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Phylogeny of the tribe Colletieae (Rhamnaceae) – a sensitivity analysis of the plastid region *trn*L-*trn*F combined with morphology

L. Aagesen[1,2], **D. Medan**[3], **J. Kellermann**[4], and **H. H. Hilger**[5]

[1]Division of Invertebrate Zoology, American Museum of Natural History, New York, USA
[2]Instituto de Botánica Darwinion, San Isidro, Argentina
[3]Cátedra de Botánica, Facultad de Agronomía de la U.B.A., Buenos Aires, Argentina
[4]School of Botany, The University of Melbourne, Australia
[5]Institut für Biologie, Systematische Botanik und Pflanzengeographie, Freie Universität Berlin, Berlin, Germany

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Abstract. The phylogenetic relationships within the tribe Colletieae (Rhamnaceae) were examined combining data from a previous morphological analysis with data from the *trn*L intron and *trn*L-F spacer. Previous studies have failed to confirm monophyly of the genus *Discaria*, the only genus of the tribe with an amphiantarctic distribution. The data set was analyzed using direct optimization as implemented in the computer program POY. Direct optimization searches for multiple optimal sequence alignments and is therefore well suited for analyzing DNA sequences including ambiguous alignable regions as found in the present study. Eight different costs were used for treating the indel information. Indels were treated as single events, equal to a fifth character state, or strings of gaps were treated as single events using different costs for opening a gap and extending the gap. The optimal cost set was selected by use of both character-based and topological congruence measures. Both congruence measures agreed upon a single optimal cost set. The resulting tree generally agrees with the current taxonomic treatment of the tribe Colletieae that recognizes six genera out of which three are monotypic. However, monophyly of *Discaria* was not supported and the results strongly suggest segregating *D. nana* and *D. trinervis*, and re-establishing the genus *Ochetophila*.

Key words: Colletieae, Rhamnaceae, *trn*L intron, *trn*L-F spacer, phylogeny, direct optimization, sensitivity analysis.

Introduction

The tribe Colletieae is one of the smaller tribes of the family Rhamnaceae comprising six genera and a total of 20 species [*Adolphia* Meisn. 1, *Colletia* Comm. ex A. Juss. 5, *Discaria* Hook. 8, *Kentrothamnus* Suess. & Overkott 1, *Retanilla* (DC.)Brongn. 4, and *Trevoa* Miers ex Hook. 1 (Medan and Schirarend 2004)]. The maximum species diversity of the tribe is found south of 30° S and most of the distributions are loosely associated with the Andes in South America, but the tribe also includes a genus of Gondwanic distribution, *Discaria*, with members found in South America, Australia and New Zealand (Medan 1985, Arroyo et al. 1995). Since long-distance

dispersal seems highly improbable (Keogh and Bannister 1993), the amphiantarctic disjunction of *Discaria* may be as old as the separation of South America-Antarctica from Australia-New Zealand, i.e. well over 20 million years old (Hinojosa and Villagrán 1997).

The morphology of all members is well known (Medan and Aagesen 1995, Tortosa et al. 1996, Aagesen 1999) with the species differing primarily in flower, fruit, and inflorescence morphology. The circumscription of the Colletieae has never been disputed. Decussate leaves, abundance of spines, and presence of serial meristems in the leaf axils have traditionally been the diagnostic characters of the tribe (e.g. Miers 1860, Suessenguth 1953), and monophyly has been corroborated both by a morphological analysis of the tribe (Aagesen 1999) and by analyses at family level based on DNA sequence data using *rbc*L and *trn*L-F sequence data (Richardson et al. 2000). The phylogenetic relationship among the Colletieae species was addressed in the morphological analysis (Aagesen 1999). According to the results the Colletieae are divided in two major clades separating *Trevoa* and *Retanilla* from the rest of the tribe. The *Trevoa-Retanilla* clade is fully resolved, but the other clade lacks detailed resolution. Within this clade *Colletia* forms a well supported monophyletic group with distinct disc and inflorescence structure among other characters. Monophyly of *Discaria* could not be confirmed (Aagesen 1999). No morphological characters define the genus *Discaria* and the members of the genus have been assigned to three different genera *Discaria*, *Notophaena* Miers and *Ochetophila* Poeppig ex Endl. which mainly differ in petal and leaf characters (Miers 1860, Suessenguth 1953, Tortosa 1983).

The aim of this study is to provide a better supported and more stable phylogenetic hypothesis of the whole tribe, by adding molecular data from the *trn*L (UAA) 59 intron and the intergenic spacer between the *trn*L (UAA) 39 exon and *trn*F (GAA; Taberlet et al. 1991, Böhle et al. 1994) of the plastid genome. We do not attempt to resolve the outgroup relationships of the Colletieae; the inclusion of *Colubrina* Rich. ex Brongn., *Ceanothus* L., and *Noltea* Reichb. in the analysis serves exclusively the purpose of rooting the Colletieae.

Materials and methods

Taxon sampling. All species of the Colletieae are represented in the analyses except *Colletia spartioides* Bertero ex Colla, an endemic of Más a Tierra in the Juan Fernández Archipelago (Chile) of which no material was available. Voucher information is included in Table 1. For five of the 19 included ingroup species *trn*L intron DNA sequences were obtained from two different individuals. In only one case, *Discaria nitida*, were the sequences polymorphic with differences at 19 positions (3.7%). One sequence was very similar to the one obtained from *D. toumatou* (99.2% similarity) while the other resembled the one from *D. pubescens* (98.3% similarity). We have no reason to doubt the authenticity of the material (one voucher is deposited at BAA; the other originates from cultivated specimens from the Royal Botanic Gardens Melbourne, collected at the type locality). Mixing of samples seems unlikely as the sequences in question were produced on different dates. Consequently we have included both sequences in the analysis. All other species are represented only once in the data matrix. The *trn*L-F intergenic spacer sequences were obtained for all species except *Colletia hystrix, C. spinosissima, Retanilla ephedra,* and *R. patagonica*. Monophyly of *Colletia* was firmly established by both morphological data and the *trn*L intron sequence data. Likewise the morphological data supported monophyly of *Retanilla* while *trn*L intron sequence data supported monophyly of *R. ephedra*, *R. patagonica*, and *R. trinervis*. We therefore considered sequencing the *trn*L-F intergenic spacer of the four above mentioned species as unnecessary, and analyzed the species with data missing for the *trn*L spacer. Missing data can potentially result in lack of resolution of the final trees, but not distort the resolution as commonly believed (Kearney and Clark 2003). Outgroup choice was based on both the morphological and DNA sequence analysis. *Ceanothus coeruleus* and *Noltea africana* were included as outgroups, while *Colubrina asiatica* was used to root the cladogram.

