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Phylogenetic relationships of the Aurantioideae (Rutaceae) based on the nuclear ribosomal DNA ITS region and three noncoding chloroplast DNA regions, *atpB-rbcL* spacer, *rps16*, and *trnL-trnF*

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Abstract

The tribes and subtribes of Aurantioideae, an economically important subfamily of the Rutaceae, have a controversial taxonomic history because a phylogenetic framework has been lacking. In order to construct an evolutionary history and evaluate the most recent classification system [Swingle and Reece 1967. The botany of *Citrus* and its wild relatives, in: The Citrus Industry, vol. 1, History, World Distribution, Botany, and Varieties. University of California, Berkeley, pp. 190–430], one nuclear and three noncoding chloroplast genes were sequenced and analyzed phylogenetically along with selected non-molecular characters. Taxa representing tribes Citreae and Clauseneae and their six subtribes were sampled. In all analyses Aurantioideae is monophyletic. The majority-rule consensus tree from the combined analysis indicates that the two tribes are not monophyletic. The combined topology is not congruent with the widely used classification of Aurantioideae by Swingle and Reece (1967). The tribes and subtribes are in need of revision.

Keywords: Aurantioideae; Citreae; Clauseneae; Rutaceae; ITS; *atpB-rbcL* spacer

Introduction

The Aurantioideae (this is the correct name for 'Citroideae' or 'Limonoideae') are one of seven subfamilies in the family Rutaceae (Engler 1931). In the most recent classification (Swingle and Reece 1967), 33 genera were recognized and grouped into two tribes: the Clauseneae with five genera, and the Citreae with 28 genera, including *Citrus*.

In general, Aurantioideae can be characterized as small trees, shrubs, or rarely vines that produce fruit with a grandular peel, thin skin or hard shell and often containing pulp vesicles. The leaves and fruits have schizolysigenous oil glands that release an aroma when touched, and the flowers are typically white and fragrant. The leaves are usually persistent (Swingle and Reece 1967).

The Rutaceae are native to Africa, Australia, North and South America, and Asia. The genera of Aurantioideae occur in varied climates from equatorial hot-humid to cool maritime conditions. Generally, the trees and fruits are sensitive to frost and cold and require long, warm summers for the fruit to reach maturity. Twenty-nine of the 33 genera comprising Aurantioideae are indigenous to monsoon regions, which extend from West Pakistan to north-central

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Table 1. Tribe and subtribe classifications of the three most recent circumscriptions of Aurantioideae.

Engler (1931)	Tanaka (1936)	Swingle and Reece (1967)
Aurantieae	Aegleae	Clauseneae
Hesperethusinae	Atalantieae	Clauseninae
Citrinae	Aurantieae	Merrilliinae
	Hesperethusinae	Micromelinae
	Citropsinae	Citreae
	Citrinae	Balsamocitrinae
	Poncirinae	Citrinae
	Clauseneae	Triphasiinae
	Lavangeae	
	Balsamocitrinae	
	Feroniinae	
	Merrillinae	
	Swingleinae	
	Meropeae	
	Microcitreae	
	Micromeleae	

China and south through the East Indian Archipelago to New Guinea, the Bismarck Archipelago, Australia, New Caledonia, Melanesia, and the western Polynesian islands. Five genera are native to tropical Africa. Only one genus, *Clausena*, is native to both the monsoon regions and tropical Africa (Swingle and Reece 1967).

Tribal and subtribal classifications are in dispute, as they differ among the most recent classifications of Aurantioideae (Engler 1931; Tanaka 1936; Swingle and Reece 1967); see Table 1. Tanaka (1936) grouped the subfamily into eight tribes and eight subtribes including 28 genera. Tribes Micromeleae, Clauseneae, Aegleae, Lavangeae, Meropeae, Atalantieae, Microcitreae, and Aurantieae were divided by a suite of features that includes the number of leaflets, venation of the leaf, origin and development of the winged rachis, presence and appearance of the thorns, the number of the floral organs (stamens, locules, and ovules), the development of the pulp vesicles, the texture of the rind of the fruits, and features of the cotyledons. Engler (1931) had grouped members of the subfamily into a single tribe Aurantieae, with 16 genera in the subtribe Hesperethusinae, and 13 genera in the subtribe Citrinae. These subtribes had been distinguished mainly by the number of ovules per locule, which is 1 or 2 in the Hesperethusinae (except *Wenzelia*), greater than 2 in the Citrinae. Modern authors most commonly refer to the classification by Swingle and Reece (1967), which includes two tribes and six subtribes in the subfamily. Tribe Clauseneae contains three subtribes with five genera, whereas tribe Citreae contains three subtribes with 28 genera. Swingle and Reece (1967) believed that the Clauseneae tribe contains the more primitive genera of the subfamily. All Clauseneae lack axillary spines and have odd-pinnate leaves with leaflets alternately attached to the rachis. The fruits are usually small, semi-dry or juicy berries, except in *Merrillia*. *Merrillia* fruits are ovoid with a thick, radially lacunose leathery exocarp that is unique in the subfamily. *Merrillia* is also the only genus in the subfamily with zygomorphic flowers.

In the tribe Citreae nearly all the species develop axillary spines, single or paired, and sometimes curved as in *Luvunga* and *Paramignya*. Their simple, unifoliolate or trifoliolate leaves typically distinguish Citreae from Clauseneae, but in a few Citrinae genera the leaves are odd-pinnate with opposite leaflets (*Feronia*, *Feroniella*, and *Hesperethusa*). The Citrinae differ from all the other subtribes in the subfamily by having pulp vesicles which arise from the dorsal wall of the locule, grow into the locular cavity, and develop into sacs filled with large, thin-walled cells with watery juice. No close homologies are known in any of the higher plants. Some genera of Aurantioideae have secretory glands on the walls of the locules, giving rise to mucilaginous gum, which fills the locular cavity of the fruit. Genera of the subtribe Balsamocitrinae have woody-shelled fruits filled with resinous gum.

Chase et al. (1999) found the Aurantioideae to be a well-defined monophyletic group, and that members of the Flindersioideae (*Chloroxylon*) and Rutoideae (*Ruta*) were basal to the main Aurantioideae clade. Therefore, *Chloroxylon* and *Ruta* were used as the outgroups in the present analysis.

Many species of Aurantioideae have commercial importance. For example, the fruits of *Citrus* and *Fortunella*, including oranges, lemons, limes, and kumquats, are the most economically significant fruits in the world. Understanding the internal relationships among the different taxa of the subfamily will facilitate the improvement of both breeding techniques and conservation strategies. The most current classification by Swingle and Reece (1967) is based on traditional taxonomic methods using morphology and anatomy. The present study examines the phylogenetic relationships within the Aurantioideae using molecular characters and selected non-molecular features.

Three noncoding chloroplast regions (*trnL-trnF*, *rps16*, *atpB-rbcL* spacer) were selected, along with one nuclear region (ITS) and various non-molecular characters. The *trnL-trnF* region consists of the *trnL* intron and the *trnL-trnF* intergenic spacer (Taberlet et al. 1991). The intron of the *rps16* is a group II intron that was first used for phylogenetic studies by Oxelman et al. (1997). Various workers have found that both of these sequences give good resolution at the genus and species levels (e.g. Baker et al. 2000; Wallander and Albert 2000). The *atpB-rbcL* spacer, which lies between the large subunit of the ribulose-1-5-bisphosphate-carboxylase (*rbcL*) and the beta subunit of the chloroplast ATP-synthase (*atpB*) genes, has proven

useful in phylogenetic studies of angiosperms (e.g. Manen et al. 1994; Crayn and Quinn 2000; Powell and Kron 2001) as well as of mosses (Chiang et al. 1998, Chiang and Schaal 2000). The internal transcribed spacers (ITS) of the 18S–26S nuclear ribosomal DNA (nrDNA) separate the three gene regions coding for the 18S, 5.8S, and 26S ribosomal subunits, respectively. The ITS1 spacer is located between the 18S and 5.8S regions, the ITS2 spacer between 5.8S and 26S. The rapidly evolving ITS spacer sequences have been used extensively in phylogenetic studies at lower levels (Manos 1997; Compton et al. 1998), but have also helped to resolve intrafamily relationships (Johnson et al. 1999). The present study's choice of non-molecular characters to be considered in the analysis was based on information from the literature and on personal observations.

Preliminary studies on Aurantioideae were conducted by Samuel et al. (2001), using two plastid genes, the *atpB/rbcL* intergenic spacer and the *rps16* intron. The joint matrix contained 15 of the 33 genera recognized in Aurantioideae. For many of the branches within the phylogeny, the two gene regions did not provide enough informative characters to provide resoution or sufficient support. Araújo et al. (2003) published a phylogenetic study on the tribe Citreae, using partial sequences from the *trnL-F* region and a few morphological characters. Morton et al. (2003) used the *rps16* and *trnL-trnF* introns from 24 genera in their investigation of Aurantioideae. Once again the two gene regions did not provide enough informative characters to give good resolution or support to the phylogeny. Mabberley (1998) fused *Eremocitrus*, *Fortunella*, and *Microcitrus* with *Citrus*, based on morphological characters, and suggested that *Poncirus* should be so treated as well.

