MOLECULAR PHYLOGENETICS

Phylogeny of the tribe Cinchoneae (Rubiaceae), its position in Cinchonoideae, and description of a new genus, *Ciliosemina*

Lennart Andersson* & Alexandre Antonelli

Botanical Institute, Göteborg University, P. O. Box 461, SE-405 30 Göteborg, Sweden. alexandre.antonelli@botany.gu.se (author for correspondence)

Relationships of and within the Rubiaceae tribe Cinchoneae were estimated based on DNA sequence variation in five loci: the ITS region, the *matK* and *rbcL* genes, the *rps*16 intron, and the *trnL-F* region including the *trnL* intron and the *trnL-F* intergenic spacer. Within Cinchonoideae s.s., the tribe Naucleeae is the sister group of a clade that comprises all other taxa. Cinchoneae and Isertieae s.s., are strongly supported as sister groups. The tribe Cinchoneae is strongly supported as monophyletic in a restricted sense, including the genera *Cinchona*, *Cinchonopsis*, *Joosia*, *Ladenbergia*, *Remijia* and *Stilpnophyllum*. There is strong support that these genera are monophyletic as presently conceived, except that one species mostly referred to *Remijia* is of uncertain phylogenetic affinity. To accommodate this species and a morphologically closely similar one, a new genus, *Ciliosemina* A. Antonelli, is proposed and two new combinations are made.

KEYWORDS: *Cinchona*, Cinchoneae, *Cinchonopsis*, *Joosia*, *Ladenbergia*, *Remijia*, *Stilpnophyllum*, Rubiaceae; ITS, *matK*, *rbcL*, *rps*16 intron, *trnL-F*.

INTRODUCTION

Traditionally (e.g., Candolle, 1830; Schumann, 1891, 1897; Robbrecht, 1988), the tribe Cinchoneae has been circumscribed to include about 50 genera with ascendingly imbricate, winged seeds. Based on a cladistic analysis of morphological data, Andersson & Persson (1991) narrowed the circumscription to include only 13 genera (three of which tentatively placed): *Capirona* Spruce, *Cephalodendron* Steyerm., *Cinchona* L., *Cosmibuena* Ruiz & Pav., *Dolicholobium* A. Gray, *Ferdinandusa* Pohl, *Joosia* H. Karst., *Ladenbergia* Klotzsch, *Macrocnemum* P. Browne, *Maguireocharis* Steyerm., *Pimentelia* Wedd., *Remijia* DC., and *Stilpnophyllum* Hook. f. Based on a more detailed, morphological dataset, Andersson (1995) removed *Capirona*, *Cosmibuena*, *Dolicholobium*, *Ferdinandusa* and *Macrocnemum*. He also synonymized *Cephalodendron* under *Remijia* and established one new genus, *Cinchonopsis* L. Andersson. As a consequence, the tribe Cinchoneae is presently considered to comprise eight genera.

For a further understanding of the relationships of and within Cinchoneae, the subfamilial context must be considered. Schumann (1891) subdivided Rubiaceae into two subfamilies, Cinchonoideae with two to many ovules per ovary locule and Coffeoideae with only one. In this classification Cinchoneae was placed in Cinchonoideae. Bremekamp (e.g., 1966) revised Schumann's classification and redefined Cinchonoideae to comprise only genera without raphides, with imbricate or valvate corolla aestivation and testa cells with coarsely pitted basal walls. Bremekamp recognized five subfamilies in addition to Cinchonoideae, namely Guettardoideae, Hillioideae, Ixoroideae and Rubioideae. Robbrecht (1988) essentially followed Bremekamp, but reduced the number of subfamilies to four: Antirheoideae, Cinchonoideae, Ixoroideae, and Rubioideae.

Cladistic studies based on DNA restriction site data (Bremer & Jansen, 1991) and DNA sequence variation (e.g., Bremer & al., 1995; Andersson & Rova, 1999; Rova & al., 2002) have shown that there are three more or less strongly supported, larger clades in Rubiaceae, which in accordance with Bremer & al. (1995) may be termed Cinchonoideae s.s., Ixoroideae s.l., and Rubioideae. Since the circumscriptions of these entities are uncontroversial by now, we will henceforth refer to them without the *sensu* designations. The cladistic studies agree that Robbrecht's Antirheoideae is polyphyletic and that Bremekamp's Guettardoideae and Hillioideae are nested within Cinchonoideae. Only two genera have been sequenced that have not been supported to belong in any of these groups. *Coptosapelta* Korth. was weakly supported as sister of all other Rubiaceae in the study of Bremer & al. (1999). *Luculia* Sweet has been found in

**Prof. Andersson died suddenly while this paper was in press. He will be deeply missed by all his friends, colleagues and the scientific community.*

several studies (Bremer & al., 1995, 1999; Rova & al., 2002) to have an unresolved position outside of the three currently recognised subfamilies. A sister group relationship between Cinchonoideae and Ixoroideae is strongly supported by several studies (Andersson & Rova, 1999; Bremer & al., 1999; Rova & al., 2002).

Relationships within Cinchonoideae s.s. remain largely uncertain, in spite of a number of DNA-based studies. Rova (1999; Fig. 1A) used the most extensive sample analysed so far. Based on a combination of *rps*16 intron and *trnL-F* sequences, he identified six major subclades within this group, five of which were more or less strongly supported. One of these corresponds to the tribe Cinchoneae in Andersson's (1995) circumscription.

Rova's subclades are to a large extent identifiable in other studies such as those of Bremer & al. (1995; Fig. 1B), Andersson & Rova (1999; Fig. 1C) and Razafimandimbison & Bremer (2001; Fig. 1D), even though represented there by fewer taxa. However, no study has so far managed to find support for the relationships between these clades, and the relationships of the tribe Cinchoneae to other Cinchonoideae remain essentially unknown. Andersson & Rova (1999) found *Cinchona* to be sister of *Isertia*, and these two to form the sister group of other Cinchonoideae, although with weak support. Furthermore, these relationships based on *rps*16 intron data were not supported when Rova (1999) combined *rps*16 and *trnL-F* data. Bremer & al. (1995) and Razafimandimbison & Bremer (2001) found *Cinchona* to be the sister of other Cinchonoideae. However, in these studies bootstrap support was lacking and representatives of the Isertieae were absent.

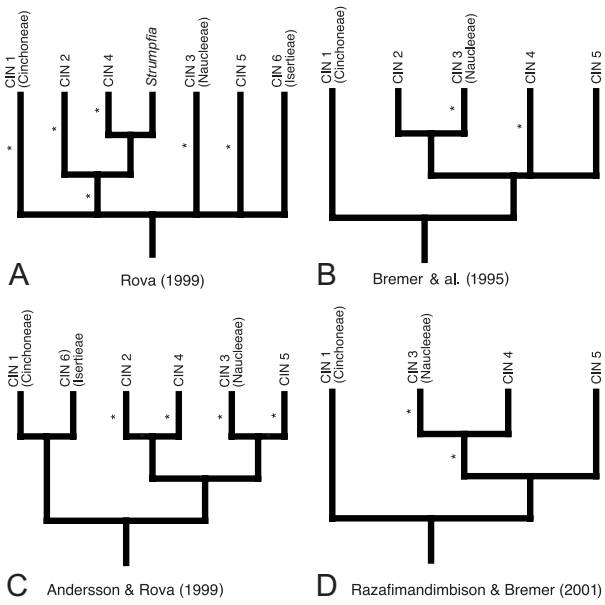

Fig. 1. Relationships among the six major clades of Cinchonoideae s.s., as inferred in four different studies. The major clades are those identified by Rova (1999): CIN 1 corresponds to tribe Cinchoneae (s.s.), CIN 2 includes the tribes Hamelieae and Hillieae, and the genus *Chione*, CIN 3 to Naucleeae (s.l.), CIN 4 to tribe Chiococceae (s.l.), CIN 5 mainly to tribes Guettardeae and Rondeletieae, and CIN 6 to Isertieae (s.s.). However, taxon representation differs among studies. Branches marked by an asterisk correspond to groups that were supported by bootstrap or jackknife values > 50% in a particular study.

