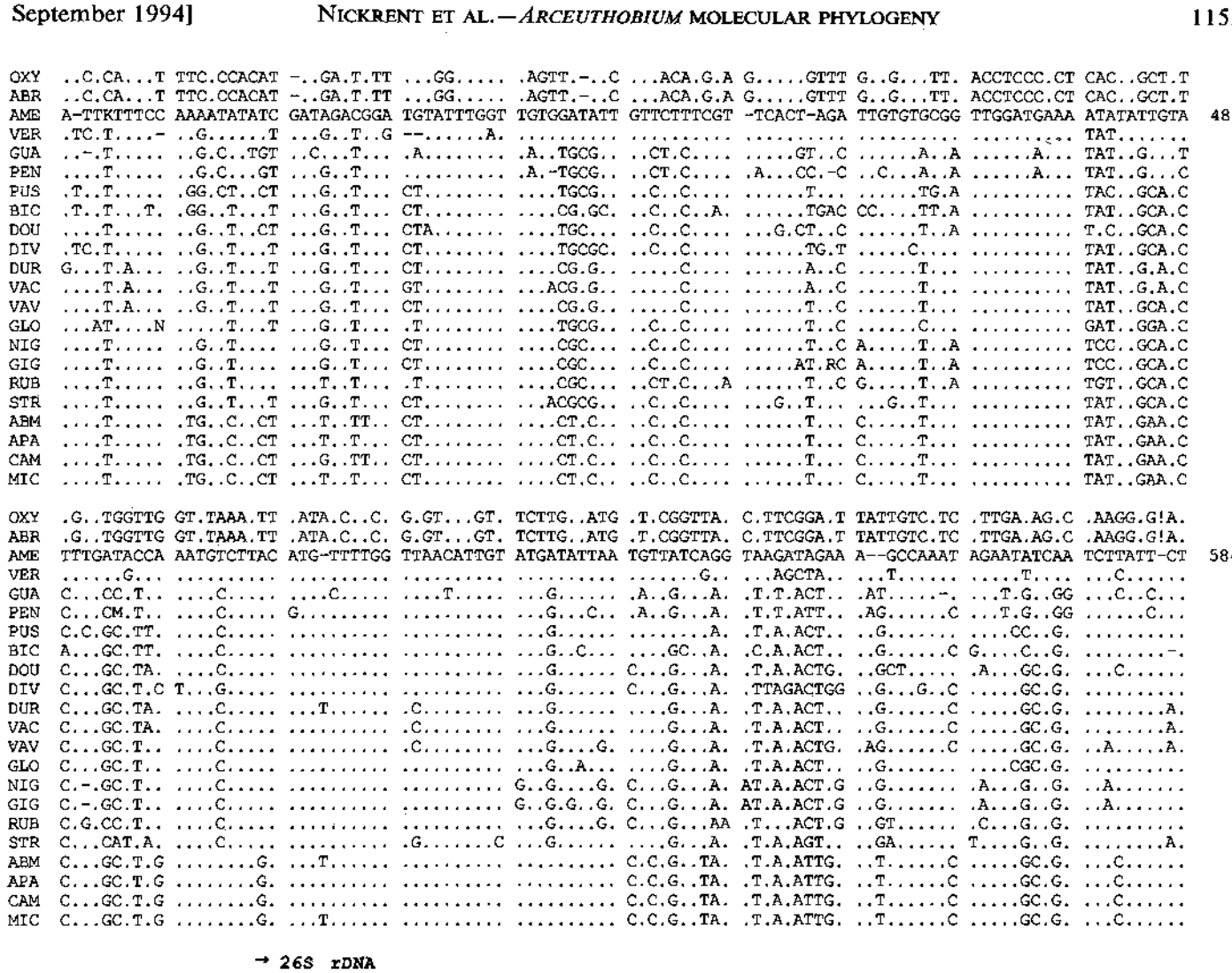

Fig. 1. Continued.

