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Phylogenetic utility of the nuclear rDNA ITS region in subfamily *Ixoroideae* (*Rubiaceae*): comparisons with cpDNA *rbc*L sequence data

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Abstract: ITS of the nrDNA were sequenced for 21 taxa in *Ixoroideae* and outgroups (*Rubiaceae*) and compared with sequences of the cp-gene *rbc*L. Separate and combined analyses were performed. ITS-variation was extensive and, because of alignment ambiguities, some sites were excluded from the analyses. Several topologies from the *rbc*L analysis that conflicted with earlier classifications are corroborated by the ITS data: 1) *Posoqueria* should be excluded from *Gardenieae*. 2) The disputed genus *Bertiera*, previously in *Gardenieae*, is basal in an extended *Coffeeae*, including *Tricalysia*. 3) *Ixora* should be excluded from *Pavetteae*. 4) *Vangueria*, (*Antirheoideae*), belongs to *Ixoroideae*. This affiliation of *Antirheoideae* tribes with *Ixoroideae* is also shown by new ITS and *rbc*L data for *Alberta*. Incongruities found between the two data sets may be caused by density of taxon sampling, different evolutionary rates, phylogenetic sorting, homoplasy caused by functional constraints, or sampling of non-orthologous ITS types.

Molecular studies using the internal transcribed spacer (ITS) region (consisting of ITS-1, 5.8S, and ITS-2) in 18S–26S nuclear, ribosomal DNA (nrDNA) have been applied widely to phylogenetic questions in flowering plants (see review in BALDWIN & al. 1995). These investigations have concentrated on lower-level taxonomic problems, but subfamily (e.g. *Apioideae*, DOWNIE & KATZ-DOWNIE 1996) and family level (e.g. *Winteraceae*, SUH & al. 1993) studies also have been conducted. Furthermore, at least in ITS-2, there are regions conserved across flowering plants; HERSHKOVITZ & ZIMMER (1996) presented an alignment in which one-third to one-half of the ITS-2 sequence is alignable above the family level in angiosperms. Hence, the ITS region has the potential for contributing phylogenetic signal at higher taxonomic levels than were earlier anticipated.

For some angiosperm groups more than one molecular region has now been investigated phylogenetically. Especially interesting are studies based on both chloroplast (cp) and nuclear (n) regions, e.g. WENDEL & al. 1991, BALDWIN 1992, WOJCIECHOWSKI & al. 1993, DOWNIE & KATZ-DOWNIE 1996, GIELLY & al. 1996, SOLTIS & al. 1996, and OXELMAN & al. 1997. In contrast to phylogenetic studies

involving two or more cpDNA regions, combined cpDNA-nDNA studies generate trees from genetically unlinked regions. Hence, they have the potential of discovering effects of hybridization events on evolutionary patterns. The finding that cpDNA is readily transferred across lineages promotes detection of ancient hybridization (see e.g. RIESEBERG & WENDEL 1993). Adding more cpDNA characters to an analysis may enhance resolution and support in the resulting trees, but cannot, because of the typically uniparental mode of inheritance and lack of recombination of cpDNA, yield the benefits of examining independent character sets (DOYLE 1992).

The circumscription of *Rubiaceae*, the fourth largest angiosperm family, is relatively clearcut, and the family, with the exception of a few aberrant taxa (e.g. *Theligonum*, see WUNDERLICH 1971), has been considered a natural group. Within the family, the classification at subfamilial and tribal levels has presented several difficulties and different classifications have been proposed (e.g. VERDCOURT 1958; BREMEKAMP 1966; ROBBRECHT 1988, 1993). BREMEKAMP recognized three major subfamilies (*Cinchonoideae*, *Rubioideae*, and *Ixoroideae*) and five minor ones. VERDCOURT recognized three subfamilies (*Cinchonoideae*, *Rubioideae*, and the small *Guettardoideae*), and ROBBRECHT recognized four (*Cinchonoideae*, *Rubioideae*, *Ixoroideae*, and *Antirheoideae*). On the basis of a cladistic analysis of *rbc*L sequence data (BREMER & al. 1995), the subfamilies *Cinchonoideae*, *Rubioideae*, and *Ixoroideae* were supported, while *Antirheoideae* (sensu ROBBRECHT 1988) were shown to be highly polyphyletic. Low variation in cpDNA data within the subfam. *Ixoroideae* (restriction enzyme analysis, BREMER & JANSEN 1991; ANDREASEN & BREMER, unpubl. data; *rbc*L sequence data, ANDREASEN & BREMER 1996) dictated that a faster evolving DNA region be analyzed to improve phylogenetic resolution and support.

ROBBRECHT (1988, 1993) included 136 genera, which constitute about 20% of all *Rubiaceae* genera, in *Ixoroideae* (= *Ixoroideae* s. str.) and distributed them in five tribes. In the family-wide *rbc*L study of *Rubiaceae* (BREMER & al. 1995), a lineage corresponding to the subfam. *Ixoroideae* was found, but it encompassed additional taxa not assigned to *Ixoroideae* by ROBBRECHT (= *Ixoroideae* s. l.). Some genera of the polyphyletic subfam. *Antirheoideae* were strongly supported as part of this clade in the analysis. In the investigation reported here we include representatives of *Ixoroideae* sensu ROBBRECHT and of *Antirheoideae*. The latter group includes taxa of the tribes *Alberteae* and *Vanguerieae*. *Vanguerieae* were part of *Ixoroideae* in BREMEKAMP's classification of *Rubiaceae* (1966), and this affiliation is supported by morphological and *rbc*L sequence data (BREMER & al. 1995, ANDREASEN & BREMER 1996). The present study has been conducted to test if variation in the ITS region is suitable for phylogenetic analysis at the subfamilial level in *Rubiaceae* and if so, to use the results from phylogenetic analysis of this DNA region to help to resolve conflicting or unresolved topologies found in the *rbc*L trees of the subfam. *Ixoroideae*.