Andersson's (1995) circumscription of Cinchoneae was based on morphological data, and monophyly of this group has not been confirmed by sequence data, except that Rova (1999) found strong support that *Cinchona*, *Ladenbergia* and *Stilpnophyllum* form a monophyletic group. On the other hand, many of the genera removed from Cinchoneae by Andersson & Persson (1991) and Andersson (1995) have indeed been confirmed to belong elsewhere. Thus, *Alseis* Schott, *Blepharidium* Standl., *Calycophyllum* DC., *Capirona*, *Dolicholobium*, *Emmenopterys* Oliver, *Ferdinandusa*, *Macrocnemum*, *Molopanthera* Turcz., *Schizocalyx* Wedd., and *Wittmackanthus* Kuntze belong in Ixoroideae (Rova, 1999; Rova & al., 2002). *Bouvardia* Salisb., *Danais* Vent., *Heterophyllaea* Hook. f., *Hindsia* Lindl., *Manettia* L., and *Schismatoclada* Baker belong in Rubioideae (e.g., Andersson & Rova, 1999; Bremer & Manen, 2000). *Corynanthe* Welw., *Crossopteryx* Fenzl., *Hymenodictyon* Wallich, *Mitragyna* Korth., *Paracorynanthe* Capuron & J.-F. Leroy, *Pausinystalia* Beille, and *Uncaria* Schreb. belong in Naucleeae (e.g., Razafimandimbison & Bremer, 2001). Finally, *Cosmibuena* belongs in Hillieae (e.g., Andersson & Rova, 1999), *Coutarea* Aubl. and *Exostema* Bonpl. belong in Rova's CIN4 clade (Rova, 1999; Rova & al., 2002), *Suberanthus* Borh. & Fernández belongs in Rova's CIN5 clade (Rova, 1999; Rova & al., 2002), and *Coptosapelta* and *Luculia* have positions outside the three major clades of Rubiaceae (Bremer & al., 1999).

Based on morphological data, Andersson (1995) concluded that the genera of Cinchoneae are monophyletic in approximately their traditional sense. However, he found that the genus *Cinchona* is better characterised by marginally villous corolla lobes than by the traditional diagnostic character, i.e., acropetal capsule dehiscence. Consequently he described a new genus, *Cinchonopsis*, for one species previously placed in *Cinchona* based on its capsule dehiscence. With more extensive ingroup sampling (but small outgroups) and still based on morphological evidence, he later confirmed monophyly of *Cinchona* (Andersson, 1997a), *Joosia* (Andersson, 1997b) and *Ladenbergia* (Andersson, 1997c). Except that Rova (1999) found strong support that his three species of *Cinchona* form a strongly supported monophyletic group, there is no molecular evidence for monophyly of genera of Cinchoneae.

The aim of this study is to (1) find better support for the placement of the tribe Cinchoneae within Cinchonoideae, (2) confirm the monophyly of the genera, and (3) infer in greater detail the relationships among them. This is accomplished by using more sequence data and a more extensive sample of genera and species than in earlier studies.

MATERIAL AND METHODS

Taxon sample. — The taxon sample was based on the following considerations. (1) We wanted as ample representation as possible of the tribe Cinchoneae as circumscribed by Andersson (1995). Therefore, we included all species of which we were able to find material suitable for sequencing. (2) In order to infer the phylogenetic position of Cinchoneae within Cinchonoideae, we wanted to have a representative sample of this subfamily. Therefore, we included one to several species representing each of the five subclades identified by Rova (1999). (3) In order to show the position of Cinchonoideae in the context of all Rubiaceae, we included a small sample of Ixoroideae and Rubioideae, and one species of the unresolved genus *Luculia* (no material of *Coptosapelta* was available to us). These taxa may be thought of as an "inner outgroup", although they were treated technically as part of the ingroup. (4) An outgroup comprising non-Rubiaceae Gentianales was used for rooting of the family. The outgroup was chosen based on evidence (e.g., Albach & al., 2001) that Rubiaceae are the sister group of other Gentianales. The choice of outgroup taxa was determined largely by availability of *matK* sequences in GenBank.

The final taxon sample comprised 55 species, four of which in the outgroup. All species are detailed in the Appendix, together with information on origin of material and GenBank accession numbers for the sequences.

Choice of markers. — Because we wanted to both place Cinchoneae in a broader context of a family level phylogeny and also get maximal resolution within the tribe, we chose to use a combination of relatively conservative and relatively fast-evolving sequences. After some trials we settled for a combination of five markers, the *matK* and *rbcL* genes (plastid DNA), the *rps*16 intron and the *trnL-F* region (plastid DNA), including the *trnL* intron and the *trnL-trnF* intergenic spacer, and the ITS tandem repeat (nrDNA).

The *rbcL* gene has proved in numerous studies (e.g., Bremer & al., 1995; Bremer & Thulin, 1998; Bremer & Manen, 2000) to offer sufficient information for well-supported resolution of larger groups within the family. It also has the advantage that a fair number of useful sequences were already available from GenBank. The *matK* gene is known from other families (e.g., Thiv & al., 1999) to offer much more information than the *rbcL*, still being relatively easy to align. The few complete sequences available in GenBank, as well as some more that were put at our disposal by Dr. Claes Persson, confirmed this impression, and *matK* was therefore added to our dataset. The *rps*16 intron (e.g., Andersson & Rova, 1999; Andersson 2002) and the *trnL-F* intergenic spacer (e.g., Persson, 2000a; Rova & al., 2002) have been shown in earlier studies to have sufficient information for supported resolution within tribes. Both are possible to align throughout the family, even though portions of the *rps*16 intron have to be deleted. Both *rps*16 and *trnL-F* sequences have the advantage that a fairly ample set of sequences is available from GenBank. The ITS tandem repeat has been shown (e.g., Nepokroeff & al., 1999; Persson, 2000b; Gustafsson & Persson, 2002) to contain information sufficient for resolution even within genera. However, ITS sequences have a high rate of substitutions combined with a high rate of length variation, making alignment extensively ambiguous outside rather narrowly circumscribed groups, and ITS was therefore sequenced only for species of Cinchoneae and Isertieae and coded as missing data for other taxa.

DNA extraction, amplification, and sequencing. — Total genomic DNA was extracted from leaf tissues collected in silica gel or taken from herbarium material. Mostly, we used the method described by Andersson & Rova (1999), but some extractions were made with the DNeasy Plant Mini Kit (QUIAGEN), according to the manufacturer's instructions.

The ITS region was mostly amplified using the primers ITS10 (Table 1) and ITS4 (White & al., 1990). In some cases the primer ITS1 (White & al., 1990) was used instead of ITS10. The *matK* gene was mostly amplified using the primer pair *trnK*2r/*trnK*3914f (Johnson &

Table 1. Primers designed or modified by us.

