RESEARCH ARTICLE

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# Conflicting results from mitochondrial genomic data challenge current views of Rubiaceae phylogeny[1]

Catarina Rydin[2,3,4], Niklas Wikström[3], and Birgitta Bremer[3]

**PREMISE OF THE STUDY:** Reconstruction of plant phylogeny has heavily relied on single-gene or multigene plastid data. New sequencing methods have led to an increasing number of studies based on data from the entire plastid, but the mitochondrion has rarely been used to infer plant phylogeny because of an assumed information poverty and demonstrated lateral transfer of mitochondrial gene regions between distantly related species.

**METHODS:** We explored phylogenetic information from the plant mitochondrion using 57 representatives of the species-rich coffee family as study system and assessed consistency with previous results based (mostly) on plastid data.

**KEY RESULTS:** We showed that the mitochondrial genome can provide structured and statistically significant information on plant phylogeny. While most of our results are consistent with those based on plastid data, some surprising and statistically significant conflicts emerge, and our study demonstrates with striking clarity that the phylogeny of Rubiaceae is far from resolved.

**CONCLUSIONS:** It appears unlikely that conflicts between results retrieved from the different genomic compartments would be restricted to Rubiaceae. Rather, they are probably a general phenomenon and an important factor behind longstanding "difficult" phylogenetic questions. The biological processes responsible for the conflicting results detected here are unclear, but some conflicts are likely caused by hybridization events that occurred tens of millions of years ago. Whether such ancient events can be reconstructed based on molecular data from extant plants remains to be seen, but future studies of the nuclear genome may provide a way forward.

**KEY WORDS** coffee family; conflicting topologies; hybridization; next-generation sequencing; NGS; phylogenomics; phylogenetics; plant mitochondrion; Rubiaceae

Today, about 30 years after the beginning of the "molecular revolution" of systematics, we have relatively good knowledge of angiosperm evolution and interrelationships among major groups (APG III, 2009). There are, however, still unanswered phylogenetic questions and unresolved nodes. Furthermore, contemporary knowledge of plant phylogeny typically derives from plastid data, which have been widely used for resolving evolutionary relationships. Early phylogenetic studies based on analysis of the rubisco gene *rbcL* (Olmstead et al., 1992; Chase et al., 1993; Bremer et al., 1995) inspired, and the availability of universal primers (e.g., Zurawski and Clegg, 1987; Taberlet et al., 1991) enhanced molecular laboratory work, resulting in numerous phylogenetic studies on land plants during the subsequent decades. Typically, these studies were single-gene or multigene studies, i.e., they were based on one to several gene regions. Following recent developments, new sequencing techniques permit time- and cost-efficient production of much more data, e.g., entire genomes, but it is nevertheless still not fully clear to what extent phylogenomics can significantly increase our understanding of land plant phylogeny.

While it was early recognized that data from the nuclear and mitochondrial genomes are desirable, such data have been much less used for the purpose of resolving evolutionary relationships in plants than have plastid data. Using nuclear genes may pose biological challenges associated with, for example, allelic variation and paralogy, as well as methodological problems with availability of general primers and requirements of fresh plant material (see e.g., Zimmer and Wen, 2012). The exception is nuclear ribosomal DNA, which is present in many copies that are assumed to be

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[2] Department of Ecology, Environment and Plant Sciences, Stockholm University, SE-106 91 Stockholm, Sweden; and

[3] Bergius Foundation, Royal Swedish Academy of Sciences, Box 50005, SE-104 05 Stockholm, Sweden

[4] Author for correspondence (e-mail: Catarina.Rydin@su.se); ORCID id: 0000-0002-3347-7820

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homogenized through the process of concerted evolution (Elder and Turner, 1995) and for which primers with wide specificity can easily be produced. Mitochondrial data pose no such biological challenges, yet more than 90% of all mitochondrial DNA deposited in GenBank is from animals (Smith, 2016), and data from the mitochondrion have rarely been used to infer phylogeny of plants (Palmer and Herbon, 1988; Palmer, 1990; Seberg and Petersen, 2006; Mower et al., 2012; Van de Paer et al., 2016). In plants, mitochondrial genes are often conserved with a slow substitution rate (Wolfe et al., 1987; Palmer and Herbon, 1988; Palmer, 1990; Hiesel et al., 1994) potentially providing little information on phylogeny within most groups. Substitution rates of mitochondrial genes may, however, be extremely heterogeneous among plant lineages, with some species having exceptionally elevated rates and others slower rates than most lineages (Mower et al., 2007), something which also may cause problems in phylogenetic reconstruction. Further, horizontal transfer of mitochondrial genes between distantly related plant species are reported (Bergthorsson et al., 2003; Won and Renner, 2003; Davis and Wurdack, 2004; Rice et al., 2013), as are intracellular transfer of genes from the mitochondrion to the nucleus (Adams et al., 2000; Ong and Palmer, 2006) and transfer of genetic material between the plastid genome and the mitochondrial genome (Hao and Palmer, 2009).

The consequence is that many phylogenetic studies of plants have relied solely on results from plastid data (e.g., discussed by Zimmer and Wen, 2012; Rothfels et al., 2015), sometimes in combination with data from nuclear ribosomal DNA. The resulting phylogeny has often been put forward as reliably representing the species phylogeny, and although this typically could be considered a reasonable assumption, it should be noted that the phylogenetic signal in the second organellar genome of plants, the mitochondrial genome, is poorly understood in most plant groups.

Here we explore the consequence of this biased employment of (mostly plastid) data, using a phylogenomic approach and one of the largest angiosperm families, the coffee family (Rubiaceae, Gentianales). Rubiaceae comprise about 13,000 species, currently divided into three subfamilies and 65 tribes. Although a number of studies during the last decades have contributed considerable knowledge on the phylogeny and early evolution in the family (Bremer, 2009) the results are based on up to about 10 molecular markers, mostly from the plastid genome. Among frequently used gene regions are the plastid genes *rbcL*, *rps16*, and *trn*(*T*)*L-F* and the internal transcribed spacers of the nuclear ribosomal DNA (Bremer, 2009). As with most plant groups, mitochondrial data have never been used to address phylogenetic relationships in Rubiaceae. Furthermore, several important phylogenetic questions are not yet answered, among them deep divergences in the family and in the three subfamilies. We therefore used a reference-guided genome skimming approach (Straub et al., 2012) to assemble the genic portion of the mitochondrial genome for 58 gentianalean taxa. These data were analyzed with two previously published genomes to infer phylogenetic relationships in Rubiaceae based on mitochondrial data. For the purpose of comparison, we also reanalyzed the plastid multigene data set from Wikström et al. (2015), pruned to include a set of ingroup taxa identical to that of our mitochondrial data set. The major aims were to seek answers to longstanding questions on deep divergences in Rubiaceae and assess the consistency of results based on mitochondrial data with those based on plastid data, and thus test currently accepted views of relationships in the Rubiaceae. We hypothesize that utilization of sufficient amounts of data from the mitochondrion will reveal previously undetected evolutionary patterns.

