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Phylogenetic relationships of Ruteae (Rutaceae): New evidence from the chloroplast genome and comparisons with non-molecular data

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ABSTRACT

Phylogenetic analyses of three cpDNA markers (*mat*K, *rpl*16, and *trn*L–*trn*F) were performed to evaluate previous treatments of Ruteae based on morphology and phytochemistry that contradicted each other, especially regarding the taxonomic status of *Haplophyllum* and *Dictamnus*. Trees derived from morphological, phytochemical, and molecular datasets of Ruteae were then compared to look for possible patterns of agreement among them. Furthermore, non-molecular characters were mapped on the molecular phylogeny to identify uniquely derived states and patterns of homoplasy in the morphological and phytochemical datasets. The phylogenetic analyses determined that *Haplophyllum* and *Ruta* form reciprocally exclusive monophyletic groups and that *Dictamnus* is not closely related to the other genera of Ruteae. The different types of datasets were partly incongruent with each other. The discordant phylogenetic patterns between the phytochemical and molecular trees might be best explained in terms of convergence in secondary chemical compounds. Finally, only a few non-molecular synapomorphies provided support for the clades of the molecular tree, while most of the morphological characters traditionally used for taxonomic purposes were found to be homoplasious. Within the context of the phylogenetic relationships supported by molecular data, *Ruta*, the type genus for the family, can only be diagnosed by using a combination of plesiomorphic, homoplasious, and autapomorphic morphological character states.

1. Introduction

Testing whether traditional taxonomic classifications based on morphology are congruent with more recent molecular phylogenetic findings has become a central task in the current systematic agenda (e.g., Simões et al., 2004; Van der Niet et al., 2005; Wiens et al., 2005; Marazzi et al., 2006; Rønsted et al., 2007; but see Grant, 2003). Disagreements between morphological taxonomies and molecular phylogenies have often been attributed to high levels of homoplasy in characters traditionally used to delimit taxa (e.g., Lavin et al., 2001; Moylan et al., 2004; Mueller et al., 2004; Simões et al., 2006) and taxon diagnoses based on plesiomorphic morphological character-states (e.g., Roalson et al., 2005; Norup et al., 2006). Incongruence between molecular phylogenies and morphological classifications has prompted the recognition of groups highly supported by molecular data, but lacking unique morphological synapomorphies (e.g., Porter and Johnson, 2000; Lavin et al., 2001; Hughes et al., 2004), or the dismantling of traditionally accepted taxa (e.g., Kim et al., 1996; Kron et al., 1999; Wiens et al., 2005).

More generally, the choice of characters for phylogenetic analysis has been a crucial and controversial issue in systematics (e.g., Hart et al., 2004; Stace, 2005) and the relative role of molecular and morphological data in reconstructing phylogenies has been extensively debated (Hillis, 1987; Patterson, 1988; Sytsma, 1990; Donoghue and Sanderson, 1992; Novacek, 1994; Baker et al., 1998; Wahlberg and Nylin, 2003; Wortley and Scotland, 2006). Directly linked to character choice is the controversy about combined versus separate analyses of different datasets (Bull et al., 1993; de Queiroz et al., 1995). For example, should morphological, molecular, and phytochemical characters for a certain group of organisms be analyzed together or separately? Advocates of separate analyses have stressed the fact that congruence among trees derived from independent sources of data can offer strong evidence for the accuracy of the inferred relationships (Swofford, 1991; Hillis, 1995; Miyamoto and Fitch, 1995; Graham et al., 1998), while incongruence can provide initial insights on important biological phenomena, ranging from hybridization to lineage sorting (e.g., Rieseberg et al., 1996; Won and Renner, 2003; Doyle et al., 2004). Conversely, advocates of global evidence have emphasized the fact that combining datasets before phylogenetic analysis grants the best opportunity to resolve relationships at different scales of divergence (Cunningham, 1997; Kluge, 1998; Gatesy and Baker, 2005).

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Ruta L. (Rutaceae Juss.) and related genera offer a primary example of the discordant systematic conclusions that can be reached by using different types of data. Below we provide the necessary background to understand the sources of such discrepancy and explain how novel evidence from molecular characters might help to clarify the discordant taxonomic treatments published until now. The paucity of diagnostic morphological traits, combined with their overlapping and contradicting nature, has hindered both a stable circumscription for *Ruta*—alternately subjected to taxonomic "lumping" (Engler, 1896, 1931) and "splitting" (Townsend, 1968, 1986)—and the unequivocal identification of relationships with other genera of Rutaceae (Townsend, 1986).

At the family level, Rutaceae (161 genera/1815 species; Stevens, 2001 onwards, Angiosperm Phylogeny Website) have been investigated morphologically (Engler, 1896, 1931; Saunders, 1934; Moore, 1936; Scholz, 1964; Tilak and Nene, 1978), molecularly (Chase et al., 1999; Scott et al., 2000; Samuel et al., 2001; Morton et al., 2003), and biochemically, owing to their remarkable diversity of secondary chemical compounds (Price, 1963; Fish and Waterman, 1973; Waterman 1975, 1983, 1990; Gray and Waterman, 1978; Waterman and Grundon, 1983; Kong et al., 1986; Ng et al., 1987; Da Silva et al., 1988; Zakaria, 2001). However, different types of characters led to contrasting systematic conclusions. For example, some taxonomic groups recognized in the most comprehensive morphological study (Engler, 1896, 1931) and the most recent chemotaxonomic survey (Da Silva et al., 1988) of Rutaceae conflict with each other and with the groups supported in the broadest molecular phylogenies available until now (Chase et al., 1999; Scott et al., 2000). The cited molecular studies, based on sparse character and taxon sampling, supported *Ruta* either as sister to a genus of subfamily Flindersioideae (Chase et al., 1999), or as sister to a clade of subfamily Citroideae (Scott et al., 2000), while Engler (1896, 1931) had placed it within subfamily Rutoideae.

In his comprehensive morphological study of Rutaceae Engler (1896, 1931) divided tribe Ruteae into two subtribes: Rutinae, comprising *Ruta*, *Thamnosma* Torrey and Frémont, *Boenninghausenia* Reichb. ex Meissner, *Cneoridium* Hook.f., and *Psilopeganum* Hemsl. ex Forb. and Hemsl.; and Dictamninae, consisting only of *Dictamnus* L. (Table 1). Furthermore, he split *Ruta* into subgenus *Euruta* Engl., housing five species, three of which were originally described by Linnaeus (1735, 1753), and subgenus *Haplophyllum* (around 50 species; see Table 1). Later systematic treatments (Mester and Vicol, 1971; Townsend, 1986; Da Silva et al., 1988; Navarro et al., 2004), however, ranked *Haplophyllum* at the generic level, as originally proposed by Jussieu (1825), reducing the number of species in *Ruta* from around 60 to 8, as currently recognized (Townsend, 1968; Bramwell and Bramwell, 2001).

The six genera included in Ruteae by Engler (1896, 1931) were each distinguished by the following morphological traits (Table 1):

Table 1
Engler's (1896, 1931) classification of Ruteae, with subsequent modifications by Townsend (1986) and Da Silva et al. (1988)

Engler (1896, 1931) Morphology	Townsend (1986) Morphology	Da Silva et al. (1988) Phytochemistry
Tribe Ruteae		
Subtribe Rutinae		*Ruta*-tribe
Boenninghausenia	—	*Boenninghausenia*
Thamnosma	*Thamnosma*	*Thamnosma*
Cneoridium	—	*Cneoridium*
Ruta		
Subgenus *Euruta*	*Ruta*	*Ruta*
Subgenus *Haplophyllum*	*Haplophyllum*	*Haplophyllum*
Psilopeganum	—	—
Subtribe Dictamninae		*Dictamnus*-tribe
Dictamnus	—	*Dictamnus*

Taxa not treated by the authors are indicated with a dash.

Ruta (around 60 species) by tetra- and pentamerous flowers, a thick cushion-shaped nectary disk, and dorsally angled seeds; *Thamnosma* (one species) by almost reniform seeds and variation in the shape of the nectary disk; *Boenninghausenia* (one species) by a cup-shaped nectary disk and filiform filaments; *Cneoridium* (one species) by one carpel, two ovules per locule, and an almost spherical stigma; *Psilopeganum* (one species) by a relatively small nectary disk with a narrow ending; and *Dictamnus* (one species) by zygomorphic flowers, lanceolate petals and sepals, club-shaped filaments with protruding glands, and three ovules per locule (see Table 2). *Psilopeganum* was analyzed in a systematic context only by Engler (1896, 1931), but its narrow occurrence in the Three Gorges Reservoir area of central China (Song et al., 2004; Tang et al., 2007) prevented its inclusion in more recent taxonomic treatments (e.g., Townsend, 1986; Da Silva et al., 1988).

