A MOLECULAR PHYLOGENY OF SANTALALES

D. L. Nickrent [1] and V. Malécot[2]

[1] *Department of Plant Biology and Center for Systematic Biology, Southern Illinois University, Carbondale, IL, 62901-6509, USA. e-mail: nickrent@plant.siu.edu*

[2] *Laboratoire de Paléobotanique, 12 rue Cuvier, 75005 Paris, France. e-mail: vmalecot@hall.snv.jussieu.fr*
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SUMMARY
We report here our continuing studies whereby greater generic sampling has allowed increasingly detailed inferences of phylogenetic relationships among genera of Santalales. Sequences of SSU rDNA and chloroplast *rbcL* have been obtained from 54 of the 155 total genera (58 species) in the order. These sequences and those from six outgroups resulted in a 64 taxon matrix which was analyzed using maximum parsimony (MP). Two trees were obtained, the strict consensus of which showed a monophyletic Opiliaceae, Loranthaceae and Viscaceae. Santalaceae (including Eremolepidaceae) and Olacaceae were paraphyletic. As previously reported, *Schoepfia* was sister to *Misodendrum* and not closely related to the remaining Olacaceae. These analyses resulted in eight clades within a paraphyletic "Olacaceae." Positive reports of parasitism and nonparasitism suggest that only two clades (tribes Olaceae and Ximeneae) are parasitic, thus indicating that this life form evolved once in Santalales. Seven clades with "Santalaceae" are discussed. Although greater sampling of taxa and genes has increased resolution of phylogenetic relationships, complete sampling and likely other gene sequences will be required to fully resolve relationships in Santalales.

INTRODUCTION
Among angiosperms, the most diverse assemblage of trophic modes is found in the sandalwood order (Santalales): nonparasites, root parasites or aerial parasites. Clarification of phylogenetic relationships among the component genera and families of this order thus presents opportunities to better understand the evolution of this heterotrophic mode. As traditionally defined, Santalales consist of seven families: Eremolepidaceae, Loranthaceae, Misodendraceae, Opiliaceae, Olacaceae, Santalaceae and Viscaceae. Previous molecular phylogenetic investigations have utilized nuclear small-subunit (SSU) rDNA sequences (Nickrent and Duff 1996) as well as sequences from plastid *rbcL* (Nickrent, et al. 1998). In each of these studies, however, sampling was limited, particularly in Santalaceae and Olacaceae. We report here our continuing studies whereby greater generic sampling has allowed increasingly detailed inferences of phylogenetic relationships among members of the sandalwood order.

MATERIALS AND METHODS
DNA extraction, PCR conditions, and sequencing methodology have been previously reported (Nickrent 1994). Six taxa were chosen as outgroups given results from global analyses using multiple genes (Soltis, et al. 2000). Sequences of nuclear SSU rDNA and plastid *rbcL* were obtained for 58 ingroup Santalales taxa and the six outgroups (64 total taxa). These sequences were aligned and combined into a single matrix and analyzed with maximum parsimony (MP) using PAUP* (Swofford 1998).

RESULTS AND DISCUSSION
Complete generic sampling was achieved for Viscaceae (7 genera), "Eremolepidaceae" (3 genera), and Misodendraceae (1 genus); sampling was incomplete for Olacaceae (17 of 26 genera) Santalaceae (20 of 37 genera), Opiliaceae (6 of 10 genera) and Loranthaceae (3 of 74 genera). Thus, 54 genera (58 species) of Santalales with both SSU rDNA and *rbcL* sequences were included in the 64 taxon data matrix (3118 characters). Upon analysis, two most parsimonious trees resulted of length 3477, the strict consensus of which is shown in Figure 1. The topology of this tree is generally congruent with ones previously reported (Nickrent and Duff 1996, Nickrent,

et al. 1998, Nickrent and Malecot 2000) with Olacaceae basal followed by a Loranthaceae/Misodendraceae clade, Opiliaceae, a grade comprising Santalaceae/Eremolepidaceae and then Viscaceae. Bootstrap support for a monophyletic Santalales is moderately strong (78%). Santalales minus Olacaceae are monophyletic with high BS support (90%). High BS support (100%) was obtained for Loranthaceae, Opiliaceae and Viscaceae whereas Olacaceae and Santalaceae (including Eremolepidaceae) are paraphyletic. These "families" each show a succession of stepwise clades with little BS support for nodes present along the "spine" of the tree. Thus, the relationships among the component clades should at present be viewed as a large polytomy. Given that the increased taxon sampling reported here mainly concerns Olacaceae and Santalaceae, detailed discussion will be made only for these two families.