Designation	Composition (5'-3')
ITS10	TGC TAA CTA GCT ATG YGG AG
*matK*49f	CAA ATC CTT CTG ATA TCG TTT G
*matK*301r	CAA CAC GGC TTT CTA TAT CCA C
*matK*1198f[1]	CTG TGT TAG ATA TAC NAA TAC CCC
*matK*1581r[1]	CTT GAT ACC TAA CAT AAT GCA T
*matK*1729f	AAG GGT CTA TAT AAA NCA ATT A
*matK*2053r[1]	TTA GCR CAA GAY AGT CGA AGT A
*rbcL*26f	ATG TCA CCA CAA ACA GAG ACT AAA GC
*rbcL*358f	CTG TTA CTA ACA TGT TTA CTT CYA
*rbcL*361r	CAA TRG AAG TAA ACA TGT TAG TAA
*rbcL*667r	CGG CAC AAA ATA MGA AAC GRT CTC TC
*rbcL*799f	GTA ATG CTA TCC TTG AGG RTT AAG AG
*rbcL*1010r	CCT TCA AGT TTM CCT ACT ACK GTA CC
*rbcL*1117f	CAC RCA AGA TTG GGT CTC TRT ACC AG
rbcLt[2]	CCT TTT AGT AAA AGA TTG GGC CGA G
*trnf*2	CAG TCC TCT GCT CTA CCA ACT GAG

[1] Modified after Thiv & al., 1999.
[2] Supposedly the same as the "3' primer" of Olmstead & al., 1992.

Soltis, 1994). In some cases we used the primer pairs *matK*49f/*matK*2053r or *matK*1198f/*matK*2053r instead (Table 1). The *rbcL* gene was amplified by the primer pair *rbcL*26f/*rbcL*t (Table 1). The *rps*16 intron was amplified by the primer pair *rps*F/*rps*R2 (Oxelman & al., 1997). The *trnL-F* region was amplified using the primer pair *trn*c/*trn*f (Taberlet & al., 1991). In some cases PCR was done using Ready.To.Go PCR beads (Amersham Pharmacia Biotech) for 25 µl reactions, using 25-100 ng of template DNA and 30 nmol of each primer. In other cases we used the MasterAmp (Epicentre Technologies) kit, using 25 µl of premix G, 30 nmol of each primer, 1U Taq polymerase, 25–100 ng of template DNA, and water to fill a reaction volume of 50 µl. PCR products were visualised by electrophoresis on a 1% agarose gel and purified on QIAquick (QUIAGEN, Hilden, Germany) spin columns.

Sequencing was performed either on an ALFexpress (Pharmacia Biotech) automated sequencer, or on a CEQ 8000 (Beckman Coulter) automated sequencer. Sequence reactions for the ALFexpress were done as described by Andersson & Rova (1999). Reactions for the CEQ 8000 were done with the Quick Start Kit (Beckman Coulter) according to manufacturer's instructions, except that we prepared 10 µl reactions, using ca. 100 fmol template and 0.15 nmol primer. Sequencing of the ITS region was done using the ITS1, ITS2, ITS3 and ITS4 primers (White & al., 1990), sometimes also ITS10 (Table 2). The *matK* gene was sequenced using the *matK*49f, *matK*301r, *matK*1198f, *matK*1581r, *matK*1729f, and *matK*2053r primers (Table 1). The *rbcL* gene was sequenced using the *rbcL*26f, *rbcL*358f, *rbcL*361r, *rbcL*667r, *rbcL*799f, *rbcL*1010r, and *rbcL*1117f primers (Table 1). The *rps*16 intron was sequenced using the PCR primers. The *trnL-F* region was sequenced using the *trn*c, *trn*d, *trn*e, and *trn*f2 primers (Taberlet & al., 1991; Table 1).

Editing and compilation of sequences was made using Sequencher (Gene Codes Corporation, Ann Arbor, Michigan) version 4.1.

Alignment and gap coding. — Alignment of *matK*, *rbcL*, *rps*16, and *trn* sequences was done manually. With ITS sequences a first alignment was done with ClustalX (Thompson & al., 1997), and this was then adjusted manually. Gaps were inserted and gap codes added in accordance with the principles specified by Andersson & Chase (2001). Some sections of the *rps* and ITS sequences could not be unambiguously aligned and were deleted prior to analysis.

Cladistic procedures. — Cladistic analyses were performed with PAUP* version 4.0b10 (Swofford, 1999). Maximally parsimonious trees (MPTs) were found using the heuristic search algorithm. Full heuristic searches were run with 10000 replicates of random addition sequence, using TBR branch swapping. The first search was run without topological constraints. Since one species of *Remijia* turned out in an unexpected and poorly supported position, we also ran a second search with a topological constraint forcing *Remijia* to be monophyletic in Andersson's (1995) sense.

Support was estimated using jackknife analysis. We ran 1000 replicates with 37% deletion, using random addition sequence (10 replicates) and TBR branch swapping, saving up to 500 trees per replicate. In order to identify conflicts among data from different loci, data from each locus were ran in a separate jackknife analysis (settings as above), and jackknife consensus trees were inspected for groups in conflict.

RESULTS

The matrix comprised a total of 5869 characters, including 47 indel codes. Of these, 631 (17 indels) were

Table 2. Comparison of some character states in the genera of tribe Cinchoneae (data mainly from Andersson, 1995). The term paniculoid is here used to describe a many-flowered, ± pyramidal inflorescence with a distinct main axis, whereas corymbose is used for a flat-topped inflorescence and cymose is used for di- or monochasial inflorescences with distinct pedicels and cyme peduncles but without a differentiated main axis. Seed size, as expressed by length including wing, is classified as follows: <4 mm = small (s.), 4–11 mm = medium (m.), >11 mm = large (l.).

Genus	Terminal infl.	Inflorescence structure	Corolla tube inside	Corolla lobe append.	Adaxial corolla lobe surface	Seed size	Seed wing margin
Ciliosemina	absent	corymbose	glabrous	absent	papillose	s. to m.	ciliate or fimbriate
Cinchona	present	paniculoid or cymose	glabrous or subglabrous	absent	villous along margins	m.	entire to dented
Cinchonopsis	present	paniculoid	distally hirsute	absent	hirtellous all over	m.	entire to dented
Joosia	present	umbelliform, cymose, or flowers solitary	glabrous	present	glabrous	m.	entire to dented
Ladenbergia	present	paniculoid or cymose	glabrous	absent	papillose	(m. to) l.	entire to laciniate
Maguireocharis	absent	paniculoid or cymose	distally hirsute	absent	villous all over	m.	entire to dented
Pimentelia	absent	paniculoid	distally hirsute	absent	hirtellous all over	m.	entire to dented
Remijia	absent	thyrsoid (to globose)	glabrous	absent	papillose	(s. to) m. (to l.)	entire to dented
Stilpnophyllum	absent	cymose	distally hirsute	absent	hirtellous all over	s.	entire to dented

derived from the ITS data set, 1623 (5 indels) from *matK*, 1398 from *rbcL*, 946 (15 indels) from the *rps*16 intron, and 1271 (10 indels) from the *trnL-F* region. Out of 2096 variable characters 963 were uninformative and 1133 parsimony informative.

No groups were identified in jackknife analyses of individual loci that were at the same time supported (frequency ≥63%) and in conflict with groups found on the basis of another locus. Nor was any group found that had a higher jackknife support based on one locus than based on the complete dataset.

