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COMPARATIVE RESTRICTION SITE MAPPING OF CHLOROPLAST DNA IMPLIES NEW PHYLOGENETIC RELATIONSHIPS WITHIN RUBIACEAE[1]

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Phylogenetic analyses of 33 species of Rubiaceae were performed using chloroplast DNA restriction site mutations. Complete cleavage maps of eight enzymes were constructed for *Psychotria bacteriophila* and used as a reference in comparisons among other species. The species examined represent 33 genera from 18 tribes and four subfamilies of the Rubiaceae. A total of 268 restriction site mutations was detected, 161 of which were phylogenetically informative. Wagner and Dollo parsimony trees were compared to the classifications of Verdcourt, Bremekamp, and Robbrecht. The Wagner analysis resulted in six equally parsimonious trees with 348 steps and 54% homoplasy. Dollo analysis resulted in a single most parsimonious tree. Most clades were identical in the two analyses. The subfamily Cinchonoideae is paraphyletic. The subfamilies Antirheoideae, Ixoroideae, and Rubioideae are monophyletic, although their circumscriptions differ from previous classifications. Several new phylogenetic relationships are indicated: the tribe Chiococceae (Ixoroideae) groups with *Exostema* and *Coutarea* (Cinchonoideae); the subfamily Ixoroideae including tribe Vanguerieae is closely related to *Pogonopus, Pinckneya, Calycophyllum,* and *Mussaenda* (Cinchonoideae); and tribe Hamelieae forms a monophyletic group outside the subfamily Rubioideae.

The Rubiaceae are one of the largest of all tropical angiosperm families, with 630 genera and 10,400 species (Mabberley, 1987). Compared to many other large families, much revisionary work is yet to be completed in the family. Many species-rich genera (e.g., *Psychotria,* the *Hedyotis-Oldenlandia* complex) are taxonomically very difficult. The most controversial problem in the Rubiaceae, however, is the subfamilial and tribal classification (cf. Table 2).

In older classifications, from De Candolle (1830) to Schumann (1891), two almost equally large subfamilies, Cinchonoideae and Coffeoideae, were recognized. This subfamilial division was based on a single character, the number of seeds in each carpel, with several in the Cinchonoideae and one in the Coffeoideae. More recently, Bremekamp (1952, 1954, 1966) and Verdcourt (1958) used more characters in their systems, but they relied primarily on floral biology, seed coat structures, the presence of raphides, and hair types.

Verdcourt (1958) recognized two large and one small subfamilies (Table 2). Bremekamp (1966) distinguished eight subfamilies, three of which were large with seven or 19 tribes each (Table 2). Bremekamp's other subfamilies were comparatively small with only one or three tribes each. Several subsequent studies (Hallé, 1961; Steyermark and Kirkbride, 1975; Kirkbride, 1979; Ridsdale, 1982; Tirvengadum, 1984; Robbrecht and Puff, 1986; Bremer, 1987; Robbrecht, 1988) have shown that many or most tribes are not well defined and many genera are of uncertain tribal placement (Darwin, 1977). Robbrecht (1988) presented an extensive survey of tropical woody Rubiaceae in which he pointed to gaps in our systematic knowledge of the family. He also proposed a modified infrafamilial classification with four subfamilies and 44 tribes (Table 2).

In addition to the lack of agreement on subfamilial and tribal circumscription, there have been few studies of relationships at higher

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taxonomic levels. One notable exception is the serological study of Lee and Fairbrothers (1978). Their results indicate affinities between taxa. However, affinity based on overall similarity cannot be considered as phylogenetic relationship. The two primary reasons for the lack of agreement on tribal and subfamilial limits and relationships in the Rubiaceae are repeated parallel development of the morphological characters and the absence of a cladistically based phylogeny for the family.

During the last several years there has been a surge of molecular data for phylogenetic reconstruction. In plant systematics, restriction site variation and structural changes of chloroplast DNA (cpDNA) have proved to be very useful (reviewed in Palmer, 1987; Palmer et al., 1988). The vast majority of the studies have focused on interspecific or, less frequently, intergeneric relationships in such genera as *Brassica* (Erickson, Straus, and Beversdorf, 1983), *Clarkia* (Sytsma and Gottlieb, 1986a, b), *Coffea* (Berthou, Matthieu, and Vedel, 1983), *Cucumis* (Perl-Treves and Galun, 1985), *Helianthus* (Rieseberg, Soltis, and Palmer, 1988), *Linum* (Coates and Cullis, 1987), *Lisianthius* (Sytsma and Schaal, 1985), *Lycopersicon* (Palmer and Zamir, 1982), *Nicotiana* (Kung, Zhu, and Chen, 1982), *Pisum* (Palmer, Jorgensen, and Thomson, 1985), *Solanum* (Hosaka et al., 1984; Hosaka, 1986), *Triticum* (Bowman, Bonnard, and Dyer, 1983; Tsunewaki and Ogihara, 1983), and *Viguiera* (Schilling and Jansen, 1989). The systematic utility of cpDNA at higher taxonomic levels has been demonstrated only recently for Asteraceae (Jansen and Palmer, 1987a, b, 1988; Jansen, Palmer, and Michaels, 1988; Palmer et al., 1988; Jansen et al., in press; Jansen, Michaels, and Palmer, in press).

This study includes the examination of cpDNA variation among 33 species and genera of Rubiaceae, representing 18 tribes. In addition to the long-term goal of clarifying the interrelationships within Rubiaceae, our study was initiated to answer the question: Is restriction site variation of cpDNA a useful and powerful source of information for phylogenetic reconstruction of Rubiaceae?

MATERIALS AND METHODS

Fresh leaves of 33 species of Rubiaceae were provided by the botanical gardens listed in Table 1. Total DNA was extracted from fresh tissue by the method of Saghai-Maroof et al. (1984) as modified by Doyle and Doyle (1987). The DNA was further purified via ultracentrifugation in a CsCl/ethidium bromide gradient (Maniatis, Fritsch, and Sambrook, 1982). DNAs were digested with eight restriction endonucleases (Table 3) following the manufacturers' specifications. DNA fragments were separated by agarose electrophoresis in 1% gels and transferred to Zetabind (AMF CUNO) nylon filters by bidirectional blotting, following Palmer (1982, 1986). Filter hybridizations were performed to construct restriction maps of the 33 species from all eight enzymes. Mapping was performed in two stages. The chloroplast genome of *Psychotria bacteriophila* was mapped using filters generated from gels 19 cm long, and gels containing all 33 species including *Psychotria* were run 12 cm and blotted. The high degree of restriction site conservation among cpDNA in the Rubiaceae allowed us to map sites in all 33 taxa by comparison to complete maps of *Psychotria.*

Sixteen cloned cpDNA restriction fragments of *Lactuca* (Jansen and Palmer, 1987a) and three of *Petunia* (Palmer et al., 1983) covering more than 95% of the chloroplast genome were used to probe for homologous regions in the Rubiaceae cpDNA (Table 3). The cloned probes were labeled with α-32p dATP via nick translation, filters were hybridized at 65 C, and the homologous fragments were visualized by autoradiography. Only fragments larger than 0.4 kilobase (kb) were visualized. Restriction sites were mapped for all 33 taxa and all eight enzymes, using the overlap hybridization method described in Palmer (1986), although most small fragments from Eco RV proved to be too difficult to unambiguously order.

Restriction maps were constructed for all taxa, and the sites of the different taxa were aligned relative to each other. Sites were also aligned to the *Nicotiana* sequence (provided by K. Shinozaki, Nagoya; Shinozaki et al., 1986). Restriction site occurrences or absences were used as characters in subsequent phylogenetic analyses (Tables 4, 5).

The phylogenetic analyses were performed on a 386 microcomputer and on a Macintosh Plus. Wagner analyses (Table 5) were performed both with Hennig86 (Farris, 1988; where the initial trees were calculated by the mhennig method, and the options were mhennig* and bb*) and PAUP 3.0 (Swofford, 1989; and the options global branch swapping and mulpars). Dollo parsimony (Table 5) was performed with PAUP test version 3.0 (Swofford, personal communication; and the options global branch swapping and mulpars).