Materials and methods

Taxon sampling. Representatives from four of the five tribes of *Ixoroideae* (Table 1; the small tribe Aulacocalyceae is not represented) and from two tribes of *Antirheoideae* (sensu

Table 1. Sequenced species and EMBL accession numbers. For new sequences source, voucher, and herbarium (UPS if not otherwise indicated) where the voucher is deposited are given. Earlier published voucher references: [1]BREMER & JANSEN 1991; [2]BREMER & al. 1995; [3]ANDREASEN & BREMER 1996. [4]Erroneous voucher reference in BREMER & al. 1995. [5]In flower. Vegetative voucher in[2]. Ingroup taxa are grouped according to subfamily and tribe/subtribe (ROBBRECHT 1988, 1993)

Species	Source & voucher	EMBL number ITS/*rbc*L
Outgroup		
Mussaenda erythrophylla SCHUMACH. & THONN.[1]		AJ224823/X83652
Cinchona pubescens VAHL[1] (=*C. succirubra* KLOTZSCH)		AJ224838/X83630
Ixoroideae		
Coffeeae		
Coffea arabica L.[3]		AJ224846/X83631
Psilanthus mannii HOOK. f.[3]		AJ224822/Z68852
Gardenieae-Gardeniinae		
Aidia micrantha (K. SCHUM.) F. WHITE[3]		AJ224835/Z68844
Bertiera guianensis AUBL.	Ecuador, BREMER & al. 3363 (UPS, QCA, QCNE)	AJ224841/AJ224845
Calochone redingii (DE WILD.) KEAY[3]		AJ224830/Z68845
Gardenia thunbergia L. F.[1]		AJ224833/X83637
Oxyanthus pyriformis (HOCHST.) SKEELS[3]		AJ224837/Z68836
Posoqueria latifolia (RUDGE) ROEM. & SCHULT.[3]		AJ224828/Z68850
Randia aculeata L.[3]		AJ224836/Z68832
R. moorei BENTH.[3]		AJ224831/Z68849
Gardenieae-Diplosporinae		
Cremaspora triflora (THONN.) K. SCHUM.[3]		AJ224824/Z68856
Tricalysia cryptocalyx BAKER[3]		AJ224827/Z68854
Octotropideae		
Ramosmania rodriguesii TIRVENG.[3]		AJ224834/Z68860
Pavetteae		
Ixora coccinea L.	cult. Uppsala Bot. Garden, BREMER 3104[4]	–/X83646
I. coccinea L.[2]		AJ224826/–
I. parviflora VAHL[1]		AJ224840/AJ224844
Leptactina platyphylla (HIERN) WERNHAM[3]		AJ224825/Z68867
Pavetta lanceolata ECKL. & ZEYH.[3]	cult. Royal Bot.Gard., Melbourne, TONKIN 405[5]	AJ224832/Z68865
Antirheoideae		
Vanguerieae		
Vangueria madagascariensis J. F. GMEL.[1]		–/X83670
V. madagascariensis J. F. GMEL.	Tanzania, BREMER 3077	AJ224839/–
Alberteae		
Alberta magna E. MEY	cult. Royal Bot. Gard., Melbourne, MIDDLETON s.n. 941013, TONKIN 200	AJ224842/AJ224843

ROBBRECHT 1988, 1993) were sampled. Including the outgroups, sequences from 21 species were sampled (Table 1). Two species were sequenced for *Ixora* because of the novel position of the genus in the *rbc*L trees. Inclusion of more than one species reduces the risk of being misled by methodological errors or long-branch attraction. The outgroup taxa chosen were *Cinchona* and *Mussaenda*, both of which were placed in the subfam. *Cinchonoideae* by ROBBRECHT (1988). In the analysis of *rbc*L sequence data (BREMER & al. 1995), however, *Mussaenda* was part of a basal group in *Ixoroideae* s. l.

Amplification and sequencing strategy. The plant material was either field-collected, grown from seeds, or provided by botanical gardens (see Table 1). Total DNAs were extracted using the CTAB procedure (SAGHAI-MAROOF & al. 1984) as modified by DOYLE & DOYLE (1987). The DNAs were further purified with cesium chloride/ethidium bromide gradient centrifugation or QiaQuick's PCR purification kit (QIAGEN) before amplification.

The ITS region of nrDNA was PCR-amplified using two synthetic primers; ITS leu.1 (5′-GTCCACTGAACCTTATCATTTAG-3′, designed by L. URBATSCH), which anneals to the 18S region at 78 basepairs (bp) from the ITS-1 spacer, and ITS 4 (WHITE & al. 1990). Our choice of PCR ingredients followed BALDWIN (1992). Instead of glycerol, DMSO (8%) was used in some PCR reactions. Double-stranded DNAs were cycle sequenced using Perkin-Elmer's FS kit and GeneAmp PCR Systems 2400 or 9600. The amount of DNA used in the 10 µl sequencing reactions varied between 15 and 25 ng. The forward sequencing primers used were ITS 5 (WHITE & al. 1990), or ITS 1-TM (5′-GGATCATTGACGAATCCTGC-3′, designed by T. MCDOWELL) and ITS 3 (WHITE & al. 1990). The primer ITS 1-TM attaches at the 18S-ITS-1 border, 9 bp from the end of 18S. Reverse sequencing primers used were ITS 4 and ITS 2 (WHITE & al. 1990). For most DNAs four primers were used to get as much sequence overlap as possible from the two complementary strands. Centri-Sep spin columns or ethanol precipitation were used to clean the sequenced products before loading the samples on an ABI Prism 377 Automated Sequencer (Perkin-Elmer).

