

## **Mistletoe phylogenetics: Current relationships gained from analysis of DNA sequences**

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### **Introduction**

The purpose of this paper to briefly summarize some of the advancements made in our understanding of evolutionary relationships of mistletoes. It is no exaggeration to say that the past decade has witnessed a virtual revolution in phylogenetic investigation, owing mainly to the application of molecular methodologies and advancements in data analysis techniques. These powerful approaches have provided a source of data, independent of morphology, that have been used to address questions in green plant evolution, including parasitic plants like mistletoes.

### **How Many Mistletoes?**

The term mistletoe is not strictly a taxonomic term but refers to shrubby branch parasites that occur in four families of the sandalwood order (Santalales). Of the total 90 genera and 1306 species of mistletoes (Table 1), Loranthaceae has the majority of genera (83%) and species (69%). Another 26% of the total mistletoe species are found in Viscaceae, mainly because of the speciose genera *Phoradendron* and *Viscum*.

### **How Many Times Did Mistletoes Evolve?**

As indicated in Table 1, molecular data have been instrumental in shaping our understanding of phylogenetic relationships in Santalales. At present, complete nuclear small-subunit (18S) rDNA and chloroplast *rbcL* sequences have been obtained from representatives of all mistletoe groups. Analyses of these genes separately and in combination give the same general topology for the order (Fig. 1). Olacaceae are basalmost and, although paraphyletic, this assemblage is sister to the remaining members of Santalales. This position is in agreement with traditional concepts and fits with the observation that Olacaceae is the only family in the order with parasitic and nonparasitic members. The two-gene tree (Fig. 1, no. 1) also shows that *Schoepfia* is not closely related to other Olacaceae but is sister to *Misodendrum*, a mistletoe that parasitizing southern hemisphere beech trees (*Nothofagus*) in Chile and Argentina. Indeed, if this

phylogeny is correct, *Misodendrum* may represent the oldest genus that evolved the mistletoe habit.

As can be deduced from the topology of the tree in Figure 1, the common ancestor of *Misodendrum* and Loranthaceae may have been an aerial parasite. Two factors argue against this interpretation. First, *Misodendron* is sister to *Schoepfia* (a root parasite), not the Loranthaceae clade. Second, three genera of Loranthaceae are root parasites (*Atkinsonia*, *Gaiadendron*, and *Nuytsia*), and it is generally thought that these plants are primitive in the family. At present sequences for both genes from all three genera are not available to test this hypothesis, however, it seems unlikely that “reversions” to root parasitism would have occurred in these three genera after the evolution of aerial parasitism. Once aerial parasitism had evolved in Loranthaceae, it appears that this habit was selectively advantageous given the explosive adaptive radiation of genera and species that subsequently ensued in the southern hemisphere. Therefore, the molecular results obtained to date suggest an independent evolution of the mistletoe habit in Loranthaceae (Fig. 1, no. 2) and Misodendraceae.

Within the sandalwood order, the next occurrences of the mistletoe habit are in Santalaceae. The number of times this evolutionary event happened cannot be stated precisely at present because molecular data are incomplete for all relevant genera. One possible case of independent evolution of aerial parasitism involves the three neotropical genera traditionally classified in Eremolepidaceae (Fig. 1, no. 3). Both 18S rDNA and *rbcL* sequences for all three genera have been obtained and upon analysis they emerge within a clade composed of *Exocarpos* and *Omphacomeria* (tribe Anthoboleae of Santalaceae). The three genera are not monophyletic, hence there is no support from the molecules for maintaining a separate family Eremolepidaceae. Indeed the entire “family” Santalaceae represents a series of grades which will require further work to arrive at an evolutionarily sound circumscription. Another possibly unique evolution of the mistletoe habit occurred within tribe Amphorogyneae and involves four genera: *Dendromyza* (including *Cladomyza*), *Dendrotrophe*, *Dufrenoya* (including *Hylomyza*), and *Phacellaria* (Fig. 1, no. 4). Recent work by Jill Macklin (unpublished, pers. com.) using nuclear ITS rDNA sequences indicates these aerial parasites are monophyletic (evolved once from a common ancestor) and that the basalmost genus is *Dendrotrophe*. Interestingly, the genus *Phacellaria* is an obligate parasite on other mistletoes (usually Loranthaceae). At present, sequences for *rbcL* are lacking for the two genera shown in Fig. 1 (*Dendrotrophe* and *Dufrenoya*), hence their position on this tree was determined from the 18S rDNA sequence alone. Efforts are being made to include a full complement of sequences for these and other Amphorogyneae because resolving their position in Santalaceae may help shed light upon the evolutionary origin of Viscaceae.

