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Molecular Systematics of *Arceuthobium*

Daniel L. Nickrent
Department of Plant Biology
Southern Illinois University
Carbondale, IL

Although thousands of references to dwarf mistletoes exist, relatively little work has been completed on the systematics or phylogenetic relationships of the genus *Arceuthobium*. Molecular analyses of intergeneric or interspecific relationships are even fewer. The intent of this paper is to review those molecular studies that have been conducted thus far.

Isozyme Analyses of Interspecific Relationships

Isozyme electrophoresis has been extensively used to address population genetics, breeding systems, and systematic relationships in plants (Soltis and Soltis 1989). Because of the extreme reduction associated with the parasitic habit and the resulting paucity of morphological characters, isozyme electrophoresis has proven especially valuable in providing useful data for examining species relationships of *Arceuthobium*. Nickrent and others (1984) and Nickrent (1986) first examined the isozymes of 19 North American taxa of *Arceuthobium*. From those studies, it was shown that the genus has remarkably high levels of genetic diversity - 67% of the loci were polymorphic and averaged 2.23 alleles per locus. These results were surprising given the relative homogeneity of the genus with regards to morphology and flavonoid composition (Crawford and Hawksworth 1979, Hawksworth and Wiens 1972).

Many of the results from this electrophoretic study were consistent with the taxonomic classification of Hawksworth and Wiens (1972, 1984), including the recognition of two subgenera (*Arceuthobium* and *Vaginata*), the close relationship among species of section *Campylopoda*, and the clustering of *A. gillii* (sensu lato) and *A. vaginatum* (sensu lato). Isozyme analysis did not, however, support placement of *A. douglasii* and *A. pusillum* together (e.g. in section *Minuta*). An unexpected result of this study was the grouping of *A. douglasii*, a parasite of *Pseudotsuga menziesii*, with *A. divaricatum*, a parasite of pinyons. This work clearly raised as many questions as it resolved, hence further analyses were needed, especially on Mexican and Central American taxa.

More recently, an electrophoretic study was completed that included genetic data on 13 populations of seven Mexican taxa that were not included in the previous study. Inclusion of these taxa was important to better understand species and sectional relationships in *Arceuthobium*. These data were combined with isozyme data for the 19 taxa previously examined (Nickrent 1986) yielding a total of 26 taxa.

Genetic variability statistics for a total of 36 dwarf mistletoe populations is presented in Table 1. The overall mean percentage of polymorphic loci is 48.7% and the mean number of alleles per locus is 2.15. These values are lower than those reported in Nickrent (1986) because several populations of Mexican dwarf mistletoes with unusually low levels of polymorphism were included - *Arceuthobium abietis-religiosa*, *A. globosum* subsp. *grandicaule*, *A. pendens*, *A. rubrum*, *A. strictum*, and *A. verticilliflorum*. The mean number of alleles per locus for these 6 taxa (1.5) is lower than the overall mean for the genus (2.15); this reduction in allele frequency

may be a consequence of their restricted distributions. However, two other Mexican taxa, *A. vaginatum* subsp. *vaginatum* and *A. durangense*, show high levels of polymorphism that are comparable to levels found in a related and more northern taxon, *A. vaginatum* subsp. *cryptopodum*. This contrast in species-to-species variation in polymorphism agrees with conclusions of Nickrent (1986) and Hawksworth and Wiens (1972) that section *Vaginata* (which includes *A. vaginatum* and *A. durangense*) is genetically and morphologically more variable than other sections in the genus. Although *A. globosum* subsp. *grandicaule* shows a low level of polymorphism and is also in section *Vaginata*, only 30 individuals from one population were examined electrophoretically, hence this sample may not be representative of the species' genetic diversity.