Despite the systematic importance of the above-mentioned diagnostic features, relationships and taxonomic boundaries among the six genera of Ruteae (Engler, 1896, 1931) remain controversial. Townsend (1986) observed that the states of some characters traditionally used to differentiate the genera overlap or suggest contradicting sister-group relationships (Table 2). For example, the ranges of the number of ovules per locule overlap across *Ruta* and allied genera. The presence of cuneate filaments favors *Cneoridium* and *Thamnosma* as sister taxa, whereas spherical seeds link *Cneoridium* with *Dictamnus*. Moreover, Townsend (1986) argued that there are no grounds for considering *Haplophyllum* to be more closely related to *Ruta* than to *Thamnosma*, as proposed by Engler (1896, 1931). In fact, while *Ruta* and *Haplophyllum* share several morphological similarities, including translucent dots on the leaves, yellowish flowers, a thick nectary disk, a short thick style, and connate carpels, they can be clearly distinguished by differences in petal margins, flower merism, seed shape, and pollen morphology. Furthermore, Townsend (1986) showed that the pollen grains of *Ruta* and *Thamnosma* are more morphologically similar to each other than to those of *Haplophyllum*.

The inclusion of *Dictamnus albus* L., the only species of the genus *Dictamnus* and subtribe Dictamninae, in Ruteae (Engler 1896, 1931; Table 1) is also contentious, for this species is distinct from all other Rutaceae due to the presence of special quinolones and limonoids and the absence of coumarins (Da Silva et al., 1988). Furthermore, Moore (1936) remarked that the floral anatomical differences between *Dictamnus* and *Ruta* are greater than those between any two genera within any other tribe of Rutaceae, thus criticizing the inclusion of *Dictamnus* and *Ruta* in Ruteae. Therefore, considering the above-mentioned criticisms towards Engler's (1896, 1931) classification of Ruteae, Townsend (1986) called for a comprehensive systematic re-examination of the entire tribe.

Among the genera of Ruteae (Engler, 1896, 1931), *Ruta* is characterized by strong-smelling ethereal oils in its leaves, greenish-yellow petals with dentate or fimbriate margins, and inflorescences with pentamerous terminal flowers and tetramerous lateral flowers (Townsend, 1968). As currently circumscribed (Townsend, 1968; Bramwell and Bramwell, 2001), *Ruta* includes eight species of perennial shrubs, with four species widely distributed in the Mediterranean (*R. chalepensis* L., *R. graveolens* L., *R. angustifolia* Pers., *R. montana* (L.) L.), one species endemic to the islands of Corsica and Sardinia (*R. corsica* DC.), and three species endemic to the Canary Islands (*R. pinnata* L.f., *R. oreojasme* Webb and Berth., *R. microcarpa* Svent.). Recently, the populations of *R. corsica* from Sardinia have been described as a ninth species, *R. lamarmorae*, based on morphological, karyological, and ecological differences with the populations of *R. corsica* from Corsica (Bacchetta et al., 2006).

Overall, morphological data have not been successful in elucidating the relationships and taxonomic boundaries of Ruteae owing to (i) the paucity of characters diagnostic for the genera within Ruteae, (ii) the conflicting and overlapping nature of the characters tradi-

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Table 2
Seven morphological characters, with their respective states, used by Engler (1896, 1931) to discriminate among the genera of tribe Ruteae

	Ruta	*Thamnosma*	*Cneoridium*	*Boenninghausenia*	*Psilopeganum*	*Dictamnus*
Flower merism	4-Merous or 5-merous	4-Merous	4-Merous	4-Merous	4-Merous	5-Merous
Seed shape	Angled dorsally	Almost reniform	Spherical	Reniform	Reniform	Spherical
Number of ovules per locule	6–12	5–6	2	6–8	5–6	3
Number of carpels	4 or 5	2	1	4	2	5
Nectary disk shape	Cushion-shaped	Variable	Ring-shaped	Cup-shaped	Small and narrow at the end	Ring-shaped
Stigma shape	Simple	Capitate	Almost spherical	Simple	Capitate	Simple
Filament shape	Broader at base	Cuneate	Cuneate	Filiform	n.a.	Club-shaped with protruding glands

The states of these characters are either overlapping or suggest different sister-group relationships (see text). n.a., not available.

tionally used to establish relationships within Ruteae, and (iii) the different taxonomic value assigned by different authors to comparative characters. With respect to Engler's (1896, 1931) classification of Ruteae, the most controversial issues are the placement of *Haplophyllum* within *Ruta* and the inclusion of *Dictamnus* in Ruteae (Townsend, 1986; Da Silva et al., 1988; see Table 1).

Phytochemical characters have also been used to generate taxonomic treatments of Rutaceae (e.g., Kong et al., 1986; Ng et al., 1987; Samuel et al., 2001; Zakaria, 2001), even though they pose specific problems that appear to limit their taxonomic value. Firstly, phytochemical information on the family is fragmentary, with only 30% of the species of Rutaceae examined (Waterman, 1990). Secondly, convergence in the production of secondary chemical compounds has been regarded as a primary source of erroneous systematic conclusions (Hegnauer, 1966; Mothes, 1981; Waterman, 1990; Waterman, 1998). For example, Waterman and Grundon (1983) argued that the synthesis of carbazole, benzophenanthridine, and quinolone alkaloids, occurring in taxa of Rutaceae with little or no immediate affinity with each other, originated by convergent evolution.

Considering the controversial interpretation of morphological (Townsend, 1986) and biochemical characters (Da Silva et al., 1988; Waterman, 1990), which produced contradictory taxonomic treatments for Ruteae (Table 1), and the conflict between traditional taxonomies (Engler, 1896, 1931) and available molecular phylogenies of Rutaceae (Chase et al., 1999; Scott et al., 2000), we performed a detailed phylogenetic study based on sequences from three chloroplast DNA markers to address the following questions: (1) Does the molecular phylogeny support Engler's (1896, 1931) circumscription of Ruteae and, specifically, the inclusion of *Dictamnus* in the tribe? (2) Does the molecular phylogeny support Engler's (1896, 1931) circumscription of *Ruta* and, specifically, the treatment of *Haplophyllum* as a subgenus of *Ruta*? (3) Does the molecular phylogeny support the newly described species *R. lamarmorae*? (4) Do phylogenetic analyses of morphological, phytochemical, and DNA sequence data yield the same or different relationships among *Ruta* and closely related genera? (5) Which morphological and/or phytochemical characters are congruent with the clades recovered from the molecular phylogenetic analysis? More generally, the discussion of our results on the phylogeny of Ruteae provides an opportunity to elaborate on the sources of discrepancy among different types of data, one of the fundamental debates in systematics.

2. Materials and methods

2.1. Taxon sampling

Ruta and its most closely related genera (Engler, 1896, 1931; Townsend, 1986; Da Silva et al., 1988), with the exception of *Psilopeganum* (1 sp.), were sampled: *Ruta* (8/8 species), *Haplophyllum* (24/66 species), *Thamnosma* (5/9 species), *Boenninghausenia* (1/1 sp.), *Cneoridium* (1/1 sp.), and *Dictamnus* (1/1 sp.; see Supplementary data 1). It was impossible to sample *Psilopeganum sinense*, because it is restricted to the Three Gorges Reservoir area, in the Hubei province of central China, and is endangered (Song et al., 2004; Tang et al., 2007). Furthermore, this taxon is poorly represented in the herbaria that were visited during the duration of the present study (i.e., W, LE, P, BR). In order to elucidate relationships within *Ruta*, different accessions from the eight species of the genus were selected. Five accessions of *R. corsica* from Corsica and Sardinia were sampled to verify the treatment of the populations from Sardinia as a separate species (i.e., *R. lamarmorae*; Bacchetta et al., 2006). To test the monophyly of Ruteae (Engler, 1896, 1931), in particular the inclusion of *Dictamnus* in the tribe, eight taxa outside the tribe, belonging to subfamilies Rutoideae and Toddalioideae, were selected. Choice of outgroups was guided by Engler's (1896, 1931) classification of Rutaceae and previous phylogenetic findings (Chase et al., 1999; Scott et al., 2000). Because the molecular phylogenetic analysis of Scott et al. (2000) placed *Ruta* as sister to members of subfamily Aurantioideae, rather than Rutoideae, as suggested by Engler (1896, 1931), and because the monophyly of the tribes and subfamilies of Rutaceae proposed by Engler (1896, 1931) has been questioned (Da Silva et al., 1988; Chase et al., 1999), taxa from Meliaceae and Simaroubaceae, closely related to Rutaceae (Gadek et al., 1996; Muellner et al., in press), were chosen as outgroups, to reduce the possibility that the rooting taxa might fall within the ingroup. The final matrix contained 73 accessions: 66 belonging to Rutaceae, four to Meliaceae, and three to Simaroubaceae. Included material, voucher information, sources, and GenBank/EBI accession numbers are listed in Supplementary data 1.