ever, recent molecular work utilizing *rbc*L sequences place the sandalwood order (Santalales) at the base of an expanded Asteridae (Chase et al., 1993). As with other studies comparing ITS sequences among congeneric plant species (Baldwin, 1993; Wojciechowski et al., 1993), most mutations in the dwarf mistletoe ITS region involve base substitutions, not indels. Unlike other plant ITS regions examined to date, interspecific distances in *Arceuthobium* vary widely and can attain levels seen in intergeneric comparisons. For example, distances among *Calycadenia* species ranged from 0 to 11.2% for ITS-1 and 0 to 8.6% for ITS-2 (Baldwin, 1993). Similarly, *Astragalus* interspecific distances reached a maximum of 10% (Wojciechowski et al., 1993). High substitution rates can also be seen in the 18S rRNA of *Arceuthobium*, which has been sequenced in its entirety for *A. verticilliflorum*, *A. oxycedri*, and *A. pendens* (Nickrent and Starr, 1994; Nickrent, in press). An increased number of substitutions were also detected in dwarf mistletoe *rbc*L sequences when compared with other Santalales parasites. The possible causes underlying increased evolutionary rates in parasitic plants (in particular holoparasites) is discussed in Nickrent and Starr (1994). It is suggested that rapid molecular evolution in *Arceuthobium* is associated with its adaptive radiation on numerous conifer hosts.

Section Campylopoda—Ten species used in this sequencing study were previously classified as members of Section *Campylopoda* by Hawksworth and Wiens (1972, 1984, Table 1). Relationships among these ten species will be discussed here; however, it is evident that the ITS