RESULTS

Chloroplast DNA structure—All 33 Rubiaceae species have the genome arrangement

TABLE 1. *Sources of living material of Rubiaceae extracted for cpDNA*

Species[a]	Source[b]	Voucher information[c]
Subfamily Ixoroideae		
tribe Gardenieae		
Gardenia thunbergia	FTG	X.4-217, Gillis 10913 (FTG)
Mitriostigma axillare	SUNIV	s.n. Bremer 2705 (S)
tribe Pavetteae		
Ixora parviflora	FTG	P.1738, Gillis 7892 (FTG)
Enterospermum coriaceum (=*Tarenna*)	MO	800736
tribe Coffeeae		
Coffea arabica	FTG	75-521, Sanders 1803 (FTG)
tribe Chiococceae		
Erithalis fruticosa	FTG	64-412B, Meagher 990 (FTG)
Chiococca alba	SUNIV	s.n., Bremer 2703 (S)
tribe Vanguerieae		
Vangueria madagascariensis	FTG	76-30, Sanders 1798 (FTG)
Subfamily Cinchonoideae		
tribe Cinchoneae		
Calycophyllum candidissimum	FTG	78-607, Sanders 1805 (FTG)
Cinchona succirubra	SUNIV	s.n.
Coutarea latiflora	FTG	70-365A, Sanders 1802 (FTG)
Exostema caribaeum	FTG	70-533, Misitis 2 (FTG)
Luculia grandifolia	SUNIV	s.n., Bremer 2713 (S)
tribe Naucleeae		
Haldina cordifolia	FTG	X.2-286, Gillis 11114 (FTG)
Cephalanthus occidentalis	UC	82.0070, Forbes s.n. (S)
tribe Condamineeae		
Pinckneya pubens	UC	81.0288, Forbes s.n. (S)
Pogonopus speciosus	FTG	X.4-95, Gillis 11168 (S)
tribe Rondeletieae		
Rogiera suffrutescens	CONN	656, Bremer 2712 (S)
tribe Catesbaeeae		
Catesbaea spinosa	FTG	X.3-286, Gillis 9569 (FTG)
tribe Iserteae		
Mussaenda erythrophylla	FTG	67-600, Gillis 10838 (FTG)
Subfamily Rubioideae		
tribe Psychotrieae		
Hydnophytum formicarum	CONN	652, Bremer 2701 (S)
Myrmecodia platyrea	CONN	653
Psychotria bacteriophila	SUNIV	s.n.
tribe Hamelieae		
Hamelia cuprea	FTG	85-233, Misitis 3 (FTG)
Hoffmannia refulgens x *ghiesbreghtii*	FTG	66-840, Misitis 4 (FTG)
tribe Hedyotideae		
Bouvardia glaberrima	UC	78.0400, Forbes s.n. (S)
Pentas lanceolata	CONN	657, Bremer 2702 (S)
tribe Anthospermeae		
Coprosma pumila	UC	71.0572, Forbes s.n. (S)
Nertera granadensis	CONN	1348
tribe Coccocypseleae		
Coccocypselum hirsutum	CONN	908, Bremer 2700 (S)
tribe Rubieae		
Galium odoratum	CONN	1298
Subfamily Antirheoideae		
tribe Guettardeae		
Antirhea lucida	FTG	80-692, Sanders 1801 (FTG)
Guettarda uruguensis	FTG	X.5-127, Gillis 9575 (FTG)

[a] Species ordered according to the classification of Bremekamp (1954, 1966) and Bridson and Verdcourt (1988) (subfamily Ixoroideae was not recognized by Bridson and Verdcourt, and tribes Coffeeae and Catesbaeeae were not recognized by Bremekamp). Circumscription and position of taxa printed in boldface are not in agreement with results from this study.

[b] CONN = University of Connecticut; FTG = Fairchild Tropical Garden; MO = Missouri Botanical Garden; SUNIV = University of Stockholm; UC = University of California Botanical Garden.

[c] The first number is the accession number of the institution supplying the material followed by collectors with their numbers and the herbarium in which vouchers are deposited (abbreviations according to Holmgren and Keuken, 1974).

TABLE 2. *Comparison of different tribal classifications of the Rubiaceae*

	Verdcourt[a]	Bremekamp[b]	Robbrecht[c]
Coptosapelteae	—[d]	IXOR[e]	—
Acrantereae	—	IXOR	—
Alberteae	CINC	—	ANTI
Cremasporeae	—	IXOR	—
Pavetteae	CINC	IXOR	=
Coffeeae	—	—	IXOR
Aulacocalyceae	—	—	IXOR
Gardenieae	CINC	IXOR	=
Hypobathreae	—	—	IXOR
Chiococceae	CINC	IXOR	ANTI
Vanguerieae	CINC	IXOR	ANTI
Catesbaeeae	CINC	—	?
Retiniphylleae	CINC	—	ANTI
Cinchoneae	CINC	=	=
Naucleeae	CINC	=	=
Cephalantheae	—	—	ANTI
Condamineeae	—	CINC	=
Rondeletieae	CINC	=	=
Sipaneeae	—	CINC	=
Iserteae	CINC	=	=
Sabiceeae	—	CINC	—
Urophylleae	RUBI	UROP	CINC
Pauridiantheae	—	UROP	CINC
Ophiorrhizeae	RUBI	?UROP	RUBI
Hedyotideae	RUBI	=	=
Cruckshanksieae	RUBI	=	—
Argostemmateae	RUBI	=	=
Coccocypseleae	RUBI	=	=
Schradereae	RUBI	=	=
Hamelieae	RUBI	=	=
Spermacoceae	RUBI	=	=
Anthospermeae	RUBI	=	=
Theligoneae	—	—	RUBI
Rubieae	RUBI	=	=
Perameae	—	RUBI	?
Psychotrieae	RUBI	=	=
Gaertnerieae	—	RUBI	—
Triainolepideae	—	RUBI	=
Lathraeocarpeae	—	RUBI	=
Coussareeae	RUBI	=	=
Paederieae	RUBI	=	=
Morindeae	RUBI	=	=
Knoxieae	RUBI	=	ANTI
Craterispermeae	RUBI	=	ANTI
Hillieae	—	HILL	CINC
Henriquezieae	—	GLEA	CINC
Pomazoteae	—	POMA	—
Guettardeae	ANTI	=	=
Hippotieae	—	—	?
Tammsieae	—	—	?
Jackieae	—	—	?
Number of tribes	29	40	44

[a] Verdcourt (1958).
[b] Bremekamp (1966).
[c] Robbrecht (1988).
[d] "—" Denotes a tribe not mentioned by the author or included in another tribe; "=" denotes the same subfamily as the previous author; "?" denotes uncertain position according to the author.
[e] The subfamilies are Antirheoideae = ANTI, Cinchonoiodeae = CINC, Gleasonioideae = GLEA, Hillioideae = HILL, Ixoroideae = IXOR, Pomazotoideae = POMA, Rubioideae = RUBI, and Urophylloideae = UROP.

typical for most angiosperms examined (Palmer, 1985), colinear with *Lactuca* cpDNA (mainly used as probes in this study), with the exception of the 22-kb inversion in the large single copy region of the *Lactuca* (Jansen and Palmer, 1987a). The cpDNA of *Psychotria* (Fig. 1) was completely mapped first to use as a reference for the other Rubiaceae taxa.

The total estimated length of the chloroplast genome of *Psychotria* for the eight enzymes ranges from 146.6 kb to 156.8 kb with an average length of 151.6 kb. Only fragments longer than 0.4 kb were identified.

The extent of the inverted repeat (IR) for *Psychotria* is approximately 26 kb, and the approximate boundaries are defined by NcoI (fragments no. 21, Table 3 and Fig. 1) and HindIII (fragment no. 15, Table 3 and Fig. 1). The small single copy region (SSC) is 19–22 kb, and the large single copy region (LSC) is approximately 84 kb.

Restriction fragment length variation—About 20 restriction fragment length variations of 200 base pairs or longer were detected. These probably represent only a small proportion of all length mutations since most of these changes are 1–10 base pairs (cf. Palmer, 1985; Jansen et al., in press). Deletions and insertions have not been used in phylogenetic comparisons because of the difficulty in determining homology of length mutations (Moritz, Dowling, and Brown, 1987; Palmer et al., 1988). Restriction site changes at a particular position have been treated as homologous although the underlying reason for a change could be due to either a base substitution or a length mutation.