2.2. Character sampling

After performing preliminary analyses with different cpDNA markers, three markers that provided sufficient resolution at our level of investigation and allowed unequivocal alignments were chosen: the *mat*K gene, the *rpl*16 intron, and the *trn*L–*trn*F intergenic spacer, which already proved effective at resolving inter-generic relationships in other groups of angiosperms (Simões et al., 2004; Guggisberg et al., 2006; Marazzi et al., 2006; Rutschmann et al., 2007).

2.3. DNA extraction, amplification and sequencing

Prior to DNA extraction, silica-dried leaf material (15–20 mg) was ground using glass beads and a MM 3000 shaker (Retsch GmbH, Haan, Germany). Total genomic DNA was extracted using the DNeasy Plant Mini Kit from QIAGEN AG (Basel, Switzerland), following the manufacturer's instructions. The *mat*K cpDNA coding region was amplified using primers 1F and 1R (Sang et al., 1997). The *rpl*16 intron was amplified using primers F71 and R1516 (Baum et al., 1998). The *trn*L–*trn*F spacer was amplified with the

primers e and f (Taberlet et al., 1991). All PCR were 20 μl in volume. Each reaction included 9.2 μl of ddH_2O, 2 μl of Taq Buffer [10×, 15 mM $MgCl_2$], 1.6 μl of $MgCl_2$ [25 mM], 3.2 μl of dNTP [1.25 mM], 0.2 μl of Taq Polymerase [5 U/μl], 1 μl of BSA, 0.4 μl of each primer (forward and reverse), and 2 μl of DNA template. Amplification of the *mat*K region consisted of 2 min at 94 °C followed by 30 cycles of: 1.5 min denaturation (94 °C), 2 min annealing (53 °C), and 3 min extension (72 °C). After the last cycle, the temperature was kept at 72 °C for the last 15 min of extension and then lowered to 4 °C. Amplification of both the *rpl*16 and *trn*L–*trn*F regions consisted of 2 min at 94 °C followed by 35 cycles of: 0.5 min denaturation (94 °C), 1 min annealing (52 °C), and 1.75 min extension (72 °C). After the last cycle the temperature was kept at 72 °C for 10 min of extension and then lowered to 4 °C. All PCR and cycle sequencing reactions were run on a TGradient thermocycler (Biometra, Goettingen, Germany). In order to detect amplified DNA target regions and possible contamination, PCR products were separated on 1% agarose gels, stained with ethidium bromide, and viewed under UV light. Successfully amplified products were purified with the GFX PCR DNA and Gel Band purification Kit (Bioscience Amersham, Otelfingen, Switzerland), following the manufacturer's recommendations.

Cycle sequencing reactions were carried out using the BigDye™ Terminator Mix (Applied Biosystems, Inc., Foster City, CA) and the same primers as above. The sequencing protocol consisted of 24 cycles of 10 s denaturation (96 °C), 5 s annealing (50 °C), and 4 min elongation (60 °C). Products were run on an ABI 3100 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol. For each region both strands were sequenced.

2.4. Alignment and phylogenetic analyses

Sequences were edited and assembled using Sequencher 4.2™ software (Gene Codes Corp., Ann Arbor, MI, USA). Base positions were individually double-checked for agreement between the complementary strands. All sequences were visually aligned in MacClade 4.06 (Maddison and Maddison, 2000). Regions of ambiguous alignment were excluded from the analysis (Kelchner, 2000). Gap positions were treated as missing data, unequivocally aligned gaps being coded as presence/absence of characters with the software GapCoder (Young and Healy, 2003) and then added as binary characters to the data matrix.

Three data partitions were defined, corresponding to the three loci of the chloroplast genome examined in this study. The individual partitions were initially analyzed separately to establish whether there were any strongly supported (i.e., >85 bootstrap percentage, BP), incongruent clades among the respective trees. Since no such incongruence was detected (see Section 3), the sequences of the three loci were combined in a single dataset. The combined matrix was then analyzed phylogenetically either with the gaps treated as missing data ("combined without gap coding") or with the gaps coded in GapCoder (Young and Healy, 2003; "combined with gap coding").

The individual partitions and the combined matrix without gap coding were analyzed using maximum parsimony (MP). The combined matrix with gap coding was analyzed using both MP and Bayesian Inference (BI). Parsimony analyses were conducted using PAUP*4.0b10 (Swofford, 2001). All changes were treated as unordered and equally weighted (Fitch, 1971). Tree search was performed using the following protocol: (i) a heuristic search was carried out with 1000 replicates of random taxon addition sequence and 10 trees held at each step, and tree bisection–reconnection branch swapping (TBR) on best trees only, with no more than 100 trees saved per replicate; (ii) the best trees found in (i) were then used as starting trees for a second heuristic search using TBR branch swapping until all swapping options were explored, and saving multiple trees (MULTREES option in effect). The STEEPEST DESCENT option was used in both (i) and (ii). Relative levels of homoplasy in all partitions were assessed using the consistency index (CI) and the retention index (RI) as implemented in PAUP*4.0b10 (Swofford, 2001).

Relative support for each node obtained by MP was assessed using bootstrap re-sampling (Felsenstein, 1985). The following protocol was employed: heuristic search, 1000 bootstrap replicates, 100 random addition sequence replicates with three trees held at each step, TBR swapping with STEEPEST DESCENT and saving no more than ten trees per replicate.

Bayesian inference was performed in MRBAYES v3.1.2 (Hulsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), after determining the model of evolution most suitable for each individual cpDNA region with the Akaike Information Criterion (AIC; Akaike, 1974) in ModelTest 3.06 (Posada and Crandall, 1998). Subsequently, the commands "lset NST = 6, RATES = gamma" and "lset coding = variable" were entered in MRBAYES v3.1.2 for the nucleotide and gap characters, respectively (Ronquist and Huelsenbeck, 2003). The analysis was performed with four Monte Carlo Markov chains (one cold and three incrementally heated) run for 5×10^6 generations, with trees sampled every 1000th generation (NGEN = 1.000.000, PRINTFREQ = 1000, SAMPLEFREQ = 1000, NCHAINS = 4). Each chain used a random tree as starting point and the default temperature parameter value of 0.2. Two independent Markov chain Monte Carlo runs were carried out to check for convergence on the same region of tree space. The first 25,000 sampled trees were discarded as "burn in" after checking for stability on the log-likelihood curves. The remaining trees were imported into PAUP*4.0b10 (Swofford, 2001) and used to build a 95% majority rule consensus tree showing the posterior probabilities (PP) of all observed bi-partitions.

2.5. Analyses of constrained topologies

Engler's (1896, 1931) classification of tribe Ruteae has been criticized with respect to two points: the placement of *Dictamnus* in the tribe (Moore, 1936; Da Silva et al., 1988) and the treatment of *Haplophyllum* as a subgenus of *Ruta* (Townsend, 1986). Two topological constraints were thus defined: (i) *Dictamnus* within Ruteae and (ii) *Haplophyllum* as sister to *Ruta*. For each constraint, a

Table 3
Results of S–H tests on two topological constraints

Topologies	No. of MP trees	MP length	$-\ln L$ scores[a]	$-\ln L$ differences[b]	*p* Values[c]
Unconstrained	6	2092	**17570.505**	–	–
Constraint (i): *Dictamnus* within Ruteae	94	2388	17626.010	55.506	0.080
Constraint (ii): *Haplophyllum* sister to *Ruta*	940	2455	17784.520	214.025	**<0.001**

[a] Highest likelihood scores assigned to MP trees; the highest likelihood score of the unconstrained trees (in boldface) was used for comparisons with the likelihood scores of all constrained trees.
[b] Differences between likelihood scores of constrained and uncontrained trees. Only the differences between the unconstrained tree with highest $-\ln L$ score and the constrained trees with highest $-\ln L$ scores are shown.
[c] *p* Values associated with differences between likelihood scores (significant values in boldface).

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heuristic search was performed to find the shortest trees, which were then assigned a likelihood score (Table 3) using the parameters previously estimated (see Table 4). The likelihood scores of the 94 MP trees with constraint (i) and of the 940 MP trees with constraint (ii) were each compared with the highest likelihood score of the MP unconstrained trees, for a total of 1034 pair-wise comparisons, and the differences between likelihood scores calculated (Shimodaira–Hasegawa (S–H) test; Shimodaira and Hasegawa, 1999; Goldman et al., 2000; Lee and Hugall, 2003). Significance levels for the differences were checked on a distribution generated by using a RELL technique (resampling estimated log likelihoods; Kishino and Hasegawa, 1989). The S–H tests were performed in PAUP*4.0b10 (Swofford, 2001) on the matrix without gap coding, because this software does not allow for likelihood estimation in datasets containing two or more partitions explained by different models of evolution.