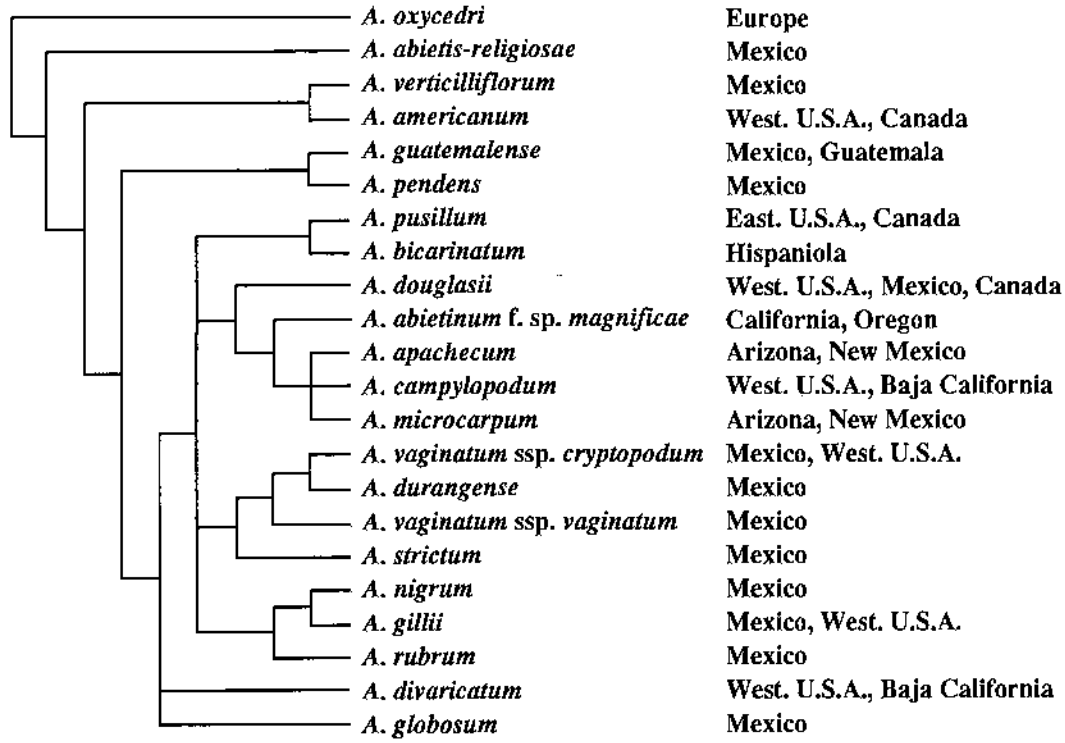

Fig. 2. Strict consensus cladogram derived from six equally parsimonious trees of length 738 produced from analysis of *Arceuthobium* ITS-1, -2, and 5.8S rDNA sequences. Consistency index = 0.730, homoplasy index = 0.270, retention index = 0.747. The tree is rooted at *A. oxycedri* and *A. abietis-religiosae* following a topology derived from analysis of complete 18S rDNA sequences. The general geographic distributions of the species are indicated.

analysis does not indicate they form a monophyletic group. Four members of Section *Campylopoda* are genetically very similar and appear on a clade with relatively short branch lengths: *A. abietinum* f. sp. *magnificae, A. apachecum, A. campylopodum,* and *A. microcarpum.* Sequences of *A. cyanocarpum, A. occidentale,* and *A. tsugense* have also been determined (Nickrent, unpublished data) but are either very similar or identical to those of the above four taxa, hence they were not included. The Section *Campylopoda* clade, comparable to Series *Campylopoda* (Hawksworth and Wiens, 1972, 1984), occurs mainly in the United States and does not include Mexican and Caribbean taxa such as *A. guatemalense, A. pendens, A. rubrum, A. bicarinatum,* and *A. strictum.* The latter three species were segregated into separate series (*Rubra* and *Stricta*) by Hawkworth and Wiens (1972), thus providing some indication of their differentiation from Series *Campylopoda* species. ITS sequence analysis does not support a close relationship between members of these series, but rather indicates relationships to various members of Section *Vaginata.*

One of the most strongly supported results of the ITS analysis (100% bootstrap) is the association of *A. pendens* with *A. guatemalense.* This clade appears basal to all other members of subgenus *Vaginata,* not as a component of Section *Campylopoda,* Series *Campylopoda* (Hawksworth and Wiens, 1972, 1984). *Arceuthobium guatemalense* is a rare mistletoe confined to the mountains of Guatemala and southern Mexico where it parasitizes *Pinus ayacahuite. Arceuthobium pendens* is known only from San Luis Potosí and Veracruz, Mexico, and is parasitic on *Pinus discolor* and *P. cembroides* var. *orizabensis* (Hawksworth and Wiens, 1980). Both of these mistletoes are narrowly endemic, as are their hosts. Given their positions on the cladogram and their restricted distributions, it can be postulated that these species represent relictual taxa that diverged early during the migration and evolution of *Arceuthobium* in the New World.

Arceuthobium divaricatum is a parasite of pinyon pine that was previously classified as a member of Section *Campylopoda,* Series *Campylopoda* (Hawksworth and Wiens, 1972, 1984). Following isozyme analysis (Nickrent, 1986), this species clustered with *A. douglasii,* a parasite of *Pseudotsuga.* This surprising relationship was supported by a large number of unique alleles shared by these species (Nickrent, 1986). In four of the six most parsimonious trees, *A. divaricatum* is sister to *A. globosum*; however, this position is unstable as seen following bootstrap analysis where these two species (and *A. douglasii*) collapse to form a polytomy. When ITS-1 alone is analyzed (tree not shown), the sister relationship of *A. divaricatum* and *A. douglasii* received high bootstrap values. Examination of the alignment shows that *A. divaricatum* has a divergent ITS-2 sequence as compared with other members of subgenus *Vaginata.* Taking the isozyme and ITS evidence together, a clade composed of these two taxa is presently favored.