The results by Samuel et al. (2001), Araújo et al. (2003) and Morton et al. (2003) were not fully congruent and did not have enough resolution to address tribal and subtribal delimitations. The aim of the present study, therefore, is to further investigate the phylogeny of the subfamily, tribes, and subtribes by including additional taxa (*Aeglopsis*, *Bergera*, and *Micromelum*), morphological data, and by studying one nuclear and three noncoding chloroplast genes.

Material and methods

DNA extraction

Plant material from 29 genera was used; the voucher information is listed in Table 2. Total genomic DNA was extracted from 0.5–1.0 g of fresh or dried leaf material. Each sample was ground into a paste using the modified CTAB procedure of Doyle and Doyle (1987). Organic compounds were removed using 24:1 chloroform: isoamyl alcohol (SEVAG), followed by DNA precipitation with ice-cold isopropanol.

rps16

The *rps16* gene regions of 29 genera were amplified using the primer pair *rpsF*/*rpsR2* (Oxelman et al. 1997) to acquire the respective entire region. The final PCR cocktail of 50 ml contained 38 ml water, 5 ml 10% buffer, 3 ml Mg^{+2}, 1 ml dNTPs, 0.25 ml Taq polymerase, and 0.5 ml of each primer. The amplifying reactions were run for 25 cycles of denaturing for 30 s at 95 °C, primer annealing for 50 s at 57 °C, and elongation for 2 min at 72 °C. Some taxa required altered amplification conditions, such as raising or lowering the $MgCl_2$ concentration, adding tetramethylammonium chloride, and changing the annealing temperature.

trnL-trnF

The *trnL* intron and the *trnL-trnF* intergenic spacer were amplified for 29 genera. PCR was performed using the universal primers trn-c, trn-d, trn-e, and trn-f as described by Taberlet et al. (1991). For some samples the entire *trnL* intron/*trnL-trnF* spacer region was amplified with trn-c and trn-f. In others, two separate amplifications were performed, one to amplify the *trnL* intron with trn-c and trn-d, the other to amplify the *trnL-trnF* spacer with trn-e and trn-f. In general, each 50 µl amplification reaction contained constituents in the same proportions as in the *rps16* reactions. PCR amplification used a 7-min denaturing step at 94 °C, followed by 30 cycles of denaturing for 1 min at 94 °C, primer annealing for 1 min at 45 °C, and elongation for 1 min at 72 °C, with a final 7-min elongation step at 72 °C.

atpB-rbcL spacer

The *atpB-rbcL* gene was amplified using primers 520F and 3158R (Crayn and Quinn 2000). Each 50 µl amplification reaction contained constituents in the same proportions as in the *rps16* and *trnL-trnF* reactions. The gene was amplified using 35 cycles of denaturation for 1 min at 95 °C, annealing for 30 s at 50 °C, and extension for 1 min at 72 °C, and a final extension for 7 min.

ITS

Amplification of the ITS gene was performed successfully for 27 genera, using oligonucleotide primers ITS1/ITS4 (White et al. 1990) to acquire the respective

Table 2. Species used in the sequence analyses (arranged by subfamily, tribe, and subtribe), voucher data, and GenBank accession numbers of the corresponding sequences.

Taxon	Voucher/source	*rps16*	*trnL-trnK*	Spacer	ITS
Aurantioideae (33/210)					
Citreae (28/124)					
Balsamocitrinae (7/14)					
Aegle marmelos (L.) Corr. Serr.	PI539142	AY295268	AY295294	FJ434197	FJ434169
Aeglopsis chevalieri Swingle	PI539143	FJ384561	FJ384562	FJ434193	–
Afraegle paniculata (Schum. & Thonn.) Engl.	PI no #	AY295269	AY295295	FJ434198	FJ434170
Balsamocitrus dawei Stapf.	PI539147	AY295252	AY295278	FJ434194	FJ434166
Feronia limonia (L.) Swingle	Morton 198	AY295273	AY295299	FJ434195	FJ434167
Feroniella oblata Swingle	PI539720	AY295263	AY295289	FJ434196	FJ434168
Swinglea glutinosa (Blanco) Merr.	PI231241	AY295259	AY295285	FJ434192	FJ434165
Citrinae (13/64)					
Atalantia ceylanica (Am.) Oliv.	Chase 1341, K	AY295262	AY295288	FJ434186	FJ434159
Citropsis gilletiana Swingle & Kellerm.	PI539149	AY295266	AY295292	FJ434199	FJ434171
Citrus paradisi Macfad.	Kew 0345903403	AY295251	AY295277	FJ434191	FJ434164
Clymenia polyandra (Tanaka) Swingle	PI263640	AY295255	AY295281	FJ434189	FJ434162
Eremocitrus glauca (Lindl.) Swingle	PI539717	AY295267	AY295293	FJ434188	FJ434161
Fortunella polyandra (Ridley) Tanaka	PI539731	AY295265	AY295291	FJ434187	FJ434160
Hesperethusa crenulata (Roxb.) M. Roem.	PI539748	AY295272	AY295298	FJ434185	FJ434158
Microcitrus garrowayi Swingle	RBG, Sydney 87285	AY295261	AY295287	FJ434190	FJ434163
Pleiospermium latialatum Swingle	PI no #	AY295257	AY295283	FJ434184	FJ434157
Poncirus trifoliata (L.) Raf.	Chase 1767, K	AY295256	AY295282	FJ434181	FJ434154
Severinia buxifolia (Poir.) Tenore	PI539793	AY295264	AY295290	FJ434183	FJ434156
Triphasiinae (8/46)					
Pamburus missionis (Wall. ex Wight) Swingle	PI539749	AY295274	AY295300	FJ434182	FJ434155
Triphasia trifolia (Burm. f.) P. Wilson	PI539800	AY295271	AY295297	FJ434200	FJ434172
Wenzelia dolichophylla (Lauterb and K.Schum.) Tanaka	PI277411	AY295260	AY295286	FJ434176	FJ434150
Clauseneae (5/86)					
Clauseninae (3/76)					
Bergera koenigii L.	PI539745	AF320262	FJ384563	AF320867	FJ434147
Clausena excavata Burm. f.	Chase 1343, K	AY295258	AY295284	FJ434179	FJ434152
Glycosmis pentaphylla (Retz.) Corr. Serr.	PI127866	AY295253	AY295279	FJ434178	FJ434151
Murraya paniculata (L.) Jack	RBG, Perth 853900	AY295254	AY295280	FJ434180	FJ434153
Merrilliinae (1/1)					
Merrillia caloxylon (Ridl.) Swingle	PI539733	AY295270	AY295296	FJ434174	FJ434149
Micromelinae (1/9)					
Micromelum minutum (G. Forst.) Wight & Arn.	PI6000637	AF320267	AF025520	FJ434175	FJ434148

Table 2. (*continued*)

Taxon	Voucher/source	*rps16*	*trnL-trnK*	Spacer	ITS
Flindersioideae (2/17)					
Flindersieae (2/20)					
Chloroxylon swietenia DC.	Chase 1291, K	AY295250	AY295276	FJ434177	–
Rutoideae (100/1204)					
Ruteae (7/84)					
Rutinae (6/83)					
Ruta gravolens L.	Chase 510, K	AY295249	AY295275	FJ434173	FJ434146

Infrafamilial groups in Aurantioideae are those recognized by Swingle and Reece (1967), in Flindersioideae and Rutoideae those recognized by Engler (1931). Numbers in parentheses following higher-taxon names are estimates of the respective numbers of genera/species included. Voucher/source abbreviations: K = obtained from Royal Botanic Gardens at Kew, UK; PI = obtained from USDA-ARS National Germplasm Repository for Citrus at Riverside, CA, USA (voucher samples are with C.M. Morton); RBG = obtained from Royal Botanic Gardens, Australia (voucher samples are with C.M. Morton).

entire region. Amplification problems occurred with the genera *Aeglopsis* and *Micromelum* (ITS1) and the *Chloroxylon* outgroup taxa; thus, these taxa are missing in the dataset. In some instances ITS2/ITS3 were used with the above primers to amplify smaller fragments. The DNA fragment amplified using these two primers is approximately 800 bp long and includes ITS1, ITS2, and the 5.8S ribosomal gene. The basic mix contained 38 µl H_2O, 5 µl 10% Mg-free buffer solution, 3–6 µl 25 mM $MgCl_2$, 1 µl 10 mM dNTPs, 0.5 µl of each primer (10 nM), and 0.25 µl Taq DNA along with 1.5 µl of DNA template for each reaction. Depending on the concentration, total extracted DNA was sometimes diluted 1:10 or 1:100. The thermal cycler was programmed to perform an initial cycle of denaturation at 95 °C for 2 min, followed by 24 cycles of 30 s at 55 °C, 90 s at 72 °C, and 30 s at 95 °C. This was followed by a 10-min extension at 72 °C to allow completion of unfinished DNA strands, which in turn links to a soak file at 4 °C. Because of multibanding patterns in the sequences, most amplification products were cloned with the pGem T Easy Vector System II (promega), following the manufacturer's instructions. Clone copies were sequenced as described below. Sequences that exhibited close genetic similarity to the samples sequenced successfully with AmpliTaq were assumed to represent functional copies of the ITS region. The point mutations in the pseudocopies made the pseudogenes easy to distinguish from functional copies.