2.6. Phylogenetic comparisons among different datasets

To evaluate whether different types of data produce congruent phylogenies, we generated comparable matrices from morphology, biochemistry, and DNA sequences. Published information was available mainly at the genus level for the morphology of Rutaceae (Engler, 1896, 1931; Saunders, 1934; Scholz, 1964), and at the species level for the secondary chemical compounds (e.g., Price, 1963; Fish and Waterman, 1973; Waterman 1975, 1983, 1990; Gray and Waterman, 1978; Waterman and Grundon, 1983; Kong et al., 1986; Da Silva et al., 1988), but not for all the species included in our molecular analyses. Therefore, comparisons among different datasets were performed at the genus level and only on *Haplophyllum*, *Thamnosma*, *Boenninghausenia*, *Cneoridium*, and *Ruta*, because the results of our molecular analyses excluded *Dictamnus* from Ruteae, in agreement with Moore (1936) and Da Silva et al. (1988) (see Section 3 below). The genus *Choisya*, belonging to the subfamily Rutoideae (Engler, 1896, 1931), was chosen to root the resulting trees, because it is closely related to Ruteae (Da Silva et al., 1988) and both morphological and phytochemical data were available for it.

Morphological characters that vary among the six genera listed above were selected from descriptions in Engler (1931) and Townsend (1986), scored for different states (i.e., multistate), and used to build a matrix (see Supplementary data 2). The number of ovules per locule was not included in the matrix of Supplementary data 2, because states overlapped extensively. Phytochemical characters were selected from Da Silva et al. (1988), the most recent and comprehensive chemotaxonomic survey of Rutaceae. These characters referred to the presence or absence of specific phytochemical compounds and consequently were scored as binary data (Supplementary data 3). The molecular dataset was built by keeping only one, randomly chosen exemplar sequence from the combined matrix with coded gaps for each of the six genera. The selected sequences (indicated by an asterisk in Supplementary data 1) were visually re-aligned in MacClade 4.06 (Maddison and Maddison, 2000). A global dataset was also constructed by combining all types of characters. For each of the four datasets, exhaustive searches were carried out using maximum parsimony with the program PAUP*4.0b10 (Swofford, 2001). Branch support was calculated with 10,000 bootstrap replicates using a Branch and Bound search strategy and an "as is" taxon addition sequence.

The trees resulting from the molecular, morphological, and phytochemical datasets were compared with each other in two ways. First, because phylogenetic incongruence should only include cases where conflicting clades are strongly supported, topologies were evaluated by direct node-to-node comparisons using branch support values (e.g., Mason-Gamer and Kellogg, 1996; Graham et al., 1998). Node-to-node comparisons were executed by using the table of "bipartitions found in one or more trees and frequency of occurrence" from the bootstrap output produced in PAUP*4.0b10 (Swofford, 2001). Secondly, the trees were compared by means of the incongruence length difference (ILD) test (Farris et al., 1994), implemented as the "partition homogeneity" test in PAUP*4.0b10 (Swofford, 2001). For the ILD test, 1000 random repartitions were used and a branch and bound tree search was implemented. Following suggestions by Cunningham (1997) and Lee (2001), the test was carried out after removing uninformative characters.

2.7. Character mapping

We investigated whether any of the morphological and/or phytochemical characters listed in Supplementary data 2 and 3 were congruent with the relationships inferred from the analysis of the 6-taxon molecular dataset. Non-molecular characters were

Table 4
Character and tree diagnostics, substitution models, and parameters estimated by ModelTest for the five data partitions

	*mat*K	*rpl*16	*trn*L–*trn*F	Combined without gap coding	Combined with gap coding
Aligned length	1564	1321	709	3594	3798
Parsimony informative ntps (% of aligned ntps)	429 (27.4)	294 (22.3)	125 (17.6)	848 (23.6)	957 (25.2)
No. of trees	600	803	70	6	16
No. of steps	1076	723	274	2092	2367
CI (CI ex)	0.744 (0.682)	0.765 (0.702)	0.803 (0.758)	0.752 (0.691)	0.751 (0.682)
RI	0.950	0.947	0.960	0.948	0.947
Model selected	TVM + G	TVM + G	TVM + G	TVM + G	TVM + G & binary model
$-\ln L$	8589.0039	6065.5581	2608.8774	17638.0703	–
Freq. [A]	0.2840	0.4054	0.4025	0.3525	–
Freq. [C]	0.1722	0.1450	0.1564	0.1575	–
Freq. [G]	0.1690	0.1522	0.1651	0.1658	–
Freq. [T]	0.3749	0.2974	0.2759	0.3242	–
R.r.o.s. [A-C]	1.1585	1.4023	0.5307	1.1031	–
R.r.o.s. [A-G]	1.5442	1.3557	0.8744	1.2461	–
R.r.o.s. [A-T]	0.2248	0.2041	0.0938	0.1931	–
R.r.o.s. [C-G]	0.8399	0.7504	0.6037	0.7632	–
R.r.o.s. [C-T]	1.5442	1.3557	0.8744	1.4942	–
R.r.o.s. [G-T]	1	1	1	1	–
I	0	0	0	0	–
Γ	0.8744	0.9871	0.5913	0.8191	–

For the "combined with gap coding" partition parameter values are not present because they were re-estimated by MrBayes. Ntps, nucleotide positions; CI, consistency index; CI ex, consistency index excluding parsimony uninformative characters; RI, retention index; Freq., frequency; R.r.o.s., relative rate of substitution; I, proportion of invariable sites; Γ, gamma distribution shape parameter.

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mapped on the molecular topology using maximum parsimony and both accelerated (ACCTRAN) and delayed (DELTRAN) character state optimizations, as implemented in MacClade 4.06 (Maddison and Maddison, 2000).

3. Results

3.1. Alignment and phylogenetic analyses

Aligned lengths, character and tree statistics, CI and RI values for all five partitions are summarized in Table 4. Forty-six and 27 nucleotide positions were excluded from the *rpl*16 and *trn*L–*trn*F partitions, respectively, owing to ambiguities in the alignment caused by strings of mononucleotides (Kelchner, 2000). Among the three cpDNA partitions, the *mat*K dataset contained the highest proportion of parsimony-informative characters (27.4%) and produced the best resolved tree, whereas the *trn*L–*trn*F partition had the highest CI and RI values (Table 4). Because no strongly supported (>85 BP) incongruent clades were detected among individual trees, the three partitions were combined, producing an alignment of 3594 characters. The gaps of the combined matrix yielded 204 additional characters, for a total of 3798 characters (Table 4). The combined matrix with gaps coded was used for final MP and Bayesian analyses, because it had a higher number of parsimony informative sites and similar CI and RI values as compared with the combined matrix without coded gaps (see Table 4).

For all three DNA regions the AIC of ModelTest selected the same model of evolution, TVM + G, which was implemented in the Bayesian analysis, with parameters estimated again from the data. The two runs of the Bayesian analysis produced identical 50% majority-rule consensus trees, suggesting convergence on the same region of tree space. Each run reached a stationary likelihood after approximately 280.000 generations, which were not used to build the Bayesian consensus tree. The 95% majority-rule consensus tree obtained from one run of the Bayesian analysis, with posterior probabilities (PP), and bootstrap percentages (BP) obtained by bootstrapping the same matrix under parsimony, is shown in Fig. 1. This tree was similar to the strict consensus tree found from the MP search of the same matrix (not shown), except that in the MP tree some groups within *Haplophyllum* were resolved differently compared to the Bayesian tree.

The main phylogenetic results are the following (Fig. 1): (1) *Ruta*, *Boenninghausenia*, *Thamnosma*, *Haplophyllum*, and *Cneoridium* form a monophyletic group with maximum support (100 BP and 1.00 PP); (2) *Dictamnus* forms a clade with *Skimmia* Thunb. and *Orixa* Thunb. (100 BP and 1.00 PP); (3) the eight species currently ascribed to *Ruta* (Townsend, 1968; Bramwell and Bramwell, 2001) form a strongly supported clade (100 BP and 1.00 PP) that is sister to a clade consisting of *Thamnosma* and *Boenninghausenia* (100 BP and 1.00 PP), while *Haplophyllum* is sister to *Cneoridium* (100 BP and 1.00 PP); (4) *R. chalepensis* and *R. angustifolia* form a clade sister to *R. graveolens* and these three species are sister to *R. corsica* (clade I); (5) the three *Ruta* species from the Canary Islands (*R. pinnata*, *R. microcarpa*, and *R. oreojasme*) form a strongly supported clade (100 BP and 1.00 PP; clade III); (6) the relationship between *R. montana* (clade II; Fig. 1) and clades I and III described above remains unresolved; (7) within *R. corsica* there is a strongly supported split between the samples from Sardinia (86 BP and 1.00 PP) and those from Corsica (99 BP and 1.00 PP).

