

A MOLECULAR PHYLOGENY OF THE ORANGE SUBFAMILY (RUTACEAE: AURANTIOIDEAE) USING NINE cpDNA SEQUENCES¹

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The breeding of new, high-quality citrus cultivars depends on dependable information about the relationships of taxa within the tribe Citreae; therefore, it is important to have a well-supported phylogeny of the relationships between species not only to advance breeding strategies, but also to advance conservation strategies for the wild taxa. The recent history of the systematics of *Citrus* (Rutaceae: Aurantioideae) and its allies, in the context of Rutaceae taxonomy as a whole, is reviewed. The most recent classification is tested using nine cpDNA sequence regions in representatives of all genera of the subfam. Aurantioideae (save *Limnocitrus*) and numerous species and hybrids referred to *Citrus* s.l. Aurantioideae are confirmed as monophyletic. Within Aurantioideae, tribe Clauseneae are not monophyletic unless *Murraya* s.s. and *Merrillia* are removed to Aurantieae. Within tribe Aurantieae, the three traditionally recognized subtribes are not monophyletic. Triphasiinae is not monophyletic unless *Oxanthera* is returned to *Citrus* (Citrinae). Balsamocitrinae is polyphyletic. *Feroniella*, traditionally considered allied closely to *Limonia* (= *Feronia*), is shown to be nested in *Citrus*. The proposed congenericity of *Severinia* and *Atalantia* is confirmed. The most recent circumscription of *Citrus* is strongly supported by this analysis, with hybrids appearing with their putative maternal parents. The genus was resolved into two clades, one comprising wild species from New Guinea, Australia, and New Caledonia (formerly *Clymenia*, *Eremocitrus*, *Microcitrus*, *Oxanthera*), but surprisingly also *Citrus medica*, traditionally believed to be native in India. The second clade is largely from the Asian mainland (including species formerly referred to *Fortunella* and *Poncirus*).

Key words: Aurantioideae; Citreae; Citrus; Clauseneae; cpDNA; molecular systematics; phylogeny; Rutaceae.

Rutaceae comprise some 158 genera and 1900 species (Mabberley, 2008). The family, Rutaceae (order Sapindales) is subcosmopolitan with major centers of diversity in southern Africa and Australia. Species of Rutaceae are distinctive and often easily recognizable because of their often opposite, usually compound, pellucid-punctate leaves; cymose inflorescences, often pachypetalous flowers with stamens with thick filaments often forming a ring, and ovaries with a conspicuous disc at the base and an expanded stigma. Although the fruits are varied, most have a glandular-punctate pericarp, with some being fleshy and indehiscent, while others are dry and dehiscent to reveal shiny black seeds. Engler (1896, 1931) produced the only comprehensive monograph of the family within the last century using gynoecial and fruit characteristics to recognize seven subfamilies: Rhabdodendroideae, Aurantioideae, Flinder-

sioideae, Spathelioideae, Dictyolomatoideae, Rutoideae, and Toddalioideae. Late twentieth-century classifications (Hutchinson, 1973; Takhtajan, 1997; Dahlgren, 1989; Thorne, 1992; Cronquist, 1993) have excluded Rhabdodendroideae from Rutaceae, now recognized as Rhabdodendraceae (Caryophyllales; APG, 1998), whereas the other six subfamilies (not always with the same circumscriptions) have been largely retained. Of recent arrangements, Hutchinson's classification (1973) was the most distinctive in that he recognized only four subfamilies: Rutoideae (including *Flindersia*), Toddalioideae, Rhabdodendroideae, and Aurantioideae, omitting *Spathelia* (Spathelioideae) and *Dictyoloma* (Dictyolomatoideae) without a stated justification. Takhtajan (1997) recognized the six subfamilies, while Thorne (1992) merged Toddalioideae with Rutoideae, making five, but neither Dahlgren (1989) nor Cronquist (1993) recognized any subfamilies.

The most recent higher-level classification of Rutaceae is that of Thorne (2000), who recognized three subfamilies: Rutoideae (including *Chloroxylon*, *Flindersia*, *Luvunga*, Toddalioideae; 120 genera), Aurantioideae (30 genera), and Spathelioideae (including Dictyolomatoideae; five genera); he excluded Rhabdodendroideae as a distinct family.

In the Thorne (2000) system, the largest groups within Rutaceae are Aurantioideae and Rutoideae (including Toddalioideae). In this paper, we are concerned with the phylogenetic relationships of the members of the citrus subfamily, Aurantioideae. Aurantioideae sensu Engler comprise 33 genera and 210 species native to the Old World tropics. Engler distinguished them by their syncarpous ovary with one or two, sometimes several, ovules per carpel; their indehiscent, fleshy fruits with a soft, parenchymatous pericarp bearing schizolysigenous oil glands (sometimes with a hard exocarp, as in *Aegle* and *Swinglea*); often with pulp vesicles in the locules; and the

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endosperm lacking; seeds often with two or more embryos. Aurantioideae have almost always been included in Rutaceae, but there has been disagreement over tribal composition. Engler (1931) recognized a single tribe (Aurantieae, i.e., Citreae), Tanaka (1936) eight (Micromeleae, Clauseneae, Aegleae, Lavangeae [i.e., Luvungeae], Aurantieae, Meropeae, Atalantieae, and Microcitreae), and Swingle (1943) and Swingle and Reece (1967) two (Clauseneae and Citreae, i.e., Aurantieae) (Table 1).

The most recent comprehensive treatment of the Aurantioideae (Swingle and Reece, 1967), has tribe Citreae, i.e., Aurantieae, divided into three subtribes, Balsamocitrinae, Citrinae, and Triphasiinae (Tables 1 and 2; taxa used Appendix 1). Swingle and Reece's (1967) subtribe Citrinae (Table 2) comprises about 13 genera and 65 species of trees and shrubs that are almost exclusively native to the Indopacific, including China and Australia. The species of this subtribe are unique in bearing fruits with pulp vesicles filling all the space in the locules of the fruit not occupied by seeds. These pulp vesicles are the basis for the most important tree fruit industry in subtropical countries (Spiegel-Roy and Goldschmidt, 1996) because, in some species and hybrids of *Citrus*, they contain juice of great commercial importance. Closely related to members of subtribe Citrinae are Triphasiinae and Balsamocitrinae (Table 2). The Triphasiinae contains eight genera and ~46 species and is found in southeast Asia and Oceania. The seven genera and ~13 species comprising Balsamocitrinae are found in tropical Africa and Asia from India to Indo-China and the northern Philippines.

Citrus itself has been variously described as comprising from 1 to 162 species. Until recently, the most widely accepted classification was that of Swingle (1943; see also Swingle and Reece [1967]), who recognized 16 species. In that classification, several genera were considered closely related: *Clymenia* (clymenia), *Oxanthera* (oxanthera or false oranges), *Eremocitrus* (Australian desert lime), *Fortunella* (kumquats), *Microcitrus* (Australian wild limes), and *Poncirus* (trifoliolate orange)—see Swingle (1914, 1915a, 1915b, 1939) and Swingle and Reece (1967). Recent morphological and chemosystematic research has shown that this system is unworkable (Mabberley, 1998, 2001, 2002, 2004; Zhang and Mabberley, 2008) such that all of these genera, except *Clymenia* and *Oxanthera*, are already reincorporated in *Citrus*. Relationships among species and genera of the group are complicated by several factors such as a long

history of cultivation and wide cross-compatibility among species (Mabberley, 2004).

Although, citrus fruits are the most widely cultivated tropical tree fruits in the world, the evolutionary origins of many of the commercial classes of cultivars, i.e., lemons, oranges, limes and grapefruits, are unclear. As a consequence, it has been exceedingly difficult to produce new cultivars by traditional hybridization/selection techniques, with difficulty often being attributed to the high heterozygosity caused by the original interspecific crosses. Several studies, using a variety of different methods, have shown that the cultivated classes of citrus fruits have perhaps originated from crosses among four or five ancestral species (Barrett and Rhodes, 1976; Moore, 2001; Zhang and Mabberley, 2008)—*C. japonica* (kumquat), *C. maxima* (pomelo), *C. medica* (citron), *C. reticulata* (mandarin) and probably *C. hystrix* (Thai lime)—though it is conceivable that some wild ancestral species have become extinct since citrus fruits first became cultivated some millennia ago. Breeding of new, high-quality *Citrus* cultivars depends on reliable information about the relationships of species in genera within the tribe Citreae (Rutaceae). Wild Citreae may be expected to possess alleles that provide a number of beneficial traits, which may be useful in *Citrus* breeding programs. It is important to have a well-supported phylogeny of the intertribal relationships between species, not only to advance breeding strategies but also to develop conservation strategies for the wild taxa.

Previous attempts to investigate relationships in Aurantioideae (Herrero et al., 1996; Fang et al., 1998; Federici et al., 1998; Nicolosi et al., 2000; Abkenar et al., 2004; Pang et al., 2007) have been constrained by restricted taxon representation, dependence on a few inferred sequences (RFLPs), or reliance on potentially unsound genetic markers for phylogenetic analysis such as isozymes, AFLPs, ISSRs, or RAPDs. Other investigations based on DNA sequencing, although including representatives of the subfamily, focused at higher taxonomic levels such as at order (Sapindales [Gadek et al., 1996]) and family (Chase et al., 1999; Poon et al., 2007; Groppo et al., 2008). Here we expand upon the studies of Samuel et al. (2001), Araújo et al. (2003) and Morton et al. (2003) by including considerably more base-pair sequence data and larger, more comprehensive, taxon representation concentrated on *Citrus*. Except for *Limnocitrus*, all recently recognized genera of Aurantioideae are represented, and 10 043 aligned bp of cpDNA sequence are used to resolve the phylogeny of this economically important group of plants.

TABLE 1. Tribal and subtribal classifications of the three most recent circumscriptions of the Aurantioideae.

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

MATERIALS AND METHODS

Outgroup selection—Outgroup taxa were selected on the basis of the analyses of Gadek et al. (1996), Chase et al. (1999), Samuel et al. (2001), and Morton et al. (2003). Seven members of the extra-aurantioid Rutaceae (*Casimiroa edulis* La Llave and Lex., *Choisya ternata* Knuth, *Flindersia australis*, *Ruta graveolens*, *Skimmia anquetilia*, *Toddalia asiatica*, and *Zanthoxylum monophyllum*, as well as *Toona ciliata* [Meliaceae]) were thus chosen as the outgroup (Table 2).

Ingroup sampling—Tribal circumscriptions and nomenclature were based on Swingle and Reece's (1967) treatment of the Aurantioideae; for species names, the recent treatments of Mabberley (1997, 1998, 2002, 2004) and Zhang and Mabberley (2008) were followed (with Swingle and Reece's [1967] names, when different, in parentheses). All genera of Aurantioideae, except *Limnocitrus*, were sampled (Table 2). Within genera, 1–17 species were sequenced including interspecific hybrids of known parentage within *Citrus*. Because the focus of our study was the tribe Aurantieae, sampling was extensive such that

TABLE 2. Swingle and Reece's (1967) tribes, subtribes, and genera of the subfamily Aurantioideae.

Tribe	Subtribe	Genus/Genera
Clauseneae	Micromelinae (very remote citroid fruit trees)	<i>Micromelum</i>
	Clauseninae (remote citroid fruit trees)	<i>Clausena</i> , <i>Glycosmis</i> , <i>Murraya</i>
	Merrilliinae (large-fruited remote citroid fruit trees)	<i>Merrillia</i>
Citreae	Triphasiinae (minor citroid fruit trees)	<i>Luvunga</i> , <i>Merope</i> , <i>Monanthocitrus</i> , <i>Oxanthera</i> , <i>Pamburus</i> , <i>Paramignya</i> , <i>Triphasia</i> , <i>Wenzelia</i>
	Citrinae (citrus fruit trees)	<i>Atalantia</i> , <i>Burkillanthus</i> , <i>Citrus</i> , <i>Citropsis</i> , <i>Clymenia</i> , <i>Eremocitrus</i> , <i>Fortunella</i> , <i>Hesperethusa</i> (= <i>Naringi</i>), <i>Limnocitrus</i> , <i>Microcitrus</i> , <i>Pleiospermium</i> , <i>Poncirus</i> , <i>Severinia</i>
	Balsamocitrinae (hard-shelled citroid fruit trees)	<i>Aegle</i> , <i>Aeglopsis</i> , <i>Afraegle</i> , <i>Balsamocitrus</i> , <i>Feronia</i> , <i>Feroniella</i> , <i>Swinglea</i>

we sampled all known species of *Clymenia*, '*Microcitrus*' (i.e., *Citrus*), *Naringi*, '*Eremocitrus*' (i.e., *Citrus*) and '*Poncirus*' (i.e., *Citrus*), with good coverage of most other genera of the tribe (Table 2). Within *Citrus*, the five important pivotal, proposed ancestral species, *C. reticulata*, *C. medica*, *C. maxima* (Scora, 1975), *C. hystrix* (Nicolosi et al., 2000), and *C. japonica* (Mabberley, 1998), were sampled, as were the major cultivar groups of *Citrus* and known hybrids (Table 2). In most cases, fresh leaves or recently collected leaves preserved in silica gel were used for DNA extraction. Materials for extraction were largely obtained from the USDA Citrus Germplasm Repository in Riverside, California, USA and from the citrus germplasm collection of the CSIRO citrus breeding program at Merbein, Victoria, Australia. See Appendix 1 for a complete list of taxa used in this study, along with voucher or repository collection numbers and GenBank accession numbers.