## MATERIALS AND METHODS

***Taxon sample***—Taxa were targeted with the objective of having all tribes currently recognized in the family (Bremer and Eriksson, 2009; Rydin et al., 2009a; Kainulainen et al., 2013; Mouly et al., 2014) represented in the analyses. Data from 55 of the 65 tribes of Rubiaceae were included, as was the genus *Glionnetia* Tirveng. from the Seychelles, currently unclassified at tribal level. Three non-Rubiaceae taxa from the remaining Gentianales were included as outgroups: *Mostuea* Didr. (Gelsemiaceae), *Asclepias* L., and *Rhazya* Decne. (Apocynaceae). Detailed species and voucher information for all included taxa is given in Appendix S1 (see Supplemental Data with this article).

***DNA extraction and sequencing***—DNA was extracted from herbarium, live, or silica-dried material using a cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987; Doyle, 1991). Extracted DNA was cleaned using the QIAquick PCR cleaning kit from Qiagen (Hilden, Germany) following the protocol specified by the manufacturer.

High-throughput sequencing was carried out at the Science for Life Laboratory (SciLifeLab, Stockholm, Sweden) following the manufacturer's instructions for the Illumina HiSeq2500 platform (Illumina, San Diego, California, USA). Pair-end runs with 300-bp insert size fragments and 2 × 150 bp read lengths were performed. Each sample was multiplexed with 14, 17, or 28 other samples in one lane in three consecutive runs. Library preparation at the SciLifeLab was done using the Illumina TruSeq DNA PCR-free library preparation kit (Illumina) for samples with high quantities of DNA, and the Thruplex DNA-seq library preparation kit from Rubicon (Rubicon Genomics, Ann Arbor, Michigan, USA) for samples with lower quantities of DNA. Demultiplexing and conversion was conducted using CASAVA v1.8.2 (Illumina).

***Mitochondrial sequence assembly***—To isolate mitochondrial sequences from the original Illumina reads, a BLAT (BLAST-like alignment tool v36) search of forward and reverse reads against a reference database including 41 protein-coding genes and two rDNA mitochondrial gene regions from the two Gentianales taxa *Asclepias syriaca* L. (NC_022796) and *Rhazya stricta* Decne. (NC_024293) was conducted (Straub et al., 2013; Park et al., 2014). Forward and reverse reads were both saved if either showed at least 70% similarity to any of the Gentianales reference sequences. Following the BLAT search, reads were extracted from the original fastq data files using pullseq v1.0.1 (http://github.com/bcthomas/pullseq) into new forward and reverse fastq data files representing a "mitochondrial subset" of the original reads. De novo assembly of the "mitochondrial subset" was performed for each taxon using the program ABySS v1.5.2 (Simpson et al., 2009) and nine *k*-mer lengths (55, 61, 67, 73, 85, 91, 97, 103). All generated contigs were pooled and mapped onto the 41 protein-coding and two rDNA reference sequences of *Rhazya stricta* (NC_024293) using the program bwa v0.7.5a-r405 (Li and Durbin, 2009). This resulted in complete or near complete draft sequences of all the protein-coding and the rDNA mitochondrial gene regions. All original

reads were subsequently mapped onto the draft sequences using bwa v0.7.5a-r405 (Li and Durbin, 2009) allowing sequencing depths to be evaluated and any unfinished gaps to be filled. Final assemblies were reviewed and edited using gap5 from the Staden Package v2.0.0b10 (Staden, 1996; Staden et al., 2000; Bonfield and Whitwham, 2010). Each gene region was annotated using the program Sequin v15.10 (available by anonymous FTP at https://www.ncbi.nlm.nih.gov/Sequin/; National Center for Biotechnology Information, Bethesda, Maryland, USA) by transferring the annotations of *Asclepias syriaca* (NC_022796) and *Rhazya stricta* (NC_024293).

***Phylogenetic analyses of mitochondrial data***—Individual gene regions were aligned using the program MUSCLE v3.8.31 (Edgar, 2004) and concatenated into a protein-coding set (CDS; 29,827 characters), an intron set (intron; 28,469 characters), and a mitochondrial ribosomal DNA set (mt rDNA; 5771 characters). A complete list of assembled gene regions, their exon/intron configurations and lengths is given in Table 1. The final data set included 57 ingroup (Rubiaceae) and three outgroup (from other Gentianales) taxa, and 64,058 aligned characters. The complete partitioned and annotated data set is available in Nexus format (online Appendix S2). Phylogenetic analyses were conducted with Markov Chain Monte Carlo methods (Larget and Simon, 1999) using nonclock models in MrBayes v3.2.5 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and relaxed-clock models in Beast v1.8.2 (Drummond et al., 2012). The protein coding set was split into three separate partitions based on codon position in all analyses. The GTR+I+Γ substitution model was used in all analyses for each of the five partitions, based on the corrected Akaike information criterion (AICc) as calculated using the programs MrAIC v.1.4.6 (Nylander, 2004) and PHYML v.3.0 (Guindon et al., 2010). Substitution models were unlinked across all partitions. MrBayes was run for 10,000,000 generations sampling trees and parameter estimates every 1000 generations. Two independent runs, each with four chains and heating parameters set to default values, were conducted for all analyses. Bayesian posterior probability values were calculated after discarding the first 50% of the trees and parameters as burnin. This was well beyond the burnin phase of the chains based on the PSRF convergence diagnostic (Gelman and Rubin, 1992) reported by MrBayes. Relaxed-clock analyses in Beast were run for 100,000,000 generations sampling trees and parameter estimates every 10,000 generations. Two independent runs were conducted, and 50% of the trees and parameter estimates were removed as burnin in each of the runs. Posterior distribution of trees and parameters were subsequently pooled yielding a final posterior of 10,000 trees and parameter estimates, which were used for calculating posterior probabilities. The uncorrelated lognormal relaxed clock model was used for all relaxed-clock analyses. A normally distributed root-age prior with a mean of 75 million years (Myr) and standard deviation of 7.7 Myr was employed corresponding to one of the Gentianales crown group age estimates reported by Wikström et al. (2015). Three fossil-based age constraints were enforced: a minimum age of 34 Myr for the *Cephalanthus* L. stem lineage (Mai and Walther, 1985, 2000), a minimum age of 38 Myr for the *Morinda* L. stem lineage (Shi et al., 2012), and a minimum age of 16 Myr for the *Scyphiphora* C.F.Gaertn. stem lineage (Leopold, 1969). All three were included as uniform priors with minimum ages set based on the fossil-based information and maximum ages set to 128 Myr (see Wikström et al., 2015, for more detailed information on the fossil-based constraints). The primary objective of the relaxed-clock analyses was not to estimate divergence-time ages within Rubiaceae but to infer relationships within a relaxed-clock framework. Three additional sets of analyses were conducted in addition to the concatenated analyses: one analyzing the protein coding set, one analyzing the intron set, and one where the mt rDNA set was analyzed. Both nonclock and relaxed-clock analyses were conducted also on these separate analyses with all settings corresponding to the ones used in the concatenated analyses.

