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# Phylogenetics of Tribe Anthocercideae (Solanaceae) Based on *ndhF* and *trnL/F* Sequence Data

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**ABSTRACT.** Tribe Anthocercideae (Solanaceae) is an Australian endemic group comprising 31 species in seven genera. Recent phylogenetic work has placed the Anthocercideae sister to *Nicotiana*. Two chloroplast DNA regions, *ndhF* and *trnL/F,* were analyzed and the phylogeny was used to test the tribe's monophyly, discover relationships within the tribe, and make inferences on character evolution and biogeography. The relationship between *Nicotiana*, *Symonanthus*, and the rest of tribe Anthocercideae is unresolved. *Anthocercis*, *Anthotroche*, *Grammosolen*, and *Symonanthus* are found to be monophyletic, while *Cyphanthera* and *Duboisia* are not (*Crenidium* is monotypic). Several characters were inferred to be derived within the Anthocercideae, including unilocular stamens with semicircular slits, ebracteolate flowers, and baccate fruits. Ancient colonization occurred in southwestern Australia followed by several radiation events eastward.

Tribe Anthocercideae as currently circumscribed comprises 31 species in seven genera: *Anthocercis* (10 spp.), *Anthotroche* (4 spp.), *Crenidium* (1 sp.), *Cyphanthera* (9 spp.), *Duboisia* (3 spp.), *Grammosolen* (2 spp.), and *Symonanthus* (2 spp.). All members are endemic to Australia, with the exception of *Duboisia myoporoides*, which is also found in New Caledonia. The region of greatest diversity for the group occurs in southwestern Australia, and most taxa are confined to the southern half of the continent. The tribe is characterized by a suite of morphological characters: woody habit, flowers with non-accrescent calyx, inflexo-valvate aestivation of the corolla lobes, a short, relatively broad actinomorphic corolla tube, extrorsely dehiscing stamens inserted low in the corolla tube, and an oblong to ellipsoid, slightly curved seed with reticulate testa in which is held a terete, slightly curved embryo (Haegi 1986).

Solanaceae tribe Anthocercideae was established by Don (1838) to contain *Anthocercis* and *Duboisia*, the only two of the present seven genera described at that time. Miers (1849) established tribe Duboiseae to include *Anthotroche* and *Cyphanthera*, along with *Anthocercis* and *Duboisia*. Bentham (1846, 1868) transferred *Anthocercis* and *Duboisia* to the Scrophulariaceae based on the presence of four didynamous stamens. Baehni (1946) resurrected the Anthocercideae, but included only *Anthocercis* (along with four genera now known to be unrelated), placing *Duboisia* in the Salpiglossideae and leaving *Anthotroche* and *Isandra* (= *Symonanthus*) *incertae sedis.* He did not recognize *Cyphanthera*. Haegi (1979, 1981, 1986, 1991) provides the most complete treatment of the Anthocercideae and established its present taxonomy, describing two new genera, *Crenidium* and *Grammosolen* (Haegi 1981) and providing a correct name for *Symonanthus* (*Isandra* was a later homonym).

Recent morphological and phytochemical studies have suggested the monophyly of the Anthocercideae (Haegi 1979, 1981, 1986; Purdie et al. 1982; Tétényi 1987; Knapp et al. 2000). A molecular phylogenetic study of the Solanaceae based on chloroplast DNA (cpDNA) restriction site variation placed the Anthocercideae sister to *Nicotiana* (Olmstead and Palmer 1992). This result was confirmed by additional studies of cpDNA restriction site, *ndhF* and *rbcL* variation (Olmstead and Sweere 1994; Olmstead et al. 1999). These studies, however, only sampled four species among the Anthocercideae: *Anthocercis viscosa*, *Cyphanthera anthocercidea*, *Duboisia myoporoides*, and *Grammosolen dixonii*. A more rigorous molecular study sampling across all genera is needed to produce a phylogenetic framework from which we can draw inferences about the group's evolutionary history. The current study includes extensive sampling within the tribe, and the phylogenies generated from molecular data are used to: (1) test the monophyly of tribe Anthocercideae, (2) elucidate relationships within the tribe, (3) infer morphological character evolution, and (4) comment on available biogeographic data.