Table 1. Sources of plant material used

Species	Voucher *Trn*L intron/*trn*L-F spacer	GenBank accession: *trn*L intron/*trn*L-F spacer
Adolphia infesta Meisn.	U.S.A.: California: Rancho Santa Ana Botanic Garden (858)	AY460408 / AY642142
Ceanothus coeruleus Lag.	Thulin et al. 1998	AJ225798
Colletia hystrix Clos	Argentina: Neuquén: D. Medan 774 (BAA)	AY460409
Colletia paradoxa (Spreng.) Escal.	Argentina: Buenos Aires: A. Mantese (BAA 22105)	AY460410 / AY642143
Colletia spinosissima Gmel.	Argentina: Buenos Aires: Hort. Bot. Facultad de Agronomía U.B.A. (607)	AY460411
Colletia ulicina Gill. & Hook.	Richardson et al. 2000	AJ390364
Colubrina asiatica Brongn.	Richardson et al. 2000	AJ390350
Discaria americana Gill. & Hook.	Germany: Berlin: Botanischer Garten und Botanisches Museum Berlin-Dahlem (048079210)	AY460413 / AY642144
Discaria articulata (Phil.) Miers	Argentina: Río Negro: S. C. de Bariloche, leg. E. Chaia, June 1997 (no herbarium voucher)/ Argentina: Neuquén: lago Huechulafquen, leg. H.H. Hilger s.n. 1995 (BSB)	AY460414 / AY642145
Discaria chacaye (G. Don) Tort.	Argentina: Neuquén: D. Medan 775 (BAA)	AY460415 / AY642146
Discaria nana (Clos) Weberb.	Argentina: Mendoza: D. Medan 840 (BAA)	AY460416 / AY642147
Discaria nitida Tort.	Sample 1: Australia: Royal Botanic Gardens (Melbourne 915497).	AY460418 / AY642148
	Sample 2: Australia: N.H. Scarlett 80-47 (BAA).	AY460417
Discaria pubescens (Brongn.) Druce	Australia: Royal Botanic Gardens (Melbourne), from wild-sourced plants at Bendock, eastern Victoria, leg. Neville Walsh 1997	AY460419 / AY642149
Discaria toumatou Raoul	Denmark: Botanic Garden of the University of Copenhagen (P 1981-5496)	AY460420 / AY642150
Discaria trinervis (Hook. & Arn.) Reiche	Argentina: Buenos Aires: J.J. Valla (BAA 23793) / Argentina: Neuquén: Catán Lil a Las Coloradas, leg. H.H. Hilger s.n. 1995 (BSB)	AY460421 / AY642151
Kentrothamnus weddellianus (Miers) Johnst.	Argentina: Jujuy: D. Medan 777 (BAA) / Argentina: Jujuy: Tafna, leg. H.H. Hilger s.n. 1995 (BSB)	AY460422 / AY642152
Noltea africana (L.) Reichenb.	Richardson et al. 2000	AJ390357
Retanilla ephedra (Vent.) Brongn.	Argentina: Buenos Aires: D. Medan (BAA 21960)	AY460423

Table 1 (continued)

Species	Voucher *Trn*L intron/*trn*L-F spacer	GenBank accession: *trn*L intron/*trn*L-F spacer
Retanilla patagonica (Speg.) Tort.	Argentina: Neuquén: D. Medan 776 (BAA)	AY460424 / AY642153
Retanilla stricta Hook. & Arn.	Chile: Colchagua: D. Medan 790 (BAA)	AY460425
Retanilla trinervia (Gill. & Hook.) Hook. & Arn.	Chile: Quillota: D. Medan et al. (BAA 21957)	AY460426 / AY642154
Trevoa quinquenervia Gill. & Hook.	Chile: Quillota: D. Medan et al. (BAA 22003)	AY460427 / AY642155

Morphological matrix. The morphological analysis has been published elsewhere (Aagesen 1999). The matrix used in the combined analysis differs from the one published by the exclusion of three dubious characters (characters 5, 23, and 53 in Aagesen 1999) which were not properly scored in non-American *Discaria* species due to the sparse material (see Aagesen 1999 for details). The original morphological matrix included ten *Ceanothus* species, *Noltea africana* and one species of *Colubrina*. When pruned to include only the taxa used in the combined analysis six characters became phylogenetically uninformative and were excluded. The morphological matrix contains 54 informative characters, with 4.7% of the cells coded as lacking or inapplicable.

DNA isolation. Sources of plant material used for sequencing are listed in Table 1. DNA was extracted from silicagel-dried green tissue, 70% (v/v) ethanol-fixed green tissue (the former was preferred whenever possible), or herbarium material following the method by Doyle and Doyle (1990), except that only 70% (v/v) ethanol was used to wash the pellets after precipitation with cold isopropanol.

DNA amplification and sequencing. Amplification and sequencing of the trnL intron and the *trn*L-F intergenic spacer were carried out separately in two different laboratories. The *trn*L intron was sequenced by D. Medan and H. Hilger while the *trn*L-F intergenic spacer was sequenced by J. Kellermann.

***trn*L intron:** 2 µl of the DNA extract was amplified in 25 µl 67mM Tris-HCl ph 9.0, 50 mM KCl, 2 mM $MgCl_2$, 1 µM of each primer, 200 µM of each dNTP, and 0.02 U/µl Taq DNA polymerase (Eurogentec). The primers used were C (5'-CGAAATCGGTAGACGCTACG-3'), D (5'-GGGGATAGAGGGACTTGAAC-3') (Taberlet et al. 1991), and C_1 (5'-AAGGATAGGTGCAGAGACTC-3'), which were designed and kindly provided by U.-R. Böhle (Berlin). The PCR reactions were performed in a Biometra thermocycler using a protocol of: 5 min 94 °C, 35 cycles (1 min 94 °C denaturation, 1 min 50 °C annealing, 1 min 72° extension), and 2 min 72 °C. PCR products were purified using the QIAquick purification kit (Qiagen). Cycle sequencing reactions (2 min 94 °C denaturation, 35 cycles [30 s 94 °C denaturation, 30 s 55 °C annealing, 30 s 72 °C extension], 1 min 72 °C extension) were done in a Perkin Elmer thermocycler using biotinylated primers. Sequencing reactions were separated in the GATC-1500-system (MWG Biotech), transferred to Nylon membranes (Qiagen, Pall Filtron) and visualized using standard protocols with Streptavidin-Alkaline Phosphatase (Promega) and BCIP/NBT (Roth) treatment. Sequences were read manually from the membranes.

***trn*L-F intergenic spacer:** 1–3 µl of DNA was amplified in 50 µl reactions containing 200 µM of each dNTP, 200 µM of each primer, 1.25 U HotStart Taq DNA polymerase (Qiagen) and 5 µl 10x PCR buffer (containing 15 mM $MgCl_2$; Qiagen). The primers used were E (5'-GGTTCAAGTCCCTCTATCCC-3') and F (5'-ATTTGAACTGGTGACACGAG-3') from Taberlet et al. (1991). Thermal cyling was performed on an Eppendorf Mastercycler gradient thermal cycler with one hold at 95 °C for 15 min preceeding 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and followed by one hold at 72 °C for 5 min. Products of the amplification were purified using the QIAquick PCR purification kit (Qiagen). Purified DNA was used as a template for direct sequencing with Applied Biosystems (ABI) Prism Ready Reaction BigDye Terminator Cycle

Sequencing Ready Reaction Kit (v3.1). Sequencing reactions were analyzed on an ABI 3730xl automatic capillary DNA sequencer by the Australian Genome Research Facility (Brisbane). Sequences were edited in Sequencher v3.0 (GeneCodes).