***Phylogenetic analyses of plastid data***—For the purpose of comparison, we reanalyzed the 6-gene plastid data set from Wikström et al. (2015). Twelve taxa were pruned from their Gentianales data

**TABLE 1.** Mitochondrial protein regions (CDS) including introns and mitochondrial ribosomal DNA (mt rDNA) gene regions assembled using the *Asclepias syrica* (NC_022796) and the *Rhazya stricta* (NC_024293) mitochondrial genomes as reference sequences.

| CDS region | No. of exons | Length of exons (bp) | No. of introns | Length of introns (bp) |
|---|---|---|---|---|
| *atp1* | 1 | 1530 | 0 | |
| *atp4* | 1 | 609 | 0 | |
| *atp6* | 1 | 906 | 0 | |
| *atp8* | 1 | 489 | 0 | |
| *atp9* | 0/1 | 285 | 0 | |
| *ccmB* | 1 | 621 | 0 | |
| *ccmC* | 1 | 762 | 0 | |
| *ccmFc* | 1/2 | 1332 | 0/1 | 1034 |
| *ccmFn* | 1 | 1758 | 0 | |
| *cob* | 1 | 1186 | 0 | |
| *cox1* | 1/2 | 1584 | 0/1 (excl.) | 971 (excluded) [a] |
| *cox2* | 1/2 | 807 | 0/1 | 1801 |
| *cox3* | 1 | 798 | 0 | |
| *matR* | 1 | 1959 | 0 | |
| *mttB* | 1 | 887 | 0 | |
| *nad1* (*exon 1*) | 1 | 385 | 0 | |
| *nad1* (*exon 2–3*) | 2 | 276 | 1 | 1572 |
| *nad1* (*exon 5*) | 1 | 261 | 0 | |
| *nad2* (*exon 1–2*) | 2 | 546 | 1 | 1372 |
| *nad2* (*exon 3–5*) | 3 | 924 | 2 | 4667 |
| *nad3* | 1 | 357 | 0 | |
| *nad4* | 4 | 1488 | 3 | 7993 |
| *nad4L* | 1 | 303 | 0 | |
| *nad5* (*exon 1–2*) | 2 | 1446 | 1 | 950 |
| *nad5* (*exon 4–5*) | 2 | 546 | 1 | 1312 |
| *nad6* | 1 | 618 | 0 | |
| *nad7* | 4 | 1185 | 3 | 4631 |
| *nad9* | 0/1 | 573 | 0 | |
| *rpl5* | 1 | 564 | 0 | |
| *rps1* | 0/1 | 624 | 0 | |
| *rps10* | 0/2 | 342 | 1 | 1002 |
| *rps12* | 1 | 378 | 0 | |
| *rps13* | 1 | 375 | 0 | |
| *rps3* | 2 | 1791 | 1 | 2126 |
| *rps4* | 1 | 876 | 0 | |
| *rps7* | 0/1 | 456 | 0 | |
| | | ∑ = 29,827 | | ∑ = 28,469 |
| *rrn18-rrn5* [b] | | 2181 | | |
| *rrn26* [b] | | 3590 | | |
| | | ∑ = 5771 | | |

[a] The *cox1* intron was excluded from the phylogenetic analyses because it is absent from the majority of the included taxa.
[b] Mitochondrial rDNA region.

set (available at https://doi.org/10.1371/journal.pone.0126690.s005) resulting in a 60-taxon data set with an identical set of ingroup taxa as used in our mitochondrial analyses. Following Wikström et al. (2015) , data were partitioned into a coding (6187 chars) and a noncoding (5283 chars) partition. The GTR+Γ substitution model was used for the coding partition and GTR+I+Γ for the noncoding based on the corrected Akaike information criterion (AICc) as calculated using MrAIC v.1.4.6 (Nylander, 2004) and PHYML v.3.0 (Guindon et al., 2010).

## RESULTS

***Assembled sequences***—A total of 38 of the original 43 mitochondrial gene regions searched for were successfully assembled for almost all of the taxa included. Among gene regions included but not assembled for all taxa are *atp9*, *nad9*, *rps1*, *rps10*, and *rps7*, which appeared to be missing or otherwise not possible to assemble in 3, 1, 4, 1, and 18 taxa, respectively. Gene regions *rpl2*, *rpl10*, *rps19*, *sdh3*, and *sdh4* appeared missing or not possible to assemble for a majority of the taxa included and were excluded from further analyses. The total number of sequenced fragments varied across the samples from 1.5 × 106 (*Cephalanthus*) to 16.6 × 106 (*Prismatomeris* Thwaites) with an average number of 8.7 × 106. Sequencing depths, as indicated in the final mapping stage of the assembly using bwa v0.7.5a-r405, displayed corresponding variation but were never shallow enough to indicate the presence of nuclear mitochondrial DNA segments (NUMT:s). Establishing that the assembled sequences are not relocated to the plastid genome (PTMT:s) is more complicated based solely on the data presented here, but complete plastid genome assemblies of the investigated taxa (unpublished data) do not indicate any such presence. Assembled sequences are deposited in GenBank. A comprehensive list of accession numbers for all deposited sequences is given in Appendix S1.