The reconstructed phylogenies presented here are based on sequence variation from two cpDNA markers, *ndhF* and *trnL/F. ndhF* is a gene that encodes a subunit of the NADH dehydrogenase complex. It exhibits almost twice the average substitution rate of *rbcL* (Sugiura 1989; Olmstead and Sweere 1994; Soltis and Soltis 1998), and has been used previously in determining phylogenetic relationships in the Solanaceae (Olmstead and Sweere 1994; Bohs and Olmstead 1997, 2001). The *trnL/F* region, also used previously in phylogenetic analyses of other genera in Solanaceae (e.g., Fukuda et al. 2001), includes an intron and spacer flanking the 3′ exon of the *trnL* gene. Because it is mostly non-coding,

TABLE 1. Voucher and GenBank accession data for taxa examined in this study. For each voucher GenBank accession numbers are given in the following sequence: *ndhF, trnL/F*.

*Anthocercis angustifolia* F. Muell., *R. G. Olmstead 94-05* (WTU) AY098704, AY098671. *A. gracilis* Benth., *H. Stace s. n.*; herbarium unknown, AY098705, AY098672. *A. ilicifolia* Hook., *H. Stace s. n.* (UWA) AY098706, AY098673. *A. intricata* F. Muell., *H. Stace s. n.* (KPBG) AY098707, AY098674. *A. littorea* Labill., *H. Stace s. n.*; herbarium unknown, AY098708, AY098675. *A. sylvicola* Macfar. & Ward., *T. Middleton s. n.* (PERTH) AY098709, AY098676. *A. viscosa* R. Br., *D. Symon 14835* (AD) U08914, AY098677.

*Anthotroche blackii* F. Muell., *H. Stace s. n.* (KPBG) AY098711, AY098678. *A. myoporoides* C.A. Gardner, *H. Stace s. n.* (KPBG) AY098810, AY098679. *A. pannosa* Endl., *H. Stace s. n.* (KPBG) AY098712, AY098680. *A. walcottii* F. Muell., *D. R. & B. Bellairs 2035* (PERTH) AY098713, AY098681.

*Crenidium spinescens* Haegi, *B. J. Lepschi & T. R. Lally 1672* (CANB) AY098714, AY098682.

*Cyphanthera albicans* (A. Cunn.) Miers, *B. J. Lepschi & T. R. Lally 1722* (CANB) AY098715, AY098683. *C. anthocercidea* (F. Muell.) Haegi, *L. Haegi 1456* (AD) AY098716, AY098684. *C. microphylla* Miers, *B.J. Lepschi 2170* (PERTH) AY098717, AY098685. *C. myosotidea* (F. Muell.) Haegi, *Alcock 9117* (AD) AY098686. *C. odgersii* (F. Muell.) Haegi, *Chinnock 3100* (AD) AY098718, AY098687. *C. racemosa* (F. Muell.) Haegi, *L. Haegi 1959* (AD) AY098688.

*Duboisia leichhardtii* (F. Muell.) F. Muell., *L. Haegi 2056* (AD) AY098719, AY098689. *D. myoporoides* R. Br., *D. Symon 14832* (AD) AY098720, AY098690.

*Grammosolen dixonii* (Muell. & Tate) Haegi, *D. Symon 14833* (AD) AY098721, AY098691. *G. truncatus* (Ising) Haegi, *Canty 2429* (AD) AY098722, AY098692.

*Symonanthus aromaticus* (C.A. Gardner) Haegi, *J. McKinney s. n.*; herbarium unknown, AY098723, AY098693. *S. bancroftii* (F. Muell.) Haegi, *H. Stace s. n.* (KPBG) AY098724, AY098694.