Cycle sequencing

The PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN Inc., Chatsworth, CA), following the manufacturer's protocols. Cleaned products were directly sequenced using the ABI PRISM dye terminator cycle sequencing ready kit with AmpliTaq DNA Polymerase (Applied Biosystems Inc., Foster City, CA). Unincorporated dye terminators were removed using the DyeEx dye-terminator removal system (QIAGEN Inc.), following the manufacturer's recommendations. Samples were then loaded into an ABI 3100 DNA sequencer. The sequencing data were analyzed and edited using the Sequencher software program (Gene Codes Corporation, Ann Arbor, MI).

Non-molecular characters

In general, character states used in phylogenetic analysis should be discrete and without overlap (Stevens 1991). Hawkins et al. (1997) and Kitching et al. (1998) have discussed in detail the problem of conflicting characters encountered during the coding. The current study adopted coding method 'C' from Kitching et al. (1998). This method treats separate attributes as separate independent characters and adds another code for the presence and absence of the features; question marks indicate inapplicable observations for the absence of the feature. This overcomes the problem of overscoring the state's absence when many different characters are perceived as connected to a feature that is absent in some taxa (Maddison 1993). Potentially useful characters had to be discarded, because data were available for only a few genera. Twenty parsimony-informative characters were included in the analysis (Tables 3 and 4). A number of characters initially considered for inclusion in the analysis were ultimately excluded because of problems associated with coding characters states, the high frequency of polymorphism within species, or the large extent of missing data. Of the 20 characters 9 are binary and 11 have been coded as unordered multistate. All but three characters were

Table 3. Non-molecular characters and their states used in phylogenetic analysis of Aurantioideae.

Character	States (matrix scores)	Source
01: Spine presence	Absent (0); present (1)	Swingle and Reece (1967)
02: Spine number	Single (0); paired (1)	Swingle and Reece (1967)
03: Rachis articulation	Absent (0); present (1)	Swingle and Reece (1967)
04: Leaf division	Pinnate (0); odd-pinnate (1); trifoliolate (2); unifoliolate (3)	Swingle and Reece (1967)
05: Stamens per petal	2 (0); 4 (1); 6–8 (2); 10–20 (3)	Swingle and Reece (1967)
06: Locule number	2–6 (0); 8–20 (1)	Swingle and Reece (1967)
07: Ovules per locule	1–2 (0); 4 or more (1)	Swingle and Reece (1967)
08: Pericarp appearance	Thin (0); thick (1)	Swingle and Reece (1967)
09: Pulp vesicles	Absent (0); mucilaginous pulp (1); rudimentary pulp (2); pulp vesicles (3)	Swingle and Reece (1967)
10: Seed surface	Wrinkled (0); smooth (1); hairy (2)	Swingle and Reece (1967)
11: Seed shape	Flattened (0); oval (1); globose (2); oblong (3)	Swingle and Reece (1967)
12: Inflorescence	Panicle (0); solitary (1); raceme (2); corymb (3)	Swingle and Reece (1967)
13: Colpus length	Long (0); short (1)	Grant et al. (2000)
14: Sculpturing pattern	Perforate (0); reticulate (1); striate (2)	Grant et al. (2000)
15: Aperture number	3 (0); 4 (1); 5 (2)	Grant et al. (2000)
16: Endexine thickening	Slight (0); distinct (1); none (2)	Grant et al. (2000)
17: Stamen traces	Unbranched (0); branched (1)	Tillson and Bamford (1938)
18: Vascular anatomy	Sepal and petal midribs independent from axis (0); sepal midribs extend to lateral petal, then fuse (1); sepal lateral bundles fused to petal midribs (2)	Tillson and Bamford (1938)
19: Carbazole	Absent (0); present (1)	Samuel et al (2001)
20: Chromosome number	9 (0); 13 (1); 18 (2); 27 (3); 10 (4)	Stace et al. (1993)

Table 4. Matrix of morphological character states.

	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20
Ruta	0	?	0	0	0	0	0	1	0	0	0	0	0	?	0	?	?	?	?	4
Chloroxylon	0	?	0	0	0	0	1	1	0	?	0	0	0	?	0	?	?	?	?	4
Glycosmis	0	?	1	1/2/3	0	0	0	0	1	1	1	2	?	3	0	0	?	?	1	0/3
Clausena	0	?	0	1	0	0	0	0	1	1	1/2	3	0	3	0	0	?	?	1	0/2
Murraya	0	?	1	1	0	0	0	1	1	0	2	0	0	3	0	1	?	?	0/1	0/2
Triphasia	1	1	0	2/3	0	0	0	?	1	?	?	1	0	3	0	1	?	?	0	0
Pamburus	1	0	0	3	0	0	0	1	1	?	1/2	2	0	1	2	0	?	?	0	?
Severinia	0/1	0	0	3	0	0	0	0/1	2	1	1	0/3	0	2	1	1	?	1/2	0	0
Pleiospermium	1	0/1	1	2/3	0	0	0	1	2	?	1	0	1	1	1/2	1	1	1/2	0	?
Hesperethusa	1	0	1	1	0	0	0	?	2	1	?	2	0	1	1/2	1	0	1/2	0	0
Citropsis	1	0/1	1	2/3	0	0	0	0	3	1	1	0	1	1	1/2	2	1	1	0	0
Atalantia	0/1	0	0	3	0	0	0	0	3	?	3	0/2/3	?	?	?	?	1	1	0	0
Fortunella	1	0	0/1	2	1	0	0	1	3	1	1	1	0	2	1	1	1	1	0	0/2
Eremocitrus	1	0	0	3	1	0	0	0	3	0	1	1	0	2	1/2	1	1	1	0	0
Clymenia	0	?	0	3	3	1	1	0	3	?	1	1	?	?	?	?	1	1	0	?
Microcitrus	1	0/1	0	3	1	0/1	1	1	3	1	1	1	1	2	1	1	1	0/1	0	0/2
Citrus	1	0	1	3	1	1	1	1	3	0/1	1	1/2	1	2	1/2	0	0/1	1/2	0	0/2/3
Swinglea	1	0/1	1	2	0	1	1	1	1	2	1	1	0	?	1	1	1	1/2	0	0
Aegle	1	0	1	2	2	1	1	1	1	2	3	1	1	2	1	1	1	0	0	0/2
Afraegle	1	0	0	2	1	0/1	1	1	1	?	1	2/3	0	1	1/2	1	1	0	0	0
Aeglopsis	1	0	0	3	0	0	1	1	1	1	1	0	1	1	1/2	1	1	0	0	0
Balsamocitrus	1	0	0	2/3	0	1	1	1	1	1	3	0	1	1	1	0	?	?	0	?
Feronia	1	0	0/1	1	0	0	1	1	1	2	3	0	1	2	1	2	1	1	0	0
Feroniella	1	0	1	1	1	0	1	1	1	1	1	0	0	3	0	2	1	1	0	0
Merrillia	0	?	1	0	0	0	1	1	1	2	1	1	0	3	0	?	?	?	0	0
Wenzelia	0/1	0/1	0	3	0	0	1	0/1	1	1	1	1	?	?	?	?	1	1	0	?
Poncirus	1	0	1	2	1	0/1	1	1	3	?	1	1	0	2	1/2	1	1	1	0	0/1/2

? = missing data

variable within the Aurantioideae. The invariant characters were included, because this was thought to be important in testing the monophyly of the subfamily. All analyses were conducted as stated in the section on molecular data analysis. Character states of taxa of Aurantioideae not observed directly by the author were derived from Tillson and Bamford (1938), Swingle and Reece (1967), Stace et al. (1993), Grant et al. (2000), and Samuel et al (2001).

Phylogenetic analysis

Boundaries of the *trnL* intron and the *atpB-rbcL* spacer, *rps16*, and ITS nuclear regions were determined by comparison with sequences in GenBank. The following alignment criteria and methodology were used: (1) when two or more gaps were not identical but overlapping, they were scored as two separate events; (2) phylogenetically informative indels (variable in two or more taxa) were scored as one event at the end of the dataset. All DNA sequences reported in the analyses have been deposited in GenBank (Table 2).

All phylogenetic analyses employed maximum parsimony with the heuristic search option in PAUP* 4.0b8 (Swofford 2000), with uninformative characters excluded. Searches were conducted with 100 random-taxon-addition replicates with TBR branch swapping, steepest descent and MulTrees selected, and with all characters and states weighted equally and unordered. All trees from the replicates were then swapped onto completion, all shortest trees were saved, and a strict-consensus tree was computed. Relative support for individual clades was estimated with the bootstrap method (Felsenstein 1985). One thousand pseudoreplicates were performed with uninformative characters excluded. Ten random-taxon-addition heuristic searches for each pseudoreplicate were performed, and all minimum-length trees were saved for each search. To reduce bootstrap search times, branches were collapsed if their minimum length was zero (“amb-”).