Olacaceae, clade A. Clade A (Fig. 1), represented by *Erythropalum*, is shown in this analysis as the basalmost member of Olacaceae and the order Santalales. This position did not receive high BS support and the position of this genus changes depending upon the choice of outgroup. For example, when using only *Glycine* and *Arabidopsis* as outgroups, *Erythropalum* is sister (with low BS support) to *Heisteria* in a monophyletic Olacaceae (BS support only 30%). It appears that the basal position is being influenced primarily by *rbcL*, possibly demonstrating a case of incongruence between the two process partitions. *Erythropalum* is aberrant in Olacaceae, and has therefore at times been classified in its own family (Erythropalaceae Blume). However, the genus does possess the free-central, pendulous placentation that is characteristic of Olacaceae and its position in the family and order is supported by molecular data.

Olacaceae, clade B. This clade received 100% BS support and is here composed of four genera traditionally classified in tribe Anacoloseae: *Diogoa, Strombosiopsis, Strombosia*, and *Scorodocarpus* (but not *Anacolosa* and *Phanerodiscus* as circumscribed by Sleumer (1984)). Morphological synapomorphies for this clade can be found in pollen morphology, specifically tricolporate pollen with rectangular endoaperture (Lobreau-Callen 1981, Feuer 1977). Three of these genera (*Strombosia, Strombosiopsis, Diogoa*) show anatomical similarities including presence of epidermal crystals, petiole vascularization, and ray type (Baas 1982, van den Oever 1984). Moreover, all four genera share an insertion of two to four additional amino acid codons at the 3' end of the *rbcL* gene, just 5' of the stop codon (Nickrent and Malecot 2000).

Olacaceae, clade C. Moderate BS support (64%) was obtained for a clade composed of *Coula* and *Minquartia*. Morphological synapomorphies for the tribe include the presence of dendritic hairs, lignified epidermal cells, laticifers (Baas 1982), long vessels (van den Oever 1984) and the absence of an infratectum on the pollen (Lobreau-Callen 1980). This tribe is traditionally recognized as very homogeneous and is often considered the most primitive one in the family (Michaud 1966, Stauffer 1961b), a result that is not incompatible with this molecular analysis.

Olacaceae, clade D. This clade is strongly supported by this molecular analysis (98% BS) as well as by several morphological and anatomical features such as inflorescence type, stamen number, and wood characteristics. It may be mentioned that Sleumer (1984) placed *Malania* in tribe Olaceae (but with some doubts) whereas the Chinese descriptors (Lee 1980, Ling 1982) clearly assigned *Malania* to tribe Ximenieae. Analysis of a data set with greater taxon density places this clade between clades F and G. If this position is correct, it suggests that haustorial parasitism arose just once in Santalales (only in clades D and G of Olacaceae).

Olacaceae, clade E. The position of *Heisteria* among the Olacaceae clades is variable depending upon the choice of gene and/or outgroup. Analyzed separately, the *rbcL* and nuSSU rDNA partitions differ in their placement of this taxon. Palynological features in *Heisteria* are similar to Couleae (Feuer 1977, Lobreau-Callen 1980), yet wood anatomy links the genus to *Brachynema* (van den Oever 1984). Its leaf anatomy is intermediate between tribes Couleae on the one hand and clade F (Aptandreae) on the other (Baas 1982). A relationship with *Chaunochiton* is not supported by this study. This morphological variability and the mobile nature of the *Heisteria* clade in these molecular analyses call for additional data to test the validity of tribe Heistereae.

Olacaceae, clade F.   This clade received moderately high (81%) BS support and here consists of three genera: *Aptandra*, *Ongokea*, and *Chaunochiton*, equivalent to tribe Aptandeae (Sleumer 1984).   These molecular data suggest adopting an expanded concept for the tribe to include *Chaunochiton* (formerly Heistereae) and *Cathedra* and *Phanerodiscus* (formerly Anacoloseae).   These taxa all have fruits with expanded floral structures.   In the case of *Aptandra*, *Ongokea,Harmandia,* and *Chaunochiton* the fruit is surrounded by an accrescent calyx.   For *Phanerodiscus*, the fruit is surrounded by membranous sack derived from the receptacular cupule (not the discal cupule).   In *Cathedra*, the fruit is closely enveloped by the accrescent discal cupule.

Olacaceae, clade G. This well-supported clade (100% BS) is composed of *Anacolosa*, *Olax*, and *Ptychopetalum*.   The latter two genera were classified in tribe Olaceae by Sleumer (1984), but molecular data do not support the inclusion of *Malania* (in clade D, above).   The strong association of *Anacolosa* with *Olax* (100% BS) is surprising given the absence of any obvious palynological, leaf, or wood anatomical characters that link these genera.