Alignment. The boundaries between the spacers and adjacent nrRNA genes (18S, 5.8S, and 26S) were determined by comparisons with earlier published angiosperm sequences (BALDWIN 1992). Alignment of the resulting sequences was done by eye after sequential pairwise comparisons (using Goroh-Myers comparative alignment option as implemented in the program Sequence Navigator, Perkin-Elmer). In general, the manual part of the alignment and coding of gaps into characters (Table 2) were undertaken with the following criteria in mind (partly following GOLENBERG & al. 1993 and OXELMAN & al. 1997): 1) Gaps were placed in positions that minimize the number of substitutions in that sequence region. 2) When gaps occurred in more than one of the sequences they were considered to represent the same indel event only if they were of equal length. 3) If multiple alignments with different phylogenetic implications (i.e. support for different groupings) were possible the region was omitted from phylogenetic analysis. 4) Gaps were treated as missing data in the sequences and coded as additional indel characters (gap absent or present in Table 2). If a position which contained a gap was variable, but not phylogenetically informative, in the present nucleotides, the autapomorphic nucleotide was treated as an additional state (state 2 in Table 2) to avoid underscoring the heterogeneity. 5) Indels and substitutions were given equal character weighting and analysed simultaneously.

Data analyses. Pairwise distances between taxa were calculated in PAUP 3.1.1 (SWOFFORD 1993) after removal of non-alignable positions. Cladistic analyses were carried out using heuristic searches with random addition sequences of taxa (1000 replicates), MULPARS option in effect, and Steepest Descent off with TBR branch-swapping. Bootstrap analysis (FELSENSTEIN 1985) with 10000 bootstrap replicates, random addition sequences of taxa (10 replicates), and TBR branch-swapping was also performed. Bremer

support values (BREMER 1988, KÄLLERSJÖ & al. 1992) were obtained using the program Autodecay 2.9.6 (ERIKSSON 1996).

Sequences from the chloroplast gene *rbc*L were analysed similarly for the same set of taxa examined in the ITS study. Different specimens were used for the ITS and *rbc*L sequences of *Ixora coccinea* and *Vangueria madagascariensis*. Most of the *rbc*L sequences used in this study have been published (Table 1). For the three new sequences the same amplification and sequencing strategies as in BREMER & al. (1995) were employed. Incongruence between the ITS and *rbc*L matrices was tested using the incongruence length difference test (10000 replicates), as implemented in a beta-version of the program Arn (FARRIS & al. 1994), and a combined *rbc*L-ITS analysis was performed. Incongruence within data sets (between spacers for ITS and between arbitrary divisions of *rbc*L) was tested. The sequential Bonferroni test (RICE 1989) was used to correct for the number of a posteriori tests performed. Character incongruence within and among data sets also was evaluated by the method proposed by MICKEVICH & FARRIS (1981). Cladistic analyses of the *rbc*L and combined *rbc*L-ITS data sets were performed as for the ITS character matrix.

Results

ITS size variation. Spacer size in the sampled members of *Ixoroideae* varies from 206 to 264 for ITS-1 and from 195 to 226 for ITS-2. This is within the range reported earlier for other angiosperms (ITS-1: 187–298, ITS-2: 187–252; BALDWIN & al. 1995). ITS-1 is longer than ITS-2 for all taxa but *Mussaenda* (ITS-1: 206, ITS-2: 209). Presence of a longer ITS-1 than ITS-2 is consistent with reports from several angiosperm families, e.g. the *Asteraceae* (BALDWIN 1992). The 5.8S subunit is invariable in size for the sequenced taxa: 164 basepairs (163 or 164 bp have been reported for most sampled angiosperms, BALDWIN & al. 1995).

ITS divergence. Divergence between pairs of sequences for sampled taxa of *Ixoroideae* ranges from 2.6 to 17.6% (up to 27.7% for comparisons with *Vangueria*, which has an aberrant ITS-1) for ITS-1 and from 1.6 to 13.2% for ITS-2 (excluding non-alignable positions). For the two spacers taken together the divergence values vary between 2.1 and 19.5%. Divergence values for 5.8S are low, from 0 to 3% between pairs of sequences.

When aligned, the sequences yielded a matrix of 695 positions (the EMBL alignment accession number is DS33446; accession numbers for the sequences are given in Table 1). Because of alignment ambiguities, 93 ITS-1 positions had to be excluded from the analyses. In addition, 45 positions were excluded from ITS-2. Of the remaining 557 positions, 202 (36.3%) are variable and of these 102 (50.5%) are potentially informative for parsimony analysis. ITS-1 contributes 50 of the informative characters and ITS-2 contributes 47. The 5.8S region contains five potentially informative characters only. When gaps containing phylogenetic information were recoded, 13 additional characters resulted (Table 2).

Sequence variants. For one of the taxa, *Leptactina*, a partial sequence, quite divergent from the others obtained from the same genomic sample, was produced from one of three amplification reactions. If included in the phylogenetic analysis (not shown), the partial sequence of *Leptactina* comes out as a basally divergent lineage in the tree. No other evidence of obvious sequence variants was found, except that some of the taxa are polymorphic for a minor proportion of sites.

Table 2. Gap characters for the aligned ITS sequences from *Ixoroideae*. Character numbers refer to positions in the aligned sequences. 0 gap, 1 no gap, 2 additional state present, – inapplicable. *outgroup taxa, *Cinchonoideae*