DNA isolation, amplification, and sequencing—A total of 710 new sequences were generated for this study (Appendix 1). Total DNA was isolated as outlined in Bayer et al. (1996). Recalcitrant DNAs were purified with Qiaquick PCR Purification Columns (Qiagen, Doncaster, Victoria, Australia). The target sequences were amplified via the polymerase chain reaction (PCR) using *Taq* DNA polymerase. The PCR reaction mixture consisted of 10 μ L of 10 \times reaction buffer, 6 μ L of 25 mM magnesium chloride solution, 4 μ L of 1.25 mM dNTP solution in equimolar ratio, 20 pmol of each primer, 10–50 ng of template DNA, and 0.5 unit of polymerase, made up to a total volume of 100 μ L with mH_2O . The PCR samples were heated to 94°C for 3 min before the addition of DNA polymerase to denature unwanted proteases and nucleases. The double-stranded PCR products were produced via 30 cycles of denaturation (94°C for 1 min), primer annealing (48°C for 1 min), and extension (72°C for 2 min). A 7-min final extension cycle at 72°C followed the 30th cycle to ensure the completion of all novel strands.

Primer details are as follows: the *trnL-F* region was usually amplified as a single piece with Taberlet et al.'s (1991) primers "c" and "f" to amplify across the *trnL* intron and *trnL-trnF* spacer. In some instances, recalcitrant DNA was amplified as two separate regions with Taberlet's (Taberlet et al., 1991) primers "c" with "d" and "e" with "f". The *atpB-rbcL* spacer was amplified as a single piece using primers 2F and 5R (Table 3). Primers S2[F] and S1494R of Hoot et al. (1995) were standardly used to amplify the *atpB*-coding region, but, as with the *trnL-F* region, recalcitrant DNA was amplified as two separate regions using Hoot's (Hoot et al., 1995) primers S2[F] with S766R or S611[F] with S1494R (Table 3). The *rps16* intron was amplified with the *rpsF* and *rps2R* primers of Oxelman et al. (1997); however, if that did not succeed, then *rpsF* was used with *rps555R* and *rps555F* (C. Morton, unpublished primers; Table 3) with *rps2R*. Double-stranded PCR products were cleaned by column purification using Qiaquick PCR Purification Columns (Qiagen) before sequencing.

The double-stranded PCR products were then used as templates in cycle sequencing reactions employing the *trnL-F* primers "c" and "f" and "d" and "e". The *atpB-rbcL* spacer sequencing was conducted using primers 2F, 5R, 7R, and 8F (Table 3) of Manen et al. (1994) and 3F and 4F, which are Aurantioideae-specific primers designed by us for use on difficult-to-sequence templates (Table 3). For the sequencing of *rps16*, primers *rpsF* and *rps2R* were used, but construction of a contig sequence was often supplemented by sequences produced using *rps555F* and *rps555R* (Table 3). Primers SS2[F], S1494R, S611[F], and S766R (Table 3) were routinely used to sequence the *atpB*-coding region. The double-stranded PCR products were sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Wellesley, Massachusetts, USA) and an ABI 3730 capillary DNA Analyzer in the John Curtin School of Medical Research, Biomolecular Resource Facility, Australian National University. Sequencing reactions for all primers were produced by 25 cycles of denaturation (96°C for 10 s), primer annealing (57°C for 5 s)

and extension (60°C for 4 min) and cleaned using standard ethanol precipitation methods. Sequences were assembled using the program Sequencher version 4.2.2 (Gene Codes Corp., Ann Arbor, Michigan, USA). New sequences have been submitted to GenBank (Appendix 1).

Alignment of sequences proceeded by hand following the principles of non-coding sequence alignment discussed in Bayer et al. (2000). Gaps were inserted to maintain sequence homology. Consideration was given to the mutational mechanisms that may have resulted in the observed-length mutations. Indels were aligned and scored by the methods outlined in Simmons and Ochoterena (2000). This procedure was used to minimize the number of inferred length mutations, except where there was clear evidence that particular length mutation events were homogenetic.

Phylogenetic analysis—The final matrix (TreeBase matrix accession number M4245, available at <http://www.treebase.org>) comprised 10264 characters encoded for 88 taxa. After exclusion of ambiguously aligned regions, columns containing gaps due to indels and columns used to separate regions for convenience during alignment, 8115 characters remained. Columns containing gaps were eliminated due to possible detrimental effects caused by the inclusion of large numbers of missing values in the analysis (Freudenstein and Chase, 2001; Wiens, 1998). Indels were recoded as binary characters at the end of the matrix (using nucleotide characters), with the original positions excluded. We analyzed the matrix using the optimality criteria of equal-weight maximum parsimony (MP), and Markov chain Monte Carlo (MCMC) Bayesian analysis (BA; Yang and Rannala, 1997) with the software PAUP* version 4.10b (Swofford, 2002) and MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) respectively, with support for clades estimated using nonparametric bootstrap proportions (Felsenstein, 1985) and posterior probabilities.

The MP analysis was not used to search for globally optimal tree(s) alone, instead we only performed a bootstrap analysis to compare support from a parsimony perspective with the Bayesian posterior probabilities. The bootstrap analyses used 8115 characters in a single partition, as well as three separate analyses, comprising (1) protein-coding regions only, (2) tRNA coding and noncoding regions only, and (3) indels only. This same partitioning was used as the basis of a partition homogeneity test (Farris et al., 1995) to explore differences in phylogenetic signal among regions. The bootstrap analyses used 200 bootstrap replicates, with two random addition sequence replicates per replicate, holding a maximum of 100 trees per random addition sequence replicate. The same random addition sequence replicate settings were used for each of 100 partition homogeneity replicates.

The BA was conducted using a single partition, three partitions (as described), and four partitions, the last separating (1) protein coding, (2) tRNA coding and group 2 introns, (3) other noncoding, and (4) indel character partitions. The model used for the single partition analysis was HKY+I+G. The models used for the three partition analysis were (protein coding) HKY+I+G, (tRNA and noncoding) GTR+I+G, and (indels) a single substitution type +G but with the coding = variable setting to account for no invariable characters being sampled. These models were selected subjectively to reflect models commonly required to adequately model these kinds of data. For example, protein-coding regions typically require G and at least transitions vs. transversions to be distinguished in the rate matrix (e.g., Pfeil et al., 2005), whereas stem-loop containing regions such as tRNAs and noncoding regions (especially those with group II introns) can show variation in substitution types that suggest additional parameters are required in the rate matrix (Kelchner, 2002). Model choice may not be especially important here given the large amount of nucleotides analyzed, the low degree of variation and our predominant aim being to reconstruct the topology rather than goals that require a more precise model fit (Kelchner and Thomas, 2007). We tested the effect of complexity of model choice to some

TABLE 3. List of primer sequences and references used in this study.

Target sequence	Primer name	5'-3' primer sequence	Reference (if applicable)
<i>rps16</i>	rpsF	GTG GTA GAA AGC AAC GTG CGA CTT	Oxelman et al., 1997
<i>rps16</i>	rps555R	CCT TGT TCC AGG ATC CTT	C. Morton, unpublished data
<i>rps16</i>	rps555F	AAG GAT CCT GGA ACA AGG	C. Morton, unpublished data
<i>rps16</i>	rpsR2	TCG GGA TCG AAC ATC AAT TGC AAC	Oxelman et al., 1997
<i>atpB</i>	S2[F]	TAT GAG AAT CAA TCC TAC TAC TTC T	Hoot et al., 1995
<i>atpB</i>	S1494R	TCA GTA CAC AAA GAT TTA AGG TCA T	Hoot et al., 1995
<i>atpB</i>	S611[F]	AAC GTA CTC GTG AAG GAA ATG ATCT	Hoot et al., 1995
<i>atpB</i>	S766R	TAA CAT CTC GGA AAT ATT CCG CCAT	Hoot et al., 1995
<i>trnL-F</i>	c	CGA AAT CGG TAG ACG CTA CG	Taberlet et al., 1991
<i>trnL-F</i>	d	GGG GAT AGA GGG ACT TGA AC	Taberlet et al., 1991
<i>trnL-F</i>	e	GGT TCA AGT CCC TCT ATC CC	Taberlet et al., 1991
<i>trnL-F</i>	f	ATT TGA ACT GGT GAC ACG AG	Taberlet et al., 1991
<i>atpB-rbcL</i> spacer	2F	GAA GTA GTA GGA TTG ATT CTC	Manen et al., 1994
<i>atpB-rbcL</i> spacer	5R	TAC AGT TGT CCA TGT ACC AG	Manen et al., 1994
<i>atpB-rbcL</i> spacer	7R	CCC TAC AAC TCA TGA ATT AAG	Manen et al., 1994
<i>atpB-rbcL</i> spacer	8F	GAC ATG AGA GTT AAC AAC	Manen et al., 1994
<i>atpB-rbcL</i> spacer	3F		R. Bayer and C. Miller, unpublished
<i>atpB-rbcL</i> spacer	4F		R. Bayer and C. Miller, unpublished
<i>matK-5'trnK</i> spacer	matK5' R	GCA TAA ATA TAY TCC YGA AAR ATA AGT GG	Shaw et al., 2005
<i>matK-5'trnK</i> spacer	matK6	TGG GTT GCT AAC TCA ATG G	Johnson and Soltis, 1994
<i>rps4-trnT</i> spacer	trnTR	AGG TTA GAG CAT CGC ATT TG	Shaw et al., 2005
<i>rps4-trnT</i> spacer	rps4R2	CTG TNA GWC CRT AAT GAA AAC G	Shaw et al., 2005
<i>trnS-trnG</i> spacer- <i>trnG</i> intron	trn ^S G ^{CU}	AGA TAG GGA TTC GAA CCC TCG GT	Shaw et al., 2005
<i>trnS-trnG</i> spacer- <i>trnG</i> intron	3'trnG ^{UUC}	GTA GCG GGA ATC GAA CCC GCA TC	Shaw et al., 2005
<i>trnS-trnG</i> spacer- <i>trnG</i> intron	5'trnG2G	GCG GGT ATA GTT TAG TGG TAA AA	Shaw et al., 2005
<i>trnS-trnG</i> spacer- <i>trnG</i> intron	5'trnG2S	TTT TAC CAC TAA ACT ATA CCC GC	Shaw et al., 2005
<i>psbM-trnD^{GUC}</i> spacer	psbMF	AGC AAT AAA TGC RAG AAT ATT TAC TTC CAT	Shaw et al., 2005
<i>psbM-trnD^{GUC}</i> spacer	trnD ^{GUC} R	GGG ATT GTA GYT CAA TTG GT	Shaw et al., 2005 (modified from Demesure et al., 1995)

degree using our partitioning strategy as well as the use of more complex models for some partitions within the partitioned analyses. Our three BAs were compared using Bayes factors (as per Kass and Raftery, 1995). Additionally, we set the prior probability of the state frequencies for the indel partition to be that of the empirical state frequencies, to account for our use of only A/T nucleotides when encoding indels. The shape parameter, proportion of invariant sites and the state frequencies were unlinked across all relevant partitions. The models used for the four-partition analysis were identical to the three-partition analysis, except that both tRNA/group 2 intron and other noncoding partitions had separate GTR+I+G models applied (separate in the sense that the same parameters were now also unlinked across the additional partition).

The BA typically required between one and two days to complete for 1 000 000 generations, with samples taken every 1000 generations using paired runs for each BA, each with 10 chains, to allow a thorough search of possible parameters. We attempted to apply a codon model to the protein-coding partition but discovered that each analysis would require over a week using a parallel implementation of MrBayes (version 3.1.2) that utilizes 20 processors and was therefore not feasible. One such analysis was aborted after over 700 000 generations and gave similar results to other analyses (not shown).