To determine the combinability of the two datasets, their data structure were compared using methods outlined by Mason-Gamer and Kellogg (1996), who discussed various ways to assess conflict between datasets. In one method the combination of independent datasets is possible if the trees do not conflict or if conflict receives low bootstrap support. Therefore, each node on the independent trees is tested for congruence against the other. If the nodes do not contain conflicting information, they are congruent and the datasets are combinable. Where there are incongruent nodes, the bootstrap values for each node are examined. If the support is less than 70%, then there is no hard conflict and the incongruence is interpreted as being due to chance. In this study the different datasets were also analyzed in combination, to see how each dataset confirmed or differed from the tree topologies from the other datasets, and to adopt a hypothesis of phylogenetic relationships for the subfamily.

Results

The inclusion of gap coding in all datasets containing molecular data resulted in more homoplasy and lack of resolution; therefore, gap coding was not used in the following results. GenBank sequences FJ384561–FJ384563 and FJ43146–FJ434200 were generated specifically for this study.

trnL-trnF

The length of the *trnL* intron ranged from 461 to 476 bp among species of Aurantioideae, and from 449 to 452 bp among the outgroups. The length of the *trnL-trnF* intergenic spacer ranged from 248 to 388 bp among the taxa of Aurantioideae, and from 363 to 383 bp among the outgroups. Multiple sequence alignment of Aurantioideae and all outgroups resulted in a matrix of 965 characters (512 characters in the intron, 453 in the spacer), of which 335 (35%) included at least one accession with a gap [100 of 512 positions (19%) in the intron, 235 of 453 positions (51%) in the spacer]. Unweighted pairwise sequence divergence among species of Aurantioideae ranged from 0 to 3.5% in the intron and from 0 to 7.4% in the spacer; that between species of Aurantioideae and the outgroups ranged from 4.6% to 7.8% in the intron and from 7.0% to 17.0% in the spacer. A total of 37 gaps were required for proper alignment of the *trnL-trnF* sequences. These gaps ranged from 1 to 27 bps, the average size being about 5–9 bps. Sixteen gaps were scored as binary characters. Regions excluded because of missing data and/or homoplasy concerned positions 552–575, 678–690, 840–862, and 945–965. Mean G + C content was 37% in the intron and 39% in the spacer.

Of the 965 positions constituting the aligned *trnL-trnF* sequences, 238 (24.7%) were variable and 64 (6.6%) were parsimony-informative. The analysis recovered 609 equally optimal trees of 91 steps (CI = 0.79, RI = 0.82; Fig. 1).

The Aurantioideae were supported as monophyletic in the strict consensus of these trees (bootstrap support BS 100). The polytomy within the subfamily clade included four minor clades. The first of these clades, containing some members of the tribe Citreae, subtribe Balsamocitrinae, consisted of *Feroniella* + *Feronia* (BS 71). The second clade consisted of taxa from the subtribe Citrinae (*Severinia* + *Atalantia*; BS 98). The third clade was unresolved, containing members

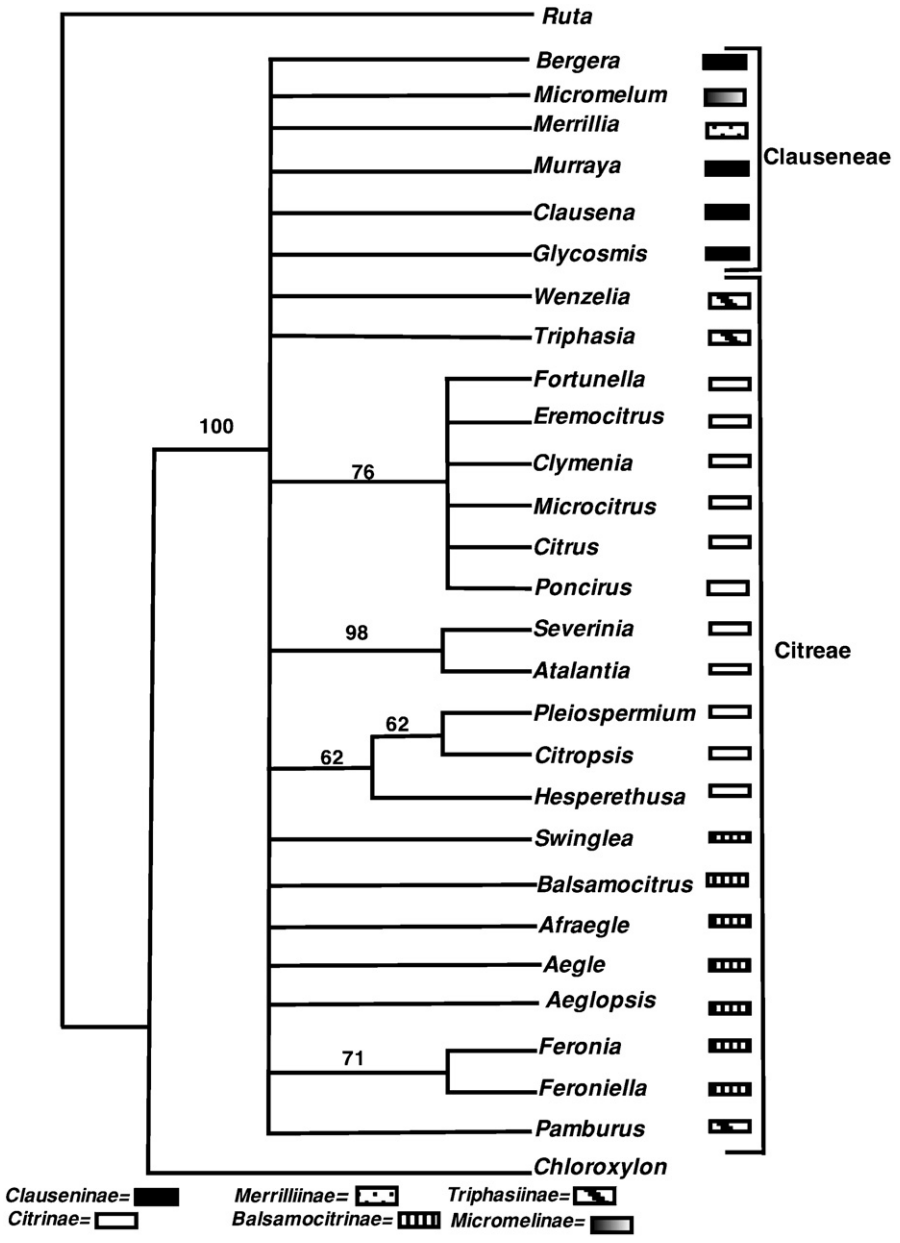

Fig. 1. Phylogeny of subfamily Aurantioideae reconstructed from *trnL-trnF* data; strict consensus of 609 most-parsimonious trees (tree length = 91 steps, CI = 0.79, RI = 0.82). Numbers above branches are bootstrap values. Current subtribe and tribe assignments indicated at bottom.

of the Citreae, subtribe Citrinae (*Citrus*, *Clymenia*, *Eremocitrus*, *Fortunella*, *Microcitrus* and *Poncirus*; BS 76). The fourth clade contained *Pleiospermium* + *Citropsis* as sister to *Hesperethusa* (BS 62), from the subtribe Citrinae. The remaining taxa were interdigitated among the unresolved polytomy in Aurantioideae.

rps16

The length of the *rps16* ranged from 811 to 914 bp among species of Aurantioideae, and from 851 to 857 bp among the outgroups. Multiple sequence alignment of Aurantioideae and all outgroups resulted in a matrix of 978 characters, of which 281 (29%) include at least one accession with a gap. Unweighted pairwise sequence divergence among species of Aurantioideae ranged from 0% to 7.0%; that between species of Aurantioideae and the outgroups ranged from 1.4% to 11.0%. Approximately 30 gaps were required for proper alignment of the *rps16* sequences. These gaps ranged from 1 to 36 bp, the average size being about 6 bp. Eight gaps were scored as binary characters. Characters excluded