***Phylogenetic analyses of mitochondrial data***—Phylogenetic relationships were generally resolved and well supported by both the nonclock and the relaxed-clock analyses (Figs. 1, 2; Appendices S3–S8). Nodes indicated by a black dot in the figures are well supported with a posterior probability (BPP) of 0.95 or more (Alfaro et al., 2003). All support values less than 1.00 are given in the figures and nodes with a BPP of less than 0.50 are collapsed.

*Concatenated analyses*—Relationships within subfamily Rubioideae are all well supported and consistent between the nonclock and the relaxed-clock analyses (Figs. 1, 2), with Colletoecemateae+Urophylleae sister to the remaining members of the subfamily, followed by Ophiorrhizeae. Lasiantheae and Coussareeae are successive sisters to a clade comprising the Psychotrieae and Spermacoceae alliances. Schizocoleeae are sister to the remaining Psychotrieae alliance followed by Craterispermeae sister to a clade comprising Prismatomerideae sister to Mitchelleae+Morindeae and Gaertnereae sister to Psychotrieae+Palicoureeae. A clade comprising Danaideae sister to Knoxieae+Spermacoceae is sister to the remaining Spermacoceae alliance, comprising Dunnieae+Anthospermeae, Paederieae, Argostemmateae and Putorieae as successive sisters to Theligoneae+Rubieae. Luculieae are, with poor support, resolved as sister to subfamily Rubioideae in the nonclock analysis (Fig. 1). In the relaxed-clock analysis, the position of Luculieae is unresolved (Fig. 2).

Coptosapelteae are well supported as sister to a clade comprising subfamilies Ixoroideae and Cinchonoideae, but Ixoroideae and Cinchonoideae as commonly recognized are not monophyletic. The two tribes Cinchoneae and Isertieae from subfamily Cinchonoideae are with strong support nested in Ixoroideae and sister to Posoquerieae. In the remaining "Cinchonoideae", a Chiococceae+Hillieae+Hamelieae clade is sister to a clade comprising Rondeletieae+Guettardeae and Hymenodictyeae+Naucleeae. Within subfamily "Ixoroideae", Sipaneeae are sister to remaining taxa, followed by the clade comprising Posoqueireae+Isertieae+Cinchoneae, Mussaendeae+Sabiceeae, Steenisieae and Retiniphylleae, all placed with strong support. The Coffeeae and Vanguerieae alliances are paraphyletic. Jackieae from the Vanguerieae alliance are resolved sister to Airospermeae of the Coffeeae alliance, and together they are sister to a clade in which the remaining Vanguerieae alliance is resolved sister to the remaining Coffeeae alliance. In the (paraphyletic) "Vanguerieae alliance", we see different results between the nonclock and the relaxed-clock analyses (although there are no supported conflicts). *Glionnetia* is resolved sister to a well-supported Trailliaedoxeae+Vanguerieae clade and Scyphiphoreae are sister to Greeneeae+Ixoreae (these two clades being sisters) in the nonclock analysis (Fig. 1). Neither relationship is present in the relaxed-clock analysis (Fig. 2), in which relationships in the "Vanguerieae alliance" are mostly collapsed. In the (paraphyletic) "Coffeeae alliance", Alberteae are sister to remaining tribes followed by Augusteae and a clade including Gardenieae, Cordiereae, Bertiereae, Pavetteae, Sherbournieae, and Octotropideae. Relationships among the latter six tribes are poorly supported in both the nonclock and the relaxed-clock analyses, the only supported clade being Pavetteae+Sherbournieae+Octotropideae.

*Separate analyses*—Results from the nonclock and relaxed-clock separate analyses of the protein coding (CDS), the intron, and the mt rDNA partitions are reported in Appendices S3–S8. The results are, for the most part, expected with the majority of relationships supported by the concatenated analyses also recovered in the separate analyses, although sometimes with less support. Conflicting results are seen, however, primarily in the Coffeeae+Vanguerieae alliances. Pavetteae, for example, group with Octotropideae in the nonclock CDS analysis (Appendix S3), with Octotropideae+Sherbournieae in the relaxed clock CDS analysis (Appendix S4), with Gardenieae in the analyses of intron data (Appendices S5, S6), and with Traillaedoxeae in the mt rDNA analyses (Appendices S7, S8). The latter two results were obtained in both the nonclock and relaxed-clock analyses of respective data sets, but are strongly supported only in the nonclock analyses. The exception is the sister relationship between Pavetteae and Traillaedoxeae seen in analyses of mt rDNA, which is poorly supported both in the nonclock and the relaxed-clock analyses. A second example is Scyphiphoreae, supported as sister to Greeneeae+Ixoreae in the nonclock CDS analysis (Appendix S3) but supported as sister to Vanguerieae in the analyses of intron data (Appendices S5, S6). Most conspicuous conflicts are seen in the analyses of the mt rDNA data (Appendices S7, S8). Here, Vanguerieae are well supported as sister to Airospermeae, which together are sister to Jackieae; Posoquerieae are grouped sister to Cinchoneae with first Isertieae and then Sabiceeae supported as successive sisters; Rubieae are supported as sister to Danaideae in a well-supported group also including Spermacoceae, Dunnieae, and Paederieae; and Gaertnereae are supported as sister to Schizocoleeae. None