The heuristic search without topological constraints found 90 equally maximally parsimonious trees (MPTs), 4172 steps long, with an ensemble consistency index of 0.52 (uninformative characters excluded), a homoplasy index of 0.35 and retention index of 0.71. The strict consensus of all MPTs is shown in Fig. 2. This consensus tree shows 43 internal branches within the ingroup. One of these had a support below 50% in the jackknife analysis, four were ambiguously (<63%) supported, four weakly (63–75%), four moderately (76–90%), and 30 strongly (≥91%).

The three subfamilies, Rubioideae, Ixoroideae and Cinchonoideae, are strongly supported. There is strong support that Ixoroideae and Cinchonoideae are sister groups and this clade appears at a basal trichotomy, the other branches of which are Rubioideae and *Luculia*. There is moderate support that Naucleeae s.l. is the sister group of other Cinchonoideae. The remainder of Cinchonoideae forms a trichotomy, one strongly supported branch of which (Fig. 2: I) corresponds to Rova's (1999) "CIN 5" clade and comprises our two taxa of Guettardeae and Rondeletieae. The other branch (Fig. 2: II) of the trichotomy, weakly supported, corresponds to Rova's "CIN 2" clade, including our sample of the tribes Chiococceae s.l., Hamelieae and Hillieae. The third branch (Fig. 2: III) of the trichotomy, strongly supported, includes our sample of the tribes Isertieae s.s. and Cinchoneae s.s. Cinchoneae and Isertieae are sister groups and both are strongly supported as monophyletic.

Within Cinchoneae there are four successive branches branching off in a ladder-like way, and a crown group (Fig. 2: IV). The successive branches are formed first by two species of *Joosia*, second by two species of *Stilpnophyllum*, third by the single species of *Cinchonopsis*, and fourth by four species of *Remijia*. The clade sister to *Joosia* is strongly supported, but the relationships between these genera are otherwise weakly supported. *Joosia*, the four species of *Remijia*, and *Stilpnophyllum* are each strongly supported as monophyletic. The crown group, supported only by a decay index ≥1, is formed by *Cinchona*, *Ladenbergia*, and *Remijia pedunculata*. *Cinchona* and *Ladenbergia* are strongly supported, but the relationships between these genera and *Remijia pedunculata* are unresolved. However, in a search constrained to make *Remijia* monophyletic including *R. pedunculata*, the shortest trees found were five steps longer than the shortest ones found without constraints.

DISCUSSION

Comparison of results from individual markers with one another, and with results based on the complete data set, do not indicate serious conflicts between data from different markers. Therefore, the following discussion will be based entirely on results obtained from the complete set.

Relationships among major clades of Rubiaceae are the same in our analysis as in most other ones based on sequence data (e.g., Andersson & Rova, 1999; Bremer & al., 1999; Bremer & Manen, 2000; Rova & al., 2002). Within Cinchonoideae, there is moderate support that Naucleeae s.l. is sister to the rest. This relationship is new to this study, having better support than any alternative presented so far. In the study of Bremer & al. (1995; Fig. 1B), Naucleeae are nested within a clade comprising, among others, *Chiococca*, *Cubanola* and *Exostema*, but this relationship had no bootstrap support and was only supported by a decay index of 2. Razafimandimbison & Bremer (2001; Fig. 1D) found Naucleeae to be sister to Chiococceae s.l., but with weak bootstrap support. Rova & al. (2002) found Naucleeae to be one of five major subclades of Cinchonoideae forming a basal polytomy. The sister group relationship presently found is biogeographically interesting because Naucleeae s.l. are predominantly paleotropical, whereas the rest of Cinchonoideae are predominantly neotropical.

Cinchoneae are strongly supported as monophyletic, including the genera *Cinchona*, *Cinchonopsis*, *Joosia*, *Ladenbergia*, *Remijia* and *Stilpnophyllum*. Only two genera referred to Cinchoneae by Andersson (1995) are absent from the present sample, i.e., *Maguireocharis* and *Pimentelia*. Thus, present results strongly corroborate monophyly of Cinchoneae in Andersson's (1995) sense. Monophyly of Cinchoneae was also supported by Rova (1999), but then only for *Cinchona*, *Ladenbergia* and *Stilpnophyllum*, and by Rova & al. (2002), but then only for *Cinchona* and *Ladenbergia*. Further, there is strong support that the sister group of Cinchoneae is the tribe Isertieae (in the restricted sense of Bremer & Thulin, 1998) and that *Kerianthera* forms a monophyletic group with *Isertia*. Thus, this study strongly corroborates Delprete's (1996b) inclusion of *Kerianthera* in Isertieae. The only study so far implying a relationship between *Isertia* and Cinchoneae is that of Andersson & Rova (1999), in which the relationship was not supported by