*Jaltomata procumbens* (Cav.) J.L. Gentry, *R. G. Olmstead S-24* (WTU) U47429, AY098695.

*Lycium cestroides* Schltdl., *R. G. Olmstead S-34* (WTU) U08920, AB036578 & AB036607.

*Nicotiana acuminata* (Graham) Hook., *R. G. Olmstead S-39* (WTU) U08923, AY098696. *N. attenuata* Torr., *R. G. Olmstead S-41* (WTU) AY098697. *N. excelsior* (Black) Black, *R. G. Olmstead S-44* (WTU) AY098725, AY098698. *N. glutinosa* L., no voucher, AY098726, AY098699. *N. gossei* Damin., *R. G. Olmstead S-48* (WTU) AY098727, AY098700. *N. paniculata* L., *R. G. Olmstead S-53* (WTU) AY098728, AY098701. *N. tabacum* L., no voucher, L14953, Z00044.

*Petunia axillaris* (Lam.) Britton, Stern & Poggenb., *R. G. Olmstead S-60* (WTU) U08926, AY098702.

*Solanum lycopersicum* L., no voucher, U08921, AY0987.

it exhibits a higher substitution rate than *ndhF*, thus it was selected with hopes that it might help to resolve relationships among closely related members of the tribe.

## MATERIALS AND METHODS

We sampled 24 species across all seven genera in the Anthocercideae (Table 1). *trnL/F* was sequenced for all 24 species, whereas *ndhF* was only sequenced for 22 species (two species of *Cyphanthera* would not amplify for *ndhF*). Due to the hypothesized sister relationship of *Nicotiana* to the group (Olmstead and Palmer 1992; Olmstead et al. 1999), seven species of *Nicotiana* were included (*N. attenuata* would not amplify for *ndhF*). Four other species from the Solanaceae were added to broaden outgroup sampling: *Jaltomata procumbens*, *Lycium cestroides*, *Petunia axillaris*, and *Solanum lycopersicum*.

Total genomic DNA was extracted from herbarium samples, provided by David Symon (Adelaide Botanic Garden), Helen Stace (U. Western Australia), and Brendan Lepschi (Australian National Herbarium, Canberra) using the CTAB micro-extraction procedure (Doyle and Doyle 1987). All extractions were purified using the QIAquick Purification Kit (QIAGEN). Primers used for *ndhF* and *trnL/F* amplification and sequencing are described in Olmstead and Sweere (1994) and Taberlet et al. (1991), respectively. Double stranded PCR products were cleaned using polyethylene glycol (PEG) DNA precipitation, and quantified by spectrophotometry. Direct DNA sequencing was accomplished in both directions using the ABI Dye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer, Foster City, CA), and analyzed on an ABI 377 automated sequencer.

Sequences were manually edited and assembled using Sequencher 3.0 (Gene Codes, Ann Arbor, Michigan, USA). All sequences were easily aligned by eye. Parsimony informative alignment gaps were coded as binary characters. Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford 1998) using equally-weighted parsimony. A heuristic search involving 50,000 random taxon addition replicates with tree bisection reconnection (TBR) branch swapping, Collapse, and MulTrees was conducted for each data set to search for multiple islands (Maddison 1991; Page 1993). Two heuristic searches were performed on the combined dataset: the first included only taxa that were sequenced for both *ndhF* and *trnL/F*, and the second included all taxa, with missing *ndhF* sequences coded as missing characters. Support for individual branches was determined using bootstrap analysis (Felsenstein 1985) involving 100 replicates with 20 random taxon additions, TBR, Collapse and MulTrees. A decay analysis (Bremer 1988; Donoghue et al. 1992) was also conducted on the combined dataset. The incongruence length difference (ILD) test was conducted to determine whether the two cpDNA regions were significantly different from random partitions of the combined data (Farris et al. 1994). This was implemented as the partition homogeneity test in PAUP* using 1,000 replicates and 1,000 random taxon additions. The data set is available on TreeBASE (study accession number = S899; matrix accession numbers = M1468 and M1477).