The sequences are deposited in GenBank with the accession numbers listed in Table l. The *trn*L_{UAA} intron and *trn*L-F spacer sequences included in this study correspond to positions 49.347 through 49.831 and 49.884 through 50.240 respectively of the Tobacco chloroplast genome published in GenBank (accession Z00044). In the species included in this study the corresponding portions vary in length between 482 bp and 531 bp for the *trn*L intron, and between 322 bp to 350 bp for the *trn*L-F spacer.

Phylogenetic analyses

Morphology. The analysis of the morphological data matrix was performed with NONA, ver. 1.9 (Goloboff 1998). All analyses were run using equal weights and the default settings amb- (retaining a branch only when unambiguous support is available) and poly = (polytomies allowed). Searches involved 500 subsearches each constructing a Wagner tree using a random addition sequence of taxa from the datamatrix, swapping the initial tree with TBR (tree bisection and reconnection) and retaining a maximum of 2 trees in each replicate (hold/2; mult*500). Bremer supports (Bremer 1994) were calculated finding suboptimal trees 1, 2, ... 10 steps longer than the shortest tree(s) saving a maximum of 10,000 trees. This was done in NONA by a loop finding suboptimal trees of a given length by swapping previous trees held in memory and extending the tree buffer with 1,000 trees in each round of the loop. Bremer supports were calculated after all trees had been found. As starting trees for the first round of the loop, all most parsimonious trees held in memory were used (syntax: loop 1 10; set 0 #*1000; hold 0'; sub#; find*; stop; bs;). Jackknife values (Farris et al. 1996) were calculated using an available instruction file for NONA, the jak.run file. The values were based on 5,000 Wagner trees submitted to TBR swapping holding a single tree for each initial Wagner tree (syntax: run[; jak 5000 hold/1 mult*1;).

Plastid data set – sensitivity analysis. Sequences were initially aligned by eye using the alignment editor Align (Hepperle 1997). During alignment an AT rich region of ambiguous alignment including 28–61 bp was found in the *trn*L intron. Areas of ambiguous alignment or 'hypervariable areas' are often excluded from phylogenetic analyses. The underlying assumptions are that positionally homology is too difficult to sort out and that there may be more that one optimal alignment (Swofford et al. 1996), or nucleotide positions that do not align consistently over a variety of alignment parameters are seen as unreliable relative to sites that are alignment-invariant (Gatesy et al. 1993). In addition, the phylogenetic information of hypervariable areas, esspecially the common AT-rich regions, has been questioned as the phylogenetic signal may be blurred due to multiple base changes, insertions, or deletions of mainly As and Ts (Lutzoni et al. 2000). However, whether these assumptions are true is seldom explored. Ambiguous alignable regions may include several optimal alignments, but it is possible to find and analyze these different optimal alignments. If a hierarchical pattern, common to all optimal alignments, emerges we see no reason for excluding this information. The concern that the hierarchical pattern may be caused by noise and not by phylogeny is more difficult to refute. Even randomly generated data sets have been shown to provide a hierarchical signal although the resulting topology lacks strong support (Hillis and Hulsenbeck 1992). Whether an observed character distribution is correlated with the phylogeny of a group is, however, a general concern not necessarily more pertinent to hypervariable areas than to other kinds of characters being morphological, DNA sequence fragments, or other. In this particular case the hypervariable area of the present sequences has been analyzed extensively elsewhere (Aagesen 2004). It was found to behave very differently from random generated areas with approximately the same base composition and sequence length variation. Furthermore, the hypervariable area was identical in four of the five species where sequences were amplified for two different specimens, hence the variation is found at species level, not at population level. Where differences were found (among the two *Discaria nitida* sequences) the differences were not confined to the hypervariable area but found throughout the sequences. Consequently we have no reasons for excluding any regions of the DNA sequences in this study.

Few methods are suitable for including ambiguous alignable regions. A hand alignment will present one possible alignment of the area,

but there may be more than one reasonable alignment of the sequences, as hand alignments do not include a strict optimality criterion. Lutzoni et al. (2000) proposed a method that relies on aligning the sequences prior to analysis, defining, and re-coding the ambiguous areas separately in step matrices using a single cost set. Direct optimization (Wheeler 1996) will find all optimal alignments under a specific cost set and does not require any manipulation of the sequences prior to analysis.

In the present study we used direct optimization as implemented in the program POY ver. 3.0.11 (Wheeler et at. 2003, documentation by De Laet and Wheeler 2003) to avoid the alignment dilemma when analyzing the *trn*L intron and *trn*L-F spacer. POY implements direct optimization (Wheeler 1996) that constructs phylogenetic hypotheses directly without the intervening step of multiple sequence alignment. When multiple sequence alignment and tree searches are conducted in two disconnected processes, as commonly done in many phylogenetic studies, the resulting trees are optimal for the multiple alignment but the multiple alignment may not be optimal for the final tree. There may exist one or more alignments that fit the tree even better (Wheeler 2001a, b). In direct optimization insertion and deletion events are incorporated in addition to base substitutions in the character optimization procedure. This ensures that the trees are compared on the basis of alignments being optimal for the individual tree as base changes and indels are placed at nodes giving an optimal length for the tree under evaluation. Consequently, base changes and indels are minimized for each tree, and POY selects the tree that requires least changes to fit the original sequences. An exact algorithm to calculate the cost of a set of unaligned sequences on a tree given a cost matrix was published by Sankoff (1975) and Sankoff and Cedergren (1983). The algorithm, however, is too computationally intensive to be of practical value, and it is known that the problem is NP-complete. Direct optimization provides heuristic for determining the cost of optimizing the unaligned sequences on a tree and, furthermore, incorporates heuristic search strategies as known from other phylogenetic tree search programs in the search for the optimal tree which itself is an NP-complete problem (De Laet and Wheeler 2003). The output of the search is the most parsimonious trees where each tree implies its own alignment, which is the optimal alignment for the tree in question (Wheeler 2003).

As any alignment is affected by the cost of the alignment parameters, commonly insertion-deletion costs and transversion:transition cost, the sensitivity approach (Wheeler 1995) explores the outcome of varying these parameters. In lack of external evidence on how to choose parameter costs, ideally all possible cost pairs should be explored. This is, however, an impossible task and as direct optimization furthermore is computationally demanding some sampling of possible cost values must be made. In the present study we use equal costs for base changes but treat the length information in different ways, analyzing a total of eight cost sets. Gaps were treated as independent events (analogous to a fifth character state) with the cost of an indel being equal to base changes or two times as costly as a base change (costs: 1,1; 2,1). Alternatively strings of gaps were treated as a single event. This is accomplished in POY by using different costs for opening a gap and for extending the gap. Two cost series with extension gaps in use were explored. One series sets the cost of extending a gap equal to the cost of a base change while the cost of opening a gap is twice, four, or eight times as costly as a base change (costs: 2,1/1; 4,1/1; 8,1/1). The second series sets the cost of extending a gap half the cost of a base change while the cost of opening a gap is equal to the cost of a base change, or twice, four, or eight times as costly as a base change (costs: 2,2/1; 4,2/1; 8,2/1).