Olacaceae, clade H. As previously reported (Nickrent, et al. 1998, Nickrent and Malecot 2000), *Schoepfia* is sister to the mistletoe genus *Misodendrum*, and this clade is itself sister to Loranthaceae, although both of these clades receive only moderate BS support (72% and 65%, respectively).   The relationship of *Schoepfia* to Loranthaceae was noted as early as 1830 by De Candolle, and traditional infrafamilial classifications have since placed this genus in a separate subfamily (Engler 1897, Sleumer 1984) or its own family, Schoepfiaceae (Gagnepain 1910, Tieghem 1896).   Unlike any other Olacaceae, *Schoepfia* shares with Loranthaceae a reduced calyx.

Santalaceae, clade a.   Although clade a (*Comandra*) occurs at the base of a paraphyletic Santalaceae, this position did not receive high BS support.   Indeed, the overall relationships among the seven clades of Santalaceae discussed here are not resolved, hence the "spine" of this part of the tree should be viewed as a polytomy.   It seems unlikely that this north temperate herbaceous perennial represent the basalmost member of the family.   Generally, the topology of the tree shown for Santalaceae in Figure 1 derives mainly from the *rbcL* partition which has more parsimony informative sites than nuSSU rDNA.

Santalaceae, clade b.   High BS support (94%) was obtained for the clade containing *Osyridocarpus* and *Thesium*.   This relationship is in agreement with the composition of tribe Thesieae of Pilger (1935) and Stauffer (1961) and both possess heteropolar pollen (Stearn 1972).   The inclusion of *Buckleya* in this clade is tenuous and received only moderate BS support (65%).   This position appears to be arising from the *rbcL* partition; nuSSU analyzed separately places this genus with members of clade e (Santaleae).

Santalaceae, clade c.   This clade has moderate support (72% BS) and contains a well supported clade of two South American genera, *Acanthosyris* and *Jodina* and a moderately supported (72%) clade composed of *Pyrularia* (U.S. and Asia) and *Okoubaka* (Africa).   The first three genera have all been classified in tribe Osyrideae by Pilger (1935) (which should be called Santaleae DC, see Stearn 1972).   *Okoubaka* was transferred from Olacaceae to Santalaceae by Stauffer (1957) who astutely noted an affinity with *Pyrularia* (and *Scleropyrum*, not sampled here) based on habit, fruit and wood anatomy.

Santalaceae, clade d.   The clade containing *Exocarpos* and *Omphacomeria* received 100% BS support and represents two of the three genera of tribe Anthoboleae (Pilger 1935, Stauffer 1959).   These genera are distinct in the family owing to the presence of hypogynous vs. epigynous flowers and fruits that occur at ends of swollen pedicels.

Santalaceae, clade e. Four genera (*Colpoon, Osyris, Rhoiacarpos*, and *Nestronia*) formed a well supported clade (94% BS).   The first three genera are African wheres *Nestronia* is endemic to the eastern U.S.   A relationship between the African genera has been recognized (Stauffer 1961a) and all were classified in Santaleae (Osyrideae) by Pilger (1935).   Also considered part of this clade (although BS support is presently lacking) are *Santalum* and *Mida*.

Santalaceae, clade f.   Bootstrap support is low for the clade containing *Antidaphne, Eubrachion*, and *Lepidoceras*, however, these three genera have traditionally been classified as part of Eremolepidaceae (Kuijt 1988).   As has been previously discussed (Nickrent and Duff

1996, Nickrent, et al. 1998), this group of three genera is not monophyletic and is embedded within a broadly paraphyletic Santalaceae. If one chooses to recognize Eremolepidaceae as a family, other clades within "Santalaceae" would also require naming at this rank. The state of knowledge about relationships among the various clades ("tribes") of Santalaceae is currently incomplete, thus we consider it prudent to abstain from reclassification until more information is available.

Santalaceae, clade g. In the 64 taxon analysis, the only representative of tribe Amphorogyneae with both gene sequences was *Choretrum* which was sister to (albeit with low BS support) a clade representing Viscaceae. Tribe Amphorogyneae was proposed by Stauffer (1969) and validly published by Stearn (1972). Eight genera remain to be sequenced, an essential action to fully understand the evolution of the mistletoe habit in Viscaceae.