	0 3 1	0 3 5	1 5 7	1 5 8	1 7 1	2 0 2	2 5 5	2 5 6	6 0 3	6 0 6	6 2 9	6 3 9	6 6 3
*Cinchona**	1	0	1	1	1	–	0	2	0	0	–	0	1
*Mussaenda**	1	0	–	0	0	0	–	–	0	0	1	0	0
Posoqueria	1	0	–	0	0	1	0	1	0	0	1	0	0
Alberta	1	0	0	1	0	0	0	1	1	0	1	0	0
Vangueria	1	1	2	1	1	0	1	1	1	0	1	0	0
Ixora coccinea	1	0	0	1	0	1	0	1	1	1	1	0	0
Ixora parviflora	1	0	0	1	0	1	0	1	1	1	1	0	0
Oxyanthus	0	0	0	1	0	0	2	1	1	0	1	0	0
Gardenia	1	0	1	1	0	0	0	1	1	0	–	–	–
Randia moorei	0	1	0	1	0	0	0	1	1	0	1	0	0
Aidia	0	1	0	1	0	0	0	1	1	0	1	0	0
Ramosmania	1	0	0	1	0	0	0	1	1	0	1	0	0
Bertiera	1	0	0	1	0	0	0	0	1	0	1	0	–
Tricalysia	1	0	0	1	1	0	0	1	1	0	1	0	0
Psilanthus	1	0	0	1	0	0	0	1	1	0	0	1	–
Coffea	1	0	0	1	0	0	0	1	1	0	0	1	0
Cremaspora	1	0	0	1	0	0	0	1	1	0	2	–	0
Leptactina	1	0	0	1	0	0	1	1	1	0	1	0	0
Pavetta	1	0	0	1	0	0	0	1	1	0	1	0	0
Randia aculeata	1	0	0	1	0	0	0	1	1	0	1	0	0
Calochone	1	0	1	1	0	0	0	0	1	0	1	0	1

Analyses. Cladistic analysis of the 21 ITS sequences resulted in nine most parsimonious trees, 386 steps long (strict consensus in Fig. 1). The consistency index (CI) of the ITS trees is 0.48, and the retention index (RI) is 0.44. The minimum number of steps in the ITS sequences is 185. The tree topology is the same whether the gap characters (Table 2) are included or not. The *rbc*L analysis yielded 160 most parsimonious trees, each 196 steps long with a consistency index of 0.50, and retention index of 0.53. The minimum number of steps in the *rbc*L sequences is 97. The strict consensus tree is presented in Fig. 2. Combining the two molecular data sets gave one tree, 590 steps long (CI 0.48, RI 0.46, minimum number of steps 282). The tree topology (Fig. 3) is not identical to any of the trees found in the two separate analyses. Character incongruence within and among data sets differs [evaluated by the method proposed by MICKEVICH & FARRIS (1981)]. The major part of the total character incongruence is found within the ITS data set (201/308 = 65%). The *rbc*L data set contributes 32% (99/308) of the character incongruence, and the incongruence between data sets is only 3% (8/308). The incongruence length difference test (FARRIS & al. 1994) did not detect significant incongruence between the ITS-1 and ITS-2 submatrices, or between the arbitrary divisions of the *rbc*L matrix. The ITS and *rbc*L matrices were shown to be

Table 3. The incongruence length difference test results for the combined ITS and *rbc*L matrix with all taxa included and with individual taxa excluded. At least 1000 replicates were performed. Null hypothesis: the data sets are congruent. Asterisk indicate rejection of the null hypothesis at the 95% confidence level. The sequential Bonferroni test is used to adjust for a posteriori significance testing

Dataset	P		P
All taxa included	4.3%*		
All taxa excluding:			
Ramosmania	1.5%	*Coffea*	9.3%
Vangueria	1.9%	*Posoqueria*	11%
Cremaspora	2.4%	*Pavetta*	12%
Randia aculeta	3.2%	*Randia moorei*	12%
Psilanthus	4.4%	*Oxyanthus*	15%
Bertiera	4.9%	*Gardenia*	18%
Tricalysia	5.3%	*Calochone*	18%
Ixora parviflora	5.6%	*Aidia*	24%
Leptactina	5.8%	*Alberta*	37%
Ixora coccinea	6.9%	*Mussaenda*	46%
Cinchona	7.9%		

significantly incongruent with each other ($P = 0.04^*$, i.e. rejection of the null hypothesis of congruence at the 95% confidence level, see Table 3). No single taxon could be identified as causing the incongruence.

Topologies. Groupings that have support in both submatrices and that are found in all trees (Figs. 1, 2, 3) are 1) the clade consisting of the tribe *Coffeeae* s. str. (i.e. *Coffea* and *Psilanthus*, ROBBRECHT 1988) and *Tricalysia* (*Gardenieae-Diplosporinae*), 2) *Randia aculeata* and *Calochone* (both *Gardenieae-Gardeniinae*), and 3) the two *Ixora* (*Pavetteae*) species. In contrast, some groupings are in conflict between the *rbc*L trees (Fig. 2) and the ITS trees (Fig. 1). *Aidia* groups with *Randia moorei* in the ITS trees, but this grouping is not found among the 160 trees from the *rbc*L analysis where the genus *Oxyanthus* is sister-taxon to *Aidia* (all three are placed in *Gardenieae-Gardeniinae*; ROBBRECHT 1988). In some of the *rbc*L trees, however, *Randia moorei* is sister to *Oxyanthus* and *Aidia*. In the ITS trees the two *Ixora* species are a more basally divergent lineage than *Alberta* (*Alberteae*) and *Vangueria* (*Vanguerieae*). This pattern is not found among the most parsimonious trees of the *rbc*L analysis, where *Vangueria* diverges basal to both *Ixora* and *Alberta* or is sister-taxon to *Ixora* (alone or together with *Alberta*).

In the combined tree (Fig. 3) the sister clade to the *Coffea-Ramosmania* clade is a heterogeneous assemblage of taxa consisting of two representatives of the tribe *Pavetteae*, their sister group *Randia aculeata* and *Calochone*, and a basally divergent lineage, *Cremaspora*. The sister to all of the above taxa is a clade consisting of three taxa: *Aidia*, *Randia moorei*, and *Gardenia*. *Oxyanthus* diverges one node down in the tree, followed by the two *Ixora* species and then the *Vangueria-Alberta* clade.