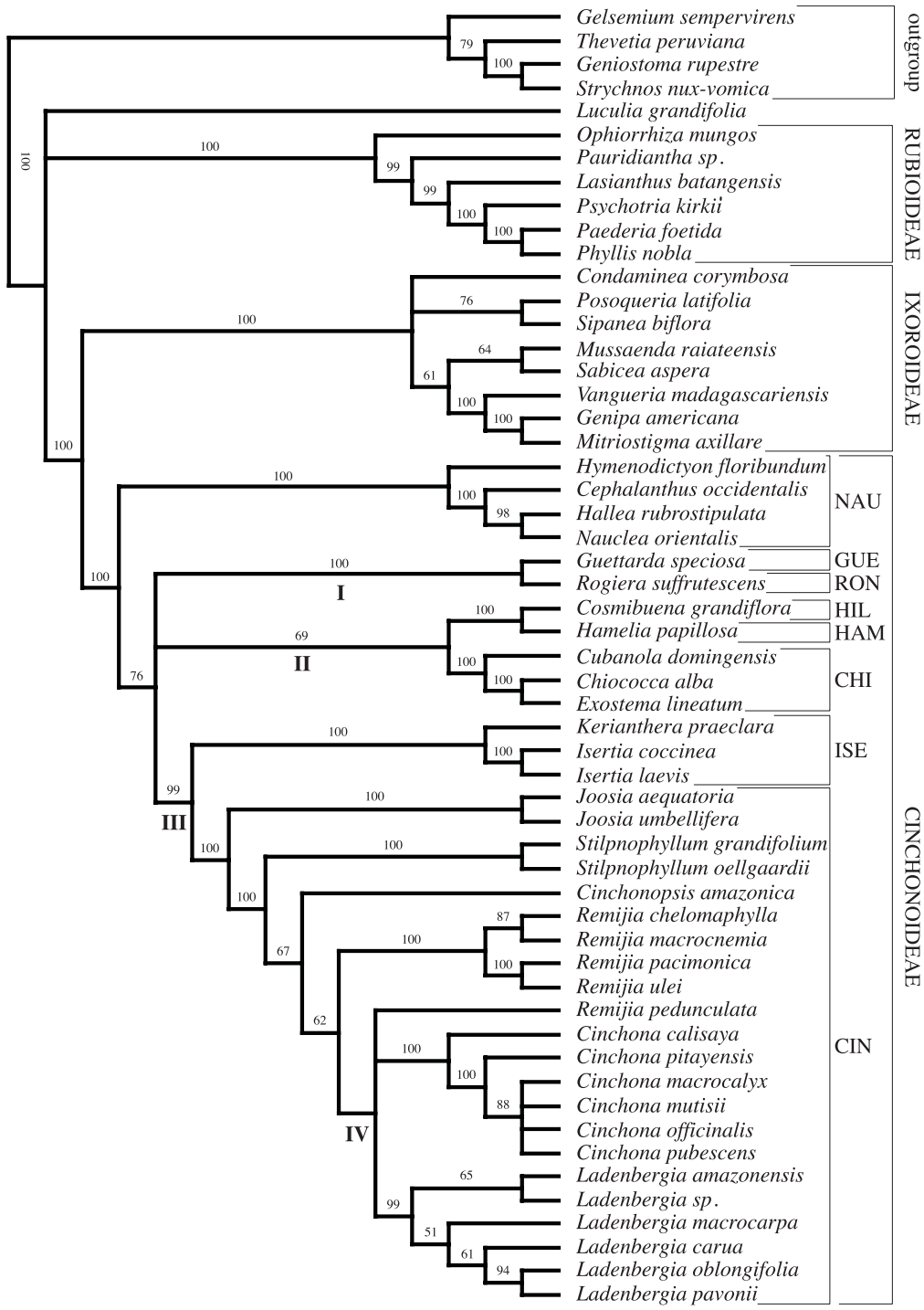

Fig. 2. Strict consensus of 90 equally maximally parsimonious trees found in the analysis based on the complete dataset. Figures above branches are jackknife (37% deletion) support values (support < 50% not shown). Roman numerals below branches identify clades discussed in the text. Major groups of Rubiaceae (Cinchonoideae, Ixoroideae and Rubioideae) are identified by brackets, as are tribes within Cinchonoideae. The following acronyms refer to tribes: CHI = Chiococceae s.l., CIN = Cinchoneae s.s., GUE = Guettardeae, HAM = Hamelieae, HIL = Hillieae, ISE = Isertieae s.s., NAU = Naucleae s.l., RON = Rondeletieae.

the bootstrap analysis.

Although only two out of 11 *Joosia* species were sampled, these two species are morphologically quite divergent and were found based on morphological evi-

dence to be distantly related (Andersson, 1995, 1997b). Because of this, the present result may be viewed as strong corroboration of monophyly of the genus, which is further supported by three non-homoplasious morphological synapomorphies: persistent stipules, monochasial partial inflorescences, and petaloid appendages on corolla lobe margins (Andersson, 1995). Based on morphological evidence, Andersson (1995) found *Joosia* to be the sister group of *Cinchona*, these two being the sister group of a clade comprising *Ladenbergia* and *Remijia*. However, those results were obtained only after successive re-weighting.

Only two species of *Stilpnophyllum* were sampled for this analysis, but there are only four species in the genus, and it is morphologically homogeneous (Andersson, 1994). Monophyly of the genus is thus strongly corroborated. However, some uncertainty rests in the fact that the monotypic genus *Pimentelia* was not included. Morphologically, *Pimentelia* is closely similar to *Stilpnophyllum* (Andersson, 1995) and differs mainly by absence of the closely-set intersecondary leaf veins that characterize *Stilpnophyllum*. Based on morphological similarity, it seems likely that *Pimentelia* belongs in the same clade as *Stilpnophyllum*, and perhaps the two should be merged. Since dense leaf venation is a synapomorphy of *Stilpnophyllum* but is lacking in *Pimentelia*, it seems probable, though, that *Pimentelia* is the sister of *Stilpnophyllum* rather than nested within it. Therefore, pending access to sequence data, we think that *Pimentelia* should remain. Andersson (1995) found *Stilpnophyllum* to form a basal clade of Cinchoneae, together with *Cinchonopsis*, *Maguireocharis* and *Pimentelia*, but, as with the position he found for *Joosia*, a resolved tree was found only after successive reweighting. The position found here is consistent with the results of Rova (1999), who found *Stilpnophyllum* to be the sister of a clade comprising *Cinchona* and *Ladenbergia*. *Cinchonopsis*, *Joosia* and *Remijia* were absent from Rova's sample.

When originally described (Standley, 1931), *Cinchonopsis amazonica* was assigned to the genus *Cinchona* presumably due to its acropetally dehiscent capsules. However, Andersson (1995) found its flowers to be quite dissimilar from those of other *Cinchona*, i.a., in their lack of marginal villi on the corolla lobes. Furthermore, in a cladistic analysis based on morphological data, he found that it was not included in the monophyletic group around the type of *Cinchona* (*C. pubescens*), but rather grouped with *Maguireocharis*, *Pimentelia* and *Stilpnophyllum*. Therefore, he established the monotypic genus *Cinchonopsis* to accommodate this species. Even though the present analysis does not corroborate a position close to *Stilpnophyllum*, it definitely supports its exclusion from *Cinchona*. In doing so, it also supports Andersson's conclusion that capsule dehiscence is not such a good generic diagnostic as previously (e.g., Schumann, 1891) assumed.

The crown group (Fig. 2: IV) revealed by the present study agrees with the one found by Andersson (1995), except that it does not include *Joosia*. In Andersson's analysis, this group was characterized by a single non-homoplasious morphological character, heterostylous flowers, the character state being unknown for one of the terminals outside the group, *Maguireocharis*. If this character is optimized on our tree it becomes homoplasious, with parallel origins for *Joosia* and the crown group.

Monophyly of *Cinchona* is strongly corroborated by the present analysis, as it was (based on a smaller sample) in that of Rova (1999). This lends some support to Andersson's (1997a) conclusion that marginally villous corolla lobes form the most important diagnostic character of the genus. However, his other conclusion, that acropetally dehiscent capsules are less reliable, is not really tested, since the sample includes neither *Cinchona* species with basipetally dehiscent capsules, nor *Ladenbergia* species with acropetally dehiscent ones. Even though the present sample is limited, it is clear that relationships within *Cinchona* were not correctly inferred by Andersson (1997a). It is biogeographically interesting to note that *C. calisaya*, the only species of our sample endemic to the southern Andes, is sister to the rest of the sample which consists of species endemic to the northern Andes, plus one, *C. pubescens*, which is widespread. However, to say with confidence that this is a case of vicariance would require a broader sample, particularly of species endemic to northern Peru.

Even though Andersson's (1995) analysis of morphological data did not identify a single non-homoplasious character to diagnose *Ladenbergia*, it suggested that the genus is monophyletic, the most consistent (CI 0.69) character beeing large seeds. Later, Andersson (1997c) said that *Ladenbergia* differs from *Remijia* mainly in plesiomorphic traits, such as presence (vs. absence) of terminal inflorescence and paniculate (rather than thyrsoid) inflorescence; the present analysis does not imply that these character states are apomorphic. Considering the lack of morphological synapomorphies, it is thus surprising that the genus is strongly supported as monophyletic in the present analysis. With respect to relationships within *Ladenbergia*, the present analysis agrees with that of Andersson (1997c) in the basal placement of *L. amazonensis*. None of the species that were placed basal to this in Andersson's analysis is represented in the present sample. Beyond this, internal relationships are in conflict between the two analyses. However, Andersson's study was based on a small number of characters and the relationships he inferred were only weakly supported.

Andersson (1995) circumscribed *Remijia* to include

all Cinchoneae with heterostylous flowers and with consistently lateral inflorescences, but found no non-homoplasious morphological synapomorphy to diagnose the genus. In his analysis, it was comprised of two clades that appeared as sister groups, a larger one characterized by thyrsoid inflorescences (CI 1.0) and a smaller one characterized by densely ciliate to fimbriate seed wing (CI 1.0). In the present analysis, *R. chelomaphylla*, *R. macrocnemia*, *R. pacimonica* and *R. ulei* have thyrsoid inflorescences, whereas *R. pedunculata* has corymbose inflorescences but ciliate-margined seeds. Whereas *Remijia* species with thyrsoid inflorescences form a strongly supported monophyletic group, *R. pedunculata* has an unresolved position in the crown group. Trees in which *Remijia* is monophyletic including *R. pedunculata* are five steps longer than the most parsimonious ones. Thus, there is no support in the present dataset that *Remijia* is monophyletic in Andersson's circumscription, but only in a more restricted one, comprising only species with thyrsoid inflorescences.