Trees produced by Bayesian methods often have some short branch lengths with unexpectedly high posterior probabilities. Lewis et al. (2005) found this to be an artifact, in some cases, of the lack of posterior probability being assigned to zero length branches (e.g., when a hard polytomy exists). Baum et al. (2004) suggested a test of these branches using a likelihood ratio test, whereby the topology and branch lengths are constrained by the analysis results, except the branch of interest, which is set to zero length. The likelihood values of these trees are compared (using PAUP*, with the same model in maximum likelihood as was used to infer the tree in the original Bayesian analysis—in our case, we used the unpartitioned HKY+G analysis results) and a lack of significant difference indicates that the branch is not substantially different from zero length. Although branch length and support are not the same thing, this test is the only available way we are aware of for attempting to double check posterior probabilities of extremely short branches. We focused these tests on branches with uniformly high posteriors (across the three models) and low parsimony bootstrap support values. All original branch lengths explained the data significantly better than when set to zero in length at $P < 0.05$, and all but one at $P < 0.01$. Given the size of the data matrix, it is possible that the power of this test is very high and that significant differences between zero and nonzero con-

straints are not necessarily indicative of support. Therefore, to be conservative, we treated as confirmed only those branches that were different at $P < 0.01$ (see Fig. 1). The greater Bayesian posterior probabilities compared to parsimony bootstrap values is interpreted in these cases as being due to a better fit between model and data, and therefore the Bayesian results are accepted as the best estimates of relationship.

RESULTS

Size and structure statistics for the sequence data set—A summary of all statistics is presented in Table 4 wherein the data are reported for the nine individual sequence regions, all coding sequences together, all noncoding sequences together, and all sequences together (hereafter referred to as the combined data). The beginning and end of each sequence is referenced (Table 4) to the recently published aligned homologous region of the orange (*Citrus ×aurantium* L. [sweet orange group] ‘Ridge Pineapple’) genome (Bausher et al., 2006). A total of 710 new sequences of 792 (90%) were generated, the others being drawn from sequences generated for other studies and available on GenBank (Table 4). Only a small proportion of the data are missing values (1.04% for the combined data set), and these generally represent small runs of nucleotide positions at the ends of sequences that were too ambiguous to be called (Table 4). The *trnS-trnG* spacer data set has the largest percentage of missing data (2.19%) because it was often a considerable challenge to generate sequence for some taxa. The smallest percentage of missing data (0.16%) was within the *rps4-trnT* spacer data set. The range in the aligned length of the nine sequences is from 674 nucleotide positions (ntp) for the *rps4-trnT* spacer to 1558 ntps for the *trnD-psbM* spacer (Table 4). Among the noncoding sequences, the *trnS-trnG* spacer has the greatest proportion of gaps (37.5%), whereas the *rbcL-atpB* spacer has

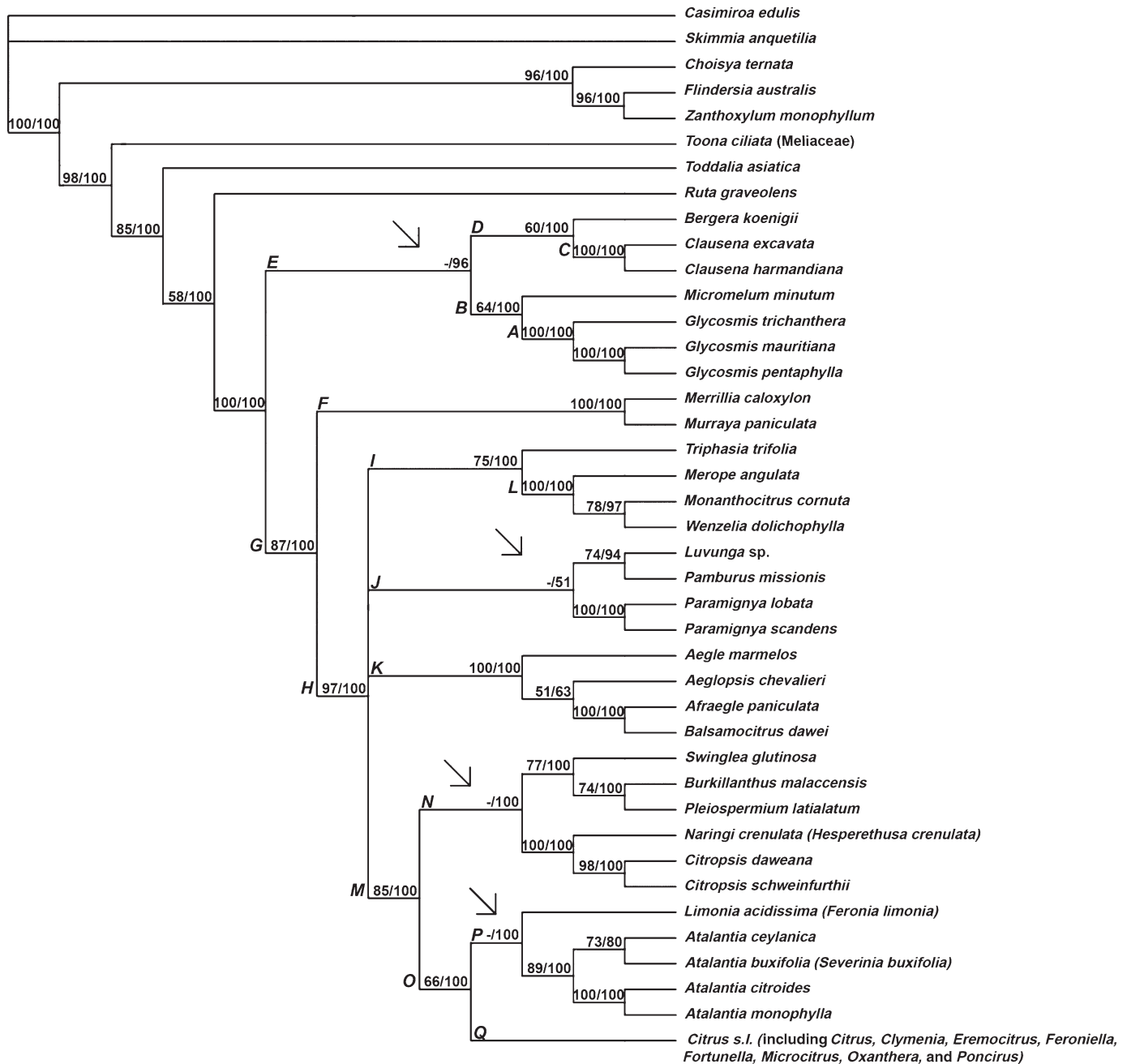


Fig. 1. Consensus tree with arbitrary rooting inferred from Bayesian analysis using three partitions comprising (1) protein-coding regions, (2) tRNA-coding and -noncoding regions, and (3) indels. Support values near branches are first, maximum parsimony bootstrap proportion percentage and, second, three-partition Bayesian analysis posterior probability expressed as a percentage. Letters indicate clades discussed in text. Part of the consensus tree showing relationships among subfamily Aurantioideae genera and outgroup taxa. The true root of the phylogeny probably lies with *Toona* (Meliaceae).

the least (13.9%). Long gaps are likely responsible for the above typical (23.4% for all noncoding sequences) percentage gapped positions for the *trnD-psbM* spacer and *trnS-trnG* spacers; e.g., a 739-bp deletion in the outgroup taxa in *trnD-psbM* spacer, a 495-bp deletion in *Citrus australasica* and *C. garrawayi* in the *trnS-trnG* spacer, and a 144-bp deletion in the ingroup taxa in *trnS-trnG* spacer. There are 10043 ntp in the combined data set (though only 8115 ntp were analyzed) of which 1766 (18%) are coding sequence (*atpB* and small segments of exons of *rbcl*, *matK*, and *rps4* at the tail ends of the intron and spacer se-

quences) and 8277 (82%) are noncoding. The noncoding regions have a GC content (33.2%) that is lower than the GC content of coding regions (44.0%), comparing favorably to the relatively narrow range in GC content (36–39%) reported in cpDNA across angiosperms (Palmer, 1991). This GC content is consistent with the established fact that exons contain higher amounts of GC than noncoding regions.

There are 1373 (13.7%) parsimony informative characters (PICs) in the combined data set. With regard to the individual noncoding sequences, the *trnS-trnG* intergenic spacer provides

TABLE 4. Genetic statistics for all nine individual sequences, all coding sequences together, all noncoding sequences together, and the combined data set.

Statistics/Partitions	<i>rmlL-F</i> region	<i>rps16</i> spacer	<i>atpB</i>	<i>rbcL-atpB</i> spacer	<i>rmlDgnc-psbM</i> spacer	<i>5' rnaK</i> intron	<i>rmlS-rnaG</i> spacer	<i>rnaG</i> intron	<i>rps4-trnT</i> spacer	All coding	All noncoding	All sequences
No. of accessions (ingroup/outgroup)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)	88 (80/8)
Unaligned* length of sequences (bp)	901–1003 1245	(736) 801–884 1058	1241–1403 1403	693–919 1037	(657) 1146–1256 1558	(659) 764–806 939	(398) 43–931 1247	606–755 882	522–560 674	1529–1753 1766	5618–6498 8277	7312–8242 10043
Aligned length of matrix (bp)	1245	1058	1403	1037	1558	939	1247	882	674	1766	8277	10043
Missing data (%)	1.25	0.76	0.57	1.21	0.67	0.30	2.19	1.51	0.16	1.48	0.89	1.04
Average proportion gaps in taxa (%)	21.5	19.9	0.2	13.9	26.0	16.9	37.5	17.1	21.0	0.2	23.4	19.3
Range in pairwise sequence divergence among all taxa (%)	0.0–12.3	0.0–12.2	0.0–4.7	0.0–9.7	0.0–13.1	0.0–9.7	0.0–25.9	0.0–12.1	0.0–10.4	0.0–4.2	0.1–11.2	0.1–9.6
Maximum sequence divergence between outgroup and ingroup	11.3	11.4	4.7	9.7	13.1	9.7	25.9	11.8	9.9	4.2	10.5	8.8
No. of variable characters	360	339	194	303	499	320	574	288	192	285	2863	3148
No. of parsimony informative characters (PICs) including indels	181	166	81	126	246	140	238	126	69	103	1270	1373
Percentage variable characters	28.9	32.0	13.8	29.2	32.0	34.1	46.0	32.7	28.5	16.1	34.6	31.3
Percentage PICs	14.5	15.7	5.8	12.2	15.8	14.9	19.1	14.3	10.2	5.8	15.3	13.7
No. of potentially phylogenetically informative indels scored	20	12	0	12	22	9	18	7	6	0	106	106
Range in length of phylogenetically informative indels (average/mode)	5–27 (8.7/6)	5–42 (9.8/5.6)	N/A	3–12 (8.8/6)	3–739 (40.5/6)	4–23 (9.6/7)	4–495 (41.9/5)	3–17 (9.8/—)	5–8 (6.5/6.7)	N/A	3–739 (21.1/6)	3–739 (21.1/6)
No. of potentially phylogenetically informative indels per 100 bp of aligned sequence	1.6	1.1	0.0	1.2	1.4	1.0	1.4	0.8	0.0	0.0	1.3	1.1
Start of sequence relative to the cotton cpDNA genome (DQ345959) ^a	50765	5268	56408	57902	32160	3630	8878	9681	49062	N/A	N/A	3630
End of sequence relative to the <i>Citrus sinensis</i> cpDNA genome (DQ864733) ^a	51456	5987	57807	58795	32855	4407	9657	10405	49587	N/A	N/A	58795

^a GenBank accession number

the highest percentage of PICs (19.1%), while the *rps4-trnT* intergenic spacer has the least (10.2%). The *atpB*-coding region has the fewest PICs (5.8%) of any of the sequences. Similarly, the combined data have 31.3% variable nucleotide positions, while the *trnS-trnG* intergenic spacer has the greatest percentage of variable nucleotide positions (46.0%) and *rps4-trnT* the least (28.5%) among the noncoding sequences. The *atpB*-coding region has 13.8% variable nucleotide positions (Table 4).

A total of 106 indels were coded as binary characters, and these resided entirely in the noncoding regions of the combined data set. The range in length of scored indels ranged from 3 to 739 bp. The *trnD-psbM* spacer, the *trnL-F* region, and the *trnS-trnG* spacer contained the highest numbers of indels and were therefore often the hardest sequences to align, whereas the *trnG* intron and *rps4-trnT* spacer have the fewest indels and were relatively easy to align.