Section Vaginata—Two strongly supported clades representing Section *Vaginata* are seen following bootstrap

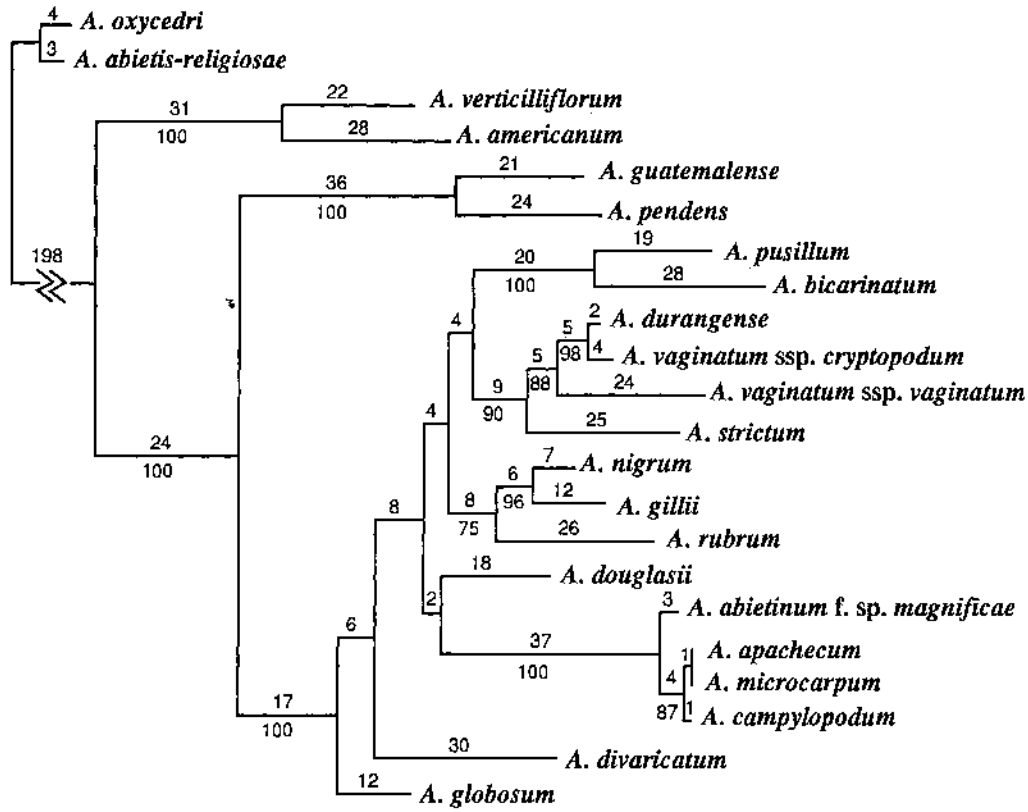

Fig. 3. One of the six equally parsimonious trees of length 738 derived from analysis of *Arceuthobium* ITS-1, -2, and 5.8S rDNA sequences. The numbers above the branches indicate branch lengths (number of nucleotide substitutions), and the numbers below the branches indicate percentage values (from 200 replications) derived from the bootstrap majority rule consensus tree. Clades with no bootstrap value indicated were not strongly supported. Consistency index = 0.755, homoplasy index = 0.286, retention index = 0.726.

analysis (Fig. 3). The first is composed of *A. vaginatum* ssp. *vaginatum*, *A. vaginatum* ssp. *cryptopodum*, *A. durangense*, and *A. strictum*. The relationships among the first three of these taxa have been shown by previous classifications (Hawksworth and Wiens, 1972) and isozyme studies (Nickrent, 1986; Nickrent, in press). The addition of *A. strictum*, an endemic species from Durango, Mexico, is somewhat surprising, although isozyme analysis showed it to be genetically divergent from other Series *Campylopoda* species. All four of these taxa are parasites of hard pines and their distributions range from the northern Sierra Madre Occidental (Durango through Chihuahua and Sonora) to the southwestern United States (Arizona, New Mexico).

The second clade is composed of *A. gillii* and its recent segregate species *A. nigrum*, as well as *A. rubrum*. The distance derived from comparing ITS sequences for the first two species is 3.2%. This value is among the smallest distances obtained when values for all species pairs are compared, but it is not as small as distances between members of Series *Campylopoda*, which are 1.3% or less. The association of *A. gillii* and *A. nigrum* is strongly supported by ITS analysis (96% bootstrap). Bootstrap support is 75% for the entire clade containing *A. rubrum*. Since the grouping of these three taxa was seen using isozyme characters, phyletic affinity is highly likely.