Restriction site variation—The approximate number of sites mapped for each taxon was 213 representing 1,278 nucleotides, or 0.78% of the total chloroplast genome. Forty mapped sites are shared by all 33 taxa and 67 are autapomorphies for single taxa.

A total of 161 phylogenetically informative sites (those shared by two taxa or more) was identified. The proportion of sites that are phylogenetically informative is 60%, which is a much higher value than 35% obtained in the Asteraceae (Palmer et al., 1988; Jansen et al., in press; Jansen, Michaels, and Palmer, in press). In the tribe Mutisieae (Asteraceae) the corresponding value is 27% (Jansen and Palmer, 1988). In studies among species in the same genus, or in closely related genera, values from 2.1% to 9.6% are found (Palmer and Zamir, 1982; Bowman, Bonnard, and Dyer, 1983; Palmer et al., 1983; Sytsma and Gottlieb,

TABLE 3. Psychotria bacteriophila *chloroplast DNA fragment identification*

Fragment number[a]	Lactuca probes	Petunia probes	SacI	BstXI	HaeII
1	10.6	15.3	18.5	24	15
2	9.1	9.2	14.5	19.5	15
3	2x 8.3[b]	9	2x 13	10.8	10
4	7.7		11.5	10	9.5
5	6.9		10	8.6	8.6
6	6.3		10	2x 8.5	8.4
7	2x 6.2		9.5	8	2x 7.5
8	5.5		9.5	7.5	7.2
9	5.4		8.5	2x 6	7
10	4.6		6.8	5.3	7
11	3.8		6.3	5	6.6
12	3.8		6.2	2x 3.4	2x 6.1
13	2x 3.7		5.4	3.2	5.3
14	3.6		5.3	3	4.3
15	2x 3.5		3.4	2.3	2.9
16	2x 1.8		1.8	2.2	2.7
17			1.8	2.2	2x 2.3
18			1.1	1.9	2.3
19				1.3	1.6
20				1.2	2x 1.2
21				2x 1.1	2x 1.1
22				1	2x 1
23				1	0.8
24				0.8	2x 0.5
25					
26					
27					
28					
29					
30					
31					
32					
Sum	114.3	33.5	156.1	156.8	153.6

Fragment number	NcoI	BamHI	HindIII	EcoRV	BclI
1	20	14	2x 11.5	42	10.8
2	13	10	9	20	8.6
3	10	8	2x 8.5	7.5	8.3
4	9.4	6.8	8.5	6.8	2x 8
5	9	6.5	8.5	6	2x 6.9
6	9	6.3	8	5.9	6.8
7	8	2x 6	7.5	4.8	6.7
8	7.9	5.8	6	4.6	6
9	7	5	5.6	4.3	5.7
10	2x 5	4.7	5.4	4.2	5.4
11	4.8	4.6	4.6	4	4.7
12	4.4	2x 4.2	4.6	3.8	4.6
13	2x 4	4	4.3	2x 2.7	4.4
14	3.8	4	3.8	2x 2.6	4
15	2x 3.4	3.5	2x 3.2	2.5	3.4
16	2x 2.7	3.5	3.2	2.3	3.3
17	1.8	2x 3.1	3.1	2x 2.2	3.2
18	2x 1.7	2.9	2.6	2	2.9
19	1.7	2.9	2.2	2x 1.6	2.9
20	1.6	2x 2.7	2.1	1.6	2.7
21	2x 1.5	2.1	1.9	1.6	2.2
22	2x 1	2	1.7	1.6	2x 1.8
23	2x 0.8	2x 1.6	2x 1.2	1.3	1.8
24		2x 1.4	1.1	1	2x 1.6
25		2x 1.4	2x 1	1	1.5
26		1.3	2x 0.9	2x 0.8	1.5
27		2x 1.2	0.8	2x 0.7	2x 1.3
28		1.2	2x 0.5	0.7	1.2
29		1.1	0.5	0.5	2x 1.1
30		2x 0.9	0.5	2x 0.4	1
31		2x 0.4			0.9
32		2x 0.4			0.7
Sum	151.61	146.8	149.1	152	146.6

[a] Maps for fragments are given in Fig. 1, fragments are in kilobases.
[b] 2x indicates fragments within the inverted repeat.

TABLE 4. *Chloroplast DNA restriction sites of eight enzymes used as characters in phylogenetic analyses of 33 species of Rubiaceae*

Character no.[a] and enzyme	Relative position[b]	Character no. and enzyme	Relative position
1. Sac I	22	82. Nco I	118
2. Sac I	22	83. Nco I	119
3. Sac I	23	84. Nco I	124
4. Sac I	42	85. Nco I	127
5. Sac I	44	86. Bam HI	7
6. Sac I	48	87. Bam HI	11
7. Sac I	52	88. Bam HI	30
8. Sac I	77	89. Bam HI	39
9. Sac I	78	90. Bam HI	45
10. Sac I	83	91. Bam HI	49
11. Sac I	84	92. Bam HI	56
12. Sac I	85	93. Bam HI	58
13. Sac I	85	94. Bam HI	59–62
14. Sac I	86	95. Bam HI	66
15. Sac I	86–96	96. Bam HI	70
16. Sac I	96.44[c]	97. Bam HI	72
17. Sac I	105.4	98. Bam HI	74
18. Sac I	108.8	99. Bam HI	77
19. Bst XI	36	100. Bam HI	78
20. Bst XI	37	101. Bam HI	80
21. Bst XI	42	102. Bam HI	84
22. Bst XI	43	103. Bam HI	91.3
23. Bst XI	44	104. Bam HI	92
24. Bst XI	50	105. Bam HI	94
25. Bst XI	51	106. Bam HI	96
26. Bst XI	52	107. Bam HI	97
27. Bst XI	54	108. Bam HI	112
28. Bst XI	58–65	109. Hind III	17
29. Bst XI	65	110. Hind III	17
30. Bst XI	67	111. Hind III	17
31. Bst XI	76	112. Hind III	19
32. Bst XI	77	113. Hind III	21
33. Bst XI	78	114. Hind III	41
34. Bst XI	94.4	115. Hind III	45
35. Bst XI	98	116. Hind III	46
36. Bst XI	104	117. Hind III	47
37. Bst XI	110.4	118. Hind III	51
38. Bst XI	112	119. Hind III	52
39. Bst XI	113	120. Hind III	52
40. Bst XI	122	121. Hind III	60
41. Hae II	12	122. Hind III	60
42. Hae II	39	123. Hind III	63
43. Hae II	40	124. Hind III	64
44. Hae II	46	125. Hind III	67
45. Hae II	52	126. Hind III	72
46. Hae II	54	127. Hind III	73
47. Hae II	55	128. Hind III	78
48. Hae II	58	129. Hind III	83
49. Hae II	60	130. Hind III	83
50. Hae II	67	131. Hind III	109.7
51. Hae II	78	132. Hind III	110
52. Hae II	81	133. Hind III	110.8
53. Hae II	85	134. Hind III	114
54. Hae II	87	135. Hind III	114
55. Hae II	88	136. Hind III	125–128
56. Hae II	93	137. Eco RV	10
57. Hae II	93	138. Eco RV	70
58. Hae II	101	139. Eco RV	73
59. Hae II	102	140. Eco RV	76
60. Hae II	107.5	141. Eco RV	78
61. Hae II	113	142. Eco RV	85
62. Hae II	119	143. Eco RV	98
63. Nco I	23	144. Bcl I	16
64. Nco I	29	145. Bcl I	21
65. Nco I	32	146. Bcl I	36
66. Nco I	35	147. Bcl I	38
67. Nco I	40	148. Bcl I	39
68. Nco I	46	149. Bcl I	45–46
69. Nco I	50	150. Bcl I	50
70. Nco I	51	151. Bcl I	53
71. Nco I	52	152. Bcl I	64
72. Nco I	56	153. Bcl I	65
73. Nco I	82	154. Bcl I	72
74. Nco I	86.9	155. Bcl I	85
75. Nco I	?94.4[d]	156. Bcl I	85
76. Nco I	95.9	157. Bcl I	95.0
77. Nco I	98.4	158. Bcl I	106
78. Nco I	99.4	159. Bcl I	108
79. Nco I	103	160. Bcl I	?109.4
80. Nco I	114	161. Bcl I	?109.4
81. Nco I	115		

[a] The character numbers correspond to those in the DNA data matrix (Table 5).