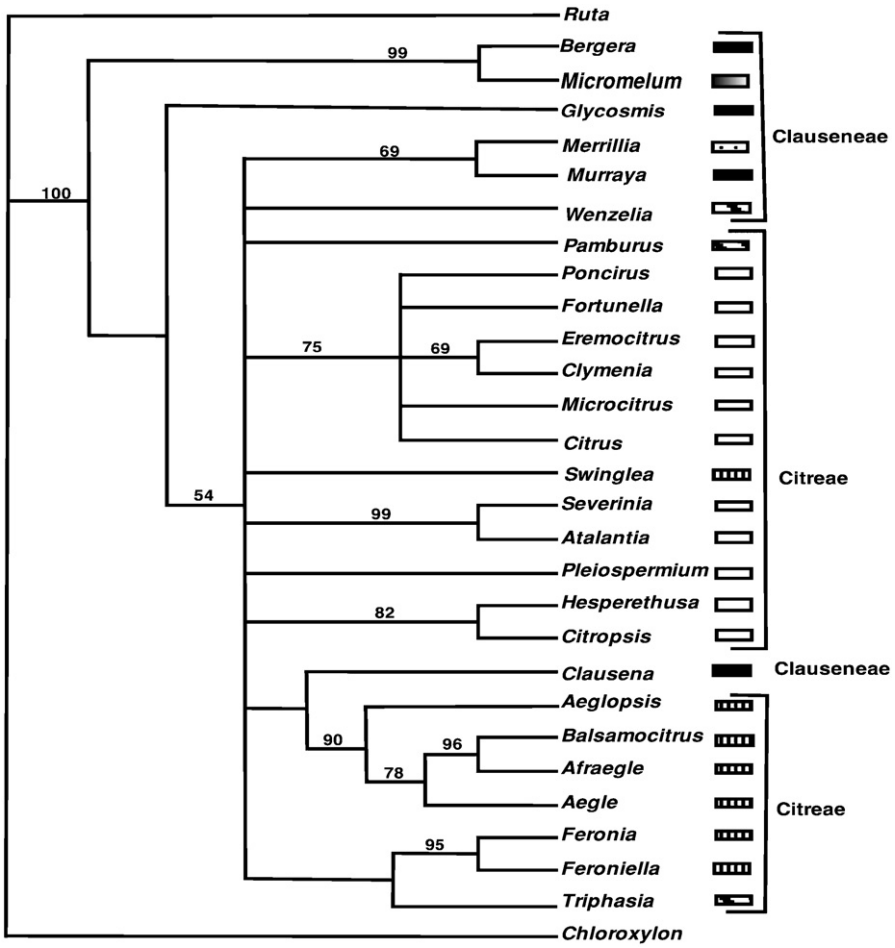

Fig. 2. Phylogeny of Aurantioideae reconstructed from *rps16* data; strict consensus of 2223 most-parsimonious trees (tree length = 155 steps, CI = 0.61, RI = 0.66). For further explanations, see Fig. 1.

because of missing data and/or homoplasy within these regions concerned positions 1–33, 109–120, 420–460, 740–769, and 935–978. Mean G + C content was 34%.

Of the 978 positions constituting the aligned *rps16* sequences, 314 (32.1%) were variable and 79 (8.0%) were parsimony-informative. The analysis recovered 2223 equally optimal trees of 155 steps (CI = 0.61, RI = 0.66; Fig. 2).

The Aurantioideae were supported as monophyletic in the strict consensus of these trees (BS 100). Within the Aurantioideae, *Bergera* + *Micromelum* (BS 99) from the subtribes Clauseninae and Micromelinae, respectively, was the first clade in the ingroup and sister to *Glycosmis* + a polytomy of the remaining taxa. Within the latter polytomy there were six clades. Three of these consisted of two-taxon clades: *Citropsis* + *Hesperethusa* (BS 82) and *Atalantia* + *Severinia* (BS 99) from the subtribe Citrinae and *Merillia* + *Murraya* (BS 69) from the subtribes Merrilliinae and Clauseninae, respectively. The fourth clade contained *Feroniella* + *Feronia* (BS 95) from the subtribe Balsamocitrinae as sister to *Triphasia* from the subtribe Triphasiinae. The fifth clade contained members of the tribe Citreae, subtribe Balsamocitrinae [*Afraegle* + *Balsamocitrus* (BS 96), *Aegle* (BS 78), and *Aeglopsis* (BS 90)], in an unsupported sister-group relationship with *Clausena* from the subtribe Clauseninae. The sixth clade was a polytomy consisting of members of the Citreae–Citrinae [*Clymenia* + *Eremocitrus* (BS 69), *Microcitrus*, *Citrus*, *Poncirus*, and *Fortunella* (BS 75)]. *Wenzelia*, *Pamburus*, *Swinglea*, and *Pleiospermium* were interdigitated among the subfamily.

atpB-rbcL spacer

The length of the *atpB-rbcL* spacer ranged from 549 to 615 bp among species of Aurantioideae, and from 587 to 603 bp among the outgroups. The total length contained a partial sequence of 166 bp from the *rbcL* gene. Multiple sequence alignment of Aurantioideae and all outgroups resulted in a matrix of 822 characters, of which 175 (21.3%) include at least one accession with a gap. Unweighted pairwise sequence divergence among species of Aurantioideae ranged from 0% to 10.3%, that between species of Aurantioideae and the outgroups

from 4.6% to 12.3%. Approximately eight gaps were required for proper alignment of the sequences. These gaps ranged from 1 to 12 bp, the average size being about 1–6 bp. Eight gaps were scored as binary characters. Characters excluded because of high homoplasy within these regions concerned positions 1–10, 66–87, 319–354, and 462–502. Mean G + C content was 35%.

Of the 822 positions constituting the aligned sequences, 238 (29.0%) were variable and 93 (11.3%) were parsimony-informative.

The analysis recovered 1080 equally optimal trees of 144 steps (CI = 0.79, RI = 0.78; Fig. 3). The Aurantioideae were supported as monophyletic in the strict consensus of these trees (BS 100). The ingroup topology was a polytomy including seven multi-taxon clades. Five of these consisted of two-taxon clades: *Citropsis* + *Hesperethusa* (BS 98) and *Atalantia* + *Severinia* (BS 99) from the subtribe Citrinae; *Feroniella* + *Feronia* (BS 94) from the subtribe Balsamocitrinae; *Murraya* + *Pamburus* (BS 59) from the respective subtribes Clauseninae and Triphasiinae; and *Bergera* + *Micromelum* (BS 100) from Clauseninae and Micromelinae. The fourth and fifth clades were polytomies containing members of Balsamocitrinae [*Aegle*, *Aeglopsis*, *Afraegle*, and *Balsamocitrus* (BS 98)] and Citrinae [*Citrus*, *Clymenia*, *Eremocitrus*, *Fortunella*, *Microcitrus*, and *Poncirus* (BS 72)], respectively.

ITS

The length of the ITS region ranges from 651 to 746 bp among species of Aurantioideae, and measures 698 bp within the outgroup. The length of the ITS1 ranges from 260 to 316 bp, that of the 5.8S subunit from 164 to 166 bp, and that of ITS2 from 180 to 280 bp among the taxa of Aurantioideae. Multiple sequence alignment of Aurantioideae and all outgroups resulted in a data matrix of 759 characters, of which 246 (32%) include at least one accession with a gap [118 of 300 positions (39%) in ITS1, two of the 166 positions (1%)

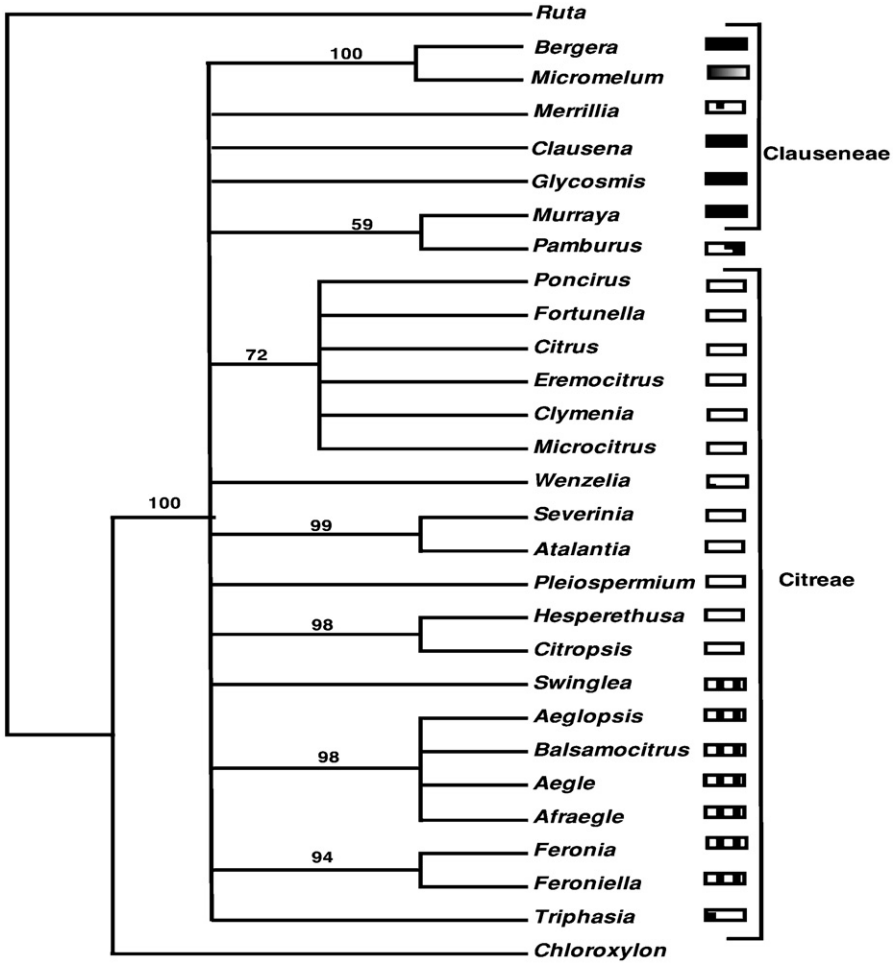

Fig. 3. Phylogeny of Aurantioideae reconstructed from *atpB-rbcL* spacer data; strict consensus of 1080 most-parsimonious trees (tree length = 144 steps, CI = 0.79, RI = 0.78). For further explanations, see Fig. 1.

in the 5.8S subunit, and 132 of 293 positions (45%) in ITS2]. Unweighted pairwise sequence divergence among species of Aurantioideae ranges from 1% to 31% in ITS1, from 2% to 37% in ITS2, from 3% to 35% in ITS1 + ITS2, and from 0% to 15% in the 5.8S subunit.