Andersson (1995) found *Cephalodendron globosum* Steyerm. to be deeply nested within *Remijia* (in the same clade as the type species) and consequently reduced *Cephalodendron* Steyerm. to a synonym of *Remijia*. Fundamental to that result was the observed absence of terminal inflorescences in *Cephalodendron* and the interpretation of its head-like inflorescence as a reduced thyrse. Although we were unable to obtain useful DNA of *Cephalodendron* for the present analysis, there is some support for the synonymization in the sense that it confirms the diagnostic value of the thyrsoid inflorescence. However, it is still dependent on Andersson's interpretation of the *Cephalodendron* inflorescence, which was not quite straightforward based on herbarium material. Further studies are certainly to be desired.

Unfortunately, we were unable to obtain useful DNA from the rather enigmatic, monotypic genus *Maguireocharis*. In Andersson's (1995) analysis this was placed as a basal branch in a clade otherwise comprising *Cinchonopsis*, *Pimentelia* and *Stilpnophyllum*. In the present analysis *Cinchonopsis* and *Stilpnophyllum* form a paraphyletic group and Andersson's (1995) clade is not supported. Furthermore, Andersson did not have access to flowering material of *Maguireocharis*, such that he had to score many of the characters of floral and pollen morphology as missing. Among the missing data was information on whether the flowers are homostylous or heterostylous, a character that appears to have had major influence on how relationships among genera were inferred in that analysis. *Maguireocharis* is similar to *Remijia pedunculata*, *Pimentelia*, *Remijia* (s.s.) and *Stilpnophyllum* in its lack of terminal inflorescences. However, it differs from *R. pedunculata* in the peculiar indumentum of its corolla and in having entire to dented rather than ciliate seed wing margin. It differs from *Pimentelia* and *Stilpnophyllum* in having buds that are not evidently resinous and larger capsules and seeds. Finally, it differs from *Remijia* in having paniculate inflorescences. Considering that the present analyses have failed to confirm the existence of the clade to which it was previously referred, and that no new evidence has been added, we must conclude that the position of *Maguireocharis* is even more uncertain than it was before.

TAXONOMIC CONCLUSION: A NEW GENUS

As shown above, *Remijia pedunculata* is not grouped with other *Remijia* in the most parsimonious trees, and trees constrained to make *Remijia* monophyletic including this species are five steps longer than the most parsimonious ones. It seems fairly clear, therefore, that *Remijia* is non-monophyletic with *R. pedunculata* included. The type species of *Remijia*, *R. ferruginea* (A. St.-Hil.) DC., was not included in our sample, but has the thyrsoid inflorescence that is a synapomorphy of the four other species (Andersson, 1995). This clearly suggests that the name *Remijia* should remain with the majority of species and that *R. pedunculata* should be transferred to another genus.

Among the most parsimonious trees, there are some in which *R. pedunculata* appears as a basal branch of *Cinchona* and some in which it appears as a basal branch of *Ladenbergia*. Molecular data would thus not be in conflict with inclusion of *R. pedunculata* in one of these. However, in whichever of these genera *R. pedunculata* were to be included, that genus would become difficult to diagnose morphologically (Table 2). *Cinchona* as circumscribed by Andersson (1995) is well characterised by the villous margins of its corolla lobes. This is a unique apomorphy of this group and one that is lacking in *R. pedunculata*. *Ladenbergia* does not have a unique and consistent synapomorphy, but it is diagnosable by the combination of presence of a terminal inflorescence, absence of hairs (both thin-walled marginal villi and long, thick-walled ones) on the adaxial side of corolla lobes, and its almost always conspicuously large seeds. *Remijia pedunculata* differs from *Ladenbergia* in two of these characters: it consistently lacks a terminal inflorescence, and the seeds are small to medium-sized (2.2–7.2 mm long, including wing). Furthermore, together with *R. purdieana*, *R. pedunculata* has a couple of unique morphological characteristics (Table 2), namely a corymbose inflorescence and a ciliate- to fimbriate-margined seed wing. These morphological considerations, namely that *R. pedunculata* is not easily accommodated either in

Cinchona, or in *Ladenbergia*, plus at the same time being readily distinguished from both, lead us to the conclusion that a new genus should be established for it.

Remijia purdieana Wedd. is morphologically very close to *R. pedunculata*. They share the same basic inflorescence structure and have the same characteristic cilia on the margins of the seeds (Fig. 3F–G). They are, furthermore, closely similar in leaf shape and floral morphology and differ mainly in size of calyx lobes (Fig. 3D–E) and seeds. It is fairly obvious that, when *R. pedunculata* is transferred to a new genus, *R. purdieana* should go with it.

Ciliosemina A. Antonelli, **gen. nov.** – Type: *Cinchona pedunculata* H. Karst. ≡ *Remijia pedunculata* (H. Karst.) Flueck.

Etymology: The generic name is composed of the Latin words *cilium* (hair) and *semen* (seed) and refers to the conspicuously fimbriate margin of the seed wings.

Absentia inflorescentia terminalis *Remijia* similis, sed ab ea differt inflorescentia corymbosa vel subcorymbosa (versus thyrsoidea) et seminum alis marginibus ciliatis vel fimbriatis; a *Cinchona*, *Cinchonopsi* et *Ladenbergia* differt absentia inflorescentia terminalis; a *Cinchona* etiam differt corollae loborum adaxialiter pag-