The groupings occurring in all trees, mentioned above, are the most strongly supported in the combined ITS-*rbc*L tree (Fig. 3): 1) The group including the two

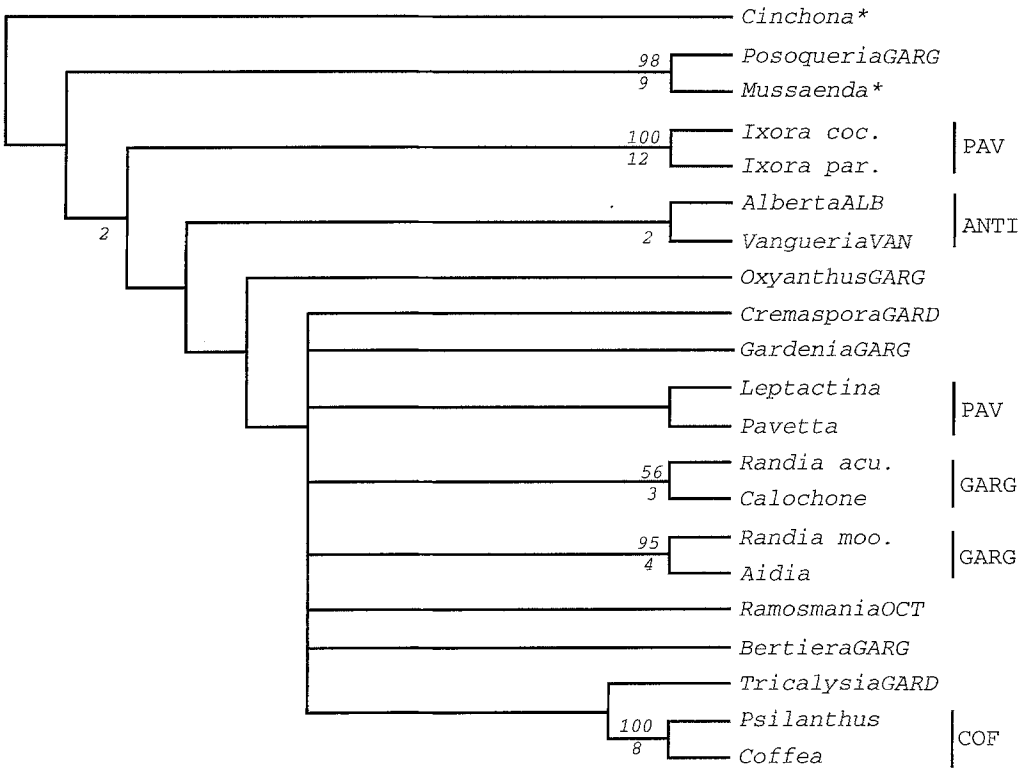

Fig. 1. Strict consensus of nine most parsimonious trees resulting from the analysis of ITS sequences in *Ixoroideae*. Support for nodes is indicated (bootstrap values above branches, Bremer support below). Bremer support of one step and bootstrap below 50% are not indicated. Taxonomic positions (following ROBBRECHT 1988) are abbreviated: *ALB Alberteae*; *ANTI Antirheoideae*; *COF Coffeeae*; *GARD Gardenieae-Diplosporiinae*; *GARG Gardenieae-Gardeniinae*; *OCT Octotropideae*; *PAV Pavetteae*; *VAN Vanguerieae*; * outgroup taxa, *Cinchonoideae*

Ixora species has a bootstrap value of 100% and Bremer support of 25. 2) The *Coffea* and *Psilanthus* clade also is supported in all bootstrap iterations (i.e. 100%) and is upheld by a Bremer support value of 12. 3) The *Randia* and *Aidia* clade has a bootstrap value of 91% and a Bremer support value of 6 steps. 4) *Posoqueria* and the outgroup taxon *Mussaenda* constitute a clade with bootstrap support of 92% and a Bremer support value of 6 steps. 5) The grouping of two Gardenieae-Gardeniinae taxa, *Randia aculeata* and *Calochone*, also has relatively strong support (bootstrap 87%, Bremer support 6).

Discussion

Variation in the ITS region of *Rubiaceae*. This study was initiated to explore the utility of the ITS region for investigating relationships at the intrafamiliar level in *Rubiaceae*. Several studies have shown that levels of variation in ITS sequences differ between angiosperm groups depending on the lineage (BALDWIN & al. 1995). Studies within some families have shown suitable levels of variation for

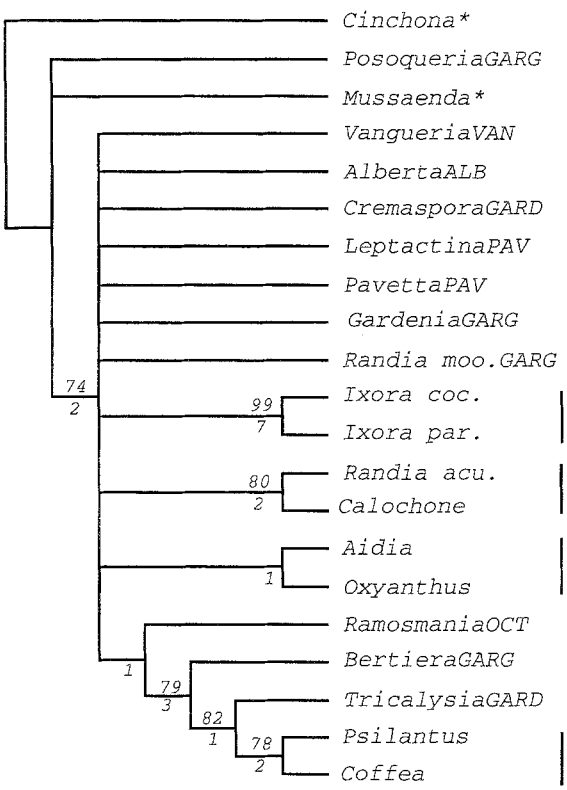

Fig. 2. Strict consensus tree of the 160 equally most parsimonious trees from the analysis with *rbc*L sequences using the same taxa as in the ITS analysis. Support for nodes is indicated (bootstrap values above branches, Bremer support below). Bremer support of one and bootstrap below 50% are not indicated. For abbreviations of taxonomic positions following ROBBRECHT (1988) see Fig. 1