With this new isozyme data, additional insight into the phylogenetic relationships of many of the dwarf mistletoe taxa occurring in North America can be gained (Fig. 1). *Arceuthobium strictum* is distantly related to most other taxa in section *Campylopoda*, but is closely related to *A. pendens*. This is not totally in conflict with the classification of Hawksworth and Wiens (1972) in which the former species was placed in its own series (Stricta). It appears, however, that the majority of species in section *Campylopoda* are found in the United States, whereas species in section *Vaginata* predominate in Mexico. The two parasites of pinyons, *Arceuthobium divaricatum* and *A. pendens*, are not closely related. This conclusion is supported by the isozyme data, markedly different flavonoid chemistry, systemic broom formation in *A. pendens*, and different hosts (Hawksworth and Wiens 1980). *Arceuthobium gillii* and *A. nigrum* are clearly related but cluster at a genetic distance value that indicates they are indeed different species. The relatives of *A. vaginatum* have high levels of genetic diversity, and cluster analysis indicates a substantial genetic differentiation between populations. Further work is required to better understand apportionment of genetic variation in the *Arceuthobium vaginatum* complex.

Subgenus *Arceuthobium* shows greater within-group heterogeneity than any of the other groups. Despite the variation, cluster analysis places *Arceuthobium abietis-religiosa*, *A. americanum*, and *A. verticilliflorum* in one group that joins the remainder of the species at a genetic distance of 0.82. Thus, isozyme data support use of verticillate secondary branching (Mark and Hawksworth 1981) as a distinguishing character for the subgenus.

Additional isozyme analyses have been conducted on various members of the *Arceuthobium campylopodum* complex (Nickrent and Butler 1990, 1991, Nickrent and Stell 1990). These studies utilized diploid shoot tissue as a source of isozymes, hence a larger number of population genetic analyses could be conducted. Based upon cluster analysis, California coastal populations of *Arceuthobium* parasitic on *Pinus radiata* and *P. muricata* are genetically distinct from *A. campylopodum* (sensu stricto) and are segregated at the specific level as *A. littorum* (Hawksworth and others 1992). Examination of isozymes from allopatric and sympatric populations of *A. campylopodum* and *A. occidentale* did not reveal significant genetic differentiation, hence they could be considered a single biological species.

Nickrent and Butler (1991) examined genetic relationships among dwarf mistletoes related to *Arceuthobium campylopodum* that are parasitic on *Pinus attenuata* and *P. monticola* in northwestern California and southwestern Oregon. The parasites of *P. attenuata* and *P. monticola* were genetically distinct from each other. These species were named, respectively, *A. siskiyouense* and *A. monticola* (Hawksworth and others 1992). This result is in accordance with the observation that the Klamath-Siskiyou Mountain region contains a highly diverse and endemic flora. These dwarf mistletoes likely represent the most recent evolutionary lines to diverge from the *A. campylopodum* complex.

The results from electrophoretic examination of the three host-forms of *Arceuthobium tsugense* (mountain hemlock, western hemlock, and shore pine) indicate that the population

infecting *Tsuga mertensiana* is genetically distinct and deserving of taxonomic recognition as *A. tsugense* subsp. *mertensianae* (Nickrent and Stell 1990, Hawksworth and others 1992). *Arceuthobium tsugense* subsp. *tsugense* consists of two morphologically similar host races parasitic on *Tsuga heterophylla* (western hemlock race) and *Pinus contorta* subsp. *contorta* (shore pine race). Isozyme analysis failed to detect significant genetic differentiation between these two host-forms, hence they were retained within the same subspecies.

Species Relationships From Ribosomal DNA Spacer Sequences

Many studies addressing interspecific relationships in plants have been conducted using restriction site data from chloroplast DNA (Soltis and others 1992). Few studies have used DNA sequences to examine interspecific relationships because a gene or segment of DNA that is of adequate size and that evolves at a sufficiently fast rate is required. Recently, the internal transcribed spacer (ITS) regions of the ribosomal DNA cistron have been shown to evolve at rates appropriate for examining more recently diverging lineages (Baldwin 1993, Baldwin and others 1995). Sequences of the ITS regions and the enclosed 5.8S rDNA were analyzed for 22 species of *Arceuthobium*, thus allowing a comparison with phylogenies generated from other methods.