Emerging from the series of Santalaceae grades is a strongly-supported clade that represents the family Viscaceae (Fig. 1, no. 5). This group of seven genera is clearly monophyletic and support (using bootstrap resampling techniques) is high (100%) using 18S rDNA, *rbcL* or combinations of the two molecules. As previously demonstrated empirically (Nickrent and Starr 1994), rates of molecular evolution in Viscaceae are higher than other families in the order. Surprisingly, despite these high rates,

relationships within the family are remain unresolved. *Phoradendron* is clearly sister to *Dendrophthora* and *Korthasella* sister to *Ginalloa*, however these are the only well-supported clades within the family. It is likely that more sequences from genes with fast evolutionary rates are required to tease apart the intergeneric relationships in Viscaceae.

### **Infrageneric Relationships in Viscaceae**

At present, all mistletoe infrageneric molecular phylogenetic studies have been in Viscaceae. Molecular phylogenetic analyses have been published on three of the seven genera: *Arceuthobium* (Nickrent et al. 1994), *Korthalsella* (Molvray et al. 1999), and *Phoradendron* (Ashworth 2000). The following gives a brief summary of the results from these studies as well as preliminary information on *Viscum*.

***Arceuthobium*.** Interspecific relationships in dwarf mistletoes have been addressed using a variety of biochemical methodologies beginning with flavonoids (Crawford and Hawksworth 1979) and continuing with isozymes (Nickrent 1986) and DNA sequencing (Nickrent et al. 1994). The taxonomic and systematic results from the latter two studies were summarized in Nickrent (1996). The original ITS study of *Arceuthobium* included sequences from 22 taxa, however, since that time sequences from an additional six New World taxa have been obtained. Given that these sequences are from Central American taxa, their relationships are discussed in Mathiasen et al. (2000) and will not be repeated here. In general, the topology of the dwarf mistletoe ITS tree is fully congruent with the one published in 1994, but greater resolution has been obtained by adding other taxa. If section *Pusilla* were reduced to a *Series*, the New World dwarf mistletoes would comprise five Sections: *Arceuthobium*, *Americana*, *Penda*, *Campylopoda*, and *Vaginata*. The latter section would then be divided into five *Series*: *Globosa*, *Minuta*, *Pusilla*, *Rubra*, and *Vaginata*. Nearly complete taxon sampling has been achieved for New World *Arceuthobium*, hence it is likely that the tree topology will remain stable. Sequences from seven members of Section *Campylopoda* are known and all are very similar or identical to those of the four taxa represented on the tree. Therefore, it is unlikely that additional sequences from this Section will alter the results. Only two Mexican taxa remain to be added to achieve complete sampling for the New World taxa: *A. oaxacanum* (*Series Rubra*) and *A. yecorensis* (*Series Stricta*). Continuing phylogenetic work with *Arceuthobium* involves obtaining sequences of the remaining Old World taxa (*A. juniperi-procerae*, *A. azoricum*, *A. chinense*, *A. minutissimum*, *A. pini*, *A. sichuanense*, and *A. tibetense*). Attempts are being made to extract genomic DNA from herbarium specimens with some success.

***Korthalsella*.** The study by Molvray et al. (1999) used nuclear ITS rDNA and chloroplast *trnL-F* spacer sequences from 25 populations of *Korthalsella* collected from across its range. Parsimony analyses of each data set separately and in combination were highly congruent. A species from Queensland Australia (*K. papuana*) was sister to the remaining taxa and these taxa were further resolved as two subclades with either differentiated or undifferentiated inflorescence branches. These results indicate that a classification based upon morphology (Danser 1937) is not supported by genetic data. Moreover, plants on different hosts that are genetically closely related can have markedly

different morphologies (internode shapes). This prompted the authors to propose host influence on the morphology of the parasite, an issue visited by workers looking at other viscaceous genera such as *Arceuthobium* (Gill 1935) and *Viscum* (Meyer von Freyhold 1987).