The intron and the spacer were analyzed on their own and combined. The program POY permits cutting the sequences into minor fragments as well as analyzing all or some fragments with a preconceived alignment (using the option –prealigned). However, the reason why we have chosen the program POY as analytic tool in this study is that it permits analyzing the sequences as they were obtained. We therefore avoid any cutting and prealining of the sequences.

When the DNA sequence data were combined with morphology a character state change within a morphological character was equated either to the gap cost (see Wheeler and Hayashi 1998), or when extension costs were in use the cost of a morphological transformation was equated to the cost of a base change.

Direct optimization is computationally demanding even for a small data set. We used the following search strategy that showed to be efficient for our data set: -replicates 50 -nopr -tbr -stopat

4-minstop 10-norandomizeoutgroup-seed-1-maxtrees 2. This creates a Wagner tree and submits it to TBR swapping holding a maximum of two trees. The procedure was repeated 50 times using the same outgroup with the time used as seed for the random number generator defining the input order of the taxa during the replicates. When the optimal length was found four times, the search was abandoned if a minimum of 10 searches had been completed. After the replicates were completed the resulting trees were, by default, submitted to TBR swapping, storing all optimal trees found. Bremer supports were calculated in POY by using the option –bremer and a constrain file. Jackknife values were calculated using the options: -replicates 1000 -nospr -tbr -maxtrees 5. This gives 1000 replicates each calculating five Wagner trees which are TBR swapped. The jackknife values are obtained by a majority rule consensus tree with a cut value of 50%.

All matrices and implied alignments (Wheeler 2003) from POY are available upon request from the first author.

Congruence. When several cost sets are used in a phylogenetic analysis the problem of how to choose among trees from different cost sets has to be addressed. Wheeler (1995) used congruence measures to select optimal alignment costs applying both taxonomic congruence (Nelson 1979) and character congruence (Mickevich and Farris 1981). A scaled version of the incongruent length difference measure, ILD (Mickevich and Farris 1981) is at present commonly used to select optimal cost sets in sensitivity analyses (e.g. Wheeler and Hayashi 1998, Frost et al. 2001, Giribet et al. 2001).

The ILD for data set A and B is:

$$\mathrm{ILD} = L_{AB} - (L_A + L_B)/L_{AB}$$

where L_{AB} is the length of the shortest tree from the combined data set and L_A and L_B are the lengths of the shortest tree of data set A and B respectively. The numerator gives the number of (weighted) extra steps obtained due to conflict between data set A and B and the ILD gives this number of extra steps as a fraction of the total (weighted) steps of the tree from the combined data set. The sensitivity analysis therefore picks as optimal the tree, and cost set, where the number of (weighted) extra steps that are caused by conflict between the data partition constitute the smallest possible fraction of the total (weighted) length of the tree from the combined data set.

The ILD can, under some circumstances, approach zero when the weighting schemes become disproportionate although congruence is not improved (Dowton and Austin 2002). In the example of Dowton and Austin (2002) two data partitions were combined and analyzed under the same transformation costs but one of the partitions was given an increasingly higher weight while the weight of the other partition was kept constant. In such cases the length of the tree for one of the partitions will approach the length of the tree from the combined data set and the ILD will approach zero. This behavior of the ILD is relevant in the present case. In this analysis indel cost is varied but this does not affect the weight of the morphological data set. Two conditions may, however, prevent the ILD from distortion. One is that both DNA partitions include sequence length variation, even though the *trn*L intron is more variable in length than the *trn*L-F spacer. Secondly, in the combined analysis, the morphological data set is given a weight equal to either gap cost or base change cost.

In addition to character based congruence we used a topological based congruence measure, the TILD and a rescaled version TILD_n (Wheeler 1999). Topology measures compare the optimal trees of the individual partitions of the data set and all share the drawback of not considering strength of evidence (Farris et al. 1995). However, as topology measures do not rely on tree length they are not affected by the distortion that differential weighting causes in the character based congruence measures. It is used here simply to report at which cost set the optimal trees of the individual partitions are most similar.

The TILD index (Wheeler 1999) is based on the incongruence length difference (Mickevich and Farris 1981), scaled as the ILD index (Wheeler and Hayashi 1998).

$$\mathrm{TILD} = L_{ab} - (L_a + L_b)/L_{ab}$$

The input matrices a and b used in the TILD index consists of the group inclusion characters (Farris 1973) derived from the topologies of the optimal trees found when analyzing the original data partitions A and B (if the data sets generate several optimal trees a consensus tree is used). Members of a clade receive the state 1 while the remaining terminals receive the state 0. L_a and L_b will be equal to the number of resolved groups in the optimal tree or consensus derived from the original matrices A and B respectively. When the

group inclusion character matrices a and b are combined and analyzed the homoplasy [$L_{ab} - (L_a + L_b)$] is proportional to the number of species placed differently in the optimal tree from matrix A and the optimal tree from matrix B. The metrics can be extended to include any number of input data partitions each defining an optimal tree that is converted into a group inclusion character matrix.

If some of the optimal trees are highly collapsed this can affect the index. If one of the data partitions under some costs generates several optimal trees and a highly collapsed consensus while under other costs a better resolved consensus trees is found, the index may choose the first cost set as optimal simply because there are less groups to disagree upon. To avoid this situation the TILD was modified and scaled by amount of possible disagreement.

$$TILD_n = L_{ab} - (L_a + L_b)/maxL_{ab} - (L_a + L_b)$$

where $maxL_{ab}$ is equal to G used in the retention index RI (Farris 1989).

The ILD, TILD and $TILD_n$ were applied to the combined data set to identify maximal congruence between the three individual data partitions (morphology, *trn*L intron, and *trn*L-F spacer) and between the morphological data set and the combined plastid data set.

It should be noted that the ILD measure used here to select among alternative cost sets differs from the more familiar ILD test (Farris et al. 1995), which is used to explore amount of incongruence between data partitions. We do not attempt to measure the degree of congruence between the data partitions. We agree with Nixon and Carpenter (1996) who argued that separate analyses are useful in understanding the differences among data partitions, but that simultaneous analysis provides the greatest possible explanatory power, and are therefore to preferred.

Results

Morphology. The reduced morphological matrix (see Material and Methods) resulted in two optimal trees (length L = 158, consistency index CI = 0.49, retention index RI = 0.71) shown in Fig. 1. In contrast to the previous analysis (Aagesen 1999) *Discaria* is here monophyletic, but monophyly is conditional on the exclusion of the characters mentioned in Materials and Methods.

Sensitivity analysis. The values of the different congruence measures under different cost sets are shown in Table 2.

According to the character based congruence measure, ILD, maximal congruence was obtained under cost set 4,1/1 both when congruence was measured between the three individual data partitions and when congruence was measured between the morphological data set and the combined plastid data set (Table 2). The scaled topological congruence measure $TILD_n$ supports the same cost set as the optimal one when congruence is measured between the morphological data set and the combined plastid data set. In the other cases cost set 4,2/1 or 8,2/1 are prefered. The differences between the trees obtained under the three cost sets are discussed below. The implied alignments (Wheeler 2003) under cost set 4,1/1 of the *trn*L intron and the *trn*L-F spacer contained few indels in the conservative regions (only those also required by an eye alignment). In the hypervariable area indels of one to 23 bp were inserted to sort out the homologies between the bases.