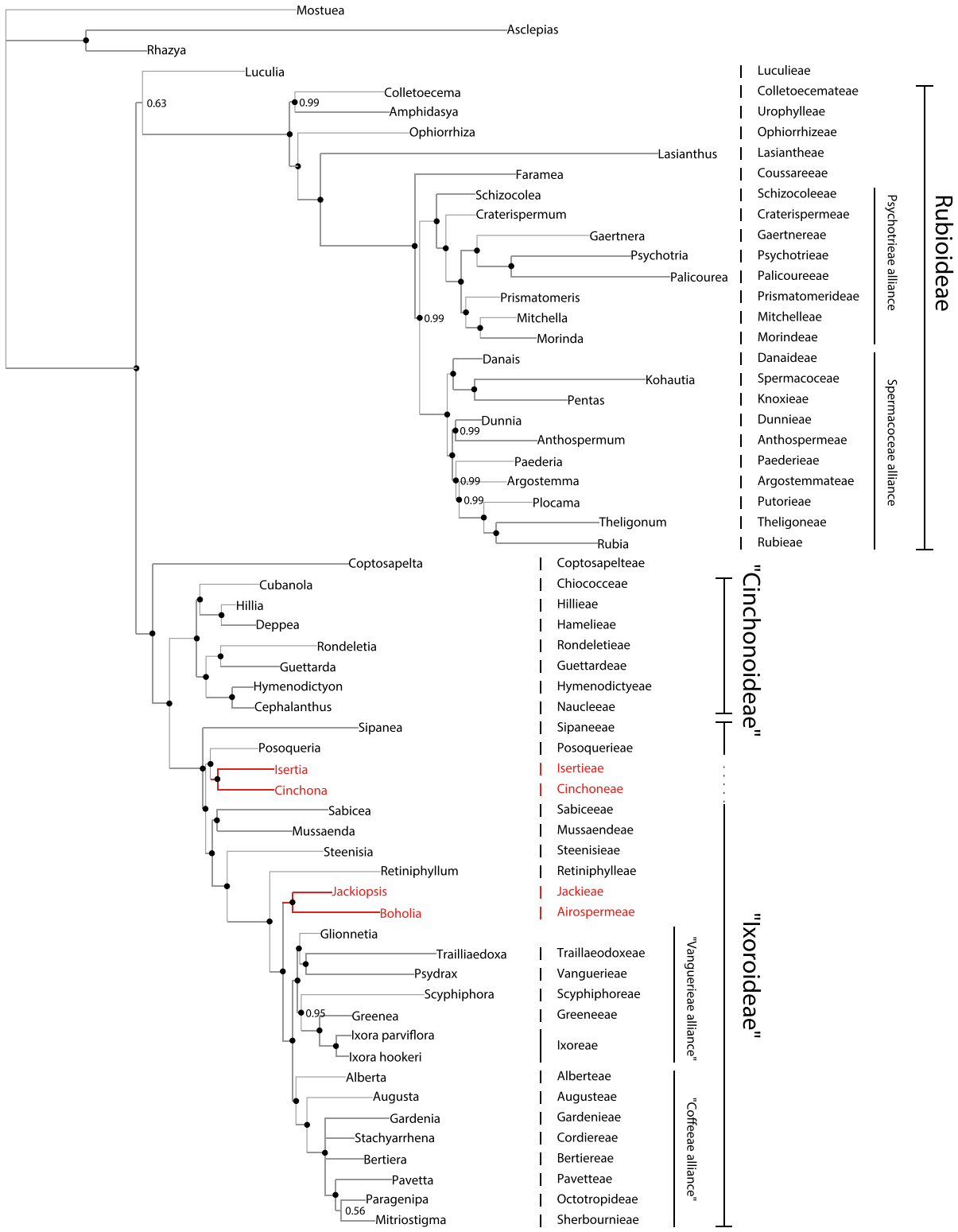


**FIGURE 1** Phylogram resulting from the nonclock analysis of the concatenated data set. Nodes indicated by a black dot are well supported and have a Bayesian posterior probability (BPP) of 0.95 or more (Alfaro et al., 2003). Support values are 1.00 for all nodes unless otherwise indicated in the figure. Nodes with BPP less than 0.50 are collapsed. Subfamilies Rubioideae, Cinchonoideae, and Ixoroideae, and the Psychotrieae, Spermacoceae (Rubioideae), and Vanguerieae, Coffeeae (Ixoroideae) alliances as traditionally recognized following analyses using plastid data are indicated on the tree. Two conspicuous conflicts between the relationships supported by mitochondrial data, as analyzed here, and "well-established" relationships supported in analyses using plastid data (see Appendices S9, S10) are highlighted in red. Current tribal assignments of included taxa are indicated to the right of the taxon names.