## RESULTS

The completed *ndhF* sequences for 32 taxa had an aligned length of 2092 nucleotides (nt), including 239 variable characters, of which 108 (5.2%) were parsimony informative (the matrix included 2.9% missing data, of which 0.2% is accounted for by alignment gaps and 2.7% by incomplete sequences). One informative gap was coded for *ndhF*. The aligned length of *trnL/F* sequences for 35 taxa was 1048 nt, including 103 variable characters, of which 42 (4.0%) were parsimony

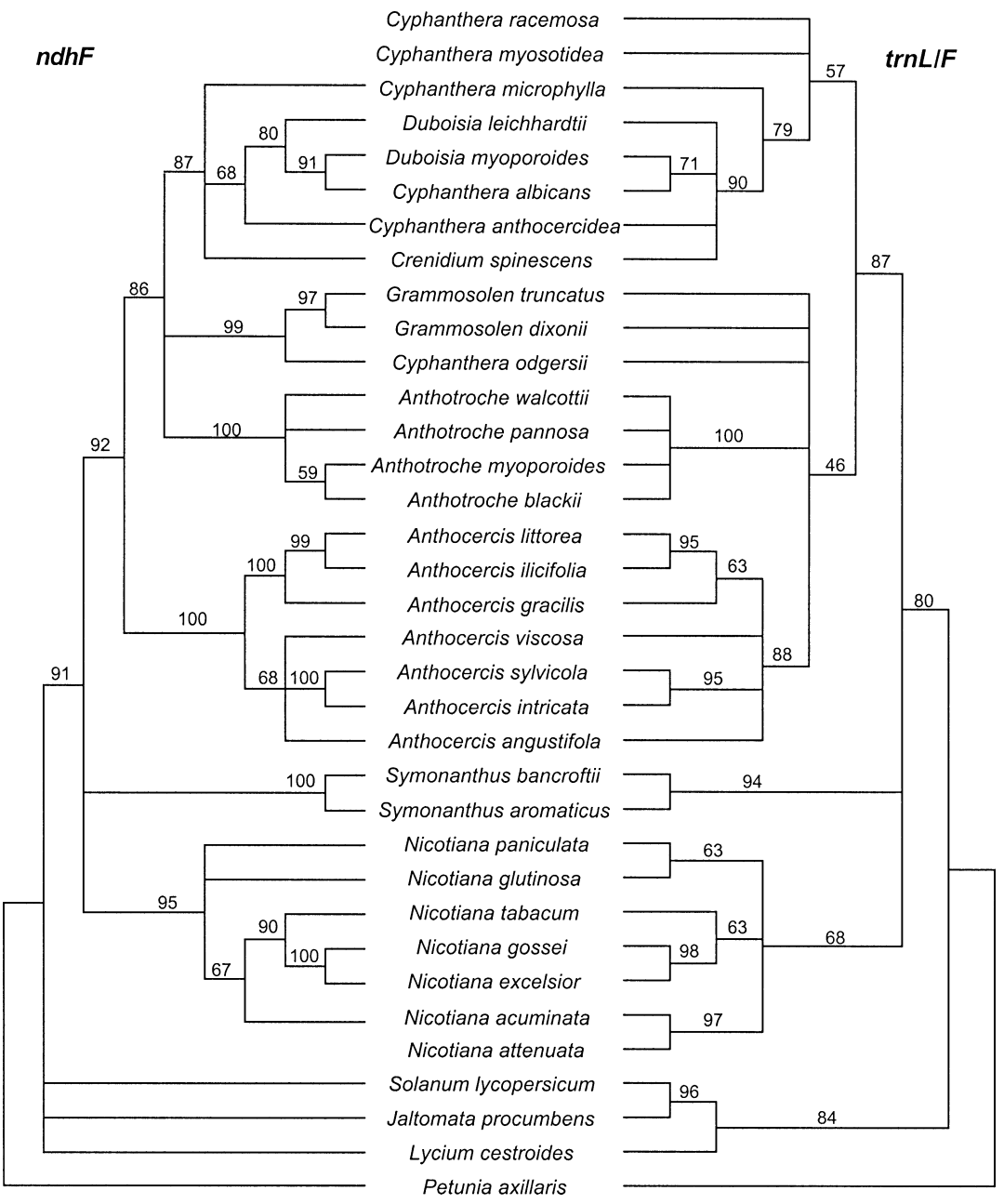


FIG. 1. Strict consensus trees of analyses of *ndhF* sequences on the left (84 trees; length = 301; CI = 0.857; RI = 0.894) and *trnL/F* sequences on the right (6 trees; length = 124; CI = 0.927; RI = 0.943). Bootstrap percentages are shown above the branches.

informative (the matrix included 9.5% missing data, of which 9.3% is accounted for by alignment gaps and 0.2% by missing nucleotides). Four informative indels were coded for *trnL/F*. The combined data matrix, including gaps, had an aligned length of 3140 nt, 338 variable characters, of which 147 (4.8%) were parsimony informative. Within the ingroup, 4.0% of the characters were parsimony informative for *ndhF* and 2.7% for *trnL/F*.

Heuristic searches using the *ndhF* data resulted in 84 most-parsimonious trees of 301 steps in two tree islands; the strict consensus tree is shown in Fig. 1. Searches using the *trnL/F* data resulted in six most-parsimonious trees of 124 steps in one tree island (Fig.