CONCLUSIONS

The addition of more sequences and taxa has further clarified relationships among genera of Santalales, particularly in Olacaceae and Santalaceae. Given the size of the order and the evolutionary diversity among its component taxa, sequence data from additional genes will be required, as well as complete or nearly complete taxon sampling, to advance our state of knowledge on this important group of parasitic plants.

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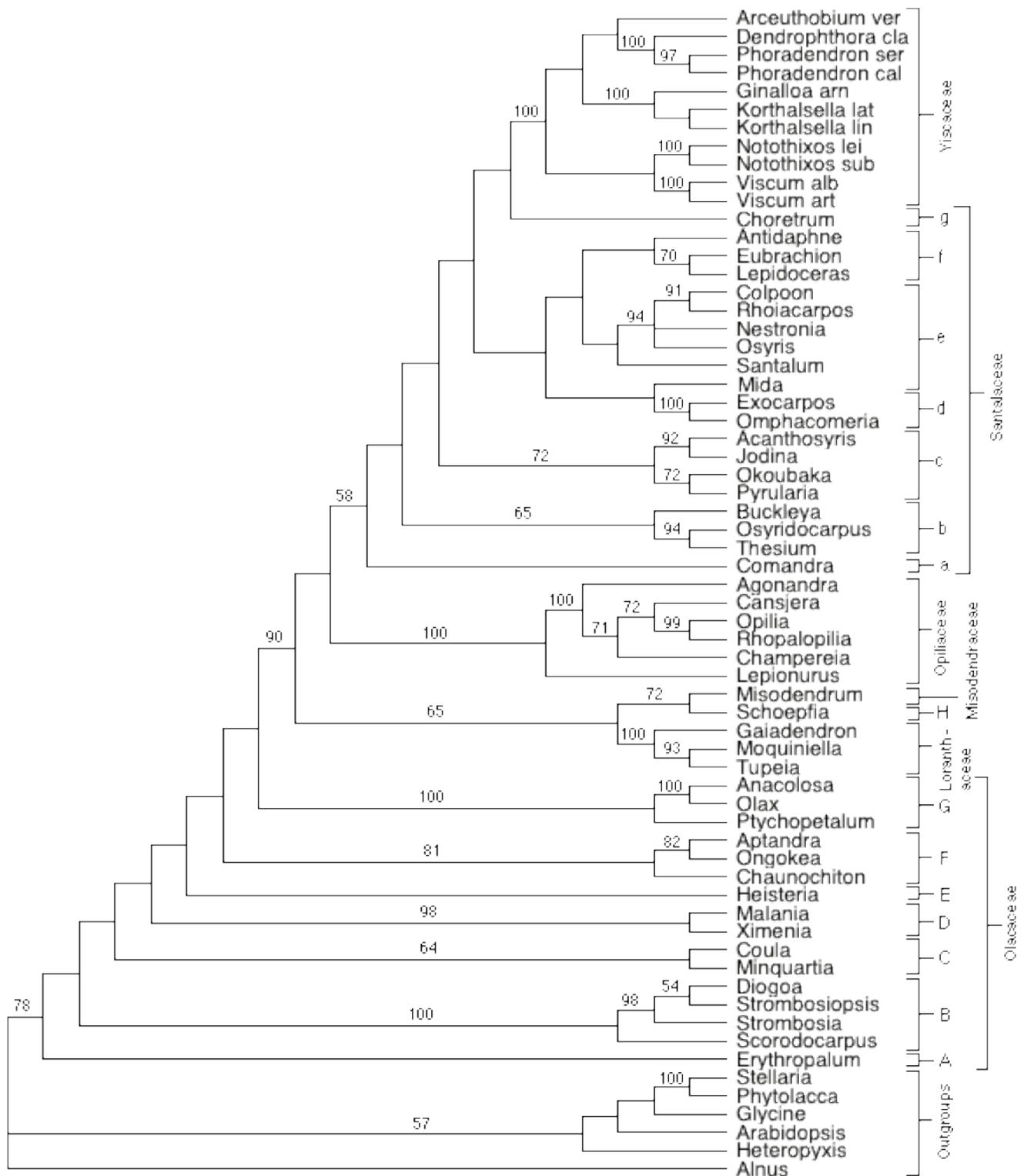
Figure 1. Strict consensus of two trees (L=3477) resulting from a MP heuristic search of a combined matrix of nuSSU rDNA and *rbcL* sequences for 58 Santalales and six outgroup taxa. Consistency index (minus uninformative sites) = 0.3612. Numbers above branches represent BS support (100 replicates). Branches without values had BS support less than 50%. See text for discussion of clades A-H (Olacaceae) and a-g (Santalaceae).