With respect to sequence divergence among taxa, we report minimum and maximum percentage sequence divergence among all taxa, as well as the maximum sequence divergence between the ingroup taxa and the eight outgroup species. The range of pairwise divergence values among all taxa for the combined analysis is 0.1–9.6% (Table 4) with a value of 8.8% for maximum divergence between the ingroup and outgroup. In several instances, maximum divergence within a sequence was not between the ingroup and outgroups, but instead between pairs within one of those groups, e.g., within the *trnG* intron the maximum divergence among all taxa was 12.1%, while that between the ingroup and outgroup was 11.8%. The maximum divergence among all pairwise comparisons of noncoding sequences is 10.5%, whereas for coding sequences it is only 4.2%. Among the noncoding sequences, the maximum percentage pairwise divergence is found among the *trnS-trnG* spacer sequences (25.9%) and in order from next highest to lowest percentage divergence are *trnD-psbM* spacer (13.1%), *trnG* intron (11.8%), *rps16* spacer (11.4%), *trnL-F* region (11.3%), *rps4-trnT* spacer (9.9%), *rbcL-atpB* spacer and 5'*trnK* intron (both with 9.7%), and the *atpB*-coding region with 4.7%.

Phylogenetic results—Results among MP and BA analyses were largely similar, differing markedly in support values at only a few nodes (Fig. 1). We present the topology of the three-partition BA, which was preferred using Bayes factors (as per Kass and Raftery, 1995) with bootstrap support (from the combined data), and posterior probabilities from the single-partition, three-partition, and four-partition BAs marked (only the three-partition results are shown (Fig. 1), but the other two analyses are shown in Appendix S1 in the Supplemental Data with the online version of this article). The finding of similar results from our use of a wide range of models and parameter partitioning for most nodes indicates that these results are not heavily dependent upon the models and methods used. Given the large amount of data we analyzed and the high likelihood that these chloroplast regions share the same history due to their linkage, the finding of similar topologies among analyses is not surprising. However, seven nodes had high posterior probabilities (1.00 BA) but low bootstrap probabilities (under 66%). We tested whether the branches below these nodes were significantly different from zero length using a likelihood ratio test.

Partition homogeneity tests revealed significant incongruence ($P = 0.01$). Examination of the separate MP analyses revealed that five taxa had anomalous positions in the coding-only analysis (although with modest bootstrap support) relative to

the noncoding MP, combined MP, and each BA result. The anomalous positions were confirmed in a BA with high support using only the protein-coding partition (HKY+I+G model; individual results not shown). These five taxa (*Burkillanthus malaccensis*, *Clausena excavata*, *Limonia acidissima*, *Citrus japonica*, and *Zanthoxylum monophyllum*) were removed and the partition homogeneity test rerun. The difference among partitions dropped to be nonsignificant ($P > 0.05$), indicating that the incongruence appears to be confined to the coding regions of these taxa. The alignment of the coding regions of these taxa was checked, but no obvious error or misalignment was detected. Given that the coding region alone has fewer changes and the signal appears to be overwhelmed in the combined analyses, we chose not to rerun these analyses with these taxa removed. The most dramatic difference in support observed between combined MP results and the noncoding-only results was an increase in the latter from 58% to 72% in the support for *Ruta graveolens* as the sister to the Aurantioideae. No changes affecting our conclusions based on the combined analyses were observed. The tree we present is well resolved, except for cultivar groups within cultivated citrus, and there is strong support (i.e., >80% bootstrap support and >0.95 posterior probability, Fig. 1) on ~63% of the clades.

Figure 1 shows that there is very strong support for a monophyletic subfam. Aurantioideae. *Ruta graveolens* (Rutoideae) is sister to Aurantioideae (58/100 BS/PP); *Ruta* and the Aurantioideae are strongly supported as sister to *Toddalia asiatica* (Toddalioidae). The *Ruta/Toddalia/Aurantioideae* clade is in a basal polytomy with the tree's real root, *Toona ciliata* (Meliaceae) and the remainder of the outgroup taxa. In the outgroup lineage, a strongly supported clade including *Zanthoxylum monophyllum* (Rutoideae), *Flindersia australis* (Flindersioideae/Rutoideae), and *Choisya ternata* (Rutoideae) is present. Throughout the remainder of this paper, it is convenient to discuss the tribes, subtribes, and groups of the Aurantioideae from the perspective of the traditional classification scheme of Swingle and Reece (1967).

There are two lineages within the Aurantioideae, (clade E; –/96 and clade G; 87/100). However, clade E does contain most of the members of the Clauseneae (sensu Swingle and Reece, 1967), which makes sense from a morphological perspective. There are two main subclades in clade E, one (clade B; 64/100) containing *Micromelum minutum* with three species of *Glycosmis* (clade A), and the other subclade (clade D; 60/100) containing *Bergera koenigii* L. [formerly *Murraya koenigii* (L.) Spreng.] sister to a monophyletic *Clausena* subclade (clade C; 100/100) with two species. The remaining species of Swingle's Clauseneae are contained in subclade F (100/100) of clade G, which is a group of two species, *Merrillia caloxylon* and *Murraya paniculata*. Therefore, Swingle's Clauseneae consist of all species in clades E and F and is nonmonophyletic as circumscribed by Swingle and Reece (1967), as was found by Samuel et al. (2001).

All species in clade H (Fig. 1; 97/100) belong to the tribe Aurantieae (Citreae sensu Swingle and Reece, 1967) and are a monophyletic group. Clades I and J (75/100 and –/51 respectively), consist of species belonging to the subtribe Triphasiinae except for *Oxanthera*, which is resolved in clade Q. The subtribe Triphasiinae is divided by Swingle and Reece (1967) into three groups, the *Wenzelia*, the *Luvunga*, and the *Triphasia* groups. *Triphasia trifolia* is sister to clade L (100/100) consisting of *Monanthocitrus cornuta*, *Merope angulata*, and *Wenzelia dolichophylla*. This clade, along with *Oxanthera*, which is

found in clade Q, is referred to as the *Wenzelia* group. *Luvunga*, *Pamburus missionis*, and *Paramignya lobata* and *P. scandens* constitute clade J belonging to the *Luvunga* and *Triphasia* groups (minus *Triphasia*). The two *Paramignya* species form a strongly supported monophyletic group in clade J.

Swingle and Reece (1967) divided the tribe Balsamocitrinae into three groups, the bael-fruit group (*Aegle*, *Aeglopsis*, *Afraegle*, and *Balsamocitrus*), the tabog group (*Swinglea*), and the wood-apple group (*Limonia* [as *Feronia*] and *Feroniella*). Our clade K (100/100) is the monophyletic bael-fruit group of Swingle and Reece, with *Aegle* sister to *Aeglopsis*, *Afraegle*, and *Balsamocitrus* (Fig. 1). *Afraegle* and *Balsamocitrus* form a strongly supported monophyletic pair, but their sister relationship to *Aeglopsis* or *Aegle* is less clearly supported. However, the other members of Swingle's Balsamocitrinae (*Swinglea*, *Limonia* [as *Feronia*], and *Feroniella*) belong to clade M, together with other members of Citrineae as circumscribed by Swingle and Reece (1967).

The Citrinae s.l. clade (clade M; 85/100) is a monophyletic lineage comprised of two subclades (clades N; -/100 and O; 66/100). Within clade N, there is a clade containing *Swinglea glutinosa*, *Burkillanthus malaccensis*, and *Pleiospermium latalatum* (77/100), and a clade comprising *Naringi crenulata* (formerly *Hesperethusa crenulata*), and two species of *Citropsis* (Engl.) Swingle and M.Kellerm., *C. daweani* Swingle and M.Kellerm. and *C. schweinfurthii* (Engl.) Swingle and M.Kellerm. (100/100). Aside from *Swinglea glutinosa*, these species constitute the majority of what Swingle and Reece (1967) referred to as the primitive-citrus and near-citrus fruit tree groups. Clade O contains two subclades (clades P [-/100] and Q, [100/100]). *Limonia acidissima* (as *Feronia limonia*) (Balsamocitrinae: wood-apple group) is sister to the "Atalantia" clade (89/100). *Atalantia citroides* Pierre ex Guillaumin and *A. monophylla* DC. are strongly supported sister taxa and are in turn sister to *Atalantia ceylanica* (Arn.) Oliv. and *A. buxifolia* (Poir.) Benth. ([formerly *Severinia buxifolia* (Poir.) Tenore] (73/80). *Atalantia* is considered as part of the near-citrus group by Swingle and Reece (1967).

Clade Q is a monophyletic clade, which contains all the genera of "true citrus fruit trees" (sensu Swingle and Reece, 1967). The exception to this is the surprising presence of *Feroniella*, which has pinnate leaves, and *Oxanthera*, which has simple leaves. We will refer to these taxa within clade Q to as *Citrus* s.l. There are two subclades in clade Q (Fig. 2): clade R (95/100) and clade S (100/74). Clade R contains all the Australasian species of *Citrus* s.l., as well as *C. medica* and *C. indica*, both native to India. In this clade, the strongly supported sister pair of *C. medica* and *C. indica* are sister to the remainder of the Australasian taxa. In the Australasian clade, the New Ireland endemic species formerly considered a distinct genus (*Clymenia*) are resolved as sister to the remainder of the group (71/93). There is strong support for most of the branches in the remainder of the well-resolved clade, with the strongly supported sister pair of New Caledonian endemics *Oxanthera neocaledonia* and *Oxanthera* sp. nov. as sister to the 'Microcitrus'/'Eremocitrus'/'Citrus gracilis' clade (clade U). Clade U comprises two subclades: V and W (100/100 and 81/100; respectively). *Citrus* (*Microcitrus*) *garrawayi*, *C. (Microcitrus) australasica*, and a hybrid (*C. australasica* as the maternal parent) comprise clade V. There is an unresolved polytomy (clade W) comprising three species pairs, *Citrus gracilis*/'Microcitrus' *winterii*, 'Microcitrus' *inodoral*/'Microcitrus' *warburgiana*, and 'Microcitrus' *australis*/'Eremocitrus' *glauca* in the other subclade.

Clade S contains most of the cultivated citrus of economic importance. *Citrus (Poncirus) trifoliata* is sister to the rest of clade S (100/74). Clade X (96/100) is sister to clade Y (-/53). *Citrus halimii*, a wild species from Malaysia and Thailand (Stone et al., 1973), is sister to clade AA (96/100), which could be regarded as the kumquat clade because it contains all the species of 'Fortunella', *C. polyandra* (*F. polyandra*), *C. japonica* (including *F. japonica* and *F. margarita*), along with *C. ×microcarpa* (calamondin), a hybrid between *C. japonica* and *C. reticulata* (Mabberley, 2004). Clade Y has two large subclades, clade Z (100/100) and clade BB (-/100). *Citrus cavaleriei*, a wild species from China, is sister to *Feroniella oblata*, which in turn is sister to the "mandarin clade" (clade CC; 82/100). The mandarin clade has *C. tachibana* (probably the wild form of *C. reticulata*) sister to the remainder of the clade (98/100), which contains a polytomy of genetically similar entities including the mandarin, *C. reticulata*, and *C. taitensis* (*C. jambhiri*—possibly a hybrid between *C. reticulata* and *C. medica*), *C. reticulata* × *Eremocitrus glauca*, 'C. limonia' (a name of uncertain application), and *C. ×junos*, probably a hybrid of *C. reticulata* with *C. cavaleriei*.

Clades DD (95/100) and EE (97/100) comprise the final clade (clade Z; 100/100). *Citrus hystrix* (*C. macroptera*) is a strongly supported sister to the remainder of clade DD, which contains a largely unresolved polytomy (clade FF; 85/100). Clade FF is the "lime clade", containing Key lime [*Citrus ×aurantifolia* (Christm.) Swingle; probably *C. hystrix* × *C. medica* Nicolosi et al., 2000], *C. celebica* (i.e., *C. hystrix*), a limequat (*C. japonica* × *C. ×aurantifolia*), the Thai lime (*C. hystrix*) and *C. "amboiensis"*. The pomelo clade (clade EE; 97/100) contains most of the commercially valuable citrus, the lemons, Tahitian lime, oranges, and grapefruits. This clade consists of a polytomy of terminal taxa and a single clade (clade GG; 81/100). Clade GG contains the lemon (*C. ×limon*, thought to be *C. ×aurantium* × *C. medica*), the Tahitian lime (*C. ×latifolia*, thought to be *C. ×aurantifolia* × ?*C. ×limon*), the sour orange (*C. ×aurantium*, sour orange group), bergamot (*C. ×limon*, bergamot group), myrtle-leaf orange (*C. ×aurantium* 'Myrtifolia') and a hybrid of known parentage (*C. maxima* × *C. medica*). The unresolved polytomy of clade EE contains the pomelo (*C. maxima*), the grapefruit (*C. ×aurantium*, grapefruit group), *C. ×aurantium* 'Goutou Chen', the orange (*C. ×aurantium*, sweet orange group), the Khasi papeda (*C. latipes*), and hybrids involving the grapefruit (backcross with *C. reticulata* and 'Minneola'), and *C. "obovoidea* Hort. ex I.Takahashi".