3.2. Analyses on constrained topologies

When *Dictamnus* was forced inside Ruteae (constraint i) and when *Haplophyllum* was constrained to be sister to *Ruta* (constraint ii), 94 MP trees of 2388 steps and 940 MP trees of 2455 steps were found, respectively, as compared to the 6 MP trees of 2092 steps resulting from the unconstrained search (Table 3). All pair-wise comparisons involving constraint (ii) produced significant differences between likelihood scores ($p < 0.001$; Table 3, in bold). However, none of the comparisons involving constraint (i) produced significant results (p values between 0.080 and 0.038; Table 3).

3.3. Comparisons among different datasets

For each six-taxon dataset, only one MP tree was found (Fig. 2; Table 5). The inter-generic relationships inferred from the six-taxon molecular dataset (Fig. 2a) were identical to those generated from the 73-accession matrix (Fig. 1), with *Ruta* sister to *Boenninghausenia*/*Thamnosma* and these three genera, in turn, sister to *Haplophyllum*/*Cneoridium*, indicating that random selection of one exemplar per genus did not change the phylogenetic pattern. All the nodes of the molecular tree were highly supported (Fig. 2a), whereas only one node each received high BP value in the morphological (Fig. 2b) and molecular trees (Fig. 2c). The relationships of the global tree were identical to those of the molecular tree and strongly supported (Fig. 2d). The *Boenninghausenia*/*Thamnosma* clade received maximum support (100 BP) from the molecular data and was found in 86% of the bootstrap replicates of the morphological data. The *Haplophyllum*/*Cneoridium* clade, with 99 BP in the molecular tree, was found in none of the bootstrap replicates of the non-molecular datasets. The phytochemical tree shared no clades with the molecular tree and its single strongly supported clade (i.e., *Ruta*/*Haplophyllum*; 97 BP) received low bootstrap support (66 BP) in the morphological tree.

Based on the results of the ILD test, the molecular dataset was found to be significantly incongruent with both the morphological ($p = 0.002$) and phytochemical ($p = 0.0001$) datasets, while the morphological and phytochemical datasets were not significantly incongruent with each other ($p = 0.41$).

3.4. Character mapping

Sixteen of the 47 non-molecular characters mapped on the molecular tree exhibited equivocal reconstructions, that is, character-state transitions occurred in different branches depending on whether ACCTRAN or DELTRAN were used for the optimization. The remaining 31 characters were optimized unequivocally and three of them changed uniquely along the branches of the simplified molecular tree (Fig. 3). Character 9 switched from a short and thick to a long and thin style along the branch leading to *Thamnosma*/*Boenninghausenia*, character 11 switched from introrse to slightly-introrse anther opening along the same branch, and character 34 switched from the absence to the presence of acridones of type H1 along the branch subtending *Ruta*/*Thamnosma*/*Boenninghausenia* (see also Supplementary data 2 and 3). In contrast, a transition from obovate to ovate petals (character 5) and a switch from the absence to the presence of 2-quinolones of type G1.1 (character 23) occurred more than once, but nonetheless supported the clades *Haplophyllum*/*Cneoridium* and *Thamnosma*/*Boenninghausenia*, respectively (Fig. 3).

4. Discussion

4.1. Circumscription of Ruteae and Ruta

In the most comprehensive classification of Rutaceae, Engler (1896, 1931) proposed the inclusion of *Dictamnus* in Ruteae. However, the inferred cpDNA phylogeny strongly supports the monophyly of a clade formed by *Ruta, Boenninghausenia, Thamnosma, Haplophyllum* and *Cneoridium*, while *Dictamnus* is embedded in a clade comprising members of Zantoxyleae, Diosmeae and Toddalioideae (Fig. 1).

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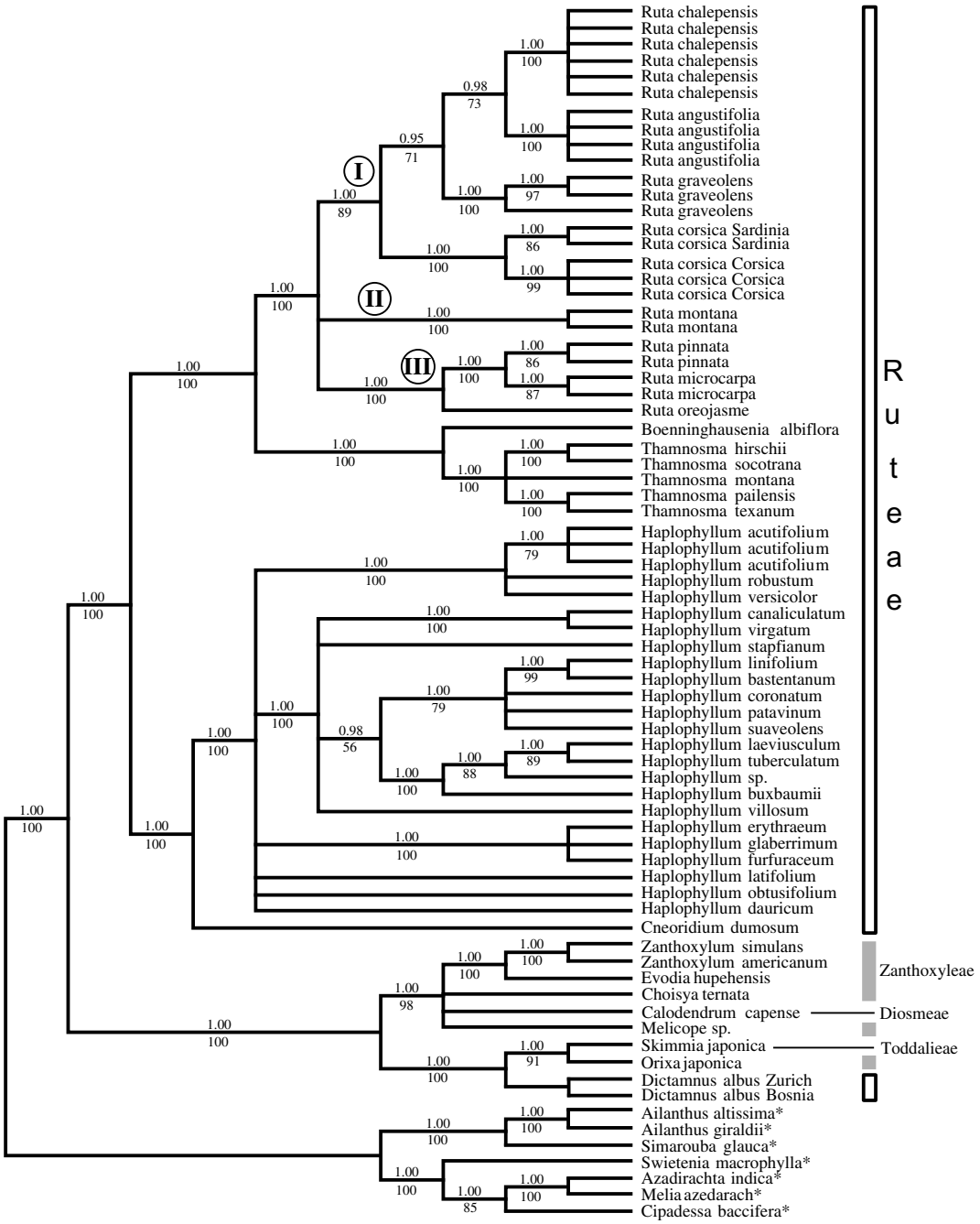

Fig. 1. Ninety five percent majority-rule consensus tree obtained from the Bayesian analysis. Posterior probabilities (PPs) and Bootstrap percentages (BPs; above 50%) are shown above and below branches, respectively. The white bar indicates members of tribe Ruteae sensu Engler (1896, 1931). The gray bar indicates members of tribe Zanthoxyleae. Tribes Ruteae, Zanthoxyleae, and Diosmeae belong to subfamily Rutoideae; tribe Toddalieae belongs to subfamily Toddalioideae. Taxa with an * were included in the outgroup.

Molecular evidence thus apparently corroborates Da Silva's (1988) interpretation of tribal boundaries (see Table 1).

The five genera sharing a single origin in the molecular tree (i.e., *Ruta*, *Boenninghausenia*, *Thamnosma*, *Haplophyllum* and *Cneoridium*; Ruteae s.s. from now on; see Fig. 1) share a number of morphological and phytochemical traits, including: the presence of actinomorphic, creamy-white to bright-yellow flowers (Engler 1896, 1931); the highest levels of lignans of the aryltetrahydronaphthalene type in Rutaceae (Waterman, 1983; Da Silva et al., 1988); specific classes of coumarins and acridones (Waterman, 1975); and, uniquely in Rutaceae, the biosynthetic pathway for acridones devoid of an oxygen substituent at the C-3 position and also, in some cases, at the C-1 position (Waterman, 1983). Therefore, these genera appear to form a cohesive taxonomic group.