A. pusillum and A. bicarinatum—A surprising but strongly supported clade (100% bootstrap, Fig. 3) contains *A. pusillum* and *A. bicarinatum*. The former species is a reduced parasite of spruce of the northern United States and Canada, whereas the latter is a relatively large parasite of *Pinus occidentalis* on the island of Hispaniola. Hawksworth and Wiens (1972) classified *A. pusillum* and *A. douglasii* in Section *Minuta*; however, this relationship was not supported by isozyme analysis (Nickrent, 1986). They also suggested that *A. bicarinatum* arrived in Hispaniola via a Central American land bridge that was connected to Honduras during the late Tertiary. This route seemed plausible given the morphological similarity between *A. bicarinatum* and *A. hondurense* (the ITS of the latter species is yet to be determined). The distance from Hispaniola to the nearest extant population of *A. pusillum* is ca. 2,300 km. *A. bicarinatum* and *A. hondurense* are presently separated by ca. 1,100 km.

Given these biogeographical distributions and the DNA sequence results, an alternate hypothesis regarding these two mistletoes is suggested. Several plant species found at high elevations in Hispaniola are known to have close relatives in the eastern United States such as *Lyonia* (Judd, 1981) and *Juniperus* (Adams, 1989). Hawksworth and Wiens (1972) suggested that *Arceuthobium* arrived in the New World in the early Tertiary via a Beringian land

bridge. If one accepts that the Neogene microthermal vegetation was largely derived from the preceding flora of that area (Wolfe, 1975), then the ancestor to *A. bicarinatum* and *A. pusillum* was likely present in (eastern) North America in the Paleogene. *Arceuthobium* pollen (likely *A. pusillum*) is known from as far south as Georgia from the Pleistocene (Watts, 1975); hence dwarf mistletoes have occupied the eastern United states in past geological times. Evidence that some *Arceuthobium* species such as *A. oxycedri* were already well differentiated by the Miocene (Stuchlik, 1964) also suggests evolution of the genus in the early or mid-Tertiary.

The configuration of the Caribbean region with respect to North and Central America during the Tertiary is an area of active research. In the later Paleocene to mid Eocene, the Greater Antilles collided with the Bahama Platform (Pindell and Barrett, 1990) connecting Cuba to the North American Plate. By the Miocene, Hispaniola was contiguous with the eastern part of Cuba, and northeastward displacement was occurring along the Oriental Fault. The connection between Honduras, Jamaica, and the Greater Antilles via the Nicaraguan rise was likely severed during the middle Cenozoic owing to subsidence. This route to Hispaniola (via Central America) was proposed by Rosen (1975) as a vicariance pathway. Given the molecular evidence, entry into Hispaniola via eastern North America and Cuba is favored over a southwest track (via Honduras). The present lack of parasitism of low elevation pines in Honduras, Cuba, Hispaniola, and the southeastern United States indicates that this ancestral species was either already adapted to high elevation hosts or that, subsequent to speciation, the low elevation parasites became extinct. If *A. hondurense* is found to be genetically similar to *A. bicarinatum*, this does not favor one migration track over the other. That species would simply be another relictual taxon derived from the originally widespread ancestor. Given the overall climatic deterioration that took place in eastern North America during the Oligocene, and the accompanying extinctions (Tiffney, 1985), this ancestral mistletoe is likely now extinct. The isolated position of *A. bicarinatum* on high elevation pines of Hispaniola is indicative of a Tertiary relic.