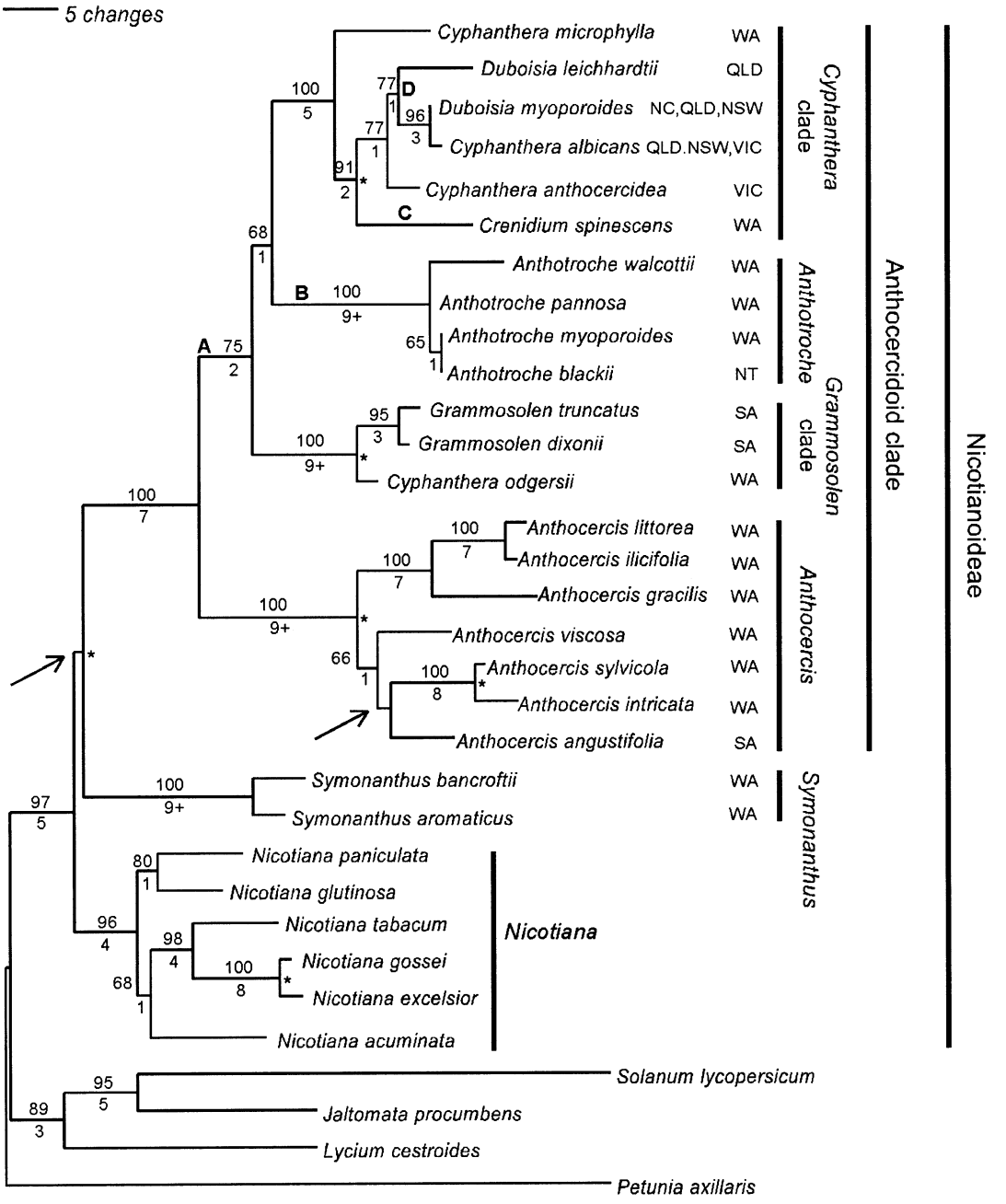


FIG. 2. One of six equally parsimonious trees of length 421 from combined analysis of *ndhF* and *trnL/F* sequences. Arrows indicate collapsed branches under strict consensus. Bootstrap percentages are shown above the branches, decay indices below. Asterisks indicate clades supported unambiguously by coded indels. Informal clade names correspond to well-supported clades. Morphological character transformations: A (unilocular stamens with semicircular slits), B (ebracteolate flowers), C (leaflessness), D (baccate fruit; see text for alternate placement). Biogeographic distributions: NC (New Caledonia), NSW (New South Wales), NT (Northern Territory), QLD (Queensland), SA (South Australia), VIC (Victoria), WA (Western Australia).

1). The *trnL/F* tree was less well-supported and had fewer resolved clades than the *ndhF* tree.

The results of the ILD Test (Farris et al. 1994) showed that patterns of character state variation between the *ndhF* dataset and the *trnL/F* dataset were not significantly different (P = 1.000). The combined dataset using taxa with both *ndhF* and *trnL/F* sequences produced six most-parsimonious trees of 421 steps in one tree island (Fig. 2). The tree topology is highly congruent with the *ndhF* tree, and includes four more

resolved clades. Complementary signals from the two cpDNA markers result in a well-resolved tree with robust support as indicated by high bootstrap values and decay indices. The combined dataset using all taxa, with missing *ndhF* sequences coded as missing characters, produced 60 most-parsimonious trees of 428 steps in one tree island (not shown). The tree topology was similar to that of *trnL/F*. *Nicotiana attenuata* was sister to *N. acuminata*. *Cyphanthera myosotidea* and *C. racemosa* were basal in the clade containing *Crenidium*, *Cyphanthera*, and *Duboisia*.

The phylogenetic relationship between *Nicotiana*, *Symonanthus*, and the rest of tribe Anthocercideae remains unresolved after the analysis of all datasets. To account for the possible effects that long branches in the outgroup may have had on this result (Fig. 2), the following analyses were conducted additively to detect any changes in the topology of the remaining taxa: (1) *Petunia* and *Solanum*, the taxa with the longest branches, were removed, (2) then *Jaltomata* and *Lycium* were also removed, and (3) then *Nicotiana* sequences were also removed. The removal of *Petunia* and *Solanum* did not resolve the trichotomy. The cumulative removal of all outgroup taxa did not change the relationships within the ingroup.