Based on the results from analysis of ITS sequences (Fig. 2), four members of section *Campylopoda* are genetically very similar - *A. abietinum* f. sp. *magnificae*, *A. apachecum*, *A. campylopodum*, and *A. microcarpum*. Sequences of *A. cyanocarpum*, *A. occidentale*, and *A. tsugense* were either very similar or identical to those of the above four taxa. Species in section *Campylopoda*, comparable to series *Campylopoda* (Hawksworth and Wiens 1972, 1984), occur mainly in the United States. Mexican and Caribbean species such as *A. guatemalense*, *A. pendens*, *A. rubrum*, *A. bicarinatum*, and *A. strictum* are not closely related to the U.S. taxa. The latter three of these species were previously segregated into series *Rubra* (*A. rubrum* and *A. bicarinatum*) and *Stricta* (*A. strictum*) by Hawksworth and Wiens (1972), thus providing some indication of their differentiation from series *Campylopoda*.

A strongly supported result of the ITS analysis is the association of *Arceuthobium guatemalense* with *A. pendens*. *Arceuthobium guatemalense* is confined to the mountains of Guatemala and southern Mexico, where it parasitizes *Pinus ayacahuite* (of subgenus *Haploxylon*). *Arceuthobium* pendens is known only from Puebla, San Luis Potosi, and Veracruz, Mexico and is parasitic on the *Haploxylon* pines, *Pinus discolor* and *P. orizabensis*. Both of these mistletoes and their hosts are narrow endemics indicating that these species could represent relictual taxa that diverged early during the migration and evolution of *Arceuthobium* in the New World.

As seen with isozymes, ITS data do not support a relationships between *A. douglasii* and *A. pusillum*. The relationship between this species and *A. divaricatum* is strongly supported by analysis of ITS-1 sequences only.

Analysis of ITS sequences strongly supports two clades representing section *Vaginata*. The first is composed of *Arceuthobium vaginatum* subsp. *vaginatum*, *A. vaginatum* subsp. *cryptopodum*, *A. durangense*, and *A. strictum*. Previous classifications (Hawksworth and Wiens 1972) and isozyme studies (Nickrent 1986) have shown relationships among the first three of these taxa. The addition of *A. strictum* is surprising, although isozyme analysis had shown genetic divergence between it and species in series *Campylopoda*. All four of these taxa parasitize pines of subgenus *Diploxylon* and their distributions range from the northern Sierra Madre Occidental (Durango through Chihuahua and Sonora) to the southwestern United States. The second section *Vaginata* clade is composed of *Arceuthobium rubrum*, *A. gillii*, and its recent

segregate species, *A. nigrum*. The association of *A. gillii* with *A. nigrum* is strongly supported by ITS analysis as is the association of *A. rubrum* with this clade. Because isozyme characters also indicate a grouping of these three species, their phyletic affinity is highly probable.

A surprising, but strongly supported clade contains *Arceuthobium pusillum* and *A. bicarinatum*. The former species is a reduced parasite of spruce of the northern United States and Canada, and the latter is a relatively large parasite of Pinus occidentalis on the island of Hispaniola. Given these geographic distributions and the DNA sequence results, it is likely that these species' ancestor was already present in eastern North America in the early Tertiary Period and was widely distributed throughout eastern North America as well as the Caribbean region. Populations of this ancestral taxon became geographically isolated during the middle Cenozoic Era. The current genetic data on *A. bicarinatum* and *A. pusillum* suggest their ancestor likely possessed a large store of potential genetic variation that became manifest following diversifying selection. Increased fitness was attained by the geographically separated populations exploiting different environments and hosts. The reduction in shoot height, systemic broom formation, spring flowering, and rapid fruit maturation seen in A. pusillum may represent adaptations to greater winter extremes, while *A. bicarinatum* retained more characteristics of the ancestral taxon because of its subtropical distribution.

Two of the most striking results of my study of ITS variation are the extreme divergence of *Arceuthobium abietis-religiosa* and *A. oxycedri* from the other taxa sampled and the similarity of these species to each other. These results require a modification of concepts regarding relationships among the three New World members of subgenus *Arceuthobium* as well as their relationship to Old World members of this subgenus.