[b] The relative positions of the sites (in kilobases) are estimated and compared to the *Nicotiana* cpDNA-sequence.

[c] Underlined positions are sites in the inverted repeat aligned to *Nicotiana* and are treated as homologous to the corresponding hexamers of *Nicotiana* cpDNA.

[d] Question marks indicate uncertainty in alignment.

1986b). These values are dependent on the number of taxa included in the study as well as the phylogenetic distance between the taxa (Sytsma and Gottlieb, 1986b).

Phylogenetic analyses of restriction sites— Only the 161 phylogenetically informative sites were used as characters (Tables 4, 5). No autapomorphies for terminal taxa, or for the family as a whole, were used in this analysis, as they contain no information for reconstruction of phylogeny using parsimony methods.

Wagner parsimony (Hennig86, Farris, 1988; PAUP, Swofford, 1989) was first run unrooted. To root the trees and polarize the characters a functional outgroup (sensu Watrous and Wheeler, 1981) was chosen from among the ingroup taxa. Two approaches were taken to identify a suitable group. The first approach was to run an extended analysis and use the cpDNA sequence of *Nicotiana* (Shinozaki et al., 1986) as an outgroup. Because *Nicotiana* (Solanaceae) is distantly related we used, in a first analysis, only those mutations from the conserved inverted repeat that we could align (33 characters). This analysis gave no clear indication of a suitable functional outgroup because the consensus tree for the several equally parsimonious solutions was more or less collapsed at the base. In a second analysis all

TABLE 5. *Data matrix of 161 phylogenetically informative restriction sites used as characters in phylogenetic analyses of 33 Rubiaceae species*

	Character[a] no.							
	1	2	3	4	5	6	7	8
	1[b] 0	0	0	0	0	0	0	0
1 Lucu	00111101100100011111111010101000010010001001000000000100100000001110001001011100
2 Cinc	00110101100101010111111010101000010010001001000000010100101000111110001001011101
3 Ceph	00110011100101000110111010100000010010001001000000000100101000111110001001011100
4 Hald	00111011100100000110111010?000000100100010010000000101001010001111100010010111100
5 Rogi	00111101100101010111111010101000010010001001000000010100101000111110001001011100
6 Erit	00111101000100010111101000000000010011001001000000100000101000111110001001011100
7 Chio	00111101000100010100000001000000010011001001000000100000101000111110001001011100
8 Exos	00111101100101010111111001000000010011001001000000110100101000111110001001011100
9 Cout	00110101100001010111111000000000010011001001000001110100101000001100001001011100
10 Pogo	10111100000101011111110010011000110010101001000001010100100000010010010101011001
11 Pinc	10110101000101011111110010011000110010101001000001010100100000011001001010101001
12 Caly	00111101000101011111110010001000110010101001000000010100100000011001001010101001
13 Muss	00110101000101011111100100010000100100110010000000101001000000001001010101101101
14 Guet	00111101100101010111111100100000010010101101001000010100101000?111000010010111100
15 Anti	00111101100101010111111100100000010010101101001000010100101000111100001001011100
16 Vang	10111101000001011111110011000000010010010001000111010100100000011001000010101100
17 Ente	10111101000011011110110010000000010010011000000111010100100000000011100101011100
18 Ixor	10111101000000011111110010000000010010001001000111000100100000110010000101101100
19 Coff	10111101000011011110110010000000010010010000000011010100100000111011100101001100
20 Mitr	10101101000011001110110010000000010010010000000010101010010001011001110010101100
21 Cate	00111101100101010110110?0100000001001001100100000011010010100011100000100101100
22 Gard	10111001000001011110110?0100000001001000000000011101010010001011001110010101100
23 Hame	01011101100101010100011100100010010010101001010000010100101000001100001001011100
24 Hoff	01011101100100010100111100100000010010101001000000010100101000110100001001011100
25 Pent	00000101010100011011000000000101011100100001010000000010010101010000001001010110
26 Bouv	0??10000010100001000000001000000011100???001110000010010000101000100001011010110
27 Gali	00010001001100001010110000100001011100100011111000001001000010100000000101001011O
28 Psyc	00010101001101010011110000110111110110010000110000000101100100100000000101010111O
29 Hydn	00010101001101111011110000?000111101100100001100000001011000001000000001010101110
30 Myrm	00010101001101111011110000010111101100100001100000001011000001000000001010101110
31 Nert	010??101010100011010110000001001011100100011010000010010000101000000001010010110
32 Copr	010??101010100011010110000010000101110010001100000001001000010100000000001010010110
33 Cocc	000??101010001001011110?000001010111001000010100000100100010001000000001011011010

[a] 1 indicates presence of site; 0 indicates absence of site; ? indicates uncertainty in mapping.

[b] Characters 1–18 are restriction sites of SacI, 19–40 of BstXi, 41–62 of HaeII, 63–85 of NcoI, 86–108 of BamHI, 109–136 HindIII, 137–143 of EcoRV, and 144–161 of BclI.

mutations were polarized relative to *Nicotiana,* and *Luculia* came out as a basal taxon.

The second approach used morphological criteria for identifying a suitable outgroup. A phylogenetic analysis of morphological characters for the same set of taxa (Bremer, unpublished data) with *Usteria* (Loganiaceae) as the outgroup identified *Luculia* as a suitable functional outgroup.

Wagner parsimony analysis using 161 characters and *Luculia* as an outgroup resulted in six equally parsimonious trees, 348 steps long with a consistency index of 0.46 (Fig. 2). Forty percent of the characters were not homoplasious (consistency index 1.0), and 72% of these were site gains and 28% were site losses. In 32% of the characters the consistency index was 0.5, and in 28% it was 0.33 or less. The resolution of the strict consensus tree (Fig. 3) into dichotomies is high as shown by the presence of polychotomies in only two instances. Unfortunately, several of the basal branchings (clades 63, 62, 58, and 34) are only weakly supported by few characters. A more inclusive analysis, incorporating all trees one or two steps longer than the shortest, yielded 905 alternative trees. In all, 48% of the nodes from the six most parsimonious trees were retained in all 905 trees. These conserved nodes are indicated by dots (Fig. 3).

There are many strongly supported clades in the trees and many of these support recognized tribes or parts of tribes, while others contradict currently accepted classifications. In two cases the clades are in agreement with larger accepted subfamilies. One of the branches (55) is strongly supported by 31 characters of which 12 are shared site gains. All of these taxa (25–33) are classified into the subfamily Rubioideae (Verdcourt, 1958; Bremekamp, 1966; Robbrecht,