phylogenetic analyses at the family level, e.g. in *Winteraceae* (SUH & al. 1993) and *Fouquieriaceae* (SCHULTHEIS & BALDWIN 1994). In other families, however, e.g. *Asteraceae* (BALDWIN 1992) and *Saxifragaceae* (SOLTIS & KUZOFF 1995), ITS sequences of distantly related taxa are too divergent to allow family-wide comparisons. One cannot expect, however, variation within families to be the same as age, diversity, and rate of evolutionary change may differ, the latter even between sister clades. In an ITS based phylogenetic study of the *Rubiaceae* genus *Exostema* (*Cinchonoideae*; MCDOWELL & BREMER 1998) sequence alignment was unambiguous. For our data set parts of the spacers had to be omitted because of alignment ambiguities and strong support for clades in the ITS tree (Fig. 1) is found mainly for groups comprising the most closely related taxa and not for larger clades. These observations suggest that ITS studies in *Ixoroideae* might be more useful for phylogenetic analysis at low taxonomic levels where the alignability of the region is less problematic and where homoplasy is less extensive.

The ITS region together with the rest of the nuclear ribosomal multigene family undergoes rapid concerted evolution (e.g. HILLIS & DIXON 1991, WENDEL & al. 1995). Unequal crossing-over and gene conversion are thought to be responsible for this phenomenon of homogenization across rDNA repeat units. Extensive sampling within and among different populations of two species of *Lomatium* (*Apiaceae*, SOLTIS & KUZOFF 1993) did not disclose any intraspecific variation in ITS-1. Nor were any major sequence variants reported in most of the angiosperm studies reviewed in BALDWIN & al. (1995). Divergent ITS paralogues have, however, been found in some species of angiosperms, e.g. in *Nicotiana*, *Tripsacum*,

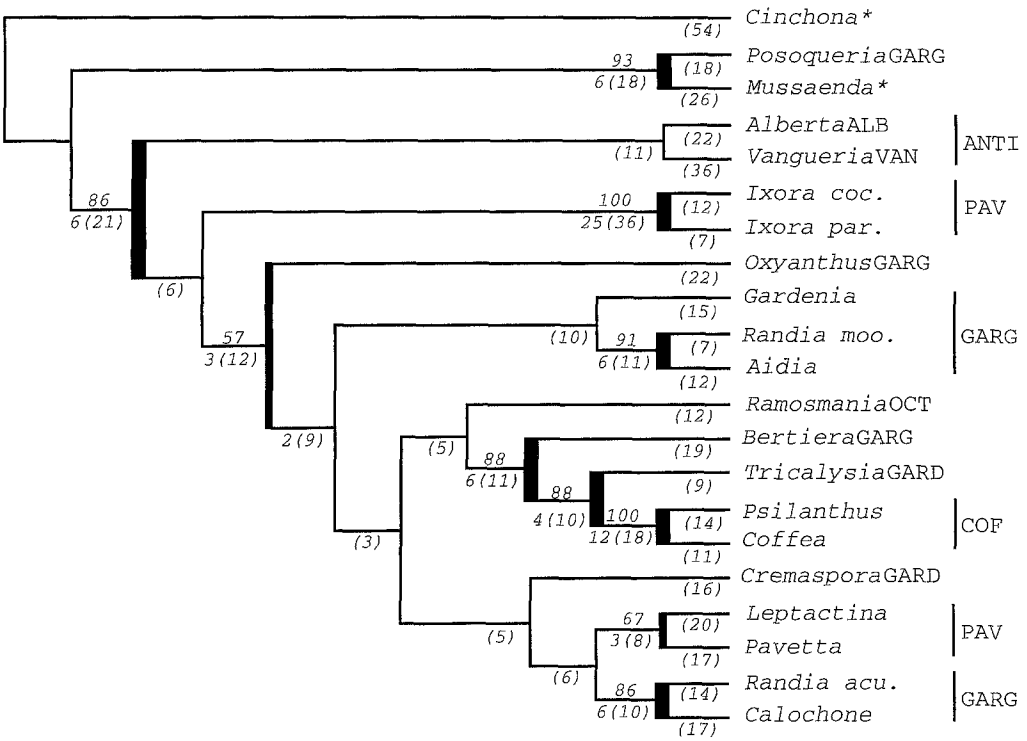

Fig. 3. The single most parsimonious tree resulting from the combined analysis of the ITS and *rbc*L sequences of *Ixoroideae*. Support for nodes is indicated (bootstrap values above branches, Bremer support below, branch lengths in parentheses). Thick branches indicate bootstrap values of $\geqslant 67\%$ and Bremer support of $\geqslant 5$; medium thick branches reflect bootstrap values over 50% and Bremer support of 3, and thin-lined branches indicate bootstrap values below 50% and Bremer support $\leqslant 2$. Bremer support of one and bootstrap below 50% are not indicated. For abbreviations of taxonomic positions following ROBBRECHT (1988) see Fig. 1

Winteraceae, and *Zea* (SUH & al. 1993, BUCKLER & al. 1997). In addition, ITS length variation within and among individuals of populations was found by restriction site mapping in *Lisianthus skinneri* (SYTSMA & SCHAAL 1990). In this study the divergent partial sequence found for the genus *Leptactina* could be another example of a divergent paralogous ITS type. However, despite repeated sequencing efforts from products of different PCR reactions, this sequence could not be rediscovered, and the possibility of contamination cannot be ruled out (checking for possible contaminant DNA among the DNAs used in the lab, however, did not reveal similar sequences). Comparisons of the conserved 5.8S region showed that it was not of fungal origin.

Combining data. The issue of combining or not combining separate data sets (e.g. from different, unlinked genes, or from morphological and molecular sources) has been the subject of much debate (reviewed in DE QUEIROZ & al. 1995, HUELSENBECK & al. 1996, NIXON & CARPENTER 1996). Different approaches have been proposed. One is the combined or simultaneous analysis of data from different sources, which is undertaken to maximize character congruence and

information content of the phylogenetic analysis by using all available evidence. "Secondary signals" which may be present in the separate data sets (but not appearing in the topologies of the separate analyses) may interact and eventually emerge in the topologies of the combined analysis (BARRETT & al. 1991). In many studies where both separate and combined analysis are performed, the combined analysis show unique topologies compared to the separately analyzed data sets (CHIPPINDALE & WIENS 1994). This is also the case in the present study (compare Figs. 1, 2, and 3).