DISCUSSION

This study is the first to sample all genera of Aurantioideae (except *Limnocitrus* an endangered species endemic to Indonesia). The use of nine cpDNA sequence regions, over 10000 bp of sequence, and 106 coded indels, provides a level of taxon sampling, tree resolution, and clade support confidence unmatched by previous single- or two-sequence studies (Samuel et al., 2001; Araújo et al., 2003; Morton et al., 2003).

Molecular evolution and the utility of cpDNA sequences in resolving phylogeny in Rutaceae—The basis on which sequences for this study were chosen was influenced by two starting points. First, we selected *atpB*, the *atpB-rbcL* intergenic spacer, and the *rps16* intron because they were found to be useful in resolving the Rutaceae phylogeny in previous studies

rantioideae. Among the nine sequences we used to resolve the phylogeny, the best were *rps16* intron, the *trnD-psbM* spacer, the *5'matK* intron, and the *trnS-trnG* spacer, because they provided the highest percentage of PICs; the worst were *atpB*, *rps4-trnT*, and *rbcL-atpB* (Table 4). The *atpB* and *rps4-trnT* regions are not highly recommended for resolving phylogenetic relationships at this level in Rutaceae. All noncoding sequences contained indels that could be coded as useful phylogenetically informative characters, but the *trnD-psbM* spacer, *trnS-trnG* spacer, and the *trnL-trnF* region gave the highest proportions of such (Table 4).

Circumscription of the Aurantioideae and its relationship to the outgroup—The Aurantioideae comprise a strongly supported monophyletic group, which confirms what has been shown in several other phylogenetic studies of Rutaceae (Chase et al., 1999; Scott et al., 2000; Samuel et al., 2001; Morton et al., 2003; Poon et al., 2007; Groppo et al., 2008). Sister to the Aurantioideae is *Ruta*, which is a relationship that has also been shown by Poon et al. (2007), Scott et al. (2000), Samuel et al. (2001), and Chase et al. (1999). The remainder of the outgroup is a mixture of species from subfamilies Toddalioidae, Flindersioideae, and Rutoideae (Rutaceae), as well as *Toona ciliata* (Meliaceae). The tree is rooted with *Toona*, as our separate analysis (see Appendix S1 in online Supplemental Data) of *atpB-rbcL* sequences of the Sapindales has confirmed it as sister to the remainder of the Rutaceae. Our analysis of the extra-Aurantioideae Rutaceae, although small, supports the conclusions of Chase et al. (1999), Gadek et al. (1996) and Poon et al. (2007) that the subfamilies Toddalioidae and Rutoideae cannot be supported and that the most appropriate action may be to follow Thorne's (2000) suggestion and subsume the Toddalioidae, along with Flindersioideae into Rutoideae.

By comparison with the other subfamilies of the Rutaceae as discussed here, the Aurantioideae comprise a very strongly supported monophyletic lineage that is also defined by a number of morphological, cytological, and chemical synapomorphies, such as leaves and fruit with schizolysigenous oil glands, flowers usually white and aromatic, fruit an unspecialized berry or hesperidium, and seeds without endosperm, sometimes with two or more nucellar embryos (Morton et al., 2003). The Aurantioideae almost uniformly possess a basic chromosome number of $x = 9$ (Morton et al., 2003) with very little natural polyploidy, which is notably different from other Rutaceae. The monophyly of the Aurantioideae is also supported by phytochemical studies carried out by Waterman (1975) using alkaloids, by Grieve and Scora (1980) via flavonoids, and by Samuel et al. (2001) using coumarins and flavonoids.

Circumscription of the Tribes of Aurantioideae—As circumscribed by Swingle and Reece (1967), Clauseneae is not monophyletic. Our results confirm the findings by Samuel et al. (2001) that this assemblage is split across two clades (clades E and F) and can only become monophyletic through the removal of *Murraya* (s.s.) and *Merrillia* (clade F) from the tribe. Our findings are supported by morphological and phytochemical differences. 3-Methyl-carbazoles are present in the Clauseneae (clade E), while they are absent from the Citreae and clade F (*Murraya* s.s. and *Merrillia*) (Kong et al., 1988b; Samuel et al., 2001). *Clausena* and *Glycosmis* are both strongly monophyletic, as was also shown by Samuel et al. (2001) in their larger scale sampling of the genera using the *atpB-rbcL* spacer and *rps16* intron sequences. *Micromelum* is apparently closely related

to *Glycosmis*, although Swingle and Reece (1967) placed these two genera in different subtribes of the Clauseneae, the Micromelinae, and Clauseninae, respectively.

It was first suggested by Tanaka (1929, 1936) that *Murraya* be divided into two groups; the large-flowered group as sect. *Murraya* (hereafter referred to as *Murraya* s.s.) and the small-flowered as sect. *Bergera*. Based on our study, that of Samuel et al. (2001), and chemical data (But et al., 1986), it is clear that species of Tanaka's sect. *Bergera* need to be recognized as belonging to the genus *Bergera* König ex L. because the genus *Murraya* s.l. is clearly nonmonophyletic when they are included. *Bergera koenigii* (*Murraya koenigii* of *Murraya* s.l.) is much more closely related to *Clausena* than it is to *Murraya* s.s. The fact that many *Murraya* species (i.e., species of section *Bergera*) are morphologically very similar to some species of *Clausena* was recognized by Swingle and Reece (1967). However, the presence of an hourglass-shaped gynophore in *Clausena* was used to justify the separation of that genus morphologically from *Bergera* (Swingle and Reece, 1967, Molino, 1994). In *Murraya* s.s. the stems are straw to light greyish-yellow, petals are relatively large, and the fruits are ellipsoidal and red, whereas in *Bergera* the stems are dark brown, the petals are much smaller, and the fruits are globular to ellipsoidal and purplish-black (But et al., 1986). Moreover, *Bergera* contains carbazoles, whereas they are absent from *Murraya* s.s. and *Merrillia* (Kong et al., 1986, 1988a), which instead contain the yuehchukene and 8-prenylated coumarins (But et al., 1986).

Merrillia caloxylon (basonym = *Murraya caloxylon* Ridl.) is unusual in the Aurantioideae in having large, zygomorphic, trumpet-shaped flowers and a thick, radially lacunate pericarp. In transferring *Murraya caloxylon* to a new monotypic genus, *Merrillia*, Swingle (1918) suggested that it might be a member of the tribe Aurantieae and placed it with *Swinglea* (Balsamocitrinae). After further study of living material, Swingle and Reece (1967) treated it as an anomalous member of the Clauseneae and close to the *Murraya paniculata* group (i.e., *Murraya* s.s.). It is clear from our study, and that of Samuel et al. (2001), that *Murraya* s.s. and *Merrillia* should be treated as closely related members of an enlarged Aurantieae tribe (clade G), as indeed Swingle (1918) had surmised for *Merrillia* itself in the first place. Therefore, the subtribe Merrillinae should be transferred from the Clauseneae s.l. to Aurantieae s.s. and *Murraya* s.s. should be moved from the Clauseninae to Merrillinae. The affinities of *Murraya* s.s. to Aurantieae is further supported by the fact that some *Citrus* cultivars are graft-compatible with *Murraya paniculata* (Swingle and Reece, 1967).

The Aurantieae s.l. (clade G) contain the Merrillinae (clade F) and the Aurantieae s.s. (clade H). Traditionally, the tribe has been circumscribed as containing three subtribes, the Triphasiinae, the Balsamocitrinae, and the Citrinae (Table 2). The Triphasiinae are not monophyletic in our analysis, appearing as two separate clades (clades I and J). Swingle and Reece (1967) attribute four genera to the *Wenzelia* group of the Triphasiinae, namely *Oxanthera*, *Merope*, *Monanthocitrus*, and *Wenzelia*. Our study is the first to investigate and confirm the phylogenetic position of the unispecific genera *Merope* and *Monanthocitrus* and the genus *Oxanthera*. The *Wenzelia* group in our tree (clade L) is strongly monophyletic and consists of *Merope*, *Monanthocitrus*, and *Wenzelia*. The genera are similar in that they are found naturally in Borneo, New Guinea, Solomon Islands, and Fiji; have unifoliate leaves; with very short wingless, nonarticulated petioles; and 4–8 ovules per locule, instead of the two per locule as found in the rest of the Triphasiinae

(Swingle and Reece, 1967). *Triphasia* is associated with the *Wenzelia* group.

One of the interesting results of our analysis of Triphasiinae is the placement of the New Caledonian endemic genus *Oxanthera* in *Citrus* s.l. (Citrinae), far removed from the Triphasiinae where it was placed by Swingle and Reece (1967). Swingle and Reece (1967) placed the genus *Oxanthera* in Triphasiinae (Table 2) because they believed that the fruits were similar to those of genera of the subtribe, i.e., berries with a soft, gland-dotted rind, but lacking the pulp vesicles. However, our examination of the reconstituted fruits (R. Bayer and T. Hartley, personal observation) from recently collected herbarium material of '*Oxanthera*', show prominent pulp vesicles. '*Oxanthera*' also has three or four times as many stamens as petals, a character state more typical of Citrinae than Triphasiinae, which usually have only twice as many stamens as petals. '*Oxanthera*' species should therefore be returned to *Citrus* (B. Pfeil and R. Bayer, unpublished nDNA data).

Paramignya is strongly monophyletic, and we are the first to show a phylogenetic position for *Luvunga* allied with *Paramignya*, the other genus of woody lianas (see also Maberley, 1998). In a previous phylogeny by Chase et al. (1999), "*Luvunga*" was shown to be misidentified through re-examination of the voucher specimen (Chase et al. 1999; note added in proof) and is instead a species of *Zanthoxylum* (Rutoideae) (see GenBank accessions AF066843, AF066815), which explains their positioning of *Luvunga* outside the Aurantioideae. The unispecific genus *Pamburus*, along with the genera *Paramignya* and *Luvunga* form a weakly supported group consisting of woody lianas (*Paramignya* and *Luvunga*) and a much branched, thorny shrub (*Pamburus*). They are all similar in that all their flowers resemble those of *Citrus*, except they have only twice as many stamens as petals, and their fruits all resemble small *Citrus* fruits with the pericarp heavily embedded with oil glands. They differ, however, from *Citrus* in that the locules are filled with mucilaginous secretions instead of pulp vesicles.

The Balsamocitrinae were considered by Swingle and Reece (1967) to comprise the genera *Aegle*, *Aeglopsis*, *Afraegle*, *Balsamocitrus*, *Limonia* (as *Feronia*), *Feroniella*, and *Swinglea*. We find that the subtribe is polyphyletic, as was previously documented by Morton et al. (2003). The Balsamocitrinae were defined by Swingle and Reece (1967) as having fruits that are large with a thick, hard, woody pericarp, except in the case of *Swinglea* in which the rind is leathery and embedded with oil-filled lacunae. It is obvious that this character is homoplastic. The Balsamocitrinae s.s. (Clade K) corresponds exactly to the bael-fruit group of Swingle and Reece (1967) and could constitute a newly circumscribed, strongly monophyletic, Balsamocitrinae. *Aegle*, which is native to Southeast Asia, is geographically isolated from the other three genera (*Aeglopsis*, *Afraegle*, *Balsamocitrus*), which are native to tropical Africa. *Aegle* is also morphologically isolated from the remainder of the group in having more numerous stamens and locules, and woolly seeds. Swingle and Reece (1967) speculated that the group dispersed from southeastern Asia to Africa, but perhaps strangely, not to Australia, New Guinea or other parts of the South Pacific.