TABLE 5. *Continued*

	Character[a] no.							
	9	10	11	12	13	14	15	16
	0	0	0	0	0	0	0	0
1 Lucu	0010001111111000000001110100???1111000000010010001011011000000000010100010011110							
2 Cinc	0010001110111000010000110100010111110000000100000010110110000000000111010101111100							
3 Ceph	0010001110111000010000100100010111110000000100100010110110000000000111011110011110							
4 Hald	0010001110111000010000100100010111110000000100110010110110000000000111011110011110							
5 Rogi	0010011110111000010000110100010111110000000100100010110110000000000101010101111100							
6 Erit	0010010100011010010010110100010101110000000100100010110110000100000101011100111110							
7 Chio	0010010100011010010010110100010101110000000100100010110110000100000101011100111110							
8 Exos	0010011100111010010010110100010111110000000100100010110000000000000101011100111110							
9 Cout	0010001100111010010010110100010111100110000000100010110110000000000101011100111110							
10 Pogo	0010100111111000100001111100010111110110000000100010110110001000100101010110110110							
11 Pinc	0010100111111000100001111100010111110110000000100010110110001000100101010110110110							
12 Caly	0010100110111000100001111100010111110110000000101010110110001000100101010110110110							
13 Muss	0010100111111000000001111100010111110000100100110010110110001000000101000010111110							
14 Guet	0010001110101000010000110100010111110000100100101000110?1000000000010101100011110							
15 Anti	0010001110111000010000110100010111110000100100101000110?1000000000010101110011?0							
16 Vang	0010001110111000000101110100010111110010000100100010110000001001000100010?011110							
17 Ente	0000001110111000000001111100001111110010001000100010110000001000000101010011110							
18 Ixor	0010001110011000000101111100010111110010001000100010110000001000000100010011011110							
19 Coff	0010001110111000000101111000001111110010000100100010110000001001000101010000111110							
20 Mitr	0010001110111000000101111100001111110010000100100010110000001001000101010011011110							
21 Cate	0010011101111000000100110100010111110010000100100010110100000000000101011000111110							
22 Gard	0010000110111000000101111100001111110010000100100010110100001001000101010011011101							
23 Hame	0100101110011000000??001101000101111100010101001000101101100000000001010111001101 0							
24 Hoff	0100101110101000000??001101000101111100010101001000101101100000000001010110001101 0							
25 Pent	1011000000001100000000111100101011110100000010110011010110011000001100?00000011001							
26 Bouv	1011000000001000000000111000100011110100000010100011010110100000001100?0000?011001							
27 Gali	1001000100011000000101110001000111101000000101000100101101000000010000000000011001							
28 Psyc	0000100100011001001001011110000011100000001000000011101000100001001000000000001010							
29 Hydn	0000000100111001001001011110000011100000001011000111010001000010010000100000001010							
30 Myrm	0000000100111001001001011110000011100000001011000111010001000010010000100000001010							
31 Nert	1011000100?0100000001111001100110001000001001000110101100110000010000000?0011001							
32 Copr	1011000100?0100000001111001100110001000000100100110101100110000010000000?0011001							
33 Cocc	0010000100011000000001110100100111100000000100000?1011010100000001000000?0011010							

1988). Two other genera, *Hamelia* (23) and *Hoffmannia* (24), usually grouped in this subfamily are on a different branch. For all taxa above clade 55, there is complete congruence among the six equally parsimonious Wagner trees (Figs. 2, 3).

Clade 54 is strongly supported by 25 characters, 14 of which are restriction site gains. This group corresponds to the tribe Psychotrieae (Verdcourt, 1958; Bremekamp, 1966; Robbrecht, 1988). Within the group two genera, *Hydnophytum* and *Myrmecodia,* share five apomorphies. The other strongly supported clade in this part of the tree (no. 51, taxa 25–27, 31–32) is supported by 15 characters of which eight are site gains. This group represents the tribes Rubieae (taxon 27), Hedyotideae (25, 26), and Anthospermeae (31, 32). The tribe Anthospermeae represented by two genera, *Nertera* and *Coprosma,* is well supported with eight synapomorphic mutations.

Clade 46 (Fig. 2) includes all genera previously placed in the Ixoroideae (Bremekamp, 1966; or the Ixoreae group, Verdcourt, 1958), except the representatives of tribe Chiococceae. Clade 42, including *Vangueria* (Vanguerieae) and *Ixora* (Pavetteae), is only weakly supported by a single site loss. Group 45 containing *Enterospermum* (Pavetteae), *Coffea* (Coffeeae), *Mitriostigma* (Gardenieae), and *Gardenia* (Gardenieae), is supported by five restriction site mutations; however, relationships among these four genera are uncertain. The unresolved relationships among the six genera in clade 46 generate all the different equally parsimonious trees found in the analysis.

Other strongly supported groups agreeing with former classifications are *Haldina* and *Cephalanthus* (clade 35), tribe Naucleeae, *Hamelia* and *Hoffmannia* (clade 60), Hamelieae, *Guettarda* and *Antirhea* (clade 59), Guettardeae, and *Erithalis* and *Chiococca* (clade 36), Chiococceae.

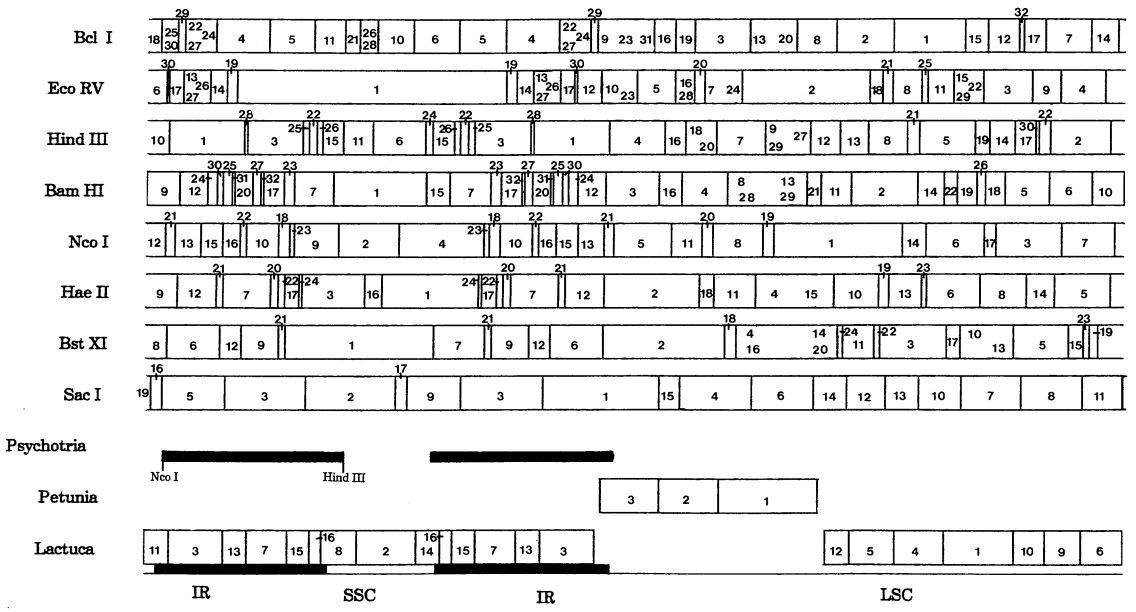

Fig. 1. Restriction maps of *Psychotria bacteriophila* cpDNA, and at the bottom the probes used. The circular maps have been linearized at the SacI site separating the 3.8 SacI-XmaI and 6.3 SacI restriction fragments of the *Lactuca* cpDNA (Jansen and Palmer, 1987a). Restriction fragment numbers correspond to those given in Table 3. The two heavy black lines below the maps indicate the extent of the inverted repeat in both the lettuce and *Psychotria* chloroplast genome.

Several well-supported clades display totally new relationships between tribes or parts of tribes. Representatives of Hamelieae are grouped together with those of the Guettardeae, a relationship never proposed (clade 61). Another interesting group (clade 38) supported by three unique gains, is comprised of representatives of the Chiococceae (clade 36, subfamily Ixoroideae) and two genera, *Exostema* and *Coutarea,* of Cinchoneae (subfamily Cinchonoideae).

Six genera of subfamily Ixoroideae (clade 46) and the four genera of clade 41 are grouped together by seven restriction site mutations, six of which are site gains. Clade 41 is composed of representatives of three different tribes of subfamily Cinchonoideae s.s., *Pogonopus* and *Pinckneya* (Condamineeae), *Calycophyllum* (Cinchoneae), and *Mussaenda* (Isertieae).

A Dollo analysis (PAUP package 3.0 test-version, Swofford, personal communication) of the same data set (Table 5) with *Luculia* as the outgroup gave one tree, 530 steps long with a consistency index of 0.30 (Fig. 4). Dollo parsimony does not allow parallel gains, just gains/losses or parallel losses. In 28% of the characters the consistency index was 1.0, all of these gains, and in 32% of the characters the consistency index was 0.5.

In the Dollo parsimony analysis most clades (24 of 30) are congruent with those from the Wagner analyses. However, incongruencies are due to different arrangements within the Ixoroideae and to the positions of the Naucleeae, the clade with Guettardeae and Hamelieae, and that of the genus *Catesbaea.*

From the Wagner analyses with *Nicotiana* as the outgroup, several clades were stable in all cladograms and also found to be congruent with the parsimony analyses using *Luculia* as an outgroup. For example, the Naucleeae and the grouping of Chiococceae together with *Exostema* and *Coutaria* were congruent between all three analyses. However, in the analysis with *Nicotiana* only one of the accepted subfamilies, the subfamily Rubioideae (excluding the Hamelieae), is supported by several unique synapomorphies. This result is congruent in both Wagner and Dollo parsimony analyses.