***Phoradendron*.** The genus *Phoradendron*, comprising at least 100 species of New World mistletoes, is closely related to *Dendrophthora*. Indeed, a single morphological character defines the two genera: one anther locule for *Dendrophthora* and two for *Phoradendron*. The monophyly of these genera has been questioned based upon molecular evidence (Nickrent and Duff 1996). More recently, a detailed molecular phylogenetic analysis of these two genera was conducted using nuclear ITS and 26S rDNA sequences (Ashworth 2000). One taxon, *D. guatemalensis*, was tentatively included in this genus despite the absence of male flowers. More recently (Kuijt, pers. com.), bilocular anthers have been observed in this taxon, hence it will be transferred to *Phoradendron* (*P. navicularis* (Standley) Kuijt). Thus, five *Dendrophthora* and 35 *Phoradendron* taxa were analyzed with parsimony using santalaceous genera as outgroups. Three major clades were identified: clade A, a morphologically heterogeneous one containing all five *Dendrophthora* species plus *P. crassifolium*, *P. piperoides*, and *P. sulfuratum*; clade B, containing seven *Phoradendron* species typically with biseriate inflorescences and one pair of basal cataphylls; and clade C containing the remaining 25 *Phoradendron* taxa (with the exception of *P. californicum*) that have biseriate or triseriate inflorescences and that generally lack basal cataphylls. As with *Arceuthobium* and *Korthalsella*, this study has demonstrated how morphological characters can be unreliable indicators of phylogenetic relationships. Although all five *Dendrophthora* species were resolved in clade A, this clade also contained three *Phoradendron* species, supporting the previous suggestion that neither genus is monophyletic.

***Viscum*.** The utility of ITS sequences for resolving phylogenetic relationships in *Arceuthobium*, *Korthalsella* and *Phoradendron* prompted an investigation of its utility in the more speciose Old World genus *Viscum* (Nickrent, unpublished). ITS sequences for five species of *Viscum* were generated: *V. album*, *V. cruciatum* (both Europe), *V. capense*, *V. continuum* (both South Africa), and *V. triflorum* (Kenya). Substitutional and insertion/deletion mutations were quite high, even in relatively conserved regions such as the 5.8S rDNA (1% of the sites polymorphic). Indels in ITS-1 and -2 prevented unambiguous alignment of the South African and European species. For this reason, variable domains of 26S rDNA were examined (portions of D2, D7 and D8 resulting in ca. 1 kb of sequence). In contrast to ITS, the 26S rDNA alignment was easily accomplished with essentially no ambiguous regions. Using the same species as above, ca. 100 potentially phylogenetically informative sites were obtained. A collaboration with V. Ashworth is currently underway to further explore 26S rDNA sequencing in *Viscum*.

### **Dwarf Mistletoe Coevolution with Their Hosts?**

The incredible proliferation of molecular phylogenetic studies may prompt one to ask “how can these results be used?” One interesting topic that can be explored when working with parasitic organisms is whether the evolutionary histories of the two sets of

organisms are related in any way. For example, it has been observed among animals that host speciation and divergence is accompanied by speciation of its parasites. This process has been called the Fahrenholtz Rule (Nobel and Nobel 1976) and was defined by Price (1980) as: “common ancestors of present-day parasites were themselves parasites of the common ancestors of present day hosts.”

Does the Fahrenholtz Rule apply to parasitic angiosperms? A requirement for answering this question is the presence of relatively detailed phylogenies for and sufficient numbers of both the host and the parasite species. Moreover, there must exist some degree of host specificity because parasites exhibiting very broad host ranges are eliminated *a priori*. Dwarf mistletoes (*Arceuthobium*) are good candidates for determining whether host tracking is taking place because numerous host and parasite combinations can be seen in nature. Several molecular phylogenies of the genus *Pinus* have been published (Krupkin et al. 1996, Liston et al. 1999, Wang et al. 1999), however, none of these approached complete sampling of the 100 or so species in the genus. Sampling in the Wang et al. (1999) paper was aimed at Eurasian pines whereas Liston et al. (1999) utilized all recognized subsections of the genus. When only species common to both studies are considered, the trees are congruent with respect to major clades, thus genes from the chloroplast and nucleus appear to be tracking the same phylogenetic signal.