The morphological differences between *A. pusillum* and *A. bicarinatum* stand in contrast to their high level of genetic similarity. High genetic variability as measured by isozymes and increased substitution rate at the nuclear ribosomal cistron suggests that *A. pusillum* is more genotypically variable than is outwardly apparent from its morphology. Sufficient diversity of pathogenicity genes apparently exists to allow this species to colonize *Larix laricina, Pinus strobus, P. resinosa,* and *P. banksiana,* in addition to spruces (*Picea mariana, P. glauca,* and *P. rubens*), its principal hosts (Hawksworth and Wiens, 1972). It is hypothesized that *A. pusillum* and *A. bicarinatum* represent the morphologically divergent endpoints of lineages that have been altered by quite different evolutionary forces. The above genetic data on the modern species suggest that their ancestor likely possessed a large store of potential genetic variation that was manifested following diversifying selection. Increased fitness was attained by the separate populations exploiting different environments and hosts. The reduction in shoot height, systemic broom formation, spring flowering, and short fruit maturation time seen in *A. pusillum* may represent adaptations to greater winter extremes, as occurred in other eastern North American plant species.

Subgenus Arceuthobium—One of the most striking results of this study of ITS variation in *Arceuthobium* is the extreme divergence of *A. abietis-religiosae* and *A. oxycedri* from the remaining sampled taxa. In addition, the sequences of these two members of subgenus *Arceuthobium* are remarkably similar to each other. Subgenus *Arceuthobium* also showed a greater range of genetic variability than subgenus *Vaginata* using isozyme markers (Nickrent, in press). These results require a reexamination of concepts regarding relationships among the three New World members of subgenus *Arceuthobium* and their relationships to the Old World species.

Arceuthobium americanum and *A. verticilliflorum* are more closely related to each other than to any other species as shown by 100% bootstrap confidence for their clade. This clade has more affinity with subgenus *Vaginatum* than with *A. abietis-religiosae* and *A. oxycedri,* which indicates a major divergence in the verticillately branched group during their evolution in the New World. Subgenus *Vaginata* was then apparently derived from an ancestor shared with *A. americanum* and *A. verticilliflorum.* The extreme divergence between *A. abietis-religiosae/A. oxycedri* and the remaining species could also be interpreted as evidence of separate migrations into the New World. Further molecular work with *Arceuthobium* would greatly benefit from the inclusion of additional Old World taxa, i.e., *A. azoricum, A. chinense, A. juniperi-procerae, A. minutissimum, A. pini,* and *A. tibetense.*

With reference to the above findings on *Arceuthobium* phylogenetic relationships, it is worth noting the observations made by Sytsma and Smith (1992) regarding concordance or discordance between morphological and molecular divergence in *Clarkia* and *Fuchsia.* These authors described four syndromes: 1) low morphological and molecular divergence; 2) high morphological and high DNA divergence; 3) low morphological but high DNA divergence; and 4) high morphological but low DNA divergence. For the first syndrome, they give as an example *Clarkia ligulata* and *C. biloba,* which is comparable to Section *Campylopoda* in *Arceuthobium.* The second syndrome was illustrated by comparing Sections *Myxocarpa* and *Eucharidum* of *Clarkia* or the Old World Section *Skinnera* with New World sections. In *Arceuthobium,* the most morphological and molecular divergence is seen between the representatives of the two subgenera, *Arceuthobium* and *Vaginata.* The third syndrome can be demonstrated by comparing *Clarkia rostrata* with *C. lewisii* and *C. cylindrica.* In dwarf mistletoes, a low amount of morphological divergence is seen between *A. pendens* and *A. guatemalense* as compared with Series *Campylopoda* species; however, DNA evidence indicates these two groups are not closely related. Finally, the fourth syndrome can be seen in comparisons of *Clarkia rostrata* and *C. epilobioides,* which differ in breeding system and floral morphology but are genetically extremely similar. For *Arceuthobium,* this syndrome is best shown by *A. pusillum* and *A. bicarinatum* or by *A. douglasii* and *A. divaricatum.*

TABLE 3. Classification of *Arceuthobium* species used for ITS sequencing.