Of the 759 positions constituting the aligned ITS sequences, 453 (59.6%) were variable and 252 (33.2%) were parsimony-informative. A total of approximately 20 gaps were required for proper alignment of the ITS sequences. These gaps ranged from 1 to 19 bp, the average size being about 1–2 bp. Twelve gaps were scored as binary characters. Regions excluded because of missing data and/or homoplasy within these regions concerned positions 1–2 and 750–759. Mean G + C content is 64%.

The analysis recovered 196 equally optimal trees of 866 steps (CI = 0.49, RI = 0.47; Fig. 4). The Aurantioideae were supported as monophyletic in the strict consensus of these trees (BS 100). Within the Aurantioideae clade there were four multi-taxon clades. The first such clade contained members of the Citreae, subtribe Citrinae [*Citrus*, *Clymenia*, *Eremocitrus*, *Fortunella*, *Microcitrus*, and *Poncirus* (BS 76)]. The second clade contained members of the Clauseneae, subtribes Merrilliinae, and Clauseninae [*Merrillia* + *Glycosmis* (BS 98)]. The third clade contained taxa from the tribe Citreae, subtribe Balsamocitrinae [*Afraegle* + *Balsamocitrus* (BS 95), and *Aegle* (BS 65)]. The fourth clade contained *Feroniella* + *Feronia* also from the Balsamocitrinae (BS 93).

Combined molecular

Following the methods outlined by Mason-Gamer and Kellogg (1996) and applied by Eldenäs and Linder (2000), the individual datasets were considered as combinable. Many of the nodes were shared among the independent strict-consensus trees. In all four analyses the subfamily was monophyletic with 100%

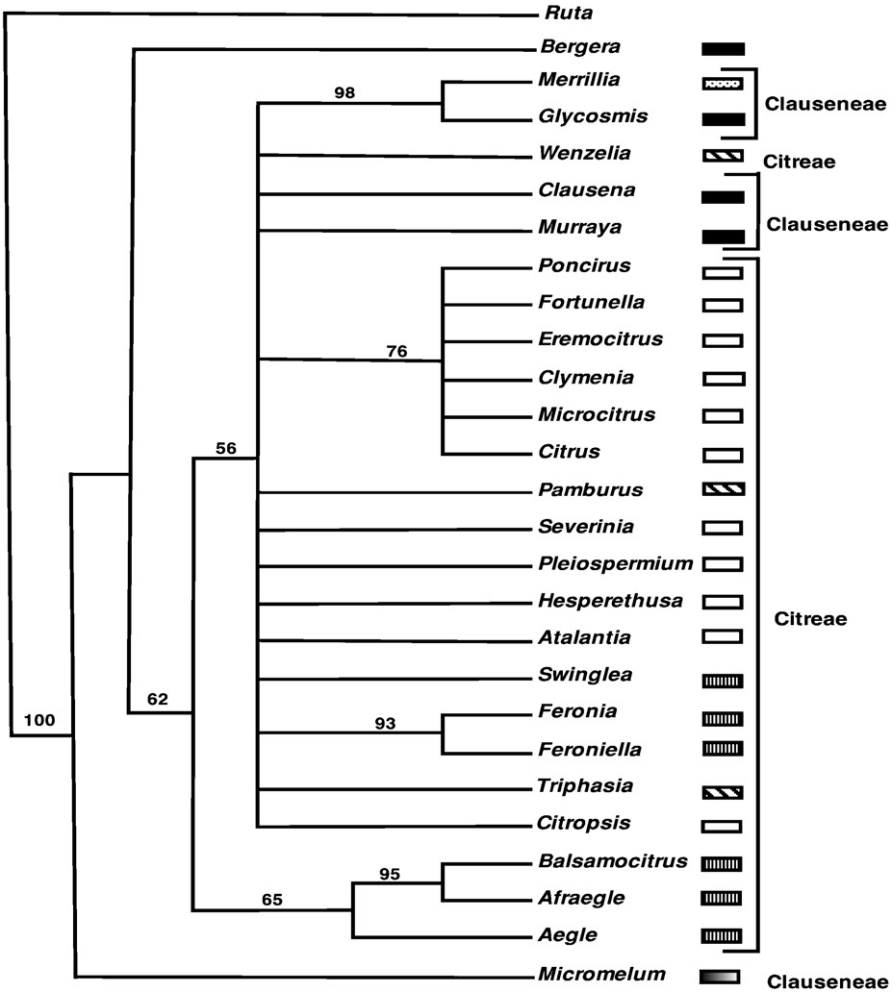

Fig. 4. Phylogeny of Aurantioideae reconstructed from ITS data; strict consensus of 196 most-parsimonious trees (tree length = 866 steps, CI = 0.49, RI = 0.47). For further explanations, see Fig. 1.

bootstrap support. There are no potential hard conflicts among the four molecular datasets.

The length of the combined matrix ranged from 2487 to 3199 bp among species of Aurantioideae, and from 2435 to 3123 bp among the outgroups. Multiple sequence alignment of Aurantioideae and all outgroups resulted in a matrix of 3524 characters, of which 1037 (29.5%) included at least one accession with a gap. Unweighted pairwise sequence divergence among species of Aurantioideae ranged from 1.0% to 8.6%, that between species of Aurantioideae and the outgroups from 5.6% to 13.3%. Mean G + C content was 41%.

Of the 3524 positions constituting the aligned sequences, 1236 (35.0%) were variable and 484 (13.7%) were parsimony-informative.

The analysis recovered 44 equally optimal trees of 2272 steps (CI = 0.70, RI = 0.51; Fig. 5). The Aurantioideae were supported as monophyletic in the strict consensus of these trees (BS 100). Within the Aurantioideae clade there were eight multi-taxon clades. The first such clade consists of *Bergera* + *Micromelum* from the respective subtribes Clauseninae and Micromelinae (BS 100). This clade resulted as sister to *Merrillia* + *Glycosmis* from the respective subtribes Merrilliinae and Clauseninae (BS 91), which latter clade was sister to *Clausena* + the remaining Aurantioideae. These remaining Aurantioideae consisted of a polytomy comprising *Wenzelia* of the subtribe Triphasiinae; a clade of taxa from the tribe Citreae, subtribes Balsamocitrinae and Triphasiinae [*Afraegle* + *Balsamocitrus* (BS 92), *Aegle*, and *Aeglopsis* (BS 100); and *Triphasia* (BS 99)]; and a clade containing the remaining taxa. The latter clade is mostly unresolved, comprising five subclades, four of which consisted of two-taxon clades: *Citropsis* + *Hesperethusa* (BS 91) and *Atalantia* + *Severinia* (BS 100) from the subtribe Citrinae; *Murraya* + *Pamburus* (BS 61) from the respective subtribes Clauseninae and Triphasiinae; and *Feroniella* + *Feronia* (BS 100) from Balsamocitrinae. The fifth clade contained members of the tribe Citreae, subtribe Citrinae [*Clymenia* + *Eremocitrus* + *Microcitrus* (BS 84); *Citrus*, *Fortunella*, and *Poncirus* (BS 100)].