The results obtained when analyzing the *trn*L-F region on its own is summarized in Table 2. Figure 2 shows the strict consensus tree obtained under cost set 4,1/1. The number above the branches indicates in how many of the eight cost sets a clade was recovered. Two groups are found under all eight cost sets. One group is the genus *Colletia* while the second group includes *Adolphia infesta* and six *Discaria* species (hereafter the *Adolphia-Discaria* p.p. clade). The Colletieae tribe is monophyletic under all cost sets except 2,1 where *Noltea* is nested within it. Disagreements among the consensus trees obtained from the different cost sets are mainly caused by cost set 2,1/1 and 8,1/1 where the *Trevoa-Retanilla* clade is collapsed due to conflict among the trees. Further disagreement concerns the relationship among the major clades: *Trevoa-Retanilla*, *Colletia*, *D. nana-D. trinervis*, and the

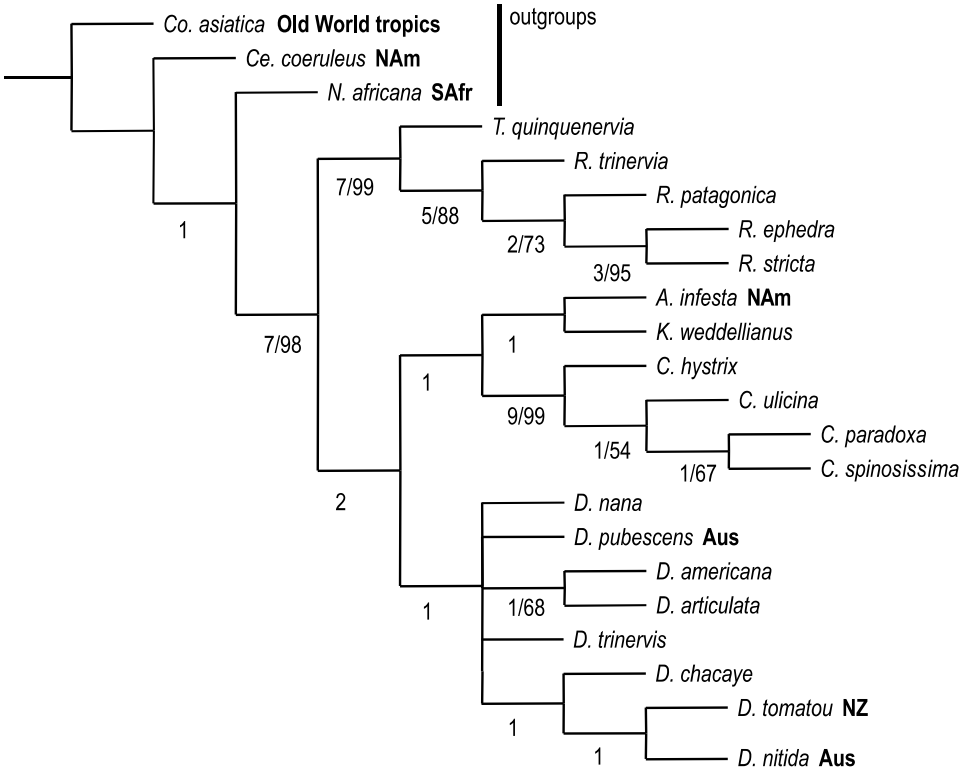

Fig. 1. Strict consensus of the two trees found when analyzing the morphological data set (L = 158, CI = 0.49, RI = 0.71). Numbers below branches refer to Bremer support/jackknife values. For all taxa not occurring in southern South America the geographic distribution has been added: *Aus* = Australia, *NZ* = New Zealand, *SAfr* = South Africa, *NAm* = North America (here southern North America and northern Mexico)

Adolphia-Discaria p.p. clade, and disagreement in the resolution within the *Adolphia-Discaria* p.p. clade.

The results from the combined sensitivity analysis are summarized in Table 2. Figure 3 shows the strict consensus tree obtained for the combined molecular and morphological data under cost set 4,1/1. This cost set was optimal according to the ILD and $TILD_n$ when congruence is measured between morphology and the plastid data set. Alternatively the topological congruence measure indicates cost set 4,2/1 or 8,2/1 as optimal (Table 2). These trees differ from the one obtained under cost set 4,1/1, either in a resolved *Colletia* with *C. hystrix* as sister to the remaining species (cost set 8,2/1), or in the basal branch among the six *Discaria* species within the *Adolphia-Discaria* p.p. clade being collapsed (cost set 4,2/1).

The tree in Fig. 3 includes many of the same clades found when analyzing the plastid data set on its own under the same cost set but the Bremer supports are improved when morphology is added. Similarly, when compared to the morphological tree (Fig. 1) the Bremer supports of clades shared with this tree are improved as well.

All costs sets agree upon monophyly of the tribe Colletieae and the resolution of the *Trevoa-Retanilla* clade. The basal dichotomy between the *Trevoa-Retanilla* clade and the remaining Colletieae species that is supported by morphological characters is also generally agreed upon, with the only exception being cost set 8,1/1. Cost set 8,1/1 represents one of the extreme cost sets within the chosen parameter space and is generally found to be among the cost sets giving the least congruent results.

Table 2. Results of the sensitivity analysis using eight different cost sets (see Material and Methods for abbreviations of the cost sets). Congruence (ILD, TILD and $TILD_n$) has been measured between the morphological data set and the combined *trn*L-F data set (morph/plastid) and between the three individual partitions: morphology, *trn*L intron and *trn*L-F spacer (3 partitions). Numbers in bold are the obtained minimum values of the corresponding congruence measures

Cost set	Length					ILD		TILD		$TILD_n$	
	morph	intron	spacer	plastid combined	all combined	morph/ plastid	3 partitions	morph/ plastid	3 partitions	morph/ plastid	3 partitions
2,1	292	295	158	463	806	0.0633	0.0757	0.2885	0.3433	0.1364	0.1742
1,1	146	192	105	301	469	0.0469	0.0554	0.2600	0.2881	0.1300	0.1604
2,1/1	146	225	116	347	511	0.0352	0.0470	0.2500	0.3553	0.1325	0.1788
4,1/1	146	277	138	423	587	**0.0307**	**0.0443**	0.2353	0.3953	**0.0984**	0.2446
8,1/1	146	345	182	545	723	0.0443	0.0692	0.2727	0.4096	0.1364	0.2636
2,2/1	292	296	168	470	803	0.0511	0.0585	0.2653	0.2969	0.1340	0.1570
4,2/1	292	361	190	559	890	0.0438	0.0528	0.2400	**0.2692**	0.1121	**0.1373**
8,2/1	292	460	234	711	1039	0.0346	0.0510	**0.2340**	0.2708	0.1058	0.1512

Four other clades are robust to changes in parameter costs: the *Colletia* clade with *C. paradoxa* and *C. spinosissima* as sistergroups, the *D. nana-D. trinervis* clade, and the *Adolphia-Discaria* p.p. clade. Within the *Adolphia-Discaria* p.p. clade *Adolphia* is sistergroup to the six *Discaria* species in all but one of the cost sets, the 8,1/1 also mentioned above. In all other cost sets the American *Discaria* species form a clade with *D. toumatou* (New Zealand) and the sample of *D. nitida* (Australia), which is similar *to D. toumatou*. Within this clade *D. americana* and *D. articulata* are sister species. In six of the eight cost sets *Discaria nana* and *D. trinervis* are sistergroups to a clade formed by *Colletia*, *Kentrothamnus* and the *Adolphia-Discaria* p.p. clade. This topology is absent in the above mentioned cost set 8,1/1 and in cost set 1,1 where the resolution among these clades is collapsed. Further resolution is more sensitive to parameter cost choice.