The conditional data combination approach (HUELSENBECK & al. 1996), on the other hand, uses a test of "homogeneity" to decide if the data sets should be combined or not. If the test result is non-significant the data should be combined. The incongruence length difference test (FARRIS & al. 1994) is one of several possible tests. Advocates of simultaneous analysis (NIXON & CARPENTER 1996) propose using this test, too, as a way to evaluate if the amount of incongruence between matrices is large or small. In contrast to the conditional data combination approach they would combine the data sets "even if the amount of incongruence is deemed 'significant'". However, if the amount of incongruence is large and, e.g. reticulation is suspected, the method of DOYLE (1992) is proposed to allow simultaneous analysis (NIXON & CARPENTER 1996).

Incongruence between data sets. When data sets are significantly incongruent, the question arises: what is the cause of incongruence and is it connected to one or a few of the taxa? Taxon correlation of incongruence can be evaluated by excluding each taxon from the matrix and rerunning the incongruence length difference test with the reduced matrix. It is not adequate just to inspect the trees from the separate analyses and exclude the taxon/taxa with different positions in the trees. Factors that can cause incongruence between data sets include chloroplast capture resulting from hybridization, inadequate density of taxon sampling, different evolutionary rates, and phylogenetic sorting which each have the potential to affect estimation of phylogeny (e.g. FELSENSTEIN 1978; SAITOU & NEI 1986; DOYLE 1987, 1992; CHASE & al. 1993; RIESEBERG & WENDEL 1993). Possible sources of error applying to multigene families like rDNA include sampling paralogues in different taxa and functional dependencies among nucleotide sites. Potential for the first type of error has been demonstrated in some groups of plants (BUCKLER & al. 1997) but the latter possibility is not yet well understood. Minimum free-energy reconstructions of ITS secondary structures are highly folded with multiple stems and loops. In order to maintain the function of a secondary structure, a mutation in a stem region may force a compensatory, and hence non-independent, mutation to a complementary nucleotide. Non-independent mutations should be weighed down in phylogenetic analyses, but so far no convincing evidence for compensatory mutations in the ITS region has been reported, at least not in ITS-2 (BALDWIN & al. 1995, HERSHKOVITZ & ZIMMER 1996). More sophisticated algorithms for reconstructing secondary structures are needed. Subminimal free-energy has experimentally been found for 5.8S rDNA in *Chlamydomonas* and yeast, indicating that basing reconstruction solely on minimum free-energy is oversimplified (THOMPSON & HERRIN 1994).

In the least problematic case, one taxon or a few taxa are found to be causing the incongruence, and the data set can be reexamined by excluding the problematic

taxon/taxa. In this study, however, most of the taxa seemed to be involved in the incongruence, as indicated by the incongruence length difference test (Table 3). The five taxa that caused a major part of the incongruence (a "major part" is here arbitrarily considered to be when $P \geqslant 18\%$ after exclusion of the taxon in question) are *Gardenia*, *Calochone*, *Mussaenda*, *Alberta*, and *Aidia*. All investigated exclusions of at least two of these taxa in different combinations changed P from 15% when all five taxa except *Alberta* was excluded to 46% when *Mussaenda* was excluded. Hence, no single taxon seems to be totally responsible for all incongruence, and the taxa interact giving different results depending on the taxon combination in the analysis. To explore the data set further the five most problematic taxa were excluded in different combinations and the cladistic analysis rerun. The trees (not shown) from these analyses are either identical to the combined tree (Fig. 3) or have a less resolved consensus tree. In the latter, some clades with bootstrap support below 50% in Fig. 3 are collapsed. In the tree excluding all five taxa, *Oxyanthus* and *Randia moorei* are sister taxa higher up in the tree and *Ramosmania* has shifted from a basal position in the clade with *Coffeeae*, *Tricalysia*, and *Bertiera* to being sister to *Cremaspora*. In trees excluding *Aidia* and *Mussaenda*, the *Ixora*-and *Antirheoideae* clades have shifted as have the *Cremaspora-Calochone* clade and *Gardenia* and *Randia moorei*. The exclusion of the five taxa then, at the most, changes the topology of clades resolved with low support in the combined analysis of all taxa.

The character incongruence between data sets constitutes only a minor part of the total incongruence (3%). The significant result of the incongruence length difference test was therefore not anticipated. If there is real heterogeneity between the data sets one reason that could be postulated is hybridization, probably involving more than one taxon based on the lack of one easily identifiable taxon as cause of the incongruence. Hybridization and chloroplast capture have been invoked as an explanation for incongruence in some phylogenetic analyses using cpDNA and nDNA regions, e.g. in the *Boykinia* and *Heuchera* groups in the family *Saxifragaceae* (SOLTIS & KUZOFF 1995, SOLTIS & al. 1996) and in *Paeonia* (SANG & al. 1995, 1997). In contrast to the *Saxifragaceae* and *Paeoniaceae*, which are well known for interspecific and even intergeneric hybridization, there are only a few reports of taxa of hybrid origin in the *Rubiaceae* (mostly from herbaceaous groups like *Galium*, see e.g. FAGERLIND 1937, but possible hybridization in *Cinchona* is reported by CAMP 1949). This scenario thus appears less likely, although ancient hybridization (or lineage sorting) cannot be ruled out.

The ITS data set contributes a higher level of homoplasy (66% of the total character incongruence) to the combined analysis than the *rbc*L data; this is evident from the Mickevich/Farris measure (MICKEVICH & FARRIS 1981). Higher levels of homoplasy in ITS compared to cpDNA data has also been found elsewhere (KIM & JANSEN 1994). A possible explanation to the high levels of homoplasy in this study is the extensive ITS-variation causing many changes on terminal branches and few characters supporting major groupings (evident from support values in Fig. 1).