Because of its broad subsectional sampling, the nuclear ribosomal ITS tree of Liston et al. (1999) was compared to the ITS phylogeny of *Arceuthobium* (Nickrent 1996, Nickrent et al. 1994) to test the Fahrenholtz Rule. As shown in Fig. 2, there is little congruence between the host and parasite trees. For example, dwarf mistletoe species within Section *Camylopoda* parasitize hosts representing all the major clades on the tree (in subgenera *Strobus* and *Pinus*). Species in *Arceuthobium* Series *Vaginata*, *Rubra* and *Globosa* and Section *Americana* are primarily parasites of *Pinus* subgenus *Pinus*. Despite this, the relationships among these series and sections do not correspond to the host phylogeny. Thus, it appears that speciation in *Arceuthobium* has not been “synchronized” with speciation in the pine hosts. It has been hypothesized that *Arceuthobium* migrated from the Old World to the New World and there encountered a great diversity of hosts that were available for colonization (Hawksworth and Wiens 1972). The high proportion of Pinaceae parasitized by dwarf mistletoes today suggests that such an adaptive radiation took place possibly after many host species had already become well-defined. The absence of any pattern of host tracking further suggests cycles of colonization and speciation that operated under different evolutionary constraints and that host fidelity was not maintained. By comparison, dwarf mistletoes have more rapid life cycles and faster evolutionary rates than conifers (Nickrent et al. 1998, Nickrent and Starr 1994), therefore they possessed high genetic potential for rapid adaptive radiation.

**Conclusions.** Molecular phylogenetic methods have proven invaluable in resolving a number of evolutionary questions about mistletoes. For example, there is now little doubt that the families Loranthaceae and Viscaceae are not closely related but represent independent “evolutionary experiments” with the mistletoe habit. These results fully support the concept of separate families first proposed from morphological, cytological, and biogeographical data (Barlow 1964). Although answering some questions, additional

data inevitably reveal new questions that require further investigation. Examples of questions that remain to be resolved include 1) the relationship between *Misodendrum*, *Schoepfia* and Loranthaceae, 2) whether the three root parasitic Loranthaceae are monophyletic and basal within the family, 3) the number of santalaceous lineages that evolved the mistletoe habit, 4) which santalaceous lineage is most closely related to Viscaceae, and 5) the phylogenetic relationships among genera of Viscaceae. Molecular methods offer exciting opportunities to directly test questions such as these.

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**Table 1. Numbers of Mistletoes**

Family	No. Genera	No. Species	Example Genera
Loranthaceae	75*	ca. 900	<i>Agelanthus, Amyema, Phthirusa, Psittacanthus, Scurrula, Struthanthus</i>
Misodendraceae	1	ca. 8	<i>Misodendrum</i>
'Santalaceae'†	4 (of 38)	37 (of 490)	<i>Dendromyza, Dendrotrophe, Dufrenoya, Phacellaria</i>
'Eremolepidaceae'*	3	11	<i>Antidaphne, Eubrachion, Lepidoceras</i>
Viscaceae	7	ca. 350	<i>Arceuthobium, Dendrophthora, Ginalloa, Korthalsella, Notothixos, Phoradendron, Viscum</i>
Totals	<u>90</u>	<u>1306</u>	

\* *Atkinsonia, Gaiadendron,* and *Nuytsia* are root parasites

† Santalaceae are paraphyletic and include Eremolepidaceae



## Legends for Figures

Fig. 1. Consensus tree resulting from an heuristic search of a data matrix composed of nuclear 18S rDNA and chloroplast *rbcL* sequences for Santalales. Details of relationships with Olacaceae and outgroups are not shown. Branches with thick lines indicate mistletoe taxa. The circled numbers refer to five potential independent evolutionary occurrences of the mistletoe habit. Sequences of *rbcL* from Amphorogyneae (*Dendrotrophe* and *Dufrenoya*) were not available, hence the position of this clade was determined solely from the 18S rDNA sequences.

Fig. 2. Comparison of ITS phylogeny of *Arceuthobium* with a phylogeny of the Pinaceae hosts. The host phylogeny is the strict consensus tree reported in an ITS study of *Pinus* by Liston et al. (1999). This tree was grafted to the intergeneric topology obtained following a multigene study of Pinaceae by Wang et al. (2000). Dwarf mistletoe - host combinations (principal hosts only) are aligned in horizontal rows. Vertical boxes enclose *Arceuthobium* species groups (Sections and Series) as determined by the ITS analyses. The inset tree gives the overall topology of the *Arceuthobium* ITS tree. This figures clearly shows that *Arceuthobium* phylogenetic relationships cannot be predicted from the host phylogeny.