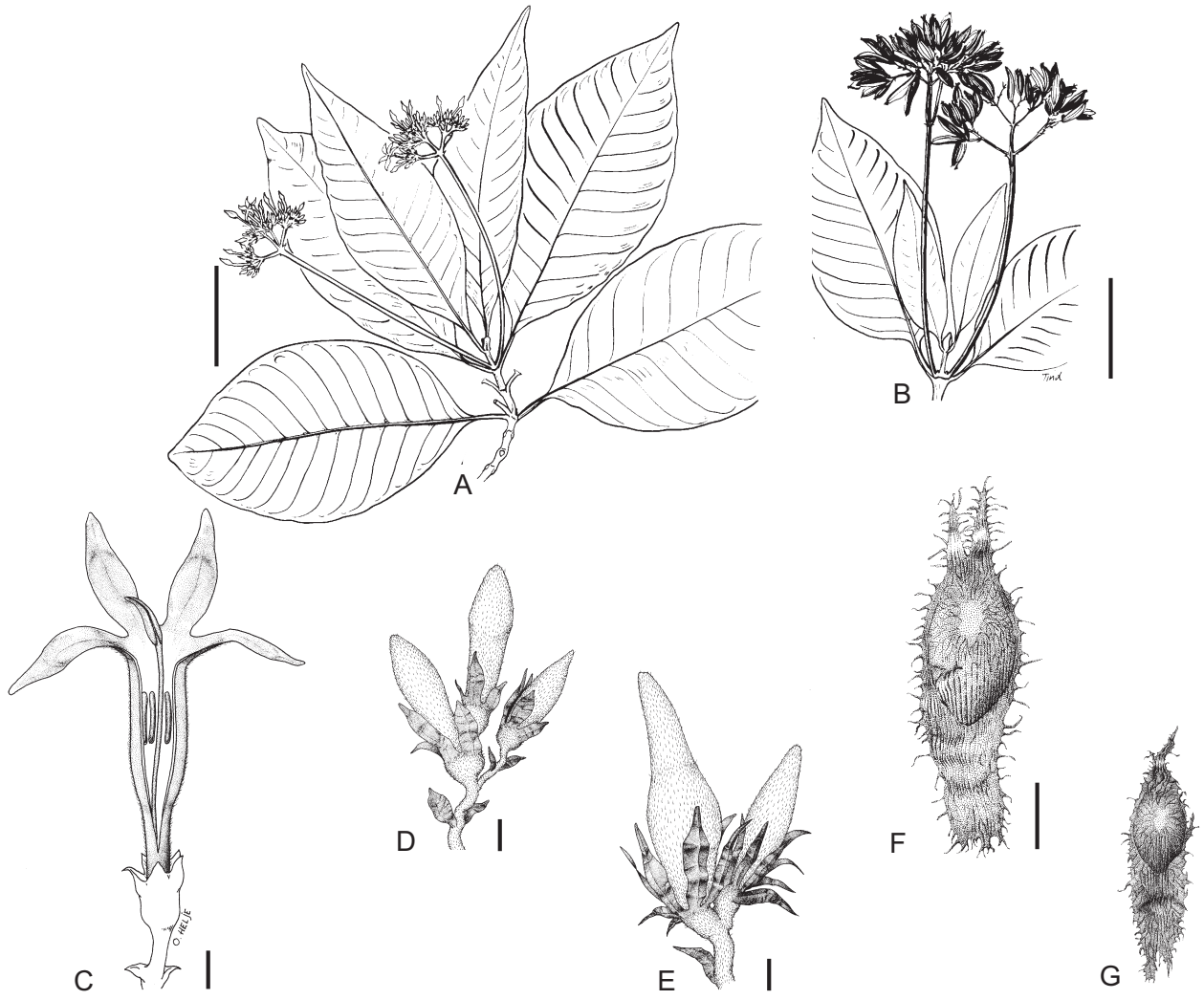

Fig. 3. *Ciliosemina pedunculata* (A–D, F) and *C. purdieana* (E, G). A, branch tip with inflorescences (*Lleras & al. P16958*, S). B, branch tip with infructescences (*Asplund 14153*, S). C, long-styled flower cut open and with one corolla lobe removed (*St. John & Arcila 20627*, GB). D, partial inflorescence with nearly mature buds, showing relatively short, unequal calyx lobes (*St. John & Arcila 20627*, GB). E, partial inflorescence with nearly mature buds, showing relatively long, equal calyx lobes (*Uribe 3443*, US). F, seed in ventral view (*Vásquez & Chávez 25003*, GB). G, seed in ventral view (*Uribe 3443*, US). Scale bars: A–B, 5 cm, C, 2 mm, D–G, 1 mm. A–B drawn by Kirsten Tind, previously published in *Flora of Ecuador* (vol. 50, 1994, as part of fig. 9); C–G drawn by Olof Helje.

ina minute papillosi (versus villosi).

Shrubs or trees, without raphides. Leaves decussate; stipules applanate-appressed. Inflorescences all lateral, from both axils of leaf pairs near branch tips, long-pedunculate and densely corymbiform or subcorymbiform, many-flowered. Flowers heterostylous; corolla white, hypocrateriform, the tube cylindrical, longer than lobes, glabrous inside, the lobes valvate in bud, the adaxial side minutely papillose, without petaloid appendages. Stamens inserted on inside of corolla tube, anthers dorsifixed near base. Ovary bilocular, each locule with numerous, ascendingly imbricate ovules. Capsules basipetally septicidal, many-seeded. Seeds lenticular, with bipolar wings along edges, wing margin densely ciliate or fimbriate.

The principal diagnostic characters of *Ciliosemina* are the long-pedunculate, corymbose or subcorymbose inflorescences (Fig. 3A), and the ciliate to fimbriate wing margins of its seeds (Fig. 3F–G). A more detailed comparison of *Ciliosemina* with other genera of Cinchoneae is given in Table 2.

Key to the two species of *Ciliosemina*.

1. Calyx lobes 0.4–1.5 mm long, distinctly unequal on one flower; colleters absent from inside of calyx; seeds 5.1–7.2 mm long including wing . . . . . *C. pedunculata*
1. Calyx lobes 3.0–6.0 mm long, ± equal on one flower; colleters present on inside of calyx at sinuses; seeds 2.2–4.5 mm long including wing . . . . . *C. purdieana*

Ciliosemina pedunculata (H. Karst.) A. Antonelli, **comb. nov.** – Basionym: *Cinchona pedunculata* H. Karst., Fl. Columb. 1: 53, Table 36. 1859. – Type: Colombia, Cundinamarca, Susumuco, s.d., *Karsten & Triana s.n.* (lectotype W, designated here).

Synonyms: *Ladenbergia pedunculata* (H. Karst.) K. Schum., Fl. Bras. 6(6): 146. 1889.

Remijia peruviana Standl., Publ. Field Mus., Bot. 8: 156. 1930. – Type: Peru, Loreto, near Morona, Iquitos, 17 Jul 1929, *Williams 1512* (holotype F; isotype C).

Ciliosemina pedunculata occurs in northwestern South America in Venezuela (state of Amazonas), Colombia (departments Amazonas, Antioquia, Boyacá, Caquetá, Cundinamarca, Meta and Santander), Ecuador (prov. Sucumbíos), Peru (departments Loreto, San Martín and Ucayali), and Brazil (western part of Amazonas state).

Ciliosemina purdieana (Wedd.) A. Antonelli, **comb. nov.** – Basionym: *Remijia purdieana* Wedd., Ann. Sci. Nat. 11: 272. 1849. – Type: Colombia, Antioquia, Cauvas, s.d., *Purdie s.n.* (holotype P; isotype, K).

Synonym: *Remijia bracteata* Standl., Publ. Field Mus., Bot. 8: 155. 1930. – Type: Colombia, "Río Magdalena, before Jul. 1888", *Weir 75* (holotype K; isotypes BM, F).

Ciliosemina purdieana seems to be a narrowly restricted endemic, occurring in the lower Magdalena valley in the Colombian departments of Antioquia, Bolívar, and Santander.

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Appendix. Sequences used in cladistic analyses, together with data on origin of sequenced material and GenBank accession numbers (in order taxon, origin, voucher, GenBank accession number: ITS, *matK*, *rbcL*, *rps*16, *trnL-F*). Data on origin and voucher are given only for newly determined sequences (indicated by *).

Outgroup

Gelsemium sempervirens (L.) J. St.-Hil., Z70195, L14397, AF004092, AF159696; *Geniostoma rupestre* J.R. Foster & G. Foster, Z70194, Z68828; *Strychnos nux-vomica* L., Z70193, L14410, AF004094, AF102484; *Thevetia peruviana* K. Schum., Z70188, X91773.