Discussion

Previous morphological analyses of the tribe Colletieae failed to obtain a detailed resolution within the tribe (Aagesen 1999). In order to improve the phylogenetic analysis we included sequence data from the *trn*L intron and *trn*L-F spacer. When visually inspecting the obtained sequences an ambiguously alignable region was found. Ambiguously alignable regions may include the fastest evolving sites of a given DNA segment and therefore potentially contain information for resolving lower level relationships within an analysis (Lutzoni et al. 2000). However, in these hypervariable areas the positional primary homologies (sensu De Pinna 1991) are uncertain due to length mutations, and gaps may be inserted in more than one optimal way. Exploring the effect of multiple optimal alignments on phylogenetic reconstruction has been recommended in textbooks, for example by Doyle and Davis (1998). In order to find and analyze all equal optimal alignments the need for explicit algorithms is

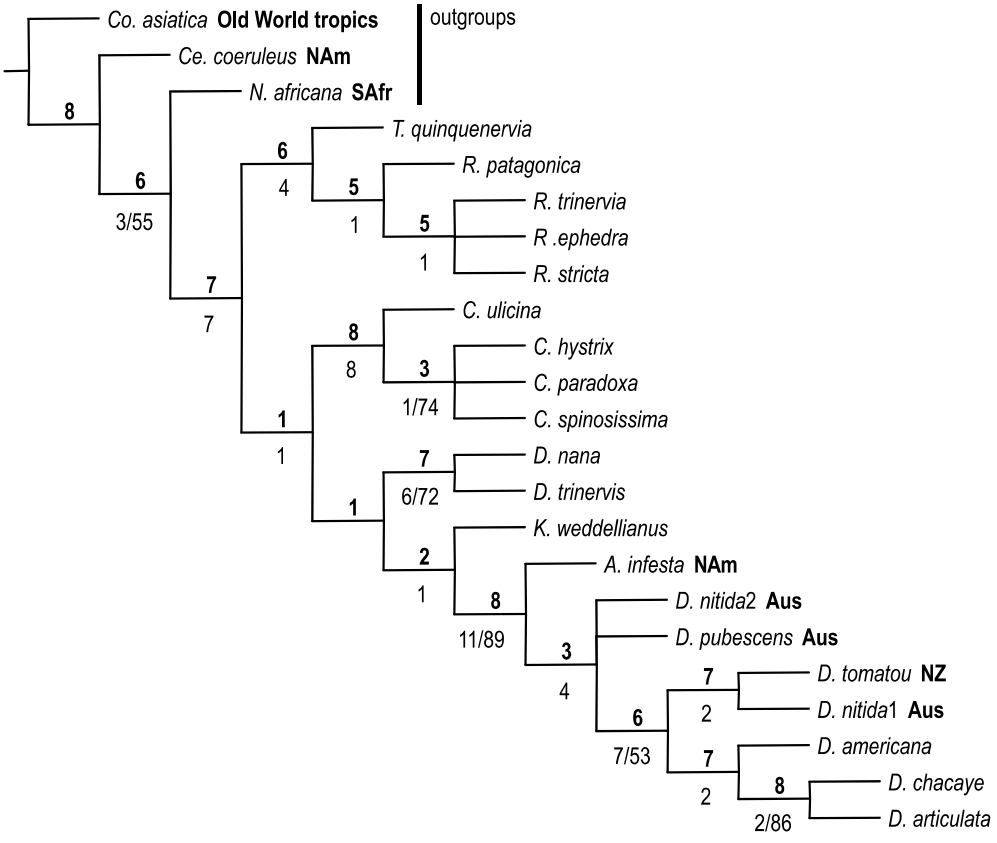

Fig. 2. Single tree found when analyzing the plastid data set under cost set 4,1/1. Numbers above branches indicate how many of the eight different cost sets supported the clade. Numbers below the branches indicate Bremer supports/jackknife values. Abbreviations as in Fig. 1

obvious. Few available alignment programs search for multiple optimal alignments. In this analysis we prefer the program POY that makes use of direct optimization (Wheeler 1996). When using direct optimization the alignment of the sequences and the search for optimal trees is done in one single step, assuring that all trees are compared on the basis of their own optimal alignment. When comparing the performance of direct optimization to analyses using multiple alignment and tree search in separate steps the former approach has been shown to be more successful in finding optimal alignments (Wheeler 2001a, b).

Eight different cost sets were used and an optimal cost set was selected by measuring congruence between the morphological data set and the plastid data set. As an alternative we also measured congruence between the three individual partitions (morphology, *trn*L intron, and *trn*L-F spacer). However, the two plastid loci do not contain sufficient phylogenetic information to yield well resolved trees under all cost sets, while the two loci combined produce well resolved trees under nearly all cost sets. These differences may affect the congruence measures. Some relevant divergences between the two approaches are discussed below.

The sensitivity approach allowed us to identify a single cost set as the optimal one when measuring congruence between morphology and plastid DNA. Both ILD and $TILD_n$ agreed upon cost set 4,1/1 as the optimal one. TILD on the other hand identified cost set 8,2/1 as optimal. The difference between these two trees is that cost set 8,2/1