Figure 1.

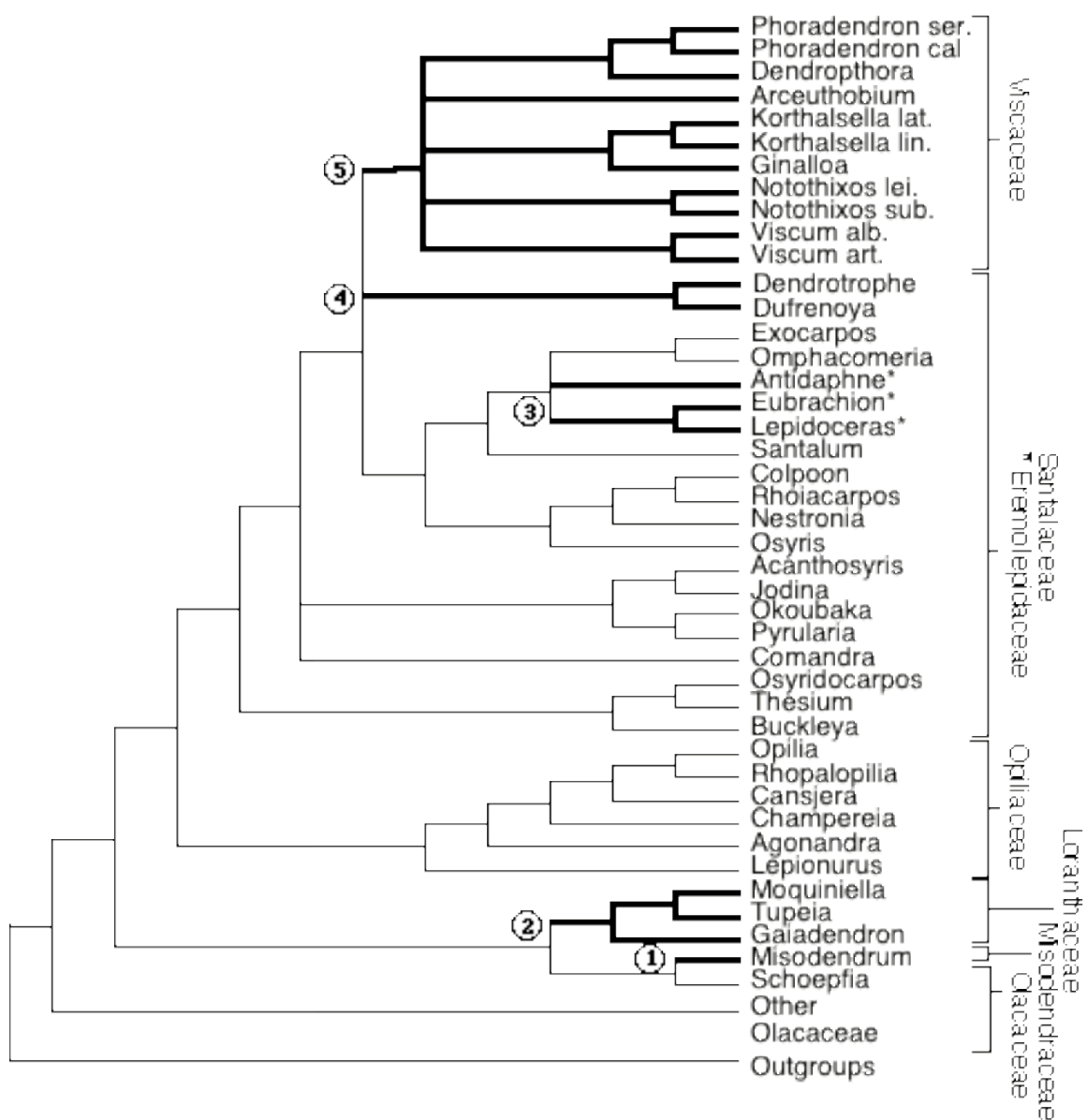


Figure 2