DISCUSSION

Phylogenetic utility of cpDNA at higher taxonomic levels—Our results demonstrate that cpDNA restriction site data are extremely informative for phylogenetic reconstruction in the Rubiaceae. This approach has been successfully used at the generic level (Sytsma and Schaal, 1985; Sytsma and Gottlieb, 1986a, b) and in one case at the tribal and subfamilial level (Jansen and Palmer, 1988; Jansen, Palmer, and Michaels, 1988; Jansen et al., in press; Jansen, Michaels, and Palmer, in press). Our study is another example of its usefulness at higher taxonomic levels.

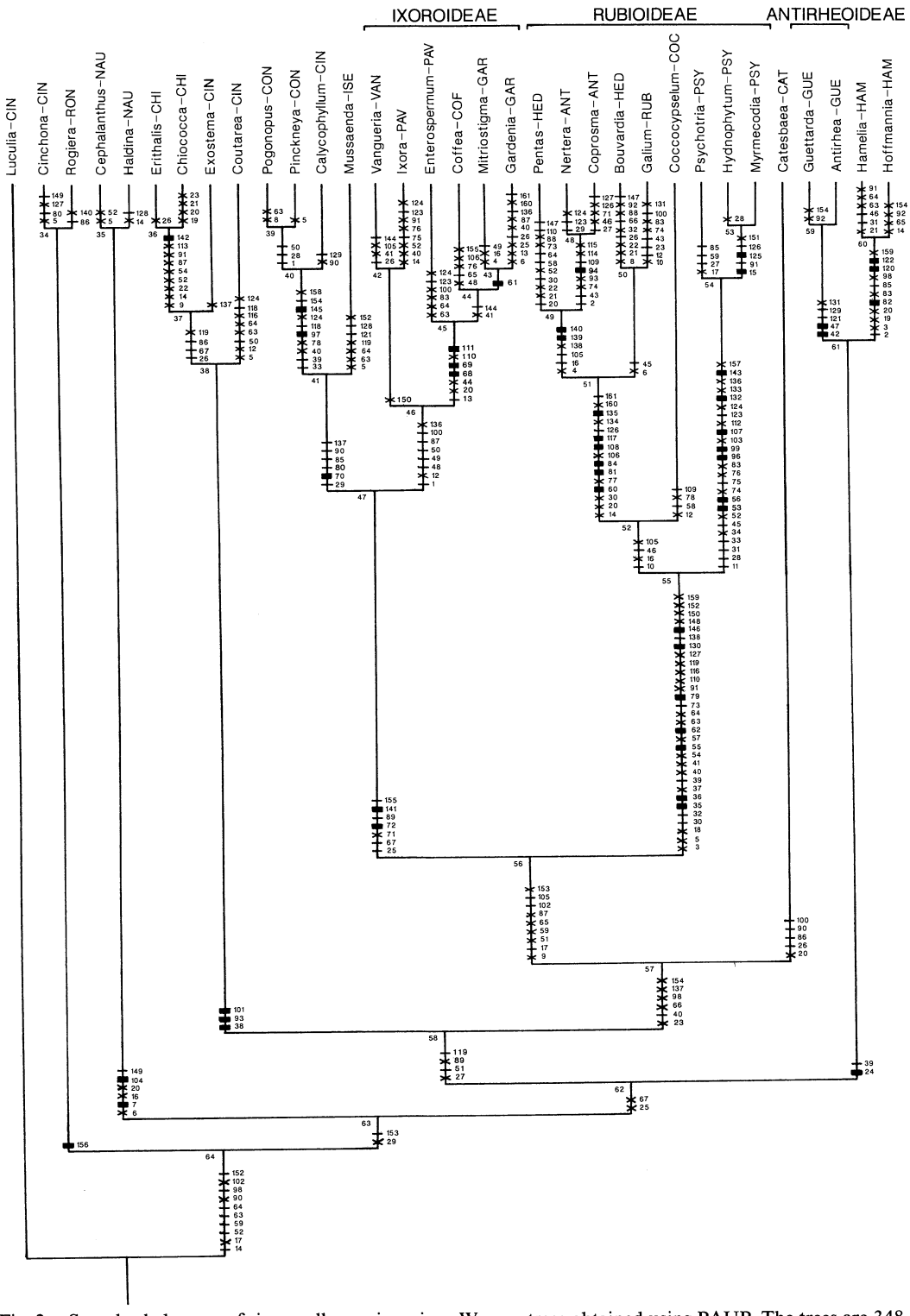

Fig. 2. Sample cladogram of six equally parsimonious Wagner trees obtained using PAUP. The trees are 348 steps long (161 characters) with a consistency index of 0.46. No autapomorphies are shown. Taxon numbers (1–33) correspond to those in Table 5. The tribal position of each taxon is indicated by a three-letter suffix corresponding to the tribes in Table 1. Gains and losses, numbered as in Table 4, are shown by bars and crosses, respectively. Heavy bars indicate gains with consistency index 1.0. The clades (nodes) are numbered 34–64. For further explanation refer to Results and Discussion.

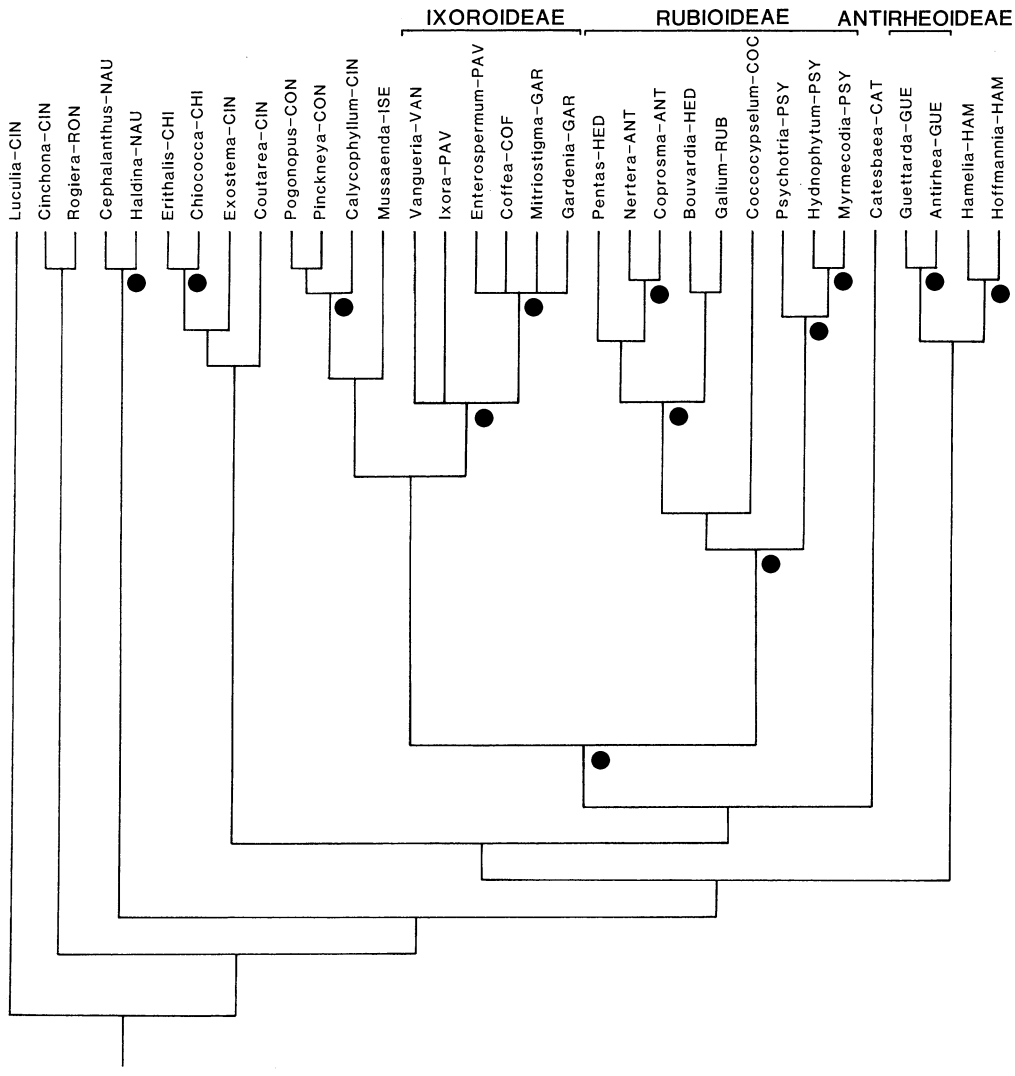

Fig. 3. Strict consensus tree for the six equally parsimonious Wagner trees. Dots indicate nodes retained within all trees one or two steps longer.