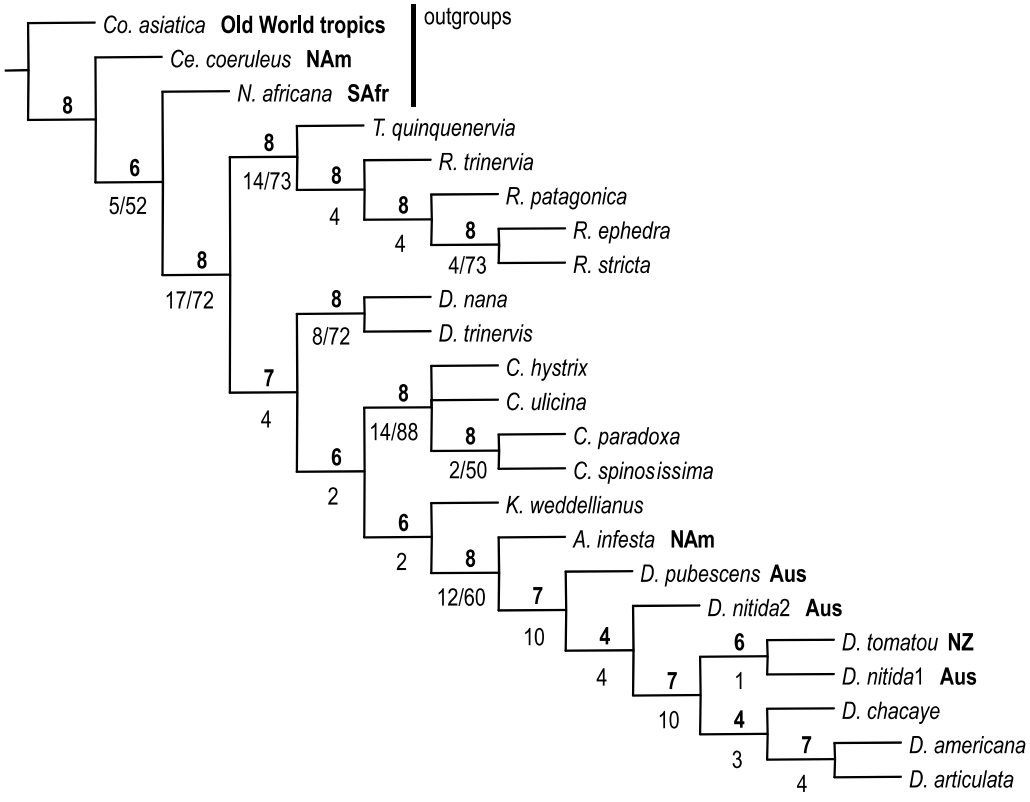

Fig. 3. Strict consensus of two optimal trees found when analyzing the morphological data set combined with the plastid data set under cost set 4,1/1. Numbers above branches indicate how many of the eight different cost sets supported the clade. Numbers below the branches indicate Bremer supports/jackknife values. Abbreviations as in Fig. 1

resolves the trichotomy in *Colletia* by placing *C. hystrix* as sistergroup to the remaining *Colletia* species. When measuring congruence among the three individual partitions ILD still selects 4,1/1 as the optimal cost set while the topological congruence measures agree on cost set 4,2/1 as the optimal one. The only difference between the consensus trees from the two cost sets is that the basal branch among the six *Discaria* species within the *Adolphia-Discaria* p.p. clade is collapsed in cost set 4,2/1.

Colletieae. Monophyly of Colletieae was supported in all analyses except when the plastid data set was analyzed on its own under cost set 2,1. The Bremer supports are high, especially in the combined analysis where the jackknife values, however, are somewhat lower that in the morphological analysis.

A strongly supported monophyletic Colletieae was also found by Richardson et al. (2000) although only four Colletieae species were included in their family level analysis. Despite the strongly supported monophyly the Colletieae are not defined by unique synapomorphies not found in the remaining Rhamnaceae species (Aagesen 1999). However, the tribe Colletieae with its densely armed shrubs or small trees with decussate leaves and serial meristems appear morphologically homogeneous and monophyly of the tribe has not been disputed.

Within the Colletieae the morphological data set supports a basal dichotomy between an almost entirely Chilean *Trevoa-Retanilla* clade and a second clade including all remaining genera (Fig. 1). The same basal dichotomy is found by analyzing the plastid data set under

cost set 4,1/1 (Fig. 2). Other cost sets partly support the same two clades but include a basal trichotomy also including *Kentrothamnus* (cost set 1,1 and cost set 2,2/1). In the remaining cost sets the consensus trees show a polytomy including several of the major clades.

When combined with morphology the basal dichotomy appears under all cost sets except cost set 8,1/1 which is not among the optimal cost sets (see Table 2 and Fig. 3). The clade containing *Adolphia*, *Colletia*, *Discaria*, and *Kentrothamnus* is only moderately supported. Although the Bremer support is higher in the combined analysis, the clade does not survive jackknifing the data set.

Trevoa-Retanilla. The *Trevoa-Retanilla* clade is supported by morphology (Fig. 1) and the plastid data set under all cost sets except 2,1/1 and 8,1/1 (Fig. 2). When all data are combined the *Trevoa-Retanilla* clade as well as its internal resolution are robust to all changes in parameter cost choice and among the groups with highest Bremer support and jackknife value (Fig. 3).

Miers (1860) perceived *Trevoa* and *Retanilla* as a distinct subset of the Colletieae, and established the division *Clithrocarpae* to include the species. However, *Clithrocarpae* has not been given formal rank as a subtribe.

Several morphological characters distinguish the group from the remaining genera of the tribe; inflorescences with a terminal flower, petals and stamens integrated as pollen-dosing units, secondary pollen dispersal, indehiscent – or tardily dehiscent – fruits, and a distinctive aril type (Medan and Aagesen 1995, Aagesen 1999).

Kentrothamnus. The morphological data set places *Kentrothamnus weddellianus* and *Adolphia infesta* in a clade sister to the *Colletia* species. In the plastid data set *Kentrothamnus* is mostly placed in a basal polytomy within the tribe, but when this polytomy is resolved *Kentrothamnus* is placed as sister group to the *Adolphia-Discaria* p.p. clade as seen in Fig. 2. This topology remains when all data are combined. *Kentrothamnus* is again placed as sister to the *Adolphia-Discaria* p.p. clade except under cost set 8,/1 and 1,1 (Fig. 3).

Colletia. Monophyly of *Colletia* is supported by both morphology and the plastid data set under all eight cost sets (Figs. 1–3). In the combined analysis both Bremer support and jackknife values are high.

Within the genus *Colletia*, *C. paradoxa* and *C. spinosissima* always appear as sister species, when morphology is included, but the position of C. *hystrix* and *C. ulicina* is unresolved, either one being the sister to the rest of the genus.

Morphological characters that define *Colletia* within the tribe include paracytic stomata, a revolute disc, and several characters related to the inflorescence (Aagesen 1999).

Discaria. The monophyly of *Discaria* could not be confirmed in the analysis of Aagesen (1999). In the present analysis three dubious morphological characters were excluded from the original matrix. The resulting morphological matrix weakly supports monophyly of the genus with a Bremer support of 1 and no jackknife value.

The plastid data set supports paraphyly or polyphyly but not monophyly of *Discaria*. *Adolphia* groups with six of the *Discaria* species in all cost sets (Fig. 2) and this topology is retained when morphology is added (Fig. 3).

The phylogenetic information of the *trn*L-F sequence data seriously challenges the current circumscription of *Discaria*, which was already questioned by the earlier morphological analysis. This is not the first time that *Discaria* has been perceived as a nonhomogeneous entity. Miers (1860) assigned the species to three different genera (*Discaria*, *Notophaena* and *Ochetophila*). Suessenguth (1953) considered the differences were of sectional rank only [sections *Eudiscaria* nom. illeg. (= sect. *Discaria*), *Ochetophila* and *Notophaena*].

Polyphyly of the genus *Discaria* is a very firm result of our analysis due to the robustness of the *Adolphia-Discaria* p.p. clade (excluding *D. nana* and *D. trinervis*) to variation in analytic parameter.

Adolphia-Discaria p.p. This clade includes *Adolphia infesta*, *Discaria americana*, *D. articulata*, *D. chacaye*, *D. nitida*1 + 2, *D. pubescens*, and *D. toumatou*. The clade is supported by the plastid data and is retained throughout the parameter space without exception when *trn*L-F sequence data is analyzed alone or in combination with morphology. When adding morphology the Bremer support is slightly improved while the jackknife value is lower. Nevertheless, the group is well supported by a Bremer support of 12 and a jackknife value of 60. No morphological characters support the group. In the combined analysis *Adolphia* is the sister group to the *Discaria* p.p. species in all cost sets except 8,1/1 (Fig. 3).