The subtribe Citrinae (Swingle and Reece, 1967) is represented by clade M and is not monophyletic as currently circumscribed because it contains the remaining members of the Balsamocitrinae, i.e., *Swinglea*, *Limonia* (as *Feronia*), and *Feroniella* and *Oxanthera* from the Triphasiinae. Therefore, we advocate moving these genera to Citrinae, which is monophyletic (clade M; 85/100). We resolved the phylogenetic relation-

ships among *Pleiospermium* and the unispecific genera *Burkillanthus* Swingle and *Swinglea* (77/100) for the first time. They have somewhat similar fruit morphologies in that they possess different evolutionary stages in development of the unspecialized pulp vesicle. *Swinglea* lacks true pulp vesicles, but possesses what Swingle and Reece (1967) termed "mucilage glands," which line the walls of the dorsal and radial walls of the locules. They likened the fruit of *Swinglea* to *Burkillanthus*, which has rather long, thick-walled, pulp vesicles and suggested that the mucilage glands of *Swinglea* might be homologous to the pulp vesicles of *Burkillanthus*. They are also similar in having a thick, coriaceous pericarp. The five species of *Pleiospermium* have a range of evolutionary types of pulp vesicles ranging from short, squat ones 1–2 mm long in *P. sumatranum* to long, pointed ones in other species (Swingle and Reece, 1967). The pulp vesicles of this group differ from the more specialized ones of *Citrus* s.l. in that they are corticated and usually break down or otherwise secrete mucilage into the locule. Our results support the ideas of Swingle (1939) that *Burkillanthus* is a close relative of *Pleiospermium*. *Limnocitrus littoralis* (Miq.) Swingle is very poorly known, but is notable for its remarkable terminal corymbs of flowers. As Swingle and Reece (1976) point out, it appears to share some features with *Pleiospermium*, but seems very distinct from that genus. The phylogenetic position of *Limnocitrus* remains controversial until material can be obtained for sequencing.

Citropsis is monophyletic and is sister to *Naringi crenulata* (formerly *Hesperethusa crenulata*) in a strongly supported clade. Swingle and Reece (1967) suggested a close relationship between *Naringi* and *Citropsis* and Morton et al. (2003) have likewise shown them to be sister genera. The genera are similar in that they have odd-pinnate leaves with winged petioles and rachis segments; they both have relatively small fruits with large seeds. The pulp vesicles of *Naringi* are rudimentary, those of *Citropsis* broad at the base and gradually tapering to the tip and, as in the *Swinglea* group, appear relatively unspecialized compared to those of *Citrus*. The phylogeography of the *Citropsis* group (clade N) parallels that of the Balsamocitrinae s.s., in which one species (*Naringi crenulata*) in the group is Asian and the greater diversity of species (*Citropsis*) is in tropical Africa.

In the *Atalantia* clade (clade P) are the genera that form the sister group to *Citrus* s.l. Swingle and Reece (1967) divided the approximately 17 species of *Atalantia* into two sections: sect. *Atalantia* with well-developed, juicy pulp vesicles and sect. *Risso* with reduced, almost vestigial, pulp vesicles. *Atalantia citroides* and *A. monophylla* form a strongly supported sister group and are from sect. *Atalantia*, whereas *A. ceylanica*, from sect. *Risso* is sister to *A. buxifolia* (formerly *Severinia buxifolia*), which has reduced pulp vesicles as in sect. *Risso*. Swingle and Reece (1967) have presumed that *Severinia* was quite distantly related to *Atalantia* and *Citrus* based on characteristics of the pulp vesicles, but our analysis shows that *Severinia* is closely allied to them and almost certainly congeneric with *Atalantia*. Additional species of *Atalantia* should be analyzed to re-evaluate the relationships of these two groups of species. Both *Atalantia* and '*Severinia*' make strong graft unions with *Citrus* (Yoshida, 1996), which is consistent with their close relationship to *Citrus*. *Limonia acidissima* is sister with the *Atalantia* clade (–/100). It has characteristics in common with other members of the non-*Citrus* Citrinae in that its flowers have twice as many stamens as petals, similar to all the other members of clades N and P. Likewise, its leaves, like those of

Feroniella, have paired opposite leaflets on a rachis that is composed of segments articulated at each leaf pair, as in *Citropsis* and *Naringi*. *Feroniella* has four times as many stamens as petals, more like the stamens of *Citrus*, which have four or more times as many stamens as petals. Although *Limonia* and *Feroniella* have been treated as sister genera (Swingle and Reece, 1967), it now seems more likely that they are distantly related.

Clade Q is *Citrus* s.l., the expanded concept of the genus, which now contains all genera referred to by Swingle and Reece (1967) as “true citrus fruit trees,” plus *Feroniella* and *Oxanthera*, perhaps the most remarkable finding in this work. Indeed, all the satellite genera, currently represented in *Citrus*, were once taxa within *Citrus* until Rafinesque (*Poncirus*), and later Swingle moved them [i.e., *Clymenia*, Swingle, 1939; *Eremocitrus*, Swingle, 1914; *Fortunella*, Swingle, 1915a; *Microcitrus*, Swingle, 1915b) to segregated genera, often on the basis of single characters. Within this framework, however, the taxonomy of *Citrus* at a detailed level, remains, on the whole, in a state of confusion, although recent preliminary treatments by Mabberley (1997, 1998, 2001, 2002, 2004) are vindicated at all levels by our results.

Many of the outstanding taxonomic problems arise because cultivars of the main cultivar groups (i.e., oranges, lemons, grapefruits, and limes) are thought to be ancient anthropogenic hybrids or selections of wild species (Mabberley, 2004). In addition, many cultivars of *Citrus* are facultatively agamosperous via adventitious embryony derived from nucellar cells. *Citrus* species are mostly diploid and cross easily with each other both naturally and artificially. Agamospermy allows hybrids to gain full fertility such that unconscious human selection and recent artificial selection has changed some of these insignificant hybrids agamospecies into widespread cultivars of great commercial importance. Many species of *Citrus* still exist in nature as wild plants or as little altered land races. Examples of these “wild”, amphimictic, diploid species include *C. maxima*, *C. reticulata*, *C. medica*, *C. halimii*, *C. cavaleriei*, and *C. hystrix*. These wild species, perhaps with extinct species, have given rise to the diversity of *Citrus* cultivars known today (Barrett and Rhodes, 1976; Moore, 2001; Mabberley, 2004). It is important to realize that the taxonomy of *Citrus*, and particularly the cultivated forms in the genus, is therefore not at all straightforward. The analysis of putative hybrid origins of these taxa is largely beyond the analysis we have here because it tells only the maternal (chloroplast) side of the phylogeny. However, the maternal parent is clearly confirmed in several known hybrids that we have in our analysis. For example, in clade V, *C. australasica* × *C. japonica* × *C. reticulata* clearly has the *C. australasica* cpDNA haplotype. Likewise, *C. reticulata* × *C. glauca*, *C. ×aurantiifolia* × *C. japonica*, *C. ×aurantium* grapefruit group × *C. reticulata* (2 tangelos), and *C. maxima* × *C. medica* are all close to their maternal parents. Therefore, this type of analysis can be quite reliable for determining the maternal parent of hybrids.

In our analysis (Fig. 2), *Citrus* appears as two main groups, clades R and S. Clade R (hereafter, the “largely southern clade”) contains primarily wild species from New Guinea and Australia (formerly *Microcitrus* and *Eremocitrus*), New Caledonia (formerly *Oxanthera*), New Ireland (formerly *Clymenia*), and, from India, *Citrus medica* and *C. indica*. This clade has species with simple leaves that nearly all lack winged petioles and many have nonarticulated petioles. The only species cultivated to any large extent is *C. medica*, the citron, and although its geographic

origin is unknown because of its spread by humans, it was known to Alexander the Great on his Indian expedition (Mabberley, 2004) and has therefore been assumed to be native in NE India, though our work seems to suggest that this supposition needs re-examining. *Citrus medica* seems to have been the paternal parent of cultigens like the lemon, *C. ×limon*, and Tahitian lime, *C. ×latifolia* (Mabberley, 2004), as well as the lime.

Clymenia polyandra is restricted to the vicinity of Namatanai, New Ireland in the Bismarck Archipelago (Papua New Guinea). Tanaka (in Swingle and Reece, 1967) suggested that it was a hybrid between *C. medica* and *C. hystrix* (*C. macroptera*). Swingle and Reece (1967) doubted that ‘*C. macroptera*’ was involved in the parentage, but noted that the leaves of *Clymenia polyandra* and *Citrus medica* are quite similar. Our analysis indeed demonstrates a close relationship between *C. medica* and *Clymenia polyandra* and the possibility that the latter had the former as one parent.

One of the taxonomically enlightening results from our analysis is the inclusion of the New Caledonian genus *Oxanthera* within *Citrus*. Based on morphology, *Oxanthera* fits into this group because its fruits have specialized pulp vesicles, simple articulated leaves, and 3–4 times as many stamens as petals. The genus differs from most *Citrus* only in the lack of thorns, but thorns are a feature commonly lost among island plants in the absence of herbivores. ‘*Microcitrus*’ (including *C. gracilis*) species are endemic to Australia (five species) and New Guinea (two species), and along with Australian *C. glauca* are the nearest relatives to ‘*Oxanthera*’. There are two well-supported lineages within Australasian *Citrus*. One clade has two species (*C. australasica* and *C. garrawayi*) with elongated finger-like fruits, and the other clade has species that have primarily oval or round fruits. Mabberley (1998) alluded to the morphological similarity between *C. wintersii* and *C. gracilis* in his protologue for *C. gracilis*, and they appear as sister taxa in our analysis.

In the other section of *Citrus* (clade S; hereafter the “northern clade”), *C. trifoliata* is in an isolated position, with no close relatives. In fact, Swingle and Reece (1967) refer to it as the most aberrant species of the true *Citrus* group, though they did not include pinnate-leaved *Feroniella* in their grouping. Although *C. trifoliata* readily crosses (to give, e.g., citrange, *C. ×insitorum*, an important rootstock; Mabberley, 2002) and is graft-compatible with other species in this clade, it is morphologically distinct in having bud scales and deciduous, trifoliolate leaves (Swingle and Reece, 1967), which are associated with its being the only species of *Citrus* s.l. to have spread far into temperate regions (China). The kumquat (‘*Fortunella*’) clade (clade X) is a strongly supported group with *C. halimii* sister to the other species. Stone et al. (1973) suggested that *C. halimii* was intermediate between *Citrus* and ‘*Fortunella*’, and our results support that idea. It has more locules and seeds per locule than ‘*Fortunella*’ species, but fewer than *Citrus* s.s. (Stone et al., 1973). The simple sequence repeat (SSR) study by Barkley et al. (2006) shows that *C. halimii* shares most of its alleles with the kumquats. The calamondin (*C. ×microcarpa*) is thought to be an orangequat (kumquat × mandarin (*C. reticulata*)) hybrid, and our analysis shows that at least the maternal parent of the calamondin must have been a kumquat species (*C. japonica*), and this result is supported by SSR data (Barkley et al., 2006). Barkley et al. (2006) also confirmed a small proportion of mandarin alleles in the calamondin, supporting an idea that the paternal parent of the calamondin may have been a mandarin.

The mandarin clade (clade BB) contains three or four species that are still known in the wild state, *Feroniella oblata*, *C. cavaleriei*, *C. reticulata*, and *C. tachibana* (probably the wild form of *C. reticulata*). *Citrus tachibana*, which occurs naturally in Japan and Taiwan, was thought by Swingle and Reece (1967) to be a "satellite" species of *C. reticulata*, and our results support this idea. The leaves and thin rinds of both species are very similar, although the fruits of *C. tachibana* are almost inedible due to bitterness. *Feroniella oblata* and *C. cavaleriei* remain as morphologically isolated species in this group. *Citrus cavaleriei* has been placed in subgenus *Papeda* (section *Papedocitrus*) by Swingle and Reece (1967), but the other members of this so-called subgenus are found in clade FF. *Citrus cavaleriei* hybridizes easily with *C. reticulata* and the hybrids, called ichandris, are probably of the same origin as the long-cultivated plants now called *C. ×junos*. The plants known as rough lemon (*C. ×taitensis*; *Citrus jambhiri*) and Rangpur lime ('*C. limonia*') are thought to have SSR alleles (Barkley et al., 2006) from *C. medica* (citron) and *C. reticulata*, and our analysis supports the idea in that their maternal genome is likely to be *C. reticulata*.