Comparison of Dollo and Wagner parsimony methods—In Wagner analyses both parallelisms and reversals are allowed, and parallel losses and gains are treated equally. On the other hand, in a Dollo analysis (Farris, 1977; PAUP 3.0, Swofford, 1989) only parallel losses are allowed, which means that parallel gains of sites are excluded from consideration. The probability of restriction site loss is much higher than the probability of gain. But this does not mean that parallel gains are impossible, as assumed in Dollo analysis. Convergent evolution of restriction sites does occur (cf. Templeton, 1983), but the estimation of the difference in probability of a loss and a gain is complicated. If the two methods, Wagner and Dollo, give different trees, how do we choose one over the other? DeBry and Slade (1985) argue in favor of Dollo analyses of restriction site data for animal mitocondrial DNA. However, Albert, Mishler, and Chase (in press) have suggested that neither Wagner nor Dollo parsimony are entirely appropriate for analysis of DNA, but that Wagner will always produce more accurate topologies than Dollo.

There is a general difference in character distribution between trees generated from the Wagner and the Dollo analysis. A Dollo analysis, not allowing parallel gains, will result in a tree with all gains having a consistency index less than 1.0 (characters with reversals) at the lower branches. The terminal branchings, on the other hand, will in many cases be defined by site losses only (cf. Bremer and Bremer, 1989). A Wagner analysis treats gains and losses equally, so they are more equally distributed

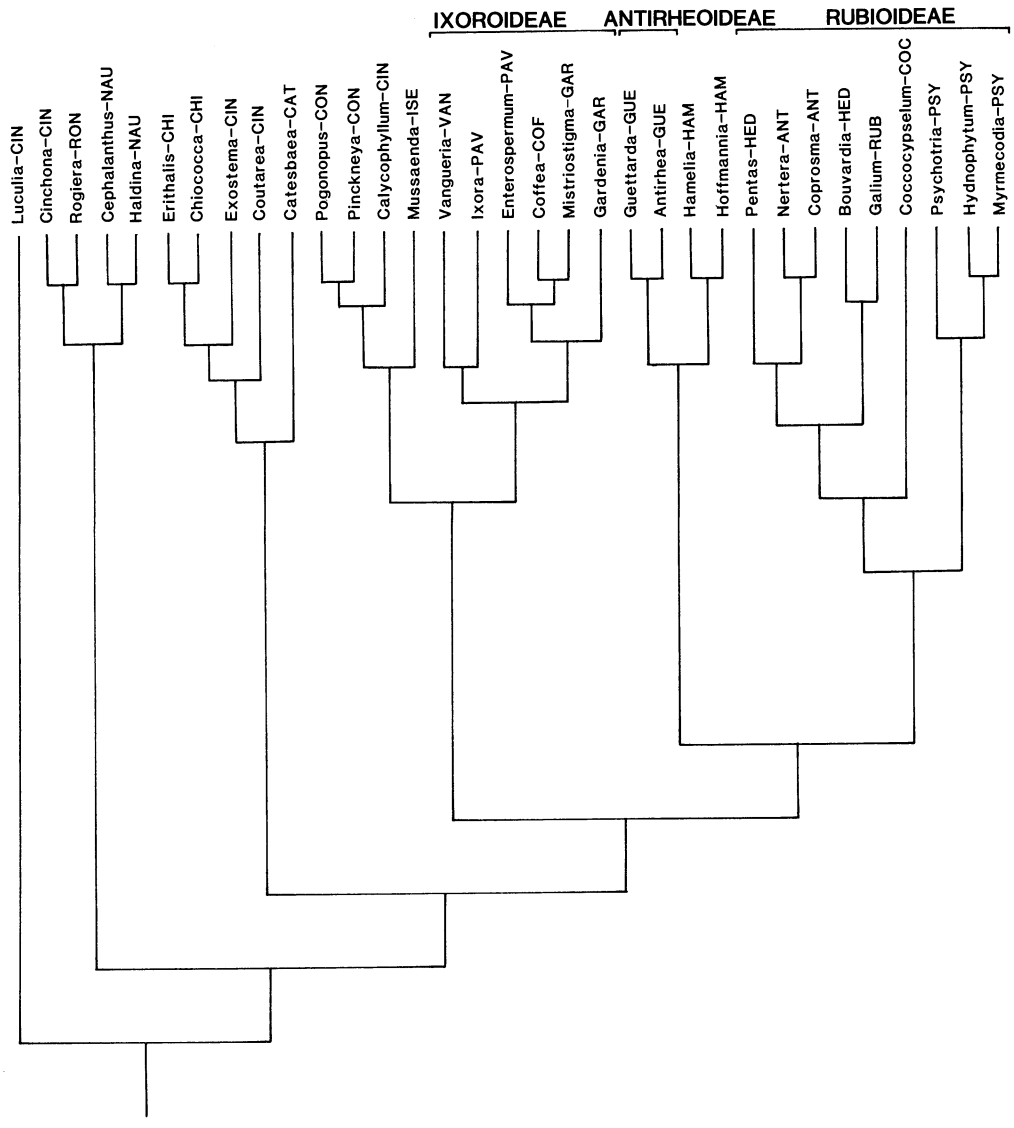

Fig. 4. Dollo parsimony cladogram allowing only single gains but multiple losses of restriction site.

on the tree.

There is no objective way of choosing which of the two methods gives a tree that best represents the "true phylogeny." The most widely used criterion to choose between two alternative trees is to choose the shortest. But tree length cannot be used to compare these two methods because a Wagner tree will never be longer than a Dollo tree based on the same data. Since the Wagner and Dollo trees presented here differ only in the weakly supported parts, as referred to above, the discussion is based on one of them, the Wagner tree.

Choice of outgroup—Selection of an outgroup is very important as it can drastically affect the basal branching (the rooting) if the wrong group is selected. In this study *Luculia* was used as a functional outgroup, but also the distantly related species *Nicotiana tabacum* was used. If the outgroup is too distantly related, many sites will be treated as apomorphic within the ingroup because they will not occur in the outgroup. If, on the other hand a more closely related outgroup is available, many sites may be detected in the outgroup and these would be plesiomorphic (cf. Maddison, Donoghue, and Maddison, 1984). If the basal branching of the tree is affected, it is important to the higher classification within the family. In the future more closely related families will

be chosen, and it will perhaps lead to a different basal tree topology.

Phylogenetic implications of cpDNA variation—The two subfamilies Rubioideae and Ixoroideae (sensu Bremekamp, 1966) correspond fairly well with two strongly supported monophyletic groups in the cpDNA phylogeny (Figs. 2–4), whereas Cinchonoideae is paraphyletic. Thus the Cinchonoideae should not be maintained as a subfamily, because most, if not all, of the other subfamilies have their closest relatives within Cinchonoideae. The most important task for the future will be to clarify the interrelationships within Cinchonoideae, as well as to identify the immediate relatives of the other subfamilies.

Few conclusions can be made concerning tribal interrelationships within the Rubioideae because cpDNAs have been examined from only a few taxa thus far. The tribe Psychotrieae is one of the most strongly supported clades in the whole analysis, as in the classifications of Verdcourt (1958) and Bremekamp (1966). It diverged early from the remaining Rubioideae, also emphasized by Robbrecht (1988). The "old-primitive" status of the Psychotrieae was proposed implicitly by Verdcourt (1958, 1976); he placed the Psychotrieae first when illustrating natural affinities between tribes. *Coccocypselum* obviously forms a lineage distinct from the remaining five examined genera of Rubioideae. The results also indicate that the Hedyotideae (Fig. 2) are polyphyletic because *Pentas* is closer to Anthospermeae than to *Bouvardia.* The monophyly of the Anthospermeae is also supported by several morphological characters, including the complex and unique structures of anemophilous flowers (Puff, 1986).