## Discussion

***Monophyly of the Anthocercideae.*** The results of these analyses raise the issue of the monophyly of tribe Anthocercideae. The relationship between *Nicotiana*, *Symonanthus*, and the rest of tribe Anthocercideae (labeled Anthocercidoid clade in Fig. 2) is not resolved. The *ndhF* analysis places *Symonanthus* sister to the Anthocercidoid clade in 36 of the 84 most-parsimonious trees. The remaining trees place *Symonanthus* sister to *Nicotiana*, or do not resolve the relationship among the three clades. All individual trees from the *trnL/F* analysis result in a trichotomy of these clades. In the combined analysis, *Symonanthus* is resolved with *Nicotiana* in two trees, with the Anthocercidoid clade in two trees and unresolved in two trees. The true placement of *Symonanthus* needs to be determined before further statements on the tribe's monophyly can be asserted. The Anthocercidoid clade, however, forms a monophyletic group with 100 percent bootstrap support.

While the results of our analyses do not provide conclusive evidence regarding whether the Anthocercideae is monophyletic, previous research suggests that the tribe is distinct. The Anthocercideae possess a unique suite of morphological characters (Haegi 1986) along with several distinctive anatomical features (Armstrong 1986). For instance, all seven anthocercid genera possess differentiated, anticlinally elongated epidermal cells showing a characteristic birefringence in the lower portions of the corolla tube, and lack a differentiated, uniseriate, adaxial calyx hypodermis found in *Nicotiana*, *Browallia*, *Nierembergia*, and *Petunia* (Armstrong 1986). The latter four genera are characterized by a vascular pattern in the calyx that is not present in tribe Anthocercideae. An anatomical study that includes more representatives from the Solanaceae is needed to determine whether these characters are synapomorphies for a monophyletic Anthocercideae or homoplasious within the Nicotianoideae.

Pollen morphology has also been used to suggest the distinctiveness of the Anthocercideae. Knapp et al. (2000) studied variation in anthocercid pollen and recognized an ''*Anthocercis* pollen type'' shared by all members of the group. They further hypothesized that a granulate colpus membrane (also observed by Gentry 1979) and absence of an endoaperature are synapomorphies of the Anthocercideae. Unfortunately, the current literature on Solanaceae pollen provides insufficient comparative data to determine the monophyly of the Anthocercideae. Basak (1967) did, however, differentiate between *Duboisia hopwoodii* and *Nicotiana* pollen types, with *Duboisia* having an ''*Atropa* type'' and *Nicotiana* with a ''*Solanum* type.''

Finally, studies have shown that the Anthocercideae have a unique phytochemical composition. All members of the tribe possess both nicotinic and tropane alkaloids (Evans 1979; Haegi 1986; Griffin and Lin 2000), a combination not found in other Solanaceae taxa. Tétényi (1987) found that the tribe's alkaloid biosynthetic pathway and the spectrum of alkaloids produced in the tribe were sufficiently different to warrant subfamilial distinction. The monophyly of the tribe remains to be determined. Perhaps a future study using a global analysis that includes morphology, anatomy, pollen, phytochemistry, and molecular evidence will resolve this uncertainty.

***Relationships within the Anthocercideae.*** *Anthocercis* (100% BS), *Anthotroche* (100% BS), *Grammosolen* (95% BS), and *Symonanthus* (100% BS) all form well-supported monophyletic groups in the combined analysis (Fig. 2). *Cyphanthera* and *Duboisia* are not monophyletic. *Cyphanthera odgersii* is most closely related to *Grammosolen* (100% BS; labeled *Grammosolen* clade in Fig. 2). The distinctiveness of *C. odgersii* from other *Cyphanthera* species is supported by its unique trichome morphology (Haegi 1991). *Cyphanthera odgersii* has verticillately branched trichomes not found in other *Cyphanthera* species, but which are present in *Grammosolen*. The other *Cyphanthera* species form a clade with *Duboisia* and the monotypic *Crenidium* (labeled *Cyphanthera* clade in Fig. 2; also see Fig. 1). *Anthotroche* is sister to the *Cyphanthera* clade. *Anthocercis* is first to diverge within the Anthocercidoid clade, followed by the divergence of the *Grammosolen* clade.