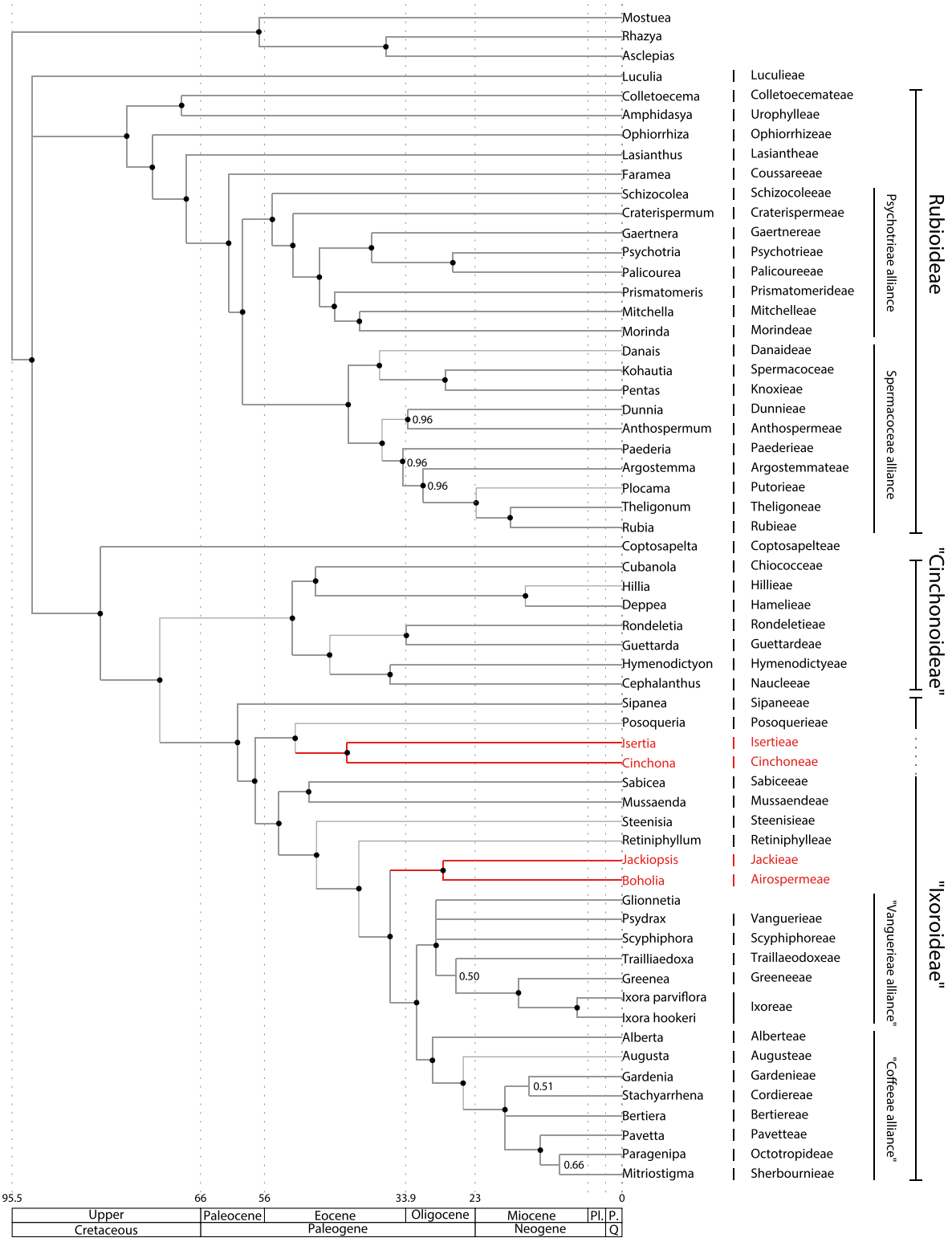


**FIGURE 2** Chronogram resulting from the relaxed-clock analysis of the concatenated data set. Nodes indicated by a black dot are well supported and have a Bayesian posterior probability (BPP) of 0.95 or more (Alfaro et al., 2003). Support values are 1.00 for all nodes unless otherwise indicated in the figure. Nodes with BPP less than 0.50 are collapsed. Subfamilies Rubioideae, Cinchonoideae, and Ixoroideae, and the Psychotrieae, Spermacoceae (Rubioideae), and Vanguerieae, Coffeeae (Ixoroideae) alliances as traditionally recognized following analyses using plastid data are indicated on the tree. Two conspicuous conflicts between the relationships supported by mitochondrial data, as analyzed here, and "well-established" relationships supported in analyses using plastid data (see Appendices S9, S10) are highlighted in red. Current tribal assignments of included taxa are indicated to the right of the taxon names.

of these relationships are seen or supported in any of our other analyses.

***Phylogenetic analyses of plastid data***—Phylogenetic relationships were generally resolved and well supported by both the nonclock and the relaxed-clock analyses (Appendices S9, S10). Relationships were also entirely consistent between the two types of analyses. Nodes indicated by a black dot in the figures are well supported with a posterior probability (BPP) of 0.95 or more (Alfaro et al., 2003). All support values less than 1.00 are given in the figures and nodes with a BPP of less than 0.50 are collapsed.

Monophyly of the three subfamilies Rubioideae, Ixoroideae, and Cinchonoideae are well supported, with Cinchonoideae resolved sister to Ixoroideae. In Rubioideae, Urophylleae are resolved sister to a poorly supported group comprising the remaining subfamily. Colletoecemateae+Lasiantheae comprise the next diverging group, followed by Ophiorrhizeae. Coussareeae are sister to a clade comprising the Psychotrieae and Spermacoceae alliances. Schizocoleeae are sister to remaining Psychotrieae alliance followed by Craterispermeae, Prismatomerideae, and a clade comprising Psychotrieae+Palicoureeae and Gaertnereae+Mitchelleae+Morindeae. A clade comprising Danaideae sister to Knoxieae and Spermacoceae is sister to the remaining Spermacoceae alliance. Anthospermeae are sister to a clade, in which Dunnieae+Argostemmateae are sister to the four tribes Paederieae, Putorieae, Theligoneae, and Rubieae. Luculieae and Coptosapelteae are resolved as successive sisters to the subfamily Rubioideae but with poor support. In Cinchonoideae, Isertieae+Cinchoneae are resolved as sister to remaining members of the subfamily. Additional and well-supported groups are Guettardeae+Rondeletieae, Hymenodictyeae+Naucleeae, and Chiococceae+Hillieae+Hamelieae, but relationships among these groups are not well supported. In subfamily Ixoroideae, Sipaneae+Posoquerieae are sister to a poorly supported clade comprising the remaining subfamily. Sabiceeae+Mussaendeae (poorly supported) are the next diverging clade, followed by Steenisieae and Retiniphylleae, which are well supported as successive sister groups to the well-supported Vanguerieae and Coffeeae alliances. The Vanguerieae alliance comprise the Scyphiphoreae, Traillaeodoxeae, Jackieae, Vanguerieae, Greenieae, Ixoreae, and the unclassified *Glionnetia*, but their interrelationships are mostly poorly supported. In the Coffeeae alliance, Airospermeae is well supported as sister to remaining taxa, followed by Augusteae and Alberteae, sister to a well-supported clade comprising Bertiereae+Octotropideae sister to Pavetteae+Gardenieae+Cordiereae+Sherbournieae. Relationships among the latter for tribes are poorly supported.

## DISCUSSION

Two important conclusions emerge from our study: (1) Mitochondrial data provide structured and statistically significant information on phylogenetic relationships in Rubiaceae, and (2) information from the mitochondrion may strongly conflict with results based (entirely or mostly) on data from the plastid genome. Our reanalysis of the plastid data set from Wikström et al. (2015) yielded results that are largely consistent with those from previous analyses of Rubiaceae using plastid data (e.g., Rydin et al., 2008, 2009a; Antonelli et al., 2009; Bremer, 2009; Bremer and Eriksson, 2009; Razafimandimbison et al., 2011; Manns et al., 2012; Kainulainen et al., 2013; Wikström et al., 2015; Janssens et al., 2016), but there are conspicuous conflicts between the mitochondrial-based results (Figs. 1, 2; Appendices S3–S8) and those based on data from the plastid (Appendices S9, S10). The conclusions are vital since phylogenetic information from the mitochondrion of plants has been largely ignored in the past and is unknown for many plant groups. We find no reason to assume that the detected conflicts between results from the mitochondrion and the plastid would be a phenomenon restricted to Rubiaceae. On the contrary, our results likely indicate a general problem. Not least for practical reasons, species phylogenies are, still, often based only or mostly on data from the plastid genome, and generally accepted hypotheses on phylogeny may unexpectedly be challenged or proven wrong in the future, as is here demonstrated for Rubiaceae.