Ingroup

Cephalanthus occidentalis L., cultivated, *Andersson 2212* (GB), AY538377*, X83629, AF004033, AF152692; *Chiococca alba* (L.) Hitchc., Cuba, *Rova & al. 2274* (GB), AY538378*, L14394, AF004034, AF102400; *Cinchona calisaya* Wedd., Bolivia, *Persson & Gustafsson 241* (GB), AY538352*, AY538379*, AY538478*, AY538447*, AF242927; *C. macrocalyx* Pav. ex DC., Ecuador, *Madsen 86030* (AAU), AY538380*, AY538479*, AF538425*, AY538448*; *C. mutisii* Lamb., Ecuador, *Madsen 85708* (AAU), AY538353*, AY538426*, AY538449*; *C. officinalis* L., Ecuador, *Andersson & Nilsson 2551* (GB), AY538354*, AY538381*, AY538480*, AY538427*, AY538450*; *C. pitayensis* Wedd., Colombia, *Andersson & al. 2109* (GB), AY538355*, AY538382*, AY538481*, AF242928, AF152684; *C. pubescens* Vahl, Ecuador, *Ståhl 3694* (GB), AY538356*, AY538451* Z70197, X83630, AF004035; *Cinchonopsis amazonica* (Standl.) L. Andersson, Brazil, *Antonelli 244* (GB), AY538357*, AY538383*, AY538482*, AY538428*, AY538452*; *Condaminea corymbosa* (Ruiz & Pav.) D.C., Colombia, *Rova & Sundbaum 2410* (GB), AY538384*, Y18713, AF004039, AF102406; *Cosmibuena grandiflora* (Ruiz & Pav.) Rusby, Colombia, *Andersson & al. 2075* (GB), AY538385*, AY538483*, AF242929, AF152686; *Cubanola domingensis* (Britton) Aiello, cultivated, *Andersson 2209* (GB), AY538386*, X83632, AF004044, AF152701; *Exostema lineatum* (Vahl) Roem. & Schult., cultivated, Rova & al. (2002), AY538387*, AY538484*, AF242944, AF152698; *Genipa americana* L., Colombia, *Persson & al. 2143* (GB), AY538388*; Z68839, AF200997, AF201045; *Guettarda speciosa* L., Seychelles, *Persson 141* (GB), AY538389*, AY538485*, AF246924, AF152725; *Hallea rubrostipulata* (K. Schum.) J.-F. Leroy, cultivated, without voucher, AY538390*, AY538486*, AF538429*, AY538453*; *Hamelia papillosa* Urb., cultivated, *Andersson 2218* (GB), AY538391*, AY538487*, AF004053, AF102439; *Hymenodictyon floribundum* Robinson, cultivated, without voucher, AY538392*, AY538488*, AY538454*, AF004058; *Isertia coccinea* (Aubl.) J.F. Gmel., French Guiana, *Andersson & al. 1912* (GB), AY538358*, AY538393*, AY538489*, AY538430*, AY538455*; *I. laevis* (Triana) Boom, Colombia, *Tuberquía & al. 524* (GB), AY538359*, AY538394*, AY538490*, AY538431*, AY538456*; *Joosia aequatoria* Steyerm., Ecuador, *Vivar & al. 3928* (AAU), AY538360*, AY538395*, AY538491*, AY538432*, AY538457*; *J. umbellifera* H. Karst., Panama, *Rova & al. 2395* (GB), AY538361*, AY538396*, AY538492*, AY538433*, AY538458*; *Kerianthera praeclara* Kirkbr., Brazil, *Prance & al. 24293* (AAU), AY538362*, AY538397*, AY538493*, AY538459*, AF242970; *Ladenbergia amazonensis* Ducke, Peru, *Persson 613* (GB), AY538363*, AY538398*, AY538494*, AY538434*, AY538460*; *L. carua* (Wedd.) Standl., Bolivia, *Persson & Gustafsson 247* (GB), AY538364*, AY538399*, AY538495*, AY538435*, AY538461*; *L. macrocarpa* (Vahl) Klotzsch, Colombia, *Andersson & al. 2103* (GB), AY538365*, AY538400*, AY538496*, AF242971, AF152683; *L. oblongifolia* (Mutis) L. Andersson, Bolivia, *Persson & Gustafsson 245* (GB), AY538366*, AY538401*, AY538497*, AY538436*, AF538462*; *L. pavonii* (Lamb.) Standl., Ecuador, *Knudsen 548* (GB), AY538367*, AY538402*, AY538437*, AY538463*, Z68801; *L.* sp. (prob. nova), Peru, *Weigend & al. 5784* (GB), AY538368*, AY538403*, AY538498*, AY538438*, AY538464*; *Lasianthus batangensis* K. Schum., Gabon, *Andersson & Nilsson 2284* (GB), AY538404*, AY538499*, AY538439*, AY538465*; *Luculia grandiflora* Ghose, Z701999, X83648, AF242974, AF102453; *Mitriostigma axillare* Hochst., cultivated, *Rova T3* (GB), AY538405*, X83650, AF201006, AF201054; *Mussaenda raiateensis* J.W. Moore, Fiji, *Rova & Gustavsson 2424* (GB), AY538406*, AY538500*, AY538466* AF242983; *Nauclea orientalis* L., Australia, without voucher, AY538407*, AY538501*, AY538440*, AY538467*; *Ophiorrhiza mungos* L., cultivated, *Andersson & Rova* (1999), AY538408*, X83656, AF004064, AF152610; *Paederia foetida* L., cultivated, without voucher, AY538409*, AF332373*, AF004065, AF152619; *Pauridiantha* sp., Cameroon, *Cable & al. 1389* (K), AY538410*, AY538502*, AF004068, AF102467; *Phyllis nobla* L., cultivated, without voucher, AY538411*, AY538468*, Z68814, AF003613; *Posoqueria latifolia* (Rudge) Roem. & Schult., French Guiana, Persson & al. 1950 (GB), AY538412*, Z68850, AF242998, AF152680; *Psychotria kirkii* Hiern, cultivated, without voucher, AY538413*, AY538469*, X83663, AF410728; *Remijia chelomaphylla* G.A. Sullivan, Ecuador, *Persson 517* (GB), AY538369*, AY538414*, AY538503*, AY538441*, AY538470*; *R. macrocnemia* (Mart.) Wedd., Peru, *Persson & Grández 616* (GB), AY538370*, AY538371*, AY538415*, AY538504*, AY538442*, AY538471*; *R. pacimonica* Standl., Brazil, *Antonelli 242* (GB), AY538372*, AY538416*, AY538505*, AY538443*, AY538472*; *R. pedunculata* (H. Karst.) Flueck., Peru, *Persson & al. 601* (GB), AY538373*, AY538417*, AY538506*, AY538444*, AY538473*; *R. ulei* K. Krause, Brazil, *Antonelli 241* (GB), AY538374*, AY538418*, AY538507*, AY538445*, AY538474*; *Rogiera suffrutescens* (Brandeg.) Borhidi, cultivated, *Bremer 2712* (S), AY538419*, X83665, AF243003, AF152738; *Sabicea aspera* Aubl., French Guiana, *Andersson & al. 1941* (GB), AY538420*, AY538508*, AY538475* AF004079; *Sipanea biflora* (L.f.) Cham. & Schltdl., French Guiana, *Rova & al. 2005* (GB), AY538421*, AY538509*, AF004085, AF152675; *Stilpnophyllum grandifolium* L. Andersson, Ecuador, *Persson 518* (GB), AY538375*, AY538422*, AY538510*, AY538446*, AY538476*; *S. oellgaardii* L. Andersson, Ecuador, *Ståhl 2099* (GB), AY538376*, AY538423*, AY538511*, AY538477*, AF243026; *Vangueria madagascariensis* J.F. Gmel., cultivated, without voucher, AY538424*, X83670, AF243033, AF152654.