The *Discaria* species included in this clade make up the sections *Eudiscaria* and *Notophaena* in the treatment of *Discaria* by Suessenguth (1953). They are morphologically diverse, having no apparent apomorphic morphological character in common. All extra-American *Discaria* species are included in the clade.

The biogeography of the clade is interesting although no clear statement can be made, and furthermore depends on which of the two *Discaria nitida* samples represent the species (see below). Recently Sanmartín and Ronquist (2004) argued that most plant-distribution patterns found in the southern hemisphere are caused by dispersal rather that by vicariance events. The authors especially doubted that the divergence between Australian and New Zealand taxa were caused by vicariance. The lack of adaptations for long-distance dispersal in *Discaria* s.s. (Medan 1985, Keogh and Bannister 1993) and, in fact, in all known genera of Colletieae (Medan and Aagesen 1995), suggest that at least part of the pattern can be attributed to vicariance events. The limited variation found in the *trn*L-F region does, however, shed some doubt on the age of the tribe. Limited variation within the same region was also found by Richardson et al. (2000) at family level, even though fossil record indicates that Rhamnaceae is considered to be 94–96 million years old (Richardson et al. 2000). However, even if *Discaria* was present in both Australia and South America before these continents drifted apart, later dispersal events may account for part of the distribution pattern, as well.

Discaria nitida. The case of *Discaria nitida* is enigmatic. The sequence found in *D. nitida*1 is very similar to that from *D. toumatou*. Within the variable area the two sequences share a seven bp indel also found in *D. americana*, *D. articulata*, and *D. chacaye*. The sequence found in *D. nitida*2 resembles that from *D. pubescens*.

Both *D. nitida* sequences were sampled from the same locality (Cobungra, Victoria). It seems likely that one of either sequences is secondarily acquired, e.g. by chloroplast capture through hybridization and introgression (Rieseberg and Soltis 1991). Rieseberg and Soltis (1991) reviewed 37 examples of supposed chloroplast capture and found several cases of unexpected cpDNA transfer. The authors suggested that the phenomenon might be common and should not be ruled out even in plant groups not noted for hybridization. Among South American *Discaria* species supposed hybrids have been found (Tortosa 1983) or artificially produced in field experiments (D. Medan and A. Basilio, unpublished data). At the above mentioned locality *Discaria pubescens* was found growing together with *D. nitida* (Hall and Parsons 1987). The flower morphology of the two species is very similar (Medan and Aagesen 1995) and their blooming periods coincide for two months (Hall and Parsons 1987). Extrapolating from data on reproductive biology of other *Discaria* spp. (*D. toumatou*, Primack 1979, Webb 1985; *D. americana*, Medan 1991, 1993), it seems reasonable to assume that both Australian species receive visits from a generalist pollinator assemblage. Therefore, it seems possible that hybridization between the two populations could take place. In fact, Wright and Briggs (2000) reported the occurrence of apparent hybrids between *D. nitida* and *D. pubescens* at five populations (of 18 surveyed) where both species are sympatric. Compared to the obtained sequence of

D. pubescens, the sequence of *D. nitida*2 differs by four autapomorphies, suggesting that if cpDNA transfer has taken place it might not be recent. Unfortunately we have no sequences of *Discaria pubescens* from the above mentioned locality. The second possibility, that cpDNA has been transferred from *D. toumatou*, seems less likely at least when considering modern geography. *Discaria toumatou* is only known from New Zealand, but if the distribution of the two species has concurred at some point in history, hybridization seems likewise possible as floral morphology and flowering time coincide (Primack 1979, Medan and Aagesen 1995). Morphologically *Discaria nitida* resembles *D. toumatou* rather than *D. pubescens*, but to confirm our findings and to trace the possible historical explanation of the divergence between the two sequences further sampling of the species in question is required. Figure 3 includes both *D. nitida* specimens. If *D. nitida*1 is excluded from the combined analysis, the same topology as the one shown in Fig. 3 appears. If, however, *D. nitida*2 is excluded, *D. chacaye* appears as sister taxon to the *D. toumatou-D. nitida*2 clade (trees not shown).

Discaria nana and D. trinervis. In the analysis based on morphological characters alone, the positions of *Discaria nana* and *D. trinervis* within Discaria, are not resolved (Fig. 1). The sensitivity analysis of the plastid data set alone supports monophyly of *D. nana* and *D. trinervis* with moderately high Bremer support and jackknife value. The placement of the clade varies with cost set. Only at cost set 4,1/1 is the clade associated with the *Adolphia-Discaria* p.p. clade and with *Kentrothamnus* (Fig. 2).

When combined with morphology *Discaria nana* and *D. trinervis* form a monophyletic group under all analyzed cost sets, still with moderately high Bremer support and jackknife value. The clade is sister group to the remaining species of the *Adolphia-Colletia-Discaria-Kentrothamnus* clade under nearly all cost sets, the only exception being cost set 8,1/1 (Fig. 3).

Discaria nana and *D. trinervis* were segregated from *Discaria* by Miers (1860) as the genus *Ochetophila* partly because of slightly different stipule morphology, not confirmed by Suessenguth (1953). The two species share several morphological character states not present in other *Discaria* species. These potential synapomorphies are leaf margin always entire, striated cuticle in leaf underside, spines with basal nodes, and the petals are not reduced as in other *Discaria* species (Medan and Aagesen 1995, Aagesen 1999).

Taxonomic considerations. The present analysis generally supports the existing taxonomic treatment of the tribe Colletieae. The three monotypic genera (*Adolphia*, *Kentrothamnus*, and *Trevoa*) were all placed in positions that did not interfere with the monophyly of other genera. The only exception is *Adolphia* which was placed as sister taxon to *Discaria americana* under cost set 8,1/1. We do, however, consider it safe to dismiss this result as cost set 8,1/1 was not included among the optimal cost sets according to the congruence measures and, furthermore, yielded several results divergent from the pattern supported by other cost sets. *Colletia* and *Retanilla* were strongly supported as monophyletic groups. *Discaria* on the other hand is still problematic. The sensitivity analysis strongly supports segregating *D. nana* and *D. trinervis* from *Discaria*, as originally proposed by Miers (1860). The remaining *Discaria* species do form a monophyletic group robust to changes in parameter cost set and well supported according to the Bremer supports. We recommend reestablishing the genus *Ochetophila* for *D. nana* and *D. trinervis*, but we will treat this topic in a separate publication.

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Addresses of the authors: Lone Aagesen (e-mail: laagesen@darwin.edu.ar), Instituto de Botánica Darwinion, C. C. 22, 1642 San Isidro, Argentina. Present address: Division of Invertebrate Zoology, American Museum of Natural History, Central Park West @ 79th St., New York, NY 10024-5192, U.S.A. Diego Medan, Cátedra de Botánica, Facultad de Agronomía de la U.B.A., Av. San Martín 4453, RA-1417 Buenos Aires, Argentina. Jürgen Kellermann, School of Botany, The University of Melbourne, VIC 3010, Australia. Hartmut H. Hilger, Institut für Biologie, Systematische Botanik und Pflanzengeographie, Freie Universität Berlin, Altensteinstrasse. 6, 14195 Berlin, Germany.