At least morphologically, *Dictamnus* can be viewed as an aberrant form of uncertain phylogenetic placement, for it shows some morphological features that cannot be readily linked with any other taxa of Rutaceae (Moore, 1936). Within Ruteae (Engler, 1896, 1931), *Dictamnus* differs from other genera in seed structure (Corner, 1976) and floral morphology, with large, zygomorphic, white to purple flowers, lanceolate petals, and unusual oil glands protruding from the carpel walls and the style (Moore, 1936; Gut, 1966). The secondary chemistry of the genus is also unique within Rutaceae. *Dictamnus* has limonoids, instead of coumarins, and special quinolones (Da Silva et al., 1988). Furthermore, early serodiagnostic studies on Rutaceae (Bärner, 1927) showed that "between *Ruta chalepensis* and *Dictamnus albus* the reaction was only slightly positive, an observation strictly in accord with floral

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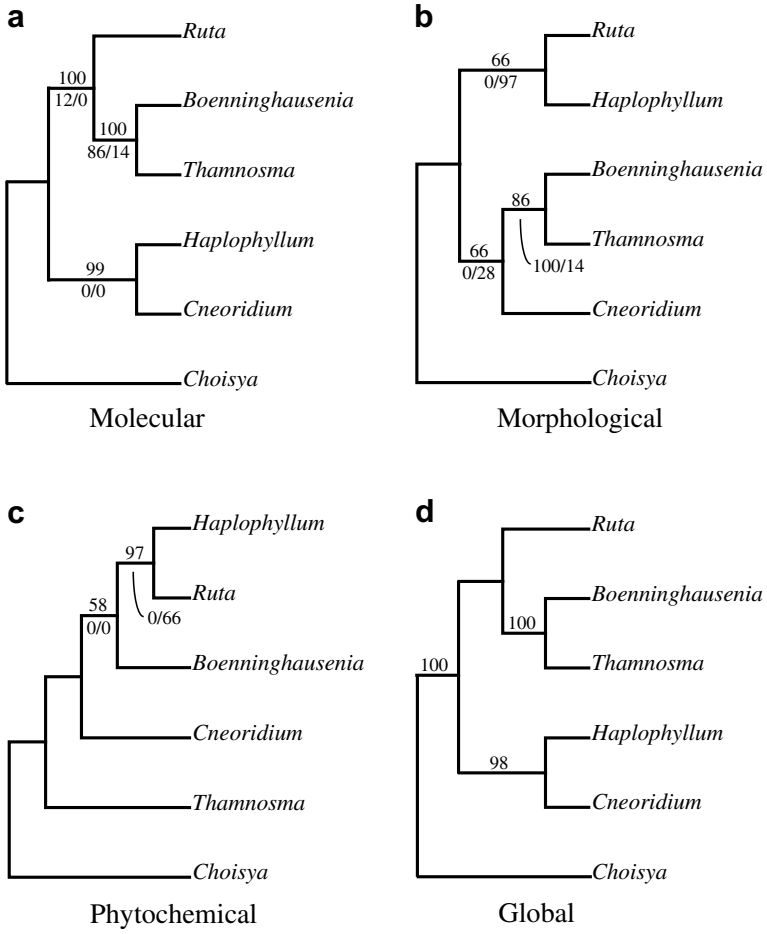

Fig. 2. Trees obtained from parsimony analyses of molecular (a), morphological (b), phytochemical (c), and global (d) datasets. *Choisya* served as the rooting taxon. Bootstrap percentages (BPs) generated by the same dataset used to infer the tree are reported above the branches; BPs generated by the rival datasets are reported below the branches: (a) BP morphology/BP phytochemistry, (b) BP molecules/BP phytochemistry, (c) BP molecules/BP morphology.

Table 5
Character diagnostics and trees resulting from the analysis of the molecular, morphological, phytochemical, and global datasets

Dataset	No. of characters	Parsimony informative characters	No. of optimal trees	No. of steps	CI (CI ex)	RI
Molecular	3176	179	1	673	0.924 (0.801)	0.791
Morphological	16	12	1	41	0.878 (0.828)	0.667
Phytochemical	31	18	1	38	0.816 (0.667)	0.667
Global	3223	209	1	769	0.899 (0.760)	0.721

CI, consistency index; CI ex, consistency index excluding parsimony uninformative characters; RI, retention index.

anatomy, but disagreeing with the taxonomists' assignment of *Ruta* and *Dictamnus* near to one another." (Moore, 1936: 322)

Despite the morphological and phytochemical differences between *Dictamnus* and the other genera of Ruteae and its distant relationship with other Ruteae in the cpDNA phylogeny (Fig. 1), the S–H test suggests that this phylogenetic result is not significantly different than the inclusion of *Dictamnus* in Ruteae (Table 3, constraint i). This result contrasts with the significant rejection of the second constraint assessed by the S–H test, i.e., forcing *Haplophyllum* to be sister to *Ruta* (see below). However, it should be noted that forcing *Dictamnus* in Ruteae applies a rather relaxed constraint compared to forcing *Haplophyllum* to be sister to *Ruta*, because in the former case all the trees with all possible placements of *Dictamnus* within Ruteae are allowed, whereas in the latter case only trees where *Haplophyllum* is sister to *Ruta* are allowed. Therefore, it is difficult to compare the significance values of intrinsically different constraints. To our knowledge, the potential influence of the stringency of the constraint on the significance levels estimated by the S–H test has not yet been investigated. Hence, while the results of the S–H test do not reject the possible inclusion of *Dictamnus* in Ruteae, the optimal cpDNA tree topology, morphological observations (Moore, 1936; Gut, 1966; Corner, 1976), and phytochemical data (Da Silva et al., 1988) all suggest that this genus may not have evolved from the same common ancestor as the other members of Ruteae (Engler, 1896, 1931).

In Engler's (1896, 1931) classification, *Ruta* comprised around 60 species, subdivided in subgenus *Euruta*, with five species, and subgenus *Haplophyllum*, with about 50 species (Table 1). If Engler's interpretation were correct, species ascribed to the two subgenera would be expected to form sister clades in a phylogeny. However, this is not the case in the cpDNA tree inferred in this study (Fig. 1), for the eight currently-recognized species of *Ruta* (*R. chalepensis, R. angustifolia, R. graveolens, R. corsica, R. montana, R. pinnata, R. microcarpa*, and *R. oreojasme* Townsend, 1968; Bramwell and Bramwell, 2001); form a strongly supported monophyletic group that is sister to *Boenninghausenia*/*Thamnosma*, while the 24 species of *Haplophyllum* constitute the sister clade of *Cneoridium*. Moreover, when *Haplophyllum* was constrained to be sister to *Ruta*, the differences between the likelihood score of the constrained and unconstrained topologies, evaluated by means of the S–H test, were statistically significant (Table 3). Hence, the molecular results corroborate Townsend's (1986) interpretation of generic boundaries (Table 1).

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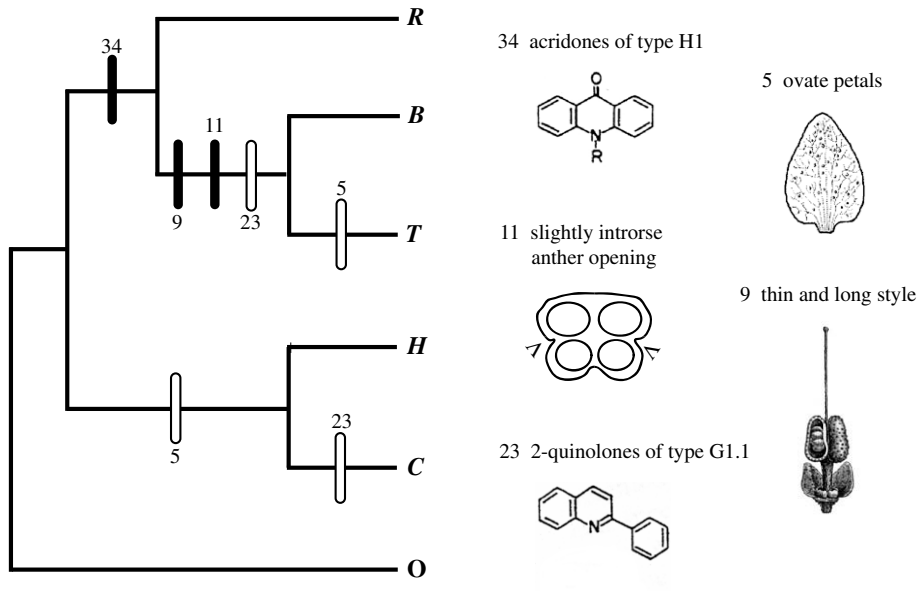

Fig. 3. The five derived non-molecular character states consistent with the clades supported by molecular data: 5, ovate petals (taken from Townsend, 1986); 9, thin and long style (taken from Engler, 1931); 11, slightly introrse anther opening (shown in cross-sectional view with the lower part facing the gynoecium and the arrows indicating the direction of pollen release; personal observation and Prof. P. Endress pers. comm.); 23, 2-quinolones of type G1.1 (taken from Da Silva et al., 1988); 34, acridones of type H1 (taken from Da Silva et al., 1988). Black and white bars indicate non-homoplasious and homoplasious transitions, respectively. *R*, *Ruta*; *B*, *Boennighausenia*; *T*, *Thamnosma*; *H*, *Haplophyllum*; *C*, *Cneoridium*, O, Outgroup (*Choisya*). See Supplementary data 2 and 3 for further details.