Arceuthobium americanum and *A. verticilliflorum* are more closely related to each other than to any other species. In addition, they have more affinity with subgenus *Vaginata* than with *A. abietis-religiosa* and *A. oxycedri*, thus indicating a major divergence in the verticillately branched group during their evolution in the New World. Subgenus *Vaginata* was apparently derived from an ancestor shared with *A. americanum* and *A. verticilliflorum*. The extreme divergence of *A. abietis-religiosa* and *A. oxycedri* from the remaining species also could be interpreted as evidence of separate migrations into the New World. Further molecular work would greatly benefit from inclusion of additional Old World species, such as *A. azoricum*, *A. chinense*, *A. juniperi-procerae*, *A. minutissimum*, *A. pini*, and *A. tibetense*.

There is no doubt that the dwarf mistletoes continue to present a challenge to the plant systematist, as do all parasitic flowering plants that follow reductional and/or convergent evolutionary paths. These plants provide the ultimate test of our abilities to reconstruct phylogenies, therefore, alternate data sets for other genes must be assembled to confirm or support proposed phylogenetic relationships.

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Table 1. Genetic Variability at 9 Isozyme Loci in 36 Populations of 25 *Arceuthobium* Species

Population No.	Coll. No.	Taxon[1]	Mean sample size per locus	Mean No. Alleles per locus	Percentage of loci polymorphic[2]	Hardy-Weinberg Expected Heterozygosity[3]
1.	1917	ABC	42.2	2.0	55.6	0.206
2.	1906	ABM	54.7	2.3	44.4	0.183
3.	1983	ABR	34.3	2.0	55.6	0.250
4.	2010	ABR	61.6	1.4	33.3	0.109
5.	1932	AME	47.7	3.0	55.6	0.200
6.	1929	AME	35.8	3.8	77.8	0.351
7.	1945	APA	61.7	2.0	44.4	0.210
8.	1937	BLU	35.1	2.0	22.2	0.133
9.	1930	CAL	49.6	2.6	66.7	0.253
10.	1924	CAM	28.8	2.1	55.6	0.211
11.	1973	CYA	17.6	1.7	44.4	0.124
12.	1953	DIV	80.3	2.7	55.6	0.209
13.	1941	DOU	27.7	2.1	44.4	0.228
14.	1949	DOU	44.6	2.4	33.3	0.192
15.	1870	DUR	26.7	2.9	66.7	0.265
16.	2049	DUR	9.3	2.3	66.7	0.299
17.	2051	DUR	26.8	3.3	77.8	0.365
18.	1938	GIG	77.8	2.8	44.4	0.198
19.	1996	GLG	27.2	1.6	11.1	0.092
20.	1801	LAR	21.4	1.7	55.6	0.170
21.	1947	MIC	19.1	1.7	33.3	0.154
22.	2041	NIG	31.7	2.4	66.7	0.269
23.	1962	OCC	37.8	2.0	55.6	0.184
24.	1992	PEN	20.8	1.8	33.3	0.106
25.	1970	PUS	50.9	2.0	22.2	0.103
26.	1971	PUS	30.8	1.7	44.4	0.166
27.	1853	RUB	8.3	1.3	33.3	0.139
28.	2061	STR	24.2	1.1	11.1	0.039
29.	1927	TSM	10.0	1.9	66.7	0.290
30.	1876	VAC	53.0	2.7	44.4	0.172
31.	1964	VAC	30.2	2.8	55.6	0.256
32.	2059	VAV	8.9	2.0	66.7	0.230
33.	1980	VAV	29.8	2.4	66.7	0.274
34.	1981	VAV	15.4	1.9	66.7	0.233
35.	2001	VER	12.8	1.7	44.4	0.175
36.	2065	VER	4.6	1.3	33.3	0.169
Means			33.3	2.15	48.7	0.200

[1] See Table 16-2 for taxon abbreviations

[2] A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95

[3] Unbiased estimate (Nei, 1978). Direct count heterozygosity not possible given the allelic data was derived from triploid genotypes