All species of *Citrus* subgenus *Papeda* sect. *Papeda* that were sampled are found in clade FF, the papeda clade. *Citrus macroptera*, *C. celebica*, and *C. hystrix* have been referred to as the papedas, and wild populations of this complex still exist in Malesia (Mabberley, 2002). These and other "species" are now considered wild and cultivated forms of *C. hystrix*, and they all have large, expanded, winged petioles; small flowers; and free staminal filaments. The fruits have acrid oil droplets, making them unpalatable. The West Indian or Mexican lime (*C. ×aurantiifolia*) has some SSR alleles from the papeda group (Barkley et al., 2006), and it is possible that one or more species of the papeda group, e.g., the Thai lime, *C. hystrix*, may be one of its maternal parents (Nicolosi et al., 2000). It seems that the recurrent parent of the Mexican lime may have been the citron (*C. medica*) because it carries a large number of SSR alleles in its genetic background.

The pomelo genome (*C. maxima*) has played a part in the parentage of many of the cultivars of *Citrus* (Barkley et al., 2006). Scora (1975) and Barrett and Rhodes (1976) were perhaps the first to suggest that all the major cultivar groups of citrus (i.e., grapefruits, sweet oranges, sour oranges, lemons, limes) were not true species, but merely hybrids derived at least in part from true wild species the pomelo, the mandarin (*C. reticulata*), and the citron (*C. medica*). The SSR work of Barkley et al. (2006) supports this idea. Clade EE contains the pomelo and all its derivatives, of which the grapefruit ('*C. paradisi*'), the sweet orange ('*C. sinensis*'), the lemon ('*C. limon*'), the Tahitian lime ('*C. latifolia*'), and the sour orange ('*C. aurantium*') are most notable. The importance of *C. medica* in the ancestry of such a diverse group of cultigens must be called into question. Such a geographically diverse group of species functioning as the other parent of these cultigens suggests that the natural distribution of *C. medica* (and *C. indica*) is not restricted to India.

Other minor cultivars/species in the clade include: bergamot ('*C. bergamia*'), a type of lemon; '*C. obovoidea*', a possible pummelo-mandarin hybrid therefore referable to *C. ×aurantium*; the myrtle-leaf orange (*C. myrtifolia* Raf.), a type of sour orange (*C. ×aurantium*, sour orange group); the Khasi papeda (*C. latipes*), thought by Swingle and Reece (1967) to be closely related to *C. cavaleriei*, but perhaps more to *C. maxima*. All the taxa in clade EE are closely related to *C. maxima* through their chloroplast (maternal) genome and no doubt have had contributions from other species from other clades in their nuclear genome.

Conclusions—The Aurantioideae have been shown to be a strongly monophyletic group within Rutaceae. The two subtribes Clauseneae and Aurantieae (Citreae) are not monophyletic as circumscribed by Swingle and Reece (1967), and it could only become monophyletic through the removal of *Murraya* (= *Murraya* sect. *Murraya* in Swingle and Reece, 1967) and *Merrillia* (subtribe Merrillinae) to Citreae s.s. and *Murraya* s.s. should be moved to Merrillinae. Aurantieae have traditionally been circumscribed as comprising three subtribes, Triphasiinae, Balsamocitrinae, and Citrinae (Table 2). None of the subtribes is monophyletic in our analysis, but rearrangement of the species in the tribes could provide monophyletic groupings. The Balsamocitrinae s.s. corresponds exactly to the bael-fruit group of Swingle and Reece (1967) and could comprise a newly circumscribed, strongly monophyletic, Balsamocitrinae. These subtribes would include *Aegle*, *Aeglopsis*, *Afraegle*, and *Balsamocitrus*. Three former members of Swingle's Balsamocitrinae, *Swinglea*, *Limonia*, and *Feroniella* should be transferred to Citrinae, rendering Citrinae monophyletic as well. Within Citrinae, four main lineages exist, the *Swinglea* group with a range of evolutionary/developmental forms of pulp vesicles, the *Citropsis* group mainly of African origin, the *Atalantia* group as sister to *Citrus*, and finally *Citrus* itself. Our work confirms that the recently proposed taxonomic circumscription of *Citrus* by Mabberley (2001) should replace that of Swingle and Reece (1967) and include not only Swingle's "microgenera", *Clymenia*, *Eremocitrus*, *Fortunella*, *Microcitrus*, and *Poncirus*, but also *Oxanthera* and *Feroniella*. In consequence, the generic description of *Citrus* will need to be expanded to take account of this.

Citrus in our sense appears to contain two main lineages; the largely southern clade contains primarily wild species from New Guinea, Australia, New Caledonia, New Ireland, and two historically considered to have arisen in India, whereas the northern clade contains most of the economically important citrus species and cultivars. Within this clade are four main lineages, the kumquat group, the mandarin group, the lime group, and the pommelo group.

It is clear that future work in the Aurantioideae should be directed to understanding the circumscription and phylogenetic relationship in some of the other genera in the group, such as *Atalantia*, *Bergera*, *Glycosmis*, *Clausena*, *Murraya*, *Micromelum*, *Paramignya*, and *Pleiospermium*. Another fruitful area of research would be a deeper investigation leading to a fuller understanding of the wild genome groups in *Citrus* and consequently the geography and chronology of the putative origins of cultivar groups of this economically important group of plants. A new taxonomic subtribal classification to accommodate the findings presented here awaits affirmation by low copy nDNA currently in progress by our Aurantioideae working group. We are expectant that the low copy, biparentally inherited nDNA will provide additional insight into the hybridization events in *Citrus*.

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APPENDIX 1. Species, sources, and GenBank accession numbers used in this study. Vouchers containing the prefix “PI” were obtained from the USDA-ARS National Germplasm Repository for Citrus in Riverside, “Merbein” numbers indication CSIRO accessions from the Merbein orchard, Victoria, Australia.

Taxon (cultivar or common name), *Voucher specimen*, Herbarium, GenBank accessions: *atpB*-coding region, *rbcL-atpB* spacer, *rps16* spacer, *trnL-F* region, *rps4-trnT* spacer, *5'trnK* intron, *trnD-psbM* spacer, and *trnG* intron

- Aegle marmelos* (L.) Correa (bael-fruit), *Chase, M.W. 1340; Rut-236, K; PI 539142; Merbein CR 034*: AF066839, AF320882, AY295268, AY295294, EF134628, EF138836, EF164808, EF176492.
- Aeglopsis chevalieri* Swingle, *PI 539143*: EF118827, EF126500, EF126567, EF126634, EF134629, EF138837, EF164809, EF176493.
- Afraegle paniculata* (Schumach. & Thonn.) Engl., *PI 103107*: EF118828, EF126501, AY295269, AY295295, EF134630, EF138838, EF164810, EF176494.
- Atalantia buxifolia* (Poir.) Oliv. [*Severinia buxifolia* (Poir.) Tenore, Chinese box-orange], *Chase, M.W. 1763, K; Rut-67; PI 539793; Merbein CR002, K*: AF066835, AF320886, EF126629, AY295290, EF134708, EF138916, EF164888, EF176572.
- Atalantia ceylanica* (Arn.) Oliv., *Chase, M.W. 1341, K; PI 539144, K*: AF066840, EF126502, EF126568, AY295288, EF134631, EF138839, EF164811, EF176495.
- Atalantia citroides* Pierre ex Guillaumin, *PI 539145*: EF118829, EF126503, EF126569, EF126635, EF134632, EF138840, EF164812, EF176496.
- Atalantia monophylla* (L.) DC., *Rut-457; PI 109613*: EF118830, AF320874, EF126570, EF126636, EF134633, EF138841, EF164813, EF176497.
- Balsamocitrus dawei* Stapf, *PI 539147*: EF118831, EF126504, EF126571, AY295278, EF134634, EF138842, EF164814, EF176498.
- Bergera koenigii* L. (curry leaf), *Rut-217; PI 539745*: EF118832, AF320867, AF320262, EF126637, EF134635, EF138843, EF164815, EF176499.
- Burkillanthus malaccensis* (Ridl.) Swingle, *FRI Malaysia Tree No. 380*: EF118833, EF126505, EF126572, EF126638, EF134636, EF138844, EF164816, EF176500.
- Casimiroa edulis* La Llave and Lex. (white sapote), *Chase, M.W. 1342; K*: AF066837, EF126506, EF126573, EF126639, EF134637, EF138845, EF164817, EF176501.
- Choisya ternata* Kunth (Mexican orange), *Bayer, R. J. GH06-033, CANB 743233*: EF118835, EF126507, EF126574, EF126640, EF134638, EF138846, EF164818, EF176502.
- Citropsis daweanae* Swingle and M. Kell., *PI 247137*: EF118837, EF126509, EF126576, EF126642, EF134640, EF138848, EF164820, EF176504.
- Citropsis schweinfurthii* (Engl.) Swingle and M. Kell., *PI 231240*: EF118838, EF126510, EF126577, EF126643, EF134641, EF138849, EF164821, EF176505.
- Citrus* sp. “Amboin, New Guinea”, *Merbein CO054*: EF118840, EF126511, EF126578, EF126644, EF134642, EF138850, EF164822, EF176506.
- Citrus aurantiifolia* (Christm.) Swingle (West Indian lime), *Rut-404; Merbein CG004, CANB 743228*: EF118841, AF320875, EF126579, EF126645, EF134643, EF138851, EF164823, EF176507.
- Citrus aurantium* L., sour orange group (rough Seville orange), *Merbein CS 010*: EF118842, EF126512, EF126580, EF126646, EF134645, EF138852, EF164824, EF176508.
- Citrus aurantium* L., sour orange group ‘Goutou Chen’, *Merbein CR143*: EF118843, EF126513, EF126581, EF126647, EF134644, EF138853, EF164825, EF176509.
- Citrus aurantium* L. (*C. xmyrtifolia* Raf., chinotto; myrtle-leaf orange), *Merbein CS004*: EF118861, EF126532, EF126600, EF126666, EF134664, EF138872, EF164844, EF176528.
- Citrus aurantium* L. (*Citrus x'obovoides'* Hort. ex I. Takahashi, grapefruit group, *Kinkôji*), *Bayer, R. J. GH06-016, CANB 743216*: EF118862, EF126533, EF126601, EF126667, EF134665, EF138873, EF164845, EF176529.
- Citrus aurantium* L., grapefruit group ‘Davis’ (grapefruit), *Chase, M.W. 2473, K (AJ); K 0345903403 (AF and AY); Merbein CG005 (EF)*: AJ238408, EF126534, AY295251, AY295277, EF134666, EF138874, EF164846, EF176530.
- Citrus aurantium* L. (= ‘*Citrus paradisi*’ × ‘*C. sinensis*’; orangelo group, ‘smooth Seville’) *Merbein CS011*: EF118863, EF126536, EF126602, EF126669, EF134667, EF138876, EF164848, EF176532.
- Citrus aurantium* L. ‘Minneola’ (tangelo), *Bayer, R.J. GH06-051, CANB 743251*: EF118834, EF126535, EF126603, EF126668, EF134668, EF138875, EF164847, EF176531.
- Citrus aurantium* L. sweet orange group ‘Valencia’ [*C. sinensis* (L.) Osbeck, sweet orange], *Merbein Valencia Clone 2*: EF118866, EF126539, EF126606, EF126672, EF134671, EF138879, EF164851, EF176535.
- Citrus australasica* F. Muell. [*Microcitrus australasica* (F. Muell.) Swingle, finger-lime], *Merbein CR019*: EF118883, EF126551, EF126619, EF126686, EF134690, EF138898, EF164870, EF176554.
- Citrus australis* (Mudie) Planch. [*Microcitrus australis* (Mudie) Swingle, Australian lime, Dooja], *Merbein CR125-1, CANB 743271*: EF118884, EF126552, EF126620, EF126687, EF134691, EF138899, EF164871, EF176555.
- Citrus cavaleriei* H. Léveillé ex Cavalerie (*C. ichangensis* Swingle), *Merbein CR145, CANB 743202*: EF118849, EF126519, EF126587, EF126653, EF134651, EF138859, EF164831, EF176515.
- Citrus xfloridana* (J. Ingram and H. Moore) Mabb. (×*Citrofortunella floridana*, Warren limequat–kumquat hybrid, *PI 539802*: EF118836, EF126508, EF126575, EF126641, EF134639, EF138847, EF164819, EF176503.
- Citrus garrawayi* F.M. Bail. [*Microcitrus garrawayi* (F. M. Bail.) Swingle, Mount White lime], *Bayer, R.J. NSW 87285, CBG 8916210*: EF118885, EF126553, AY295261, AY295287, EF134692, EF138900, EF164872, EF176556.
- Citrus glauca* (Lindl.) Burkill [*Eremocitrus glauca* (Lindl.) Swingle, Australian desert lime, *Chase, M.W. 1768; K; Br-1, PI 539717; Merbein CR101-8*: AF066847, AF320879, AY295267, AY295293, EF134676, EF138884, EF164856, EF176540.
- Citrus gracilis* Mabb. (Humpty Doo lime), *Slee, A.V. 4657, CANB 644758*: EF118846, EF126516, EF126584, EF126650, EF134648, EF138856, EF164828, EF176512.
- Citrus halimii* B.C. Stone, *FRI Malaysia s.n.*: EF118847, EF126517, EF126585, EF126651, EF134649, EF138857, EF164829, EF176513.
- Citrus* hybrid (‘*C. limonia*’, Rangpur lime), *Bayer, R. J. GH06-062, CANB 743262*: EF118856, EF126526, EF126594, EF126660, EF134658, EF138866, EF164838, EF176522.
- Citrus hystrix* DC. (*C. macroptera* Montrouz.), *Merbein CS014*: EF118857, EF126527, EF126595, EF126661, EF134659, EF138867, EF164839, EF176523.
- Citrus hystrix* DC. (*C. celebica* Koord.), *PI 539182*: EF118845, EF126515, EF126583, EF126649, EF134647, EF138855, EF164827, EF176511.
- Citrus hystrix* DC. (lime leaves), *Bayer, R.J. GH06-080, CANB 743269*: EF118848, EF126518, EF126586, EF126652, EF134650, EF138858, EF164830, EF176514.
- Citrus indica* Tanaka, *Merbein CR083*: EF118850, EF126520, EF126588, EF126654, EF134652, EF138860, EF164832, EF176516.
- Citrus inodora* F.M. Bail. [*Microcitrus inodora* (F. M. Bail.) Swingle, Russell River lime], *PI 539741*: EF118886, EF126554, EF126621, EF126688, EF134693, EF138901, EF164873, EF176557.
- Citrus japonica* Thunb. [*Fortunella japonica* (Thunb.) Swingle, round kumquat],