Townsend (1986) identified morphological differences between *Ruta* and *Haplophyllum* that had been overlooked by Engler, 1896, 1931 (Table 2). In fact, the petal margins of *Ruta* are dentate or fimbriate, whereas those of *Haplophyllum* are more or less entire; in *Ruta* the lateral flowers are 4-merous and the terminal flowers are 5-merous, whereas in *Haplophyllum* both lateral and terminal flowers have the same merism (usually 5-merous); the seeds of *Ruta* are bluntly- to sharply-angled dorsally, whereas they are reniform and dorsally-rounded in *Haplophyllum*; and finally the pollen grains of *Ruta* have elongate costae and a finely reticulate to perforate tectum ornamentation, whereas the pollen grains of *Haplophyllum* have thick costae and a closed-striate tectum. Phytochemically, while *Haplophyllum* has a predominance of alkaloids over coumarins (45%), all its most closely related genera have either more coumarins (*Ruta*, 86%; *Thamnosma*, 88%; *Boenninghausenia*, 90%) or exclusively coumarins (*Cneoridium*), an observation that, combined with additional phytochemical evidence, led Da Silva et al. (1988) to recommend that *Haplophyllum* be recognized as a distinct genus from *Ruta*. Therefore, our molecular phylogenetic results, statistical tests on constrained topologies, Townsend's (1986) detailed morphological analyses, and Da Silva et al. (1988) phytochemical data all suggest that *Haplophyllum* should be treated as a distinct genus, rather than as a subgenus of *Ruta*.

Within *Ruta*, the seven species represented by multiple accessions were all supported as monophyletic by the cpDNA genome and the main clades are congruent with morphological observations or distribution (see Fig. 1). For example, San Miguel (2003) stated that *R. angustifolia*, *R. chalepensis*, and *R. graveolens* are morphologically very similar and virtually impossible to differentiate in their vegetative parts, whereas *R. montana* is distinguished by its narrower leaves. The three species of *Ruta* from the Canary Islands are distinct from the remaining species of the genus by being taller (Townsend, 1968; Bramwell and Bramwell, 2001) and having larger leaves (G. Salvo, personal observation), consistent with the observation that insular species exhibit trends toward larger size (Lomolino et al., 2006).

Recently, the populations of *R. corsica* from Sardinia were described as a new species, *R. lamarmorae*, distinguished from the Corsican populations of *R. corsica* (i.e., *R. corsica s. str.*) by morphological, ecological and karyological differences (Bacchetta et al., 2006). *R. corsica s. str.* is diploid, has smaller flowers, stamens, and ovaries, occurs across a wider altitudinal range (1000–1900 m.a.s.l.) and its pollen matures in June. *R. lamarmorae* is tetraploid, has bigger flowers, stamens and ovaries, occurs in a more restricted altitudinal range (1500–1750 m.a.s.l.), and its pollen matures in May. In the molecular phylogeny (Fig. 1), the accessions from Corsica and Sardinia formed two strongly supported clades, rather than being interspersed, thus suggesting that the treatment of *R. lamarmorae* as a separate species might be warranted. The phylogenetic separation between *R. corsica s. str.* and *R. lamarmorae* reflects the comparatively high absolute number (seven; data not shown) of nucleotide substitutions between the respective accessions from Corsica and Sardinia, two islands in close proximity to each other. In contrast, the accessions of *R. chalepensis* from distant locations (Sicily, Greece, Corsica, Sardinia, mainland France; see Supplementary data 1) are separated at most by five nucleotide substitutions (data not shown). A more definitive assessment of the proposed specific rank of *R. lamarmorae* (Bacchetta et al., 2006) must await further evidence from the nuclear genome, molecular dating analyses, and inter-fertility studies.

4.2. Comparisons among different datasets

Phylogenies of *Ruta* and related genera inferred from six-taxon morphological, phytochemical, and molecular matrices were compared to identify and localize incongruence between the datasets (Fig. 2), rather than to argue for or against combining data (e.g., Cannatella et al., 1998). Nodes with low statistical support ambiguously represent hierarchical patterns within individual datasets, thus conflict among datasets cannot be inferred from comparisons involving weak nodes (de Queiroz, 1993; Mason-Gamer and Kellogg, 1996; Graham et al., 1998; Van der Niet et al., 2005). Following this logic, direct node-to-node comparisons of bootstrap values detected no incongruent relationships between the molecular and morphological trees (Fig. 2a and b), while the ILD test suggested significant incongruence. Beyond the known criticisms of the ILD

test (e.g., Dolphin et al., 2000; Yoder et al., 2001; Darlu and Lecointre, 2002; Quicke et al., 2007), in the case of the molecular and morphological trees of *Ruta* and related genera, the incongruence estimated by the ILD test might reflect sampling bias in the smaller morphological dataset (12 informative characters, as compared to the 179 informative characters of the molecular dataset; Table 5), rather than conflicting phylogenetic signals (e.g., Cannatella et al., 1998; Graham et al., 1998). The only significant inconsistencies among the three trees (Fig. 2a–c) involved the relationships of *Ruta* and *Haplophyllum*, for the two genera were sister to each other in the phytochemical tree (BP 97), but in the molecular tree the former was sister to *Boenninghausenia*/*Thamonosma* (BP 100) and the latter to *Cneoridium* (BP 99). What could cause the observed incongruence between the molecular and phytochemical topologies?

In the phylogeny generated from parsimony analysis of the six-taxon cpDNA matrix (Fig. 2a), the terminal branches subtending *Haplophyllum* and *Cneoridium* are the longest, with 147 and 85 steps, respectively, while the branch leading to their common ancestor is much shorter (38 steps; results not shown). Since parsimony methods are known to be particularly vulnerable to long-branch attraction (LBA), as compared to model-based methods (Felsenstein, 1978; Hendy and Penny, 1989; Lewis, 2001), it is possible that the *Haplophyllum*/*Cneoridium* clade in the molecular tree (Fig. 2a) be a product of LBA. However, the analysis of the 73-accession molecular matrix by either parsimony or Bayesian methods (Fig. 1) supported the sister relationship between *Haplophyllum* and *Cneoridium*, thus suggesting that LBA should not have biased our results for the six-taxon dataset.

Homoplasy is often invoked to explain disagreements among phylogenies inferred from different types of data (e.g., Sanderson and Hufford, 1996; Wiens et al., 2003). In secondary chemical compounds, homoplasy has been repeatedly documented, because similar selective pressures can lead to the evolution of pathways producing similar end-products in unrelated taxa (Price, 1963). Within Rutaceae, the expression of coumarin prenylation patterns, furoquinoline and acridone oxygenation patterns, and the development of the acridone and carbazole nuclei are all known to occur in unrelated taxa (Waterman, 1990). Most of the secondary chemical compounds used to infer the phytochemical phylogeny of Fig. 2c are alkaloids. Some studies have shown that alkaloid biosynthesis in plants is both plastic and labile, for the responsible genes can be repeatedly switched on and off during development and across evolutionary times (McKey, 1980; Wink and Witte, 1983; Waterman, 1998; Wink, 2003). For example, the genes responsible for the biosynthesis of quinolizidine alkaloids are widely represented in the plant kingdom, but are only expressed in a few unrelated families (Wink and Witte, 1983). Similar examples include the evolution of the benzylisoquinoline alkaloid biosynthesis (Liscombe et al., 2005), and the production of pyrrolizidine alkaloids as a defense against herbivores (Reimann et al., 2004). Waterman (1975) commented that the alkaloid types found in Rutaceae, with the exception of the canthinones and carbazoles, exhibit a highly random distribution and fail to support any of the taxonomic groups proposed by Engler (1896, 1931). Therefore, considering the well-known problem of convergence among secondary compounds (Hegnauer, 1966; Mothes, 1981), it seems more likely that the incongruent placement of *Haplophyllum* in the phytochemical and molecular trees (Fig. 2a and c) might be caused by convergence of similar alkaloids in *Haplophyllum* and *Ruta*, rather than long-branch attraction between *Haplophyllum* and *Cneoridium*.