***The mitochondrion provides structured information on phylogeny***—In contrast with the plastid DNA, the mitochondrial genome of plants varies considerably in size and organization among species and has a slow nucleotide substitution rate (Palmer and Herbon, 1988; Palmer, 1990; Mower et al., 2012) (although highly elevated rates have been detected too; Mower et al., 2007). A consequence has been a comparatively low interest in using mitochondrial data for inferring phylogenetic relationships of plants (Hiesel et al., 1994; Van de Paer et al., 2016). However, our study shows that mitochondrial DNA can be highly useful; the amount of phylogenetically informative characters in our mitochondrial data set was sufficient to resolve tribal interrelationships in Rubiaceae with very high statistical support. This is in line with results in a few earlier studies of the usefulness of the mitochondrion for phylogenetic purposes (Richardson et al., 2013; Malé et al., 2014; Van de Paer et al., 2016), although these studies used data from very few species and the possibility of making phylogenetic conclusions therefore was limited. Whether the mitochondrion can provide phylogenetic information also on more closely related species is poorly investigated. It seems, however, plausible that sufficiently variable mitochondrial regions would exist. Genome skimming methods, as adopted here, are time- and cost-efficient approaches to obtaining mitochondrial genomic data (Straub et al., 2012), and the future will likely bring an increasing number of studies exploring the usefulness of mitochondrial data for resolving phylogenies at all taxonomic levels.

***Mitochondrial data reveal conflicting results***—Our study reveals conspicuous examples showing that results from plastid data, on which consensus of plant phylogeny is often based, do not tell the whole story of plant evolution. Although a large proportion of the relationships supported in the analyses of mitochondrial data (Figs. 1, 2) are congruent with those obtained in studies using plastid data (Appendices S9, S10; Bremer, 2009; Bremer and Eriksson, 2009; Rydin et al., 2009a; Manns et al., 2012; Kainulainen et al., 2013; Wikström et al., 2015; Janssens et al., 2016), there are also significant and statistically supported differences. Several nodes, well supported by mitochondrial data, are completely incompatible with "well-established" relationships obtained in analyses using plastid data, and these incongruences should be further investigated. Among possible biological explanations for such conflicting patterns is hybridization between species with both maternal and paternal organellar inheritance (Govindarajulu et al., 2015). Plants typically have maternal inheritance of both the plastid (Corriveau and Coleman, 1988; Schneider et al., 2015; Van de Paer et al., 2016) and the mitochondrial genome (McCauley, 2013; Shen et al., 2015;

Van de Paer et al., 2016), but biparental (Corriveau and Coleman, 1988) as well as paternal inheritance are also reported (McCauley, 2013; Shen et al., 2015; Chybicki et al., 2016). This means that the mitochondrion and the plastome can have different evolutionary histories in a taxon of hybrid origin. Furthermore, if there is no strict maternal inheritance of the organellar genome(s), they are not fully linked and topological conflicts could then arise because of incomplete lineage sorting.

Among the strongly supported conflicts between phylogenetic results retrieved from analyses based on plastid vs. mitochondrial data revealed in the current study, at least two stand out as particularly striking. The first example is the rejection of monophyly of two of the three subfamilies of Rubiaceae; i.e., inclusion of the "quinine-tribe" Cinchoneae and the Isertieae of the Cinchonoideae in another subfamily (the Ixoroideae). The second example concerns Jackieae and Airospermeae, which together are placed as sister to (paraphyletic) "Vanguerieae" and "Coffeeae" alliances. To our knowledge these two results, both thus based on mitochondrial data, have never been reported before. Whether these highly surprising, yet strongly supported, conflicts between results based on plastid (Appendices S9, S10) vs. mitochondrial data (Figs. 1, 2) reflect ancient hybridization events (the Ixoroideae crown is about 47–72 Myr old, Cinchonoideae 40–62 Myr, Vanguerieae alliance 24–39 Myr, Coffeeae alliance 23–39 Myr, Wikström et al., 2015) remains to be investigated.

We further find a few possible examples where the mitochondrion itself yields supported, yet conflicting, results (see below for more details). A possible explanation for such conflicts is lateral transfer of endogenous or exogenous genes, which can result in the presence of foreign or chimeric genes in the mitochondrion of plants (Bergthorsson et al., 2003; Won and Renner, 2003; Hao et al., 2010; Rice et al., 2013). Biological mechanisms responsible for such patterns are poorly understood, but work on *Amborella* shows that donors of foreign DNA may be other angiosperms as well as green algae and mosses and that the transferring events can be millions of years old (Rice et al., 2013). As mentioned above, however, different genealogies are expected also in the absence of lateral transfer of genetic material, since the mitochondrial genome is highly recombinational and since biparental inheritance of organelles are known to occur.

***More details on the phylogeny of Rubiaceae***—The deepest splits in the family have always been uncertain, with conflicting results (see Bremer, 2009 for a review). Two species-poor East Asian lineages, the monogeneric tribe Luculieae and the digeneric tribe Coptosapelteae, are classified outside of the three generally accepted subfamilies Rubioideae, Ixoroideae, and Cinchonoideae, but relationships among these five well-supported clades have never been clarified. Here, Coptosapelteae are strongly supported as sister to the two subfamilies Cinchonoideae and Ixoroideae in all analyses of mitochondrial data, and this is a relationship that has not been seen in analyses based on plastid data (but see Robbrecht and Manen, 2006, who reported this relationship based on plastid data without statistical support). Previous studies have otherwise either resolved Coptosapelteae in an unsupported basal position outside of the three subfamilies (Rydin et al., 2008, 2009a; Bremer and Eriksson, 2009; Razafimandimbison et al., 2011) or in a weakly supported position as sister to the subfamily Rubioideae (Appendices S9, S10; Antonelli et al., 2009; Manns et al., 2012; Wikström et al., 2015).