The second largest clade (47, in Fig. 2) contains most of subfamily Ixoroideae examined and, unexpectedly, the genera representing Isertieae and parts of Cinchonoideae and Condamineeae (*Mussaenda, Calycophyllum, Pogonopus,* and *Pinckneya*). This group is strongly supported by seven restriction site mutations, six of which are site gains. Clade 47 is divided into two groups, one including genera of the Ixoroideae and the second including the genera *Mussaenda, Calycophyllum, Pogonopus,* and *Pinckneya.* The latter group is interesting because it represents three different tribes, and all the genera have one enlarged, brightly colored calyx-lobe (calycophyll or calyx-borne semaphyll). This character is sporadic and is thought to have undergone repeated parallelism (Verdcourt, 1958; Robbrecht, 1988), but here it is interpreted as a unique synapomorphy. The sole representative of the Isertieae, *Mussaenda,* is positioned at the base of this branch. Kirkbride (1979) suggested that the Isertieae show morphological similarities to Condamineeae and Rondeletieae. The other three calycophyllous genera in this analysis, *Pogonopus* and *Pinckneya* on the one hand and *Calycophyllum* on the other, may be more closely related to each other than to other genera of the tribes in which they are currently classified (Bremekamp, 1966; Robbrecht, 1988).

The subfamily Ixoroideae (fide Bremekamp, 1966) comprises "all those tribes in which the upper part of the style acts as a receptaculum pollinis" (Bremekamp, 1966 p. 18), an analogous structure to the pollen pump in the Asteraceae. The systematics of part of this subfamily have recently received much attention, especially the Gardenieae and related tribes and subtribes Aulacocalyceae, Coffeeae, Hypobathreae, Diplosporinae, and Pavetteae (Verdcourt, 1958; Robbrecht, 1980, 1984; Bridson and Robbrecht, 1985; Robbrecht and Puff, 1986). Bremekamp's (1966) circumscription of the Ixoroideae included tribes Chiococceae, Coptosapelteae, and Vanguerieae, but they were later excluded by Robbrecht and Puff (1986) and Robbrecht (1988). The two genera of the Chiococceae included in this study (*Chiococca* and *Erithalis*) certainly do not belong to the Ixoroideae (Bremekamp, 1966) or the Antirheoideae (Robbrecht, 1988). Other taxa included here and representing Coffeeae, Gardenieae, Pavetteae, and Vanguerieae are definitely clearly related as evidenced by their sharing six site gains. The cpDNA phylogeny of the Ixoroideae is not congruent with Robbrecht's and Puff's classification (1986). It differs by grouping *Coffea* (Coffeeae), *Mitriostigma, Gardenia* (Gardenieae), and *Enterospermum* (Pavetteae) together, while *Ixora* (Pavetteae) and *Vangueria* (Vanguerieae) are placed into a separate but weakly supported clade (Figs. 2–4).

The proposed circumscriptions and relationships of tribes in the Cinchonoideae have been very different (Table 2; Bremekamp, 1954, 1966; Verdcourt, 1958; Robbrecht, 1988). Bremekamp (1954, 1966) emphasized two characters (a special testa structure and the shape of the placentas) in his classification of the subfamily; however, both characters are most likely plesiomorphic within the Rubiaceae because they occur also in parts of subfamily Ixoroideae (Robbrecht, personal communication), as well as in parts of the related family Loganiaceae (Bremer, unpublished data). Verdcourt's (1958) circumscription of the Cin-

chonoideae, which included the Ixoroideae, was based entirely on the absence of characters occurring in other subfamilies. Robbrecht (1988) emphasized testa structure, but gave no unique characters for the subfamily.

The cpDNA phylogeny clearly indicates that the Cinchonoideae are paraphyletic. Although our choice of outgroup might be criticized, selecting another outgroup would not change the paraphyly of Cinchonoideae. The subfamilies Ixoroideae, Antirheoideae, and possibly the other small subfamilies would have to be included in Cinchonoideae to make it monophyletic.

The Cinchonoideae are split into several basal clades. Monophyletic groups in this part of the tree are supported by many fewer restriction site mutations than those found in clades within the more herbaceous subfamily Rubioideae. This may be due to reduced rates of cpDNA evolution among woody taxa (Bruneau, Doyle, and Neill, 1988; Schilling and Jansen, 1989), but may also be a result of incorrect rooting. Most basal clades in the tree are supported by only a few characters. Many more taxa and restriction enzymes must be added to the analysis to further resolve the circumscriptions and interrelationships of the basal tribes. However, the monophyly of four tribes (Naucleeae, Hamelieae, Guettardeae, and Chiococceae) is strongly supported.

Haldina and *Cephalanthus* of the tribe Naucleeae s.l. form a monophyletic group, although the placement of *Cephalanthus* in this tribe, and even within the subfamily Cinchonoideae, has been questioned (Bremekamp, 1966; Ridsdale 1978a, b; Robbrecht, 1988). Ridsdale placed *Cephalanthus* into a separate tribe (Cephalantheae), and Robbrecht (1988) included it in subfamily Antirheoideae. *Haldina* and *Cephalanthus* form a monophyletic group in the cpDNA phylogeny, but they are also characterized by a number of morphological synapomorphies (e.g., capituliform inflorescences, the clubshaped bracteoles).

In earlier classifications (Verdcourt, 1958; Bremekamp, 1966) Antirheoideae were restricted to the single tribe Guettardeae. Robbrecht (1988) widened its circumscription to include seven tribes. Our cpDNA results strongly contradict the idea that tribes Vanguerieae, Cephalantheae, and Chiococceae are close to the Guettardeae.

The position of Hamelieae is interesting, but not settled. It is placed among the "Cinchonoideae" tribes and not those of the Rubioideae (Fig. 2). The Hamelieae were previously placed close to the Gardenieae (Schumann, 1891) and were then moved to subfamily Rubioideae (Bremekamp, 1954) because of the occurrence of raphides in their tissues (needle-shaped calcium oxalate crystals). Much attention has been paid to the raphide character, and it is a cardinal character in Verdcourt's (1958) and Bremekamp's (1966) systems. If the position indicated by the Wagner analysis of the cpDNA data is correct, then the Hamelieae are one of the few taxa with raphides outside of the Rubioideae (Robbrecht, 1988). However, on the Dollo tree (Fig. 4) Hamelieae and Guettardeae form a sister group to the remaining Rubioideae taxa. If this placement is correct, the occurrence of raphides could be explained by a single evolutionary event.

The last strongly monophyletic clade, which is supported by three unique restriction site gains, includes representatives of tribe Chiococceae (subfamily Ixoroideae), *Exostema* (Cinchonoideae-Cinchoneae), and *Coutarea* (formerly in Condamineeae and now in Cinchoneae, Aiello, 1979). A relationship between these two tribes has never been proposed; however, several unique morphological criteria support this relationship. The stamens are basally connate forming a ring that is adnate to the corolla base. The slender filaments are villous at the base from one-quarter to halfway up and the anthers are basifixed. The stigmatic area consists of two narrow lines, usually twisted several times around the style. All these characters have been found and studied in the members of Condamineeae-Portlandiineae group (Aiello, 1979), although their rarity has not been appreciated nor has their homology to those of *Exostema* and *Coutarea* and the Chiococceae.

Conclusions—The study of Rubiaceae cpDNA indicates that several tribes within subfamily Ixoroideae and particularly in Cinchonoideae have to be redefined. Restriction site variation of cpDNA in Rubiaceae has proved to be a powerful source of information for phylogenetic reconstruction within the family, but much remains to be done, since we have only examined 5% of the known rubiaceous genera.

The following general conclusions can be drawn from this first phylogenetic cpDNA study of interrelationships within the Rubiaceae (Fig. 2): 1) Many branches (clades) are very strongly supported, as suggested by the lack of collapsing of the strict consensus tree; 2) The stability of the trees was high because during the analyses the final tree topologies appeared after fewer than 50% of the variable restriction sites had been added; 3) The outlines of the trees produced from the Wagner and Dollo analyses

correspond in part to established subfamilies in both Verdcourt's (1958) and Bremekamp's (1966) classifications; and 4) Several tribes are not monophyletic, and many tribes and genera may have to be moved or recircumscribed.

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