- PI 539727*: EF118873, EF126544, EF126611, EF126679, EF134680, EF138888, EF164860, EF176544.
- Citrus japonica* Thunb. [*Fortunella margarita* (Lour.) Swingle, oval kumquat], *Merbein CS007*: EF118874, EF126545, EF126612, EF126680, EF134681, EF138889, EF164861, EF176545.
- Citrus xjunos* Siebold ex Tanaka (Xiang cheng or Yuzu), *Merbein CRI140*: EF118852, EF126522, EF126590, EF126656, EF134654, EF138862, EF164834, EF176518.
- Citrus xlatifolia* (Tanaka ex Yu.Tanaka) Tanaka (Tahiti lime), *PI 539273*: EF118853, EF126523, EF126591, EF126657, EF134655, EF138863, EF164835, EF176519.
- Citrus latipes* (Swingle) Tanaka, *PI 230987*: EF118854, EF126524, EF126592, EF126658, EF134656, EF138864, EF164836, EF176520.
- Citrus xlimon* (L.) Osbeck 'Eureka' (lemon), *Merbein CG021*, CANB 743234: EF118855, EF126525, EF126593, EF126659, EF134657, EF138865, EF164837, EF176521.
- Citrus maxima* (Burm.) Merr. (pomelo, pummelo), *Merbein CS001*, CANB 743219: EF118839, EF126529, EF126597, EF126663, EF134661, EF138869, EF164841, EF176525.
- Citrus maxima* × *Citrus medica*, *Mabberley, D. s.n.*: EF118859, EF126530, EF126598, EF126664, EF134662, EF138870, EF164842, EF176526.
- Citrus medica* L. ([var. *ethrog* Engl.?), *Ethrog*, *Merbein CS003*, CANB 743231: EF118860, EF126531, EF126599, EF126665, EF134663, EF138871, EF164843, EF176527.
- Citrus x microcarpa* Bunge ('*C. xmadurensis*', calamondin), *Merbein CS033*: EF118858, EF126528, EF126596, EF126662, EF134660, EF138868, EF164840, EF176524.
- Citrus neocaledonica* Guillaumin [*Oxanthera neocaledonica* (Guillaumin) Tan.], *PI 539671*: EF118893, EF126559, EF126625, EF126693, EF134700, EF138908, EF164880, EF176564.
- Citrus xoliveri* Mabb. (*Citrus australasica* [*Microcitrus australasica*] × *Citrus xmicrocarpa* [*Citrus* [*Fortunella*] *japonica* × *Citrus reticulata*] sunrise lime, *Bayer, R. J. GH06-045*, CANB 743245: EF118876, EF126547, EF126613, EF126678, EF134683, EF138891, EF164863, EF176547.
- Citrus polyandra* Tanaka [*Clymenia polyandra* (Tan.) Swingle], *Rut-408*; *PI 263640*; *PI 263640*: EF118869, AF320878, AY295255, AY295281, EF134675, EF138883, EF164855, EF176539.
- Citrus reticulata* Blanco 'Emperor' (mandarin), *Merbein CO007*: EF118864, EF126537, EF126604, EF126670, EF134669, EF138877, EF164849, EF176533.
- Citrus reticulata* Blanco [*C. tachibana* (Makino) Tanaka, Tachibana orange], *PI 539679*: EF118867, EF126540, EF126607, EF126673, EF134672, EF138880, EF164852, EF176536.
- Citrus reticulata* 'Fina' × *Citrus glauca* (*Eremocitrus glauca*), *Merbein CO133-12*: EF118865, EF126538, EF126605, EF126671, EF134670, EF138878, EF164850, EF176534.
- Citrus* sp. ("*Oxanthera* sp. nov. T.G.Hartley"), *Veillon 7758*: EF118894, EF126560, EF126626, EF126694, EF134701, EF138909, EF164881, EF176565.
- Citrus swinglei* Burkill ex Harms [*Fortunella polyandra* (Ridl.) Tan.], *PI 539731*: EF118875, EF126546, AY295265, AY295291, EF134682, EF138890, EF164862, EF176546.
- Citrus xtaiensis* Risso (*C. xjambhiri* Lush., rough lemon), *Merbein CR066*, CANB 743244: EF118851, EF126521, EF126589, EF126655, EF134653, EF138861, EF164833, EF176517.
- Citrus trifoliata* L. [*Poncirus trifoliata* (L.) Raf., trifoliata orange], *Chase, M.W. 117*; *NCU (AJ)*; *HBV-1(AF)*, *Chase, M.W. 1767*, K; *Merbein CRI166*, CANB 743205: AJ238409, AF320876, AY295256, AY295282, EF134706, EF138914, EF164886, EF176570.
- Citrus warburgiana* F.M. Bail. [*Microcitrus warburgiana* (F. M. Bail.) Tan.], *PI 266043*: EF118888, EF126556, EF126623, EF126690, EF134695, EF138903, EF164875, EF176559.
- Citrus wintersii* Mabb. (*Microcitrus papuana* H.F.Winters), *PI 410943*: EF118887, EF126555, EF126622, EF126689, EF134694, EF138902, EF164874, EF176558.
- Clausena excavata* Burm. f., *Chase, M.W. 1343* (AF), K; *Rut-415* (AY); *PI 235419* (EF), K: AF066841, AF320849, AY295258, EF126674, EF134673, EF138881, EF164853, EF176537.
- Clausena harmandiana* (Pierre) Guillaumin, *PI 600640*: EF118868, AF320892, EF126608, EF126675, EF134674, EF138882, EF164854, EF176538.
- Feroniella oblata* Swingle, *PI 539720*: EF118871, EF126542, AY295263, AY295289, EF134678, EF138886, EF164858, EF176542.
- Flindersia australis* R.Br. (Australian teak), *Bayer, R. J. GH06-007*, CANB 743207: EF118872, EF126543, EF126610, EF126677, EF134679, EF138887, EF164859, EF176543.
- Glycosmis mauritiana* (Lam.) Tanaka, *Rut-215*; *PI 600641*: EF118878, AF320862, EF126614, EF126681, EF134684, EF138892, EF164864, EF176548.
- Glycosmis pentaphylla* (Retz.) Corrêa, *Merbein CR044*: EF118877, EF126548, EF126615, EF126682, EF134685, EF138893, EF164865, EF176549.
- Glycosmis trichanthera* Guillaumin, *Rut-449*; *PI RRUT 12*: EF118879, AF320855, EF126616, EF126683, EF134686, EF138894, EF164866, EF176550.
- Limonia acidissima* L. [*Feronia limonia* (L.) Swingle, wood apple], *Merbein CS061*; *PI 236991*: EF118870, EF126541, EF126609, EF126676, EF134677, EF138885, EF164857, EF176541.
- Luvunga* sp., *Triono, T. 364*, CANB: EF118880, EF126549, EF126617, EF126684, EF134687, EF138895, EF164867, EF176551.
- Merope angulata* (Willd.) Swingle, *Wong, K.M. et al. 2885*: EF118881, EF126550, EF126618, EF126685, EF134688, EF138896, EF164868, EF176552.
- Merrillia caloxylon* (Ridl.) Swingle, *Br-2*; *PI 539733*: EF118882, AF320871, AY295270, AY295296, EF134689, EF138897, EF164869, EF176553.
- Micromelum minutum* (G.Forst.) Wight and Arn., *Rut-440*; *PI 600637*: EF118889, AF320854, AF320266, EF126691, EF134696, EF138904, EF164876, EF176560.
- Monanthocitrus cornuta* (Lauterb.) Tanaka, *Hoe, T.J. s.n.*: EF118890, EF126557, EF126624, EF126692, EF134697, EF138905, EF164877, EF176561.
- Murraya paniculata* (L.) Jack (orange Jessamine), *Rut-18*, *RBG, Perth 853900*; *Merbein CR013*: EF118891, AF320868, AY295254, AY295280, EF134698, EF138906, EF164878, EF176562.
- Naringi crenulata* (Roxb.) Nicolson, *PI 539748*: EF118892, EF126558, AY295272, AY295298, EF134699, EF138907, EF164879, EF176563.
- Pamburus missionis* (Wall. ex Wight) Swingle, *Rut-002*; *PI 539749*; *PI 95350*: EF118895, AF320883, AY295274, AY295300, EF134702, EF138910, EF164882, EF176566.
- Paramignya lobata* Burkill, *PI 600642*: EF118896, EF126561, EF126627, EF126695, EF134703, EF138911, EF164883, EF176567.
- Paramignya scandens* (Griff.) Craib, *PI 109758*: EF118897, EF126562, AY295257, EF126696, EF134704, EF138912, EF164884, EF176568.
- Pleiospermium latalatum* Swingle, *PI no accession*; *PI 600643*: EF118898, EF126563, EF126628, EF126697, EF134705, EF138913, EF164885, EF176569.
- Ruta graveolens* L. (rue), *Chase, M.W. 0510*; K (AF); *HBV-2* (AF); *Chase, M.W. 0510*, K; *Bayer, R. J. GH06-114*, CANB: AF035913, AF320888, AY295249, AY295275, EF134707, EF138915, EF164887, EF176571.
- Skimmia anquetilia* N.P.Taylor and Airy Shaw, *Chase, M.W. 1766*, K: AF066846, EF126564, EF126630, EF126698, EF134709, EF138917, EF164889, EF176573.
- Swinglea glutinosa* (Blanco) Merr., *Br-3*, *PI 231241*; *PI 142571*: EF118899, AF320887, AY295259, EF126699, EF134710, EF138918, EF164890, EF176574.

- Toddalia asiatica* (L.) Lam., *Rut-229*; *Morton, C. 32-06*; *Lau, J. 2705*, (LJ) CANB 533646; EF118900, AF320889, EF126631, EF126700, EF134711, EF138919, EF164891, EF176575.
- Toona ciliata* M.Roem. (Australian red cedar), *Bayer, R. J. GH06-015*, CANB 743215: EF118901, EF126565, EF126632, EF126701, EF134712, EF138920, EF164892, EF176576.
- Triphasia trifolia* (Burm. f.) P. Wils. (limeberry), *Rut-411*; *PI 539800*: EF118902, AF320884, AY295271, AY295297, EF134713, EF138921, EF164893, EF176577.
- Wenzelia dolichophylla* (Lauterb and K.Schum.) Tanaka, *PI 277441*: EF118903, EF126566, AY295260, AY295286, EF134714, EF138922, EF164894, EF176578.
- Zanthoxylum monophyllum* P. Wilson, *Chase, M.W. 332, K*: AF035919, EF362484, EF126633, bankit 917291, EF134715, EF138923, EF164895, EF176579.
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