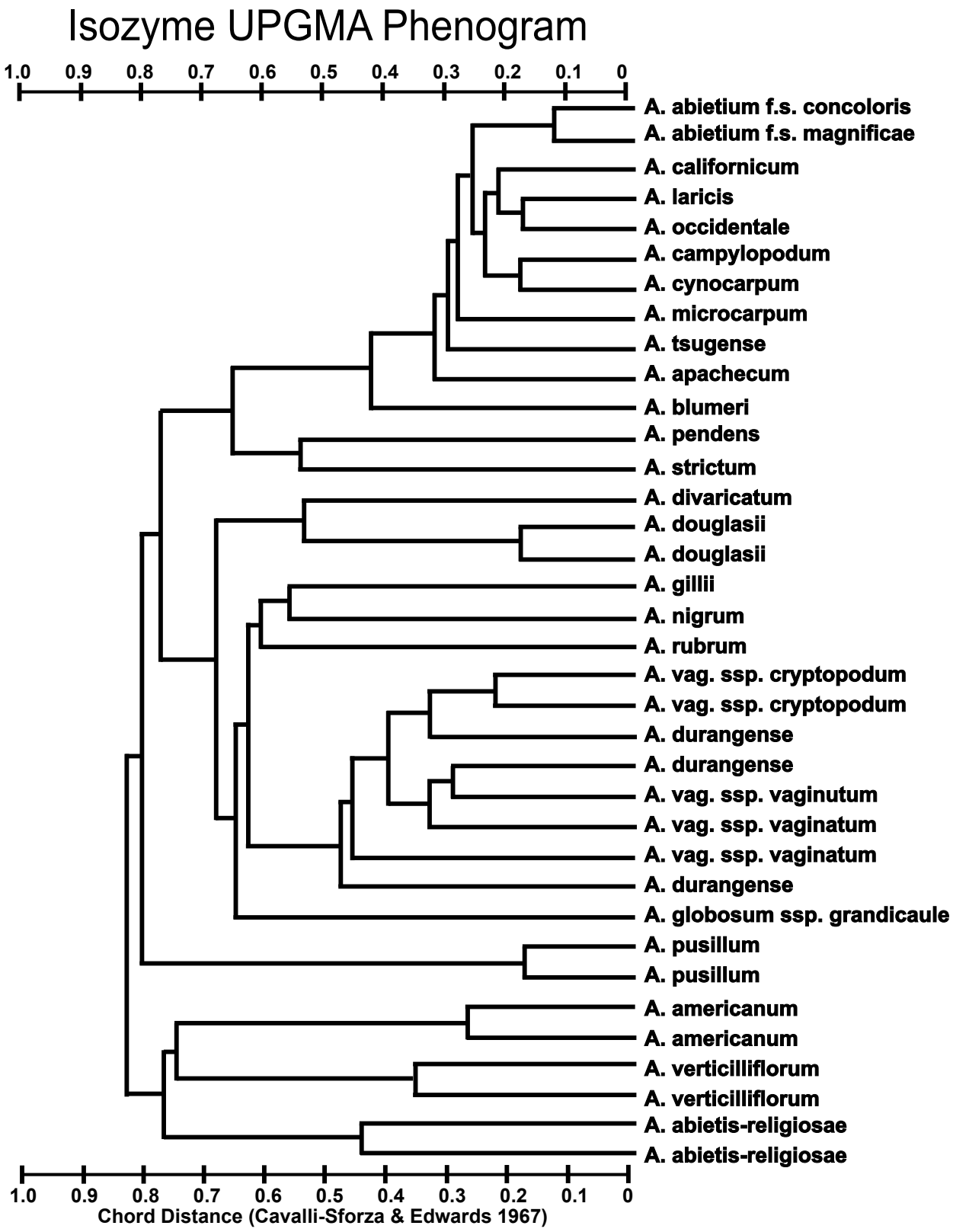

Fig. 1. The UPGMA phenogram resulting from isozyme analysis of the 36 *Arceuthobium* populations shown in Table 1. Cophenetic correlation = 0.968, standard deviation 6.6%.