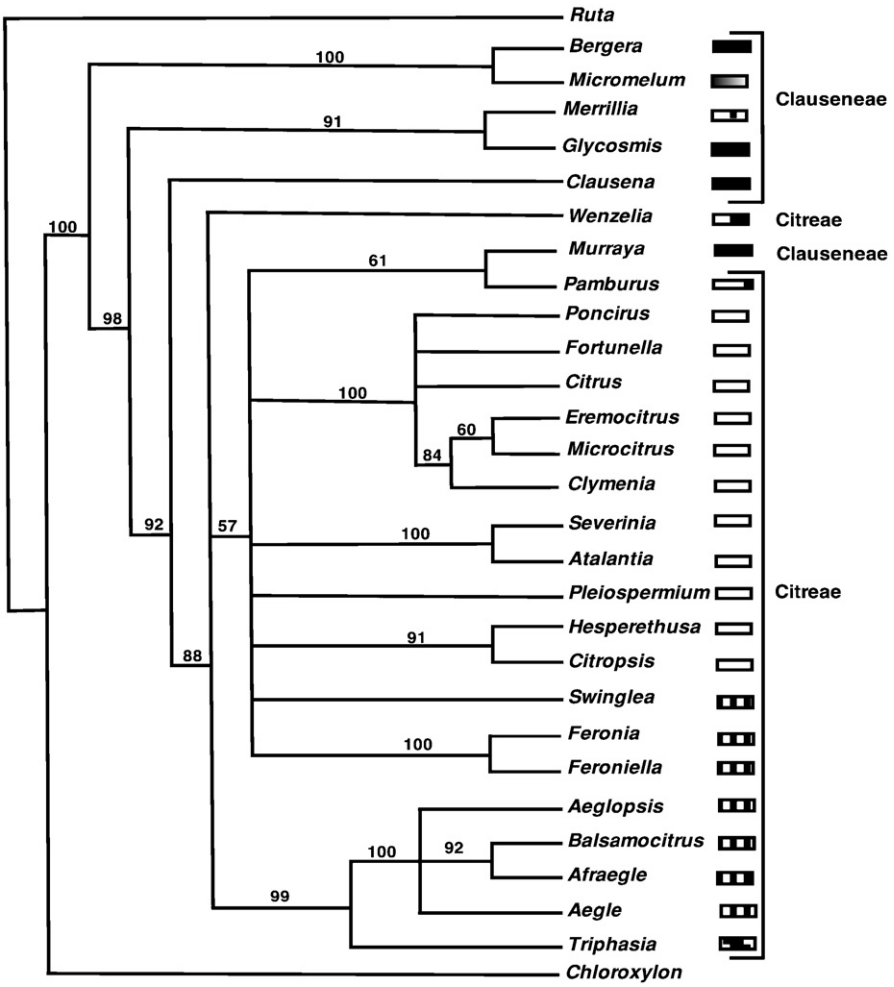

Fig. 5. Phylogeny of Aurantioideae reconstructed from the combined molecular datasets; strict consensus of 44 most-parsimonious trees (tree length = 2272 steps, CI = 0.70, RI = 0.51). For further explanations, see Fig. 1.

Non-molecular

In the non-molecular data matrix, 13.1% of the cells contained missing values. Of the 20 characters constituting the non-molecular dataset, 19 were variable and 17 (85%) were parsimony-informative.

The analysis recovered 6776 equally optimal trees of 83 steps (CI = 0.40, RI = 0.63). The Aurantioideae were monophyletic in the strict consensus of these trees (BS 77). The ingroup topology was completely unresolved.

Molecular and non-molecular data

Following the methods outlined by Mason-Gamer and Kellogg (1996) the molecular and non-molecular datasets contained no hard conflicts and were considered as combinable.

Of the 3544 positions constituting the datasets, 1236 (34.9%) were variable and 502 (14.2%) were parsimony-informative.

The analysis recovered 49 equally optimal trees of 1426 steps (CI = 0.53, RI = 0.51). The topology was similar to that obtained from the combined molecular dataset, but with better resolution. The Aurantioideae were supported as monophyletic in the strict consensus of these trees (BS 100). Within them, the first small clade branching off contains *Bergera* + *Micromelum* from the respective subtribes Clauseninae and Micromelinae (BS 100). The successive next branches off of the respective larger remainder are *Merrillia* + *Glycosmis* from the subtribes Merrilliinae and Clauseninae (BS 91), then *Clausena* from Clauseneae, then *Wenzelia* from the subtribe Triphasiinae. Within the sister group to *Wenzelia*, the first clade comprises taxa from Citreae, subtribes Balsamocitrinae and Triphasiinae [*Afraegle* + *Balsamocitrus* (BS 92), *Aegle* (BS 61), *Aeglopsis* (BS 100), and *Triphasia* (BS 89)]. Within the sister group to the *Afraegle* clade, *Swinglea* from the subtribe Balsamocitrinae is sister to a clade containing two major subclades. The first of thes subclades is unresolved, consisting of three clades (BS 61): (*Citropsis* + *Hesperethusa* (BS 82) and *Atalantia* + *Severinia* (BS 100)) from the subtribe Citrinae; *Murraya* + *Pamburus* from the respective subtribes Clauseninae and Triphasiinae; and *Pleiospermium* from Citrinae. The second remaining subclade consists of *Feroniella* + *Feronia* from Balsamocitrinae (BS 100) as sister to a clade containing members of Citreae, subtribe Citrinae [*Clymenia* + *Eremocitrus* + *Microcitrus* (BS 84), and *Citrus* + *Fortunella* + *Poncirus* (BS 74); (BS 100)].

Discussion

Utility of the four genes in the Aurantioideae

The respective numbers of variable and potentially phylogenetically informative characters in each dataset, the consistency indices and numbers of branches with bootstrap support can be found in Table 5. The number of informative characters ranged from 64 to 250; 64 were found in the *trnL-trnF* intron, 79 in the *rps16* intron, 93 in the *atpB-rbcL* spacer region, and 252 characters in the ITS region. In general, CI and RI values were lower when the number of informative characters was larger, indicating that the amount of homoplasy increased with the number of informative characters. For example, the *trnL-trnF* region has 64 informative characters and a CI of 0.79, whereas the ITS contains 252 informative characters with a CI of 0.47. The *rps16* had more branches with significant bootstrap values (9 BS > 70%), whereas the *atpB-rbcL* spacer had seven branches and the ITS gene had five. The *trnL-trnF* region had the fewest branches, four, with significant bootstrap support. Based on the number of informative characters and the number of branches with supported resolution, both the *rps16* and the *atpB-rbcL* spacer genes would make excellent candidates for a higher-level analysis. In addition, both genes produced very few alignment difficulties at the subfamilial and higher levels using the outgroup taxa as indicators. Although cloning

Table 5. Comparison of results from the various datasets.

Dataset	Total/informative characters	CI	RI	BS	BS > 70%
trnL	965/64	0.79	0.82	6	4
rps16	978/79	0.61	0.66	12	9
atpB-rbcL spacer	822/93	0.79	0.78	8	7
ITS	759/252	0.49	0.47	8	5
Combined molecular	3,524/448	0.70	0.51	17	14
Non-molecular	20/17	0.40	0.63	1	1
Total	3,544/502	0.53	0.51	17	15

CI = consistency index; RI = retention index; BS = number of branches receiving bootstrap support; BS > 70% = number of branches receiving bootstrap support greater than 70%.

was necessary in the ITS gene in order to separate the pseudogenes from the true genes, the tree topology remained consistent with the various chloroplast-derived topologies. This indicates that hybridization or polyploidization has not confounded the use of ITS for phylogeny reconstruction.

Circumscription of the Aurantioideae

The Aurantioideae clade was supported in both independent and combined analyses of molecular and non-molecular data. These results are in agreement with the previous results reported by Chase et al. (1999), Scott et al. (2000), Samuel et al. (2001), and Morton et al. (2003).

The current study examined 20 non-molecular characters, including vegetative, floral, biochemical, and chromosomal features. Most characters that needed re-evaluation from the previous study by Morton et al. (2003) were coded and included in the present one. Only one character, the basic chromosome number of $X = 9$, provided a synapomorphy for the subfamily. Many of the morphological characters (e.g. flowers white, fruit with pulp vesicles) that had been used to define the subfamily were found to be homoplasious. This study confirms the need for new morphological characters providing synapormorphies for classification at the subfamilial level. Other characters more recently suggested as potential subfamilial characters, such as the presence of coumarins, were homoplasious, too. Many of the characters traditionally used to describe the subfamily, such as that the seed is without endosperm, could not be used in the cladistic analysis because of the lack of information and the lack of plant material for examination.

Circumscriptions of the tribes

The majority-rule consensus of the combined non-molecular and molecular datasets indicates a lack of support for the two tribes Clauseneae and Citreae (Fig. 6).