Subgenus *Arceuthobium* Hawksw. & Wiens
Section *Arceuthobium* Nickrent [Sec. Nov.]
A. abietis-religiosae Heil
A. oxycedri (DC) M. Bieb.
Section *Americana* Nickrent [Sec. Nov.]
A. americanum Nutt. ex Engelm.
A. verticilliflorum Engelm.
Subgenus *Vaginata* Hawksw. & Wiens
Section *Penda* Nickrent [Sec. Nov.]
A. guatemalense Hawksw. & Wiens
A. pendens Hawksw. & Wiens
Section *Vaginata* Hawksw. & Wiens
Series *Globosa* Nickrent [Ser. Nov.]
A. globosum subsp. *globosum* Hawksw. & Wiens
Series *Rubra* Hawksw. & Wiens
A. gillii Hawksw. & Wiens
A. nigrum (Hawksw. & Wiens) Hawksw. & Wiens
A. rubrum Hawksw. & Wiens
Series *Vaginata* Hawksw. & Wiens
A. durangense (Hawksw. & Wiens) Hawksw. & Wiens
A. strictum Hawksw. & Wiens
A. vaginatum (Wild.) Presl. subsp. *vaginatum*
A. vaginatum subsp. *cryptopodum* (Engelm.) H. & Wiens
Series *Minuta* Hawksw. & Wiens
A. divaricatum Engelm.
A. douglasii Engelm.
Section *Pusilla* Nickrent [Sec. Nov.]
A. bicarinatum Urban.
A. pusillum Peck.
Section *Campylopoda* Hawksw. & Wiens
A. abietinum (Engelm.) Hawksw. & Wiens f. sp. *magnificae*
A. apachecum Hawksw. & Wiens
A. campylopodum Engelm.
A. microcarpum (Engelm.) Hawksw. & Wiens

Summary of phylogenetic relationships—The division of the genus into two subgenera (*Arceuthobium* and *Vaginata*) is supported by analyses of all types of data. From the ITS sequence data, the New World members of subgenus *Arceuthobium* comprise two groups. One species, *A. abietis-religiosae*, is genetically very similar to the Old World *A. oxycedri*. Both of these species are genetically distinct from other New World members of the subgenus. This high amount of genetic differentiation is also seen between *A. oxycedri* and both *A. verticilliflorum* and *A. pendens* when the more conservative 18S rDNA sequences are compared (Nickrent, in press). Subgenus *Vaginata*, Section *Vaginata* includes all the taxa previously classified at this rank (e.g., *A. durangense*, *A. gillii*, *A. globosum*, *A. nigrum*, and *A. vaginatum*) as well as several taxa previously placed in three series of Section *Campylopoda* (i.e., *A. divaricatum*, *A. rubrum*, and *A. strictum*). As also shown by isozyme analysis, *A. globosum* occupies a basal position within the section. A relationship between *A. douglasii* and *A. pusillum* is not supported in either the isozyme or the DNA analyses; therefore, Section *Minuta* should be reformulated. These species represent different endpoints of similar morphological grades. The association of *A. douglasii* with *A. divaricatum* receives strong support from isozyme and ITS-1 sequence data, thus indicating that these species share a common ancestor. *A. pusillum* is genetically very similar to *A. bicarinatum* but has attained a large number of morphological and physiological apomorphies, possibly as adaptations to colder climates. These two taxa are likely survivors of an ancient lineage whose most recent common ancestor became extinct during the Tertiary. Section *Campylopoda* now comprises 13 mainly U.S. species that show a high degree of morphological and genetic similarity. Two additional segregates of Section *Campylopoda* that are deserving of classification in a new Section (*Penda*) are the endemics *A. pendens* and *A. guatemalense*, also likely Tertiary relicts.

Phylogenetic relationships in *Arceuthobium* have been continually reexamined and reshaped following the introduction of new evidence from morphology, life cycles, isozymes, and now ITS sequences. By synthesizing the existing information, a new classification for the dwarf mistletoes examined in this study is proposed (Table 3). An expanded classification encompassing all species of *Arceuthobium* worldwide is given in Nickrent (in press). These plants continue to present a challenge to systematists as do all parasites that follow reductional and/or convergent evolutionary paths.

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