*Subfamily Rubioideae*—Colletoecemateae had long been accepted as sister to the remaining species of the subfamily Rubioideae (Robbrecht and Manen, 2006; Rydin et al., 2008, 2009a) but recently, Manns et al. (2012) and Wikström et al. (2015) found it nested within the Rubioideae, sister to Lasiantheae. Wikström et al. (2015) noted that this sister relationship to Lasiantheae was only indicated in analyses using relaxed-clock models (Manns et al., 2012; Wikström et al., 2015), and their reanalyses using nonclock models did resolve Colletoecemateae in a position more consistent with earlier ideas (as sister to remaining Rubioideae), although with poor support. Colletoecemateae are nested within Rubioideae also using mitochondrial data, however, not as sister to Lasiantheae but to Urophylleae (Figs. 1, 2). Dunnieae, yet another species-poor East Asian tribe, have previously been placed as sister to a larger clade within Rubioideae, comprising several tribes (Rydin et al., 2008, 2009a). Wikström et al. (2015) found instead Dunnieae sister to only one of these tribes, the Argostemmateae. Here, mitochondrial data support a sister relationship between Dunnieae and Anthospermeae, but support for this placement is lost in the analyses of individual partitions (Appendices S3–S8). Neither the placement of Colletoecemateae as sister to Urophylleae, nor that of Dunnieae as sister to Anthospermeae, have, to our knowledge, been seen in any analyses of plastid data.

The deepest splits in the Spermacoceae alliance and the position of the tribe Danaideae have been uncertain. Previous analyses have placed Danaideae as sister to the remaining species of the Spermacoceae alliance (Bremer and Manen, 2000; Bremer and Eriksson, 2009; Rydin et al., 2009b), as sister to a clade including the Anthospermeae, Argostemmateae, Paederieae, Rubieae, and Theligoneae (Robbrecht and Manen, 2006), or as sister to the Spermacoceae-Knoxieae clade (Rydin et al., 2009a; Krüger, 2014; Wikström et al., 2015). All the analyses using mitochondrial data conducted here, except for the mt rDNA analyses, support a sister group relationship between Danaideae and the Spermacoceae-Knoxieae clade, which together are supported as sister to the remaining Spermacoceae alliance (Figs. 1, 2; Appendices S3–S6). Mitochondrial ribosomal DNA instead places Danaideae as sister to Rubieae with strong support (Appendices S7, S8), possibly exemplifying horizontal transfer of individual genes or gene regions, as discussed above. Also in the Psychotrieae alliance are strongly supported conflicts between results based on mitochondrial data and plastid data detected, for example, the respective positions of Prismatomerideae and Gaertnereae.

*Subfamilies Ixoroideae and Cinchonoideae*—The most unexpected phylogenetic result of the current study is that mitochondrial data strongly reject monophyly of two of the three subfamilies of Rubiaceae: the Ixoroideae and the Cinchonoideae. In previous studies based on plastid data, Ixoroideae and Cinchonoideae are always monophyletic and sisters (Bremer, 2009), although deep divergences in both clades were only partly understood. Our results, based on the mitochondrial genome, clearly place the tribes Cinchoneae and Isertieae of the Cinchonoideae in the subfamily Ixoroideae. Their specific relationships within the Ixoroideae differ in our analyses, but the rejection of monophyly of the Cinchonoideae and the Ixoroideae as previously circumscribed is evident, strongly supported, and entirely consistent among our different analyses.

A second conflict, strongly supported and consistent among the different analyses, is the grouping of Airospermeae and Jackieae and their placement as sister to the (remaining) "Coffeeae" and

"Vanguerieae" alliances. Airospermeae comprise two genera, *Airosperma* K.Schum. & Lauterb. with six species from Fiji and New Guinea, and *Boholia* Merr. with a single species from the Philippines and Indonesia. Jackieae comprise the single monotypic genus *Jackiopsis* Ridsdale from Southeast Asia. Based on morphological data, *Boholia* and *Airosperma* were loosely associated with the Alberteae by Robbrecht (1988), whereas Jackieae have been associated with a range of different taxa from all three different subfamilies (see Razafimandimbison et al., 2011 for a discussion). Molecular data, in contrast, have consistently placed Airospermeae in the Coffeeae alliance, as sister to the remaining tribes (Kainulainen et al., 2009; Kainulainen et al., 2013; Wikström et al., 2015) (Appendices S9, S10), and Jackieae as an early-diverging member of the Vanguerieae alliance (Razafimandimbison et al., 2011; Kainulainen et al., 2013; Wikström et al., 2015). The grouping of Airospermeae with Jackieae, and its placement as sister to the remaining "Coffeeae" and "Vanguerieae" alliances, well supported by the mitochondrial data presented here, are thus clearly incompatible with previous conclusions, which were entirely based on plastid data.

## CONCLUDING REMARKS

Mitochondrial data of plants constitute a largely unexplored and potentially rich source of phylogenetic information. Our results exemplify that the mitochondrion can provide ample and structured information that result in deeper and broader understanding of plant evolution. However, our results also demonstrate some highly surprising, yet clear and well-supported, conflicts between results based on mitochondrial data and "well-established" hypotheses on relationships in Rubiaceae (mostly based on plastid data). Since studies on relationships among plants often rely on plastid data, similar phenomena are likely to be revealed for other plant groups as well in the future. One possible reason for conflicts between results obtained from the different organellar genomes is hybridization events between plants with different inheritance of the plastids and the mitochondria (i.e., maternal/paternal/biparental inheritance). Such hybridization events may be difficult to trace if they are ancient, as is indicated by our results, but an interesting start could be to further explore molecular information in the nuclear genome.

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## AUTHOR CONTRIBUTIONS

C.R., N.W., and B.B. designed the research. N.W. conducted laboratory work, processed and assembled sequence data and analyzed the data. C.R. and N.W. wrote the text with comments from B.B.

## DATA ACCESSIBILITY STATEMENT

Genetic data produced for the current study, including voucher information, are submitted to GenBank. Accessions numbers and voucher information are given in the Supplemental Data with this article (Appendix S1). The complete partitioned and annotated data set is available in Nexus format (Appendix S2).

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