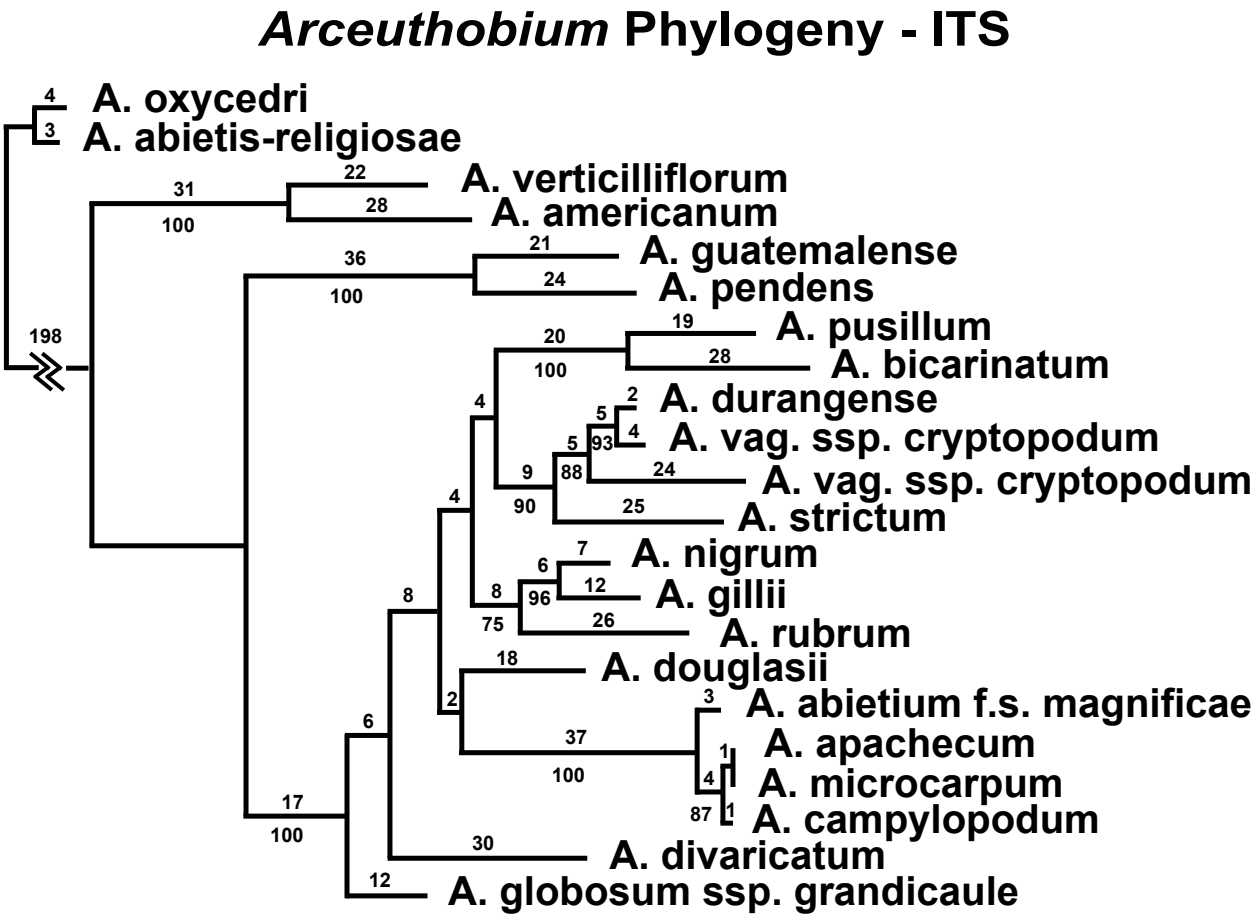

Fig. 2. One of the six shortest trees (length 738) derived from analysis of *Arceuthobium* ITS-1, -2, and 5.8S rDNA sequences. The numbers above the branches indicate branch lengths (number of nucleotide substitutions) and the numbers below the branches indicate percentage values (from 200 replications) derived from the bootstrap majority rule consensus tree. Clades with no bootstrap value indicate they were supported in less than half the trees. Consistency index = 0.755, homoplasy index = 0.286, retention index = 0.726.