The informally named clades shown in Fig. 2 are supported by most analyses (the *Grammosolen* clade is not recovered by *trnL/F*), and should be considered in

future taxonomic treatments of the tribe. Addition of the three *Anthocercis* species not included in this study (*A. anisantha*, *A. fasciculata*, and *A. genistoides*) will probably result in their placement within the *Anthocercis* clade, since this genus is distinct and well supported in all analyses. It is harder to predict the placement of *Cyphanthera miersiana*, *C. scabrella*, *C. tasmanica*, and *Duboisia hopwoodii*, since both *Cyphanthera* and *Duboisia* are found not to be monophyletic.

***Character Evolution.*** Morphological characters of each taxon (Purdie et al. 1982) were mapped onto the consensus tree from the combined data matrix (Fig. 2). Several synapomorphic characters within the Anthocercideae were discovered. *Anthotroche* and the *Cyphanthera* and *Grammosolen* clades have unilocular stamens with pollen dehiscing from semicircular slits while all other clades possess the plesiomorphic state of bilocular stamens with longitudinal slits. *Anthotroche* has evolved ebracteolate flowers, and leaflessness is an autapomorphy for *Crenidium spinescens*. All members of the tribe have a capsular fruit except for *Duboisia* which has berries. This character may have evolved once with a reversion back to capsules in *Cyphanthera albicans*, or it may have evolved independently in each *Duboisia* species. Samples of *Duboisia hopwoodii* would be important before further conclusions can be made regarding fruit evolution in this clade. Some or all of the characters shared by the Anthocercideae, but not *Nicotiana*, may represent synapomorphies that unite *Symonanthus* with the rest of the Anthocercideae. However, should convincing evidence be found that the Anthocercideae are paraphyletic, these may be convergent in *Symonanthus* and the rest of the tribe.

***Biogeography.*** Raven and Axelrod (1974) hypothesized that ancestors of the Anthocercideae reached Australia from South America via Antarctica in the Paleogene. The scattered distribution and diverse habit and morphology of the Anthocercideae also suggest its long residence in Australia (Symon 1991). By optimizing the geographic distribution of each taxon (Purdie et al. 1982) onto the consensus tree from the combined data matrix (Fig. 2), we postulate that the site of ancient colonization was southwestern Australia. Several radiation events followed colonization. Lineages in *Anthocercis* and the *Grammosolen* clade migrated eastward, penetrating present-day South Australia. Several of the most recently diverged lineages in the *Cyphanthera* clade have reached the eastern coast of Australia. *Duboisia myoporoides* also has migrated north into the rainforests in Queensland and dispersed to New Caledonia.

The phylogeny of *Nicotiana* and the lack of molecular divergence among the Australian species of *Nicotiana* strongly suggest that its origin and primary radiation was in South America and the Australian species represent a relatively recent introduction and secondary radiation (Olmstead and Palmer 1991). Thus if the Anthocercideae are monophyletic, two dispersal (or one vicariance and one dispersal) events are necessary to explain the current distribution of these two groups (a more ancient one for Anthocercideae and a recent one for *Nicotiana*). If the correct phylogenetic position for *Symonanthus* is as sister to *Nicotiana* or as sister to the clade comprising *Nicotiana* plus the rest of the Anthocercideae, then not only would the morphological similarities among the Anthocercideae be more difficult to explain, but an additional long distance dispersal event must be postulated for the ancestor of *Symonanthus*, along with the extinction of any members of that lineage in South America.

The weight of inference from shared morphological and chemical traits and biogeography argue in favor of monophyly for the Anthocercideae, but our data, by themselves, leave this question unresolved. Since the tribe is morphologically diverse, a closer examination of morphology or anatomy may also help to clarify the evolutionary history of the group.

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