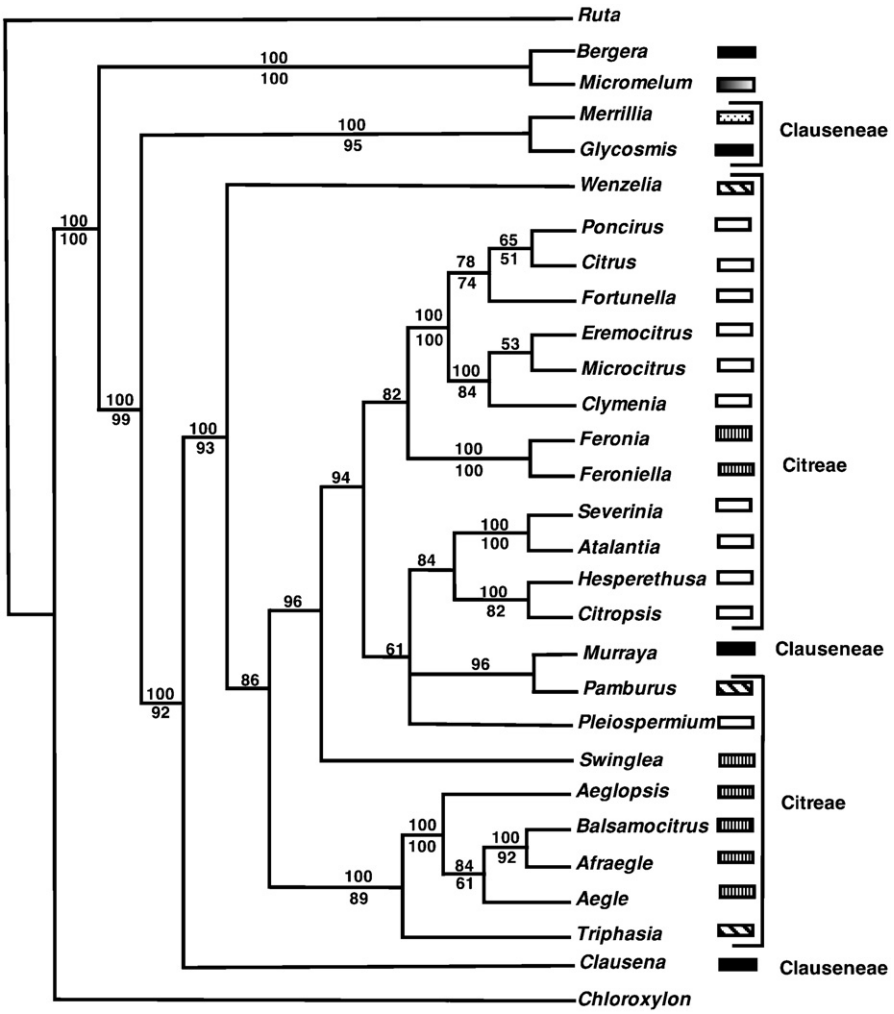

Fig. 6. Phylogeny of Aurantioideae reconstructed from the combined molecular and non-molecular datasets; majority-rule consensus of 49 most-parsimonious trees (tree length = 1426 steps, CI = 0.53, RI = 0.51). For further explanations, see Fig. 1.

Swingle and Reece (1967) believed that Clauseneae contained more unspecialized genera. Five out of the six genera (*Bergera*, *Clausena*, *Glycosmis*, *Merrillia*, and *Micromelum*) belonging to the tribe Clauseneae are placed outside of the clade representing the remainder of the subfamily, therefore provide some support to Swingle and Reece's idea. However, the position of *Murraya* from Clauseneae as sister to *Pamburus* from Citreae renders these two tribes non-monophyletic. Morphological characters used by Swingle and Reece (1967) to delimit the two tribes are revealed as failing by thorough examination of the majority-rule topology from the combined non-molecular and molecular data. Features used to distinguish Clauseneae from Citreae were mostly: (1) leaves odd-pinnate vs. mostly simple, unifoliolate or trifoliolate; (2) axillary spines lacking vs. present in most species; (3) rachis not winged vs. winged; (4) ovary with 2–5 locules vs. 2–20 locules; and (5) each locule with 1 or 2 ovules vs. 1–18 ovules. In the present analysis, all of these characters repeatedly showed transitions from one state to another; none were solely unidirectional.

The presence of a winged rachis, presence and number of thorns, numbers of the floral organs (stamens, locules, and ovules), development of the pulp vesicles, and the texture of the rind of the fruits were also used in part in the tribal classification by Tanaka (1936). Concerning Tanaka's eight tribal groups, only one of the seven examined, Atalantieae containing *Atalantia* + *Severinia*, was monophyletic in the present analysis.

This investigation demonstrates the need for additional morphological features to provide synapomorphies for these new groupings.

Circumscriptions of the subtribes

The strict-consensus trees from the independent, combined molecular, and combined non-molecular plus molecular datasets show poorly resolved topologies, thus do not allow conclusive evaluation of the subtribal delimitations in Swingle and Reece's (1967) classification. Therefore, the majority-rule tree from the combined molecular and non-molecular dataset is used to discuss the subtribal relationships (Fig. 6). None of the six subtribes are monophyletic. *Bergera* from the Clauseninae forms a sister grouping with *Micromelum* from Micromelinae. *Merrillia* from Merrilliinae forms a sister grouping with *Glycosmis* from Clauseninae. Another member of the Clauseninae, *Murraya*, forms a clade with *Pamburus* from Triphasiinae. *Triphasia* from Triphasiinae forms a well-supported clade with taxa from the Balsamocitrinae. These four mixed clades indicate that the subtribes Balsamocitrinae, Clauseninae, Merrilliinae, Micromelinae, and Triphasiinae are not monophyletic. The same is suggested for Citrinae, members of which are mixed with taxa from Balsamocitrinae, Clauseninae, and Triphasiinae.

Swingle and Reece (1967) distinguished the three subtribes in Clauseneae by the petals being valvate or imbricate, by cotyledon thickness, size of flowers and fruits, the numbers of ovaries and ovules per locule, and by the respective appearance of exocarp and seed covering. In the present study four of these features, the numbers of ovaries and ovules per locule and the appearance of exocarp and seed covering, were coded and included in the combined analysis. All of these features were homoplasious and did not provide synapomorphies.

Fruit size, texture of the exocarp, thickness of the exocarp, the presence of well-developed pulp vesicles, and the number of stamens had provided the basis for separating the subtribes of the Citrinae. The last three of these five features were coded and included in the combined analysis. All of the coded features for the Citrinae were homoplasious and once again did not provide synapomorphies.

Four characters from a palynological study by Grant et al. (2000) were coded and used in the present analysis. None of the pollen features provided synapomorhies.

Swingle and Reece (1967) divided the subtribe Citrinae into three groups, the Primitive Citrus Fruit trees (*Severinia*, *Pleiospermium*, *Burkillanthus*, *Limnocitrus*, and *Hesperethusa*), the Near-Citrus Fruit trees (*Citropsis* and *Atalantia*), and the True Citrus Fruit trees (*Fortunella*, *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus*, and *Citrus*). Only the True Citrus Fruit trees formed a monophyletic group. In addition, Swingle and Reece (1967) suggested a close relationship between *Citropsis* and *Herperethusa*; this has been confirmed by Morton et al. (2003) and the present study. The members of both genera have odd-pinnate leaves with winged petioles and rachis segments, small fruits, and large seeds.

Swingle and Reece (1967) divided the subtribe Balsamocitrinae into three groups, the Bael-Fruit Group (*Aegle*, *Aeflopsis*, *Afraegle*, and *Balsamocitrus*), the Tabog Group (*Swinglea*), and the Wood-Apple Group (*Feronia* and *Feroniella*). The present combined analysis finds the tribe Balsamocitrinae to be non-monophyletic, but supports monophyly of the Bael-Fruit Group, with *Aeglopsis* as sister to *Aegle* + (*Afraegle* + *Balsamocitrus*). The Wood-Apple group (*Feronia* + *Feroniella*) is monophyletic and sister to the True Citrus Fruit Trees. Two of the three groups belonging to the tribe Balsamocitrinae form monophyletic clades, whereas the third group, Tabog (*Swinglea*), remains unresolved.

Note on the circumscription of *Murraya*

Murraya was divided into two groups by Tanaka (1936), the large-flowered group as section *Murraya* and

the small-flowered group as section *Bergera*. Based on that study as well as on But et al. (1988) and Samuel et al. (2001), the genus *Murraya* s.l. is not monophyletic. *Bergera* is sister to *Micromelum*. In both genera the plants contain carbaxoles, whereas the latter are absent from *Murraya* s.s. and *Merrillia* (Kong et al. 1986, 1988a, b).

Conclusions

Aurantioideae as traditionally circumscribed is monophyletic; this is supported by one synapomorphy, the basic chromosome number of $X = 9$. The two tribes Citreae and Clauseneae are not monophyletic; neither is any of the six sampled subtribes. The Balsamocitrinae Bael-Fruit Group (with *Aeglopsis* sister to *Aegle* + (*Afraegle* + *Balsamocitrus*)), along with *Triphasia* from the Citreae, could comprise a newly circumscribed Balsamocitrinae. The Citrinae True Citrus Fruit trees group could comprise a newly circumscribed Citrinae. The genus *Murraya* is not monophyletic.

The previous study by Morton et al. (2003) recommended studying more genera, including more genes especially from the nuclear region, and using coded anatomical and morphological characters in the cladistic analysis before proceeding to any proposal of taxonomic alignment. Consequently, the present study has included more genera, an additional chloroplast gene and a nuclear one, and 20 non-molecular (18 morphological) characters. The phylogenetic analysis presented here provides the first study within the Aurantioideae using both chloroplast and nuclear datasets and also a non-molecular dataset. The utility of the additional chloroplast and nuclear genes has significantly increased the number of informative characters, from 143 based on the *trnL-F* and *rps 16* genes to 341 when *atpB-rbcL* spacer and ITS genes are included. That increase has provided additional support to various branches. Based on the ease of alignment and the number of branches with supported resolution, the conclusion is that both the *rps 16* and the *atpB-rbcL* spacer genes would make excellent candidates for a higher-level analysis. Topics to be addressed in a future study include determining the tribal and subtribal grouping and using additional taxa and genes to elucidate the biogeographic history of the subfamily.

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