The analysis of phytochemical data is hampered not only by problems of homoplasy, but also by methodological complications, for example: (i) a specific compound can be detected only when a sufficient amount of it is present in the plant (Price, 1963); and (ii) chemotaxonomic reports for Rutaceae rarely mention the names of the species for which certain compounds were sought but were not detected, a problem also encountered in an angiosperm-wide study (Nandi et al., 1998). Therefore, the absence of a specific compound from certain species in a phytochemical data matrix may indicate true absence, lack of detection due to technical limitations, or missing information. Hence, despite the considerable diversity of secondary chemical compounds in Rutaceae (e.g., Price, 1963; Waterman, 1975, 1983, 1990; Kong et al., 1986; Da Silva et al., 1988; Samuel et al., 2001; Zakaria, 2001), their systematic and phylogenetic value appears to be fundamentally flawed by homoplasy and methodological issues. Considering the above-mentioned problems, Waterman (1998: 547), the foremost expert on the secondary compounds of Rutaceae (Fish and Waterman, 1973; Waterman, 1975, 1983, 1990; Gray and Waterman, 1978; Waterman and Grundon, 1983), asserted that "chemical systematics remains as much an art as a science, and the most appropriate use of chemical data appears to be to test phylogenies that have arisen from the interpretation of more complete non-chemical datasets."

4.3. Character mapping analysis and genus diagnosis

The choice of the characters used to build the phylogenies that are in turn utilized to analyze the evolution of selected character sets remains a controversial issue, because, at its core, it influences assessments of homology and homoplasy in different datasets (Brooks, 1996). Considering the dearth, overlapping nature, and conflicting taxonomic value of the morphological characters traditionally used for Ruteae classifications (Townsend, 1986) and the well-known problem of convergence in the phytochemical data of Rutaceae (Waterman, 1990), it seems reasonable to use the strongly supported topology of the molecular tree (Figs. 1 and 2a) for the mapping of non-molecular characters (Figs. 3 and 4).

The optimization of character-state transitions on the molecular topology underscored the difficulty of finding non-molecular synapomorphies that are consistent with the clades of the molecular tree. Out of 47 mapped characters, only five provided character-state transitions that supported the molecular clades (characters 5, 9, 11, 23, 34; Fig. 3). Moreover, the morphological characters used by Engler (1896, 1931) to differentiate among the genera of Ruteae (i.e., characters 1, 2, 3, 4, 7, 8, 12, 14; Supplementary data 2) were either equivocally reconstructed or inconsistent with the molecular clades. Engler (1896, 1931) placed high taxonomic importance on flower merism (Table 2). However, the numbers of sepals, petals, stamens, and carpels are polymorphic in *Ruta* and the latter also in *Haplophyllum* (characters 1–4, see Supplementary data 2; Saunders, 1934; Moore, 1936). Additionally, all four characters were equivocally reconstructed (Fig. 4). Consequently, the change between 5 and 4 elements in the perianth whorls and between 10 and 8 stamens in the androecium could have occurred either by reduction or by expansion (Fig. 4), corroborating the suggestion that these characters may be evolutionarily labile (Endress, 1990, 1999), and thus of limited taxonomic value. Conversely, in the gynoecium, the transition to two carpels in *Thamnosma* and one in *Cneoridium* always occurred by reduction, regardless of the ancestral state for the clade formed by *Ruta*, *Boenninghausenia*, *Thamnosma*, *Haplophyllum*, and *Cneoridium* (Ruteae s.s.; Fig. 4). Similar reductionist trends have been previously reported in other angiosperm families and explained in terms of paedomorphic development caused by the elimination of the last initiated organ (Tucker et al., 1993; Hufford, 1996). An alternative interpretation, also consistent with the optimizations of floral merism on the molecular topology (Fig. 4), assumes that the ancestor of Ruteae s.s. was polymorphic for floral merism, thus implying that *Ruta* retained the ancestral polymorphisms for all floral whorls

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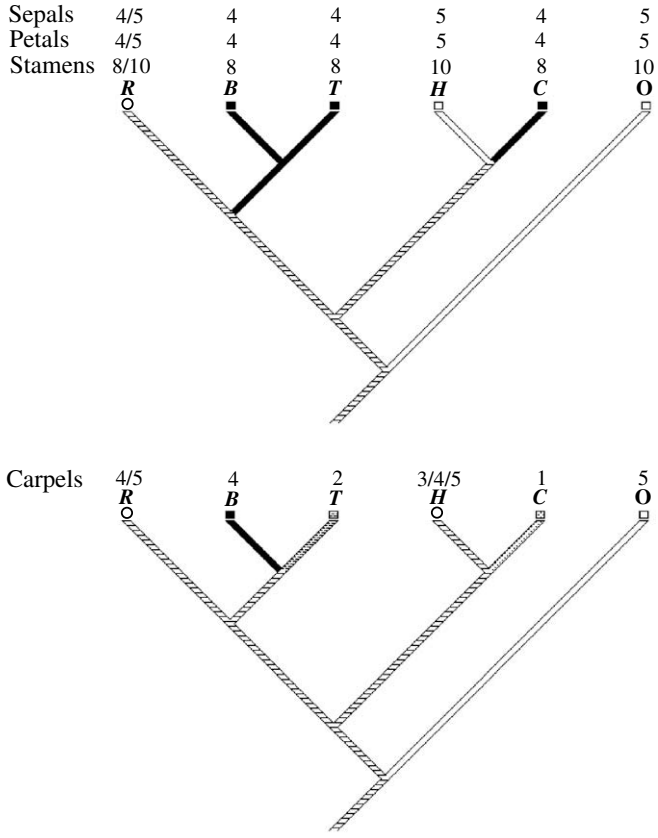

Fig. 4. Optimizations of transitions between states for the four characters associated with floral merism: number of sepals, petals, stamens, and carpels. The states of each character, representing the number of units within each whorl, are symbolized by different patterns in the boxes above of the branches; coexistence of different states for the same character within the same taxon is symbolized by a circle. Branches with horizontal lines indicate equivocal reconstructions. Name of taxa as in Fig. 3. See Supplementary data 2 for further details.

and *Haplophyllum* for the gynoecium only. Fixation of the number of organs in the perianth and androecium occurred in the lineages leading to *Boenninghausenia*/*Thamnosma* and *Cneoridium*/*Haplophyllum*, respectively, while carpel number was reduced in the ancestor of *Boenninghausenia* and *Thamnosma* and, independently, in *Cneoridium* (Fig. 4). Repeated processes of selection leading to fixation of character states from a polymorphic ancestor have indeed been proposed as a likely evolutionary explanation for multiple transitions between character states (Brooks, 1996). Thus the results of character mapping indicate that the way forward to understand patterns of homoplasy in the floral morphology of Ruteae *s.s.* may lie in detailed comparisons between the ontogenetic trajectories of floral whorls.

Within the context of the phylogenetic relationships supported by molecular data (Figs. 1 and 2a), *Ruta*, the type genus for the family, can be diagnosed by using a combination of plesiomorphic, homoplasious, and autapomorphic morphological character states, including: obovate petals and short, thick style (both plesiomorphic states, for they are also present in the outgroup); cushion-shaped nectary disk, elongate anthers, and uncurved embryo (all homoplasious states present also in *Haplophyllum*); introrse anthers' opening (a homoplasious state present also in *Haplophyllum* and *Cneoridium*); simple stigma shape and seeds angled dorsally (two homoplasious states present also in *Boenninghausenia* and *Thamnosma*, respectively); and dentate or fimbriate petal margins (an autapomorphy for *Ruta*; see Supplementary data 2).

5. Conclusion

The finding that traditionally important taxonomic characters do not provide support for the clades identified in molecular phylogenies has become a frequent occurrence with the widespread development of molecular systematics (e.g., Lavin et al., 2001; Moylan et al., 2004; Van der Niet et al., 2005; Norup et al., 2006). When mapped onto a molecular phylogeny, characters originally used to build classifications have been found to be plesiomorphic (Lavin et al., 2001), homoplasious (Moylan et al., 2004; Norup et al., 2006), or simply uninformative for diagnosing clades (Van der Niet et al., 2005). It has been remarked that the frequency of homoplasy in traditional taxonomic characters may reflect the fact that they are usually optimized on molecular topologies, thus establishing an *a priori* bias for homoplasy in non-molecular datasets (Grant, 2003). In other words, homoplasy may in part derive from inappropriate comparisons between classes of characters at different hierarchical levels of organization (Doyle, 1996; Minelli, 1998). This consideration may be especially relevant to the homoplasy observed in the mapping of phytochemical characters onto the Ruteae molecular tree, for different biogenetic pathways can produce the same compound (Hegnauer, 1966; Waterman, 1990). Consequently, the biogenetic pathways leading to these compounds, and not the compounds *per se*, should be examined and scored as character states. Rather than being viewed in a negative sense, the identification of homoplasy in non-molecular characters should be used as a starting point to study its biological and methodological causes, focusing especially on the developmental pathways underlying phenotypic traits and the conflicting assessments of homology often performed when comparing characters at different levels of organization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.09.004.

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