Corroboration of unexpected results. Several of the clades resolved in the phylogenetic analysis of *rbc*L sequences plus morphology (ANDREASEN & BREMER 1996) are in disagreement with earlier proposed classifications, but are corroborated by the ITS data.

The genus *Posoqueria* was placed in *Gardenieae-Gardeniinae*, *Ixoroideae*, by ROBBRECHT (1988), but based on both ITS and *rbc*L data *Posoqueria* is not part of *Gardenieae* or the subfam. *Ixoroideae* s. str. In the *rbc*L-morphological analysis only one outgroup was used (*Mussaenda*), so ANDREASEN & BREMER (1996) could not show whether *Posoqueria* is basal in *Ixoroideae* s. str., or should be excluded from that group. The combined ITS-*rbc*L analysis suggests a position for *Posoqueria* close to *Mussaenda*, basally divergent in *Ixoroideae* s. l. (BREMER & al. 1995), and not part of *Gardenieae*.

Non-monophyly of the tribe *Pavetteae* also is unexpected, although it was suggested in the results of the *rbc*L-morphological analysis (ANDREASEN & BREMER 1996). In the *rbc*L-morphological tree, the genus *Ixora* was basally divergent in the *Ixoroideae* s. str., while the remaining representatives of the tribe were more deeply nested in the tree. This result is corroborated in our analysis of ITS and *rbc*L data, but we find a tree topology less consistent with paraphyly of the tribe than that found by the *rbc*L-morphological analysis. However, only *Ixora*, *Leptactina* and *Pavetta* are included from *Pavetteae* and intertribal relationships are weakly supported (Fig. 3).

The taxa from the polyphyletic *Antirheoideae* (BREMER & al. 1995), *Vangueria* and *Alberta*, both belong in *Ixoroideae* s. l. according to our results. This connection has been suggested before for *Vangueria* (e.g. BREMER & JANSEN 1991, BREMER & al. 1995), but for *Alberta* this is the first time, based on molecular data, that a position close to *Ixoroideae* s. str. is shown. A position of *Alberteae* close to *Vanguerieae* also is supported by morphological characters. Both tribes have one pendulous ovule in each carpel, seeds with a superior embryo radicle, and well developed endocarps with apical splits, and both tribes are tetraploid (with $n = 22$, as in all other investigated *Ixoroideae*; SCHUMANN 1891, PUFF & al. 1984, KIEHN 1995).

The position of the genus *Bertiera* has been disputed, and has been considered uncertain or has varied between different classifications and analyses (see ROBBRECHT & al. 1993). *Bertiera* was placed in *Ixoroideae* by ROBBRECHT & al. (1993), in *Gardenieae-Gardeniinae*, a conclusion which was also tentatively drawn by PERSSON (1996). In the combined ITS-*rbc*L tree (Fig. 3) *Bertiera* is positioned within an extended *Coffeeae*, but basal to *Coffeeae* s. str. (= *Coffea* and *Psilanthus*), and the genus *Tricalysia*. *Tricalysia* has been placed in *Gardenieae-Diplosporinae* (ROBBRECHT 1988), but, as shown by *rbc*L-morphological data (ANDREASEN & BREMER 1996), and strongly corroborated here, should be excluded from *Gardenieae* and included in an extended *Coffeeae*.

The sister group to *Coffeeae* s. l., in Fig. 3, is *Ramosmania*, representing the well-delimited tribe *Octotropideae* (ANDREASEN & BREMER 1996). Like the results on other intertribal relationships, the support for this grouping is not strong (Bremer support of 1 step). Low support for intertribal groupings also was obtained in the *rbc*L sequence and morphological analysis of *Ixoroideae* (ANDREASEN & BREMER 1996). Therefore the larger clades should be interpreted with caution, especially in the trees presented here, where such a small number of *Ixoroideae* genera are represented.

In the trees obtained from ITS and *rbc*L sequence data, *Gardenieae-Gardeniinae* is not monophyletic, a result contradicting the earlier findings based

on *rbc*L plus morphological data (ANDREASEN & BREMER 1996). In the earlier study the representatives of the subtribe grouped together after successive weighting of characters, but support for the clade was not strong. To evaluate the cost of monophyly for *Gardenieae-Gardeniinae* the group was constrained as monophyletic in the ITS-*rbc*L analysis (excluding *Posoqueria* and *Bertiera*; as discussed above). Monophyly of *Gardenieae-Gardeniinae* required an additional seven steps, a result which must be considered to be rather robust if compared to the support for other groupings in the analysis. The restricted taxon sampling in this study, however, might cause problems in terms of long-branch attraction.

In conclusion, the reasons for the incongruities between the ITS and *rbc*L data sets remain to be fully understood. Our knowledge about the amount and type of heterogeneity among real data sets and the behaviour of various congruence tests is imperfect at present. Identifying heterogeneity is thus important to increase our knowledge and to stimulate further research on these issues. One approach to learn more about the evolution in *Ixoroideae* is to add more taxa, or even more pertinent, more characters from non-correlated data sets. As for ITS, sequence and length variation is large in the subfam. *Ixoroideae*; using this set of markers may be better suited for examining relationships within tribes of *Ixoroideae* from the standpoint of sequence alignability and homoplasy. The support for intertribal relationships is weak and some unclear positions of taxa in the *rbc*L trees, e.g. the position of *Ramosmania*, are still not settled. However, several unexpected topologies based on *rbc*L data are supported by the ITS data even with this limited taxon sampling: 1) the inclusion of *Vangueria* and *Alberta* in *Ixoroideae* s. l., 2) the exclusion of *Posoqueria* from *Ixoroideae* s. str., 3) the positions of *Bertiera* and *Tricalysia* near *Coffeeae* s. str., and 4) a basally divergent position of *Ixora* in the subfamily.

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