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# Molecular cytogenetics of *Dictyoloma vandellianum* A. Juss. and the ancestral karyotype of Rutaceae

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## ABSTRACT

Determination of the chromosome base number of a taxon is fundamental to understanding karyotypic variation and its implications for the evolution of that group. This usually requires careful evaluation of cytological literature and robust phylogenetic support. The base number for the family Rutaceae ($x$ = 9 or $x$ = 18) has long been the subject of debate. Here, we analyzed the banding pattern, rDNA sites, and genome size of *Dictyoloma vandellianum*, subfamily Cneoroideae, the sister group of the remaining Rutaceae, and revised critical points about the chromosome base number of the family. We found that this species has $n$ = 9, which differs from the $n$ = 18 possessed by other cytologically known Cneoroideae species. Thus, $n$ = 9 occurs in the main clades of Rutaceae and is the most probable base number of the family. The hypothesis of $x$ = 18 as the base number is no longer sustainable, although $n$ = 18 is very common in Rutaceae. Moreover, the fluorescent banding pattern and the relatively large genome size (1C = 1.3 pg) of *D. vandellianum* suggest that its chromosomal organization is highly divergent from Aurantieae, the only large Rutaceae clade where species with $n$ = 9 are greatly dominant.

**Keywords:** Aurantieae, chromosome base number, Cneoroideae, *Dictyoloma vandelianum*, genome size, heterochromatin, rDNA sites

## Introduction

The Rutaceae is a highly diversified and nearly cosmopolitan plant family comprising 150–162 genera and 1500–2096 species (Groppo *et al*. 2012). The systematic treatment of Engler (1931) split Rutaceae into seven subfamilies, based mainly on fruit and floral characters, although molecular phylogenetic studies suggest the exclusion of the monogeneric subfamily Rhabdodendroideae and the inclusion of some genera from other families (*Harrisonia* (Simaroubaceae), *Cneorum* (Cneoraceae), and *Ptaeroxylon* (Ptaeroxylaceae)) to ensure its monophyletic status (Chase *et al*. 1999; Groppo *et al*. 2008; Morton & Telmer 2014). That infrafamilial division appears unacceptable, however, as no other subfamily having more than one genus is monophyletic (except the subfamily Aurantioideae (Chase *et al*. 1999), which includes *Citrus* and related genera).

Currently, only two subfamilies of Rutaceae are recognized (Groppo *et al*. 2012): Cneoroideae, comprising only eight genera (Appelhans *et al*. 2011), including *Cneorum* and *Dictyoloma*; and Rutoideae, which congregates four former Englerian subfamilies (Rutoideae, Aurantioideae, Toddalioideae, and Flindersioideae). Because of the large economic, agricultural, and ethnobotanical interest in *Citrus*

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species, Aurantieae (formerly subfamily Aurantioideae) was, for many years, the only tribe extensively investigated (*e.g.*, Groppo *et al*. 2012; Wu *et al*. 2018; Nagano *et al*. 2018). The karyology and cytotaxonomy of Aurantieae were well-explored by both classical (Guerra 2009) and molecular methods (Wu *et al*. 2018; Costa Silva *et al*. 2019; He *et al*. 2020; Mendes *et al*. 2020), although the ancestral or base chromosome number of the family is still a matter of debate (Shan *et al*. 2006).

It became clear at the beginning of the last century that *Citrus* and related genera shared the stable chromosome number $n = 9$, except for a few polyploids (Guerra *et al*. 2000; Mou & Zhang 2012). Smith-White (1954) analyzed 69 Australian Rutaceae species and concluded that the base number of the family was $x = 9$, although polyploids with $n = 18$, or multiples of 18, were widespread in the family. A critical genus for his assumption was the highly variable *Boronia*, with species having $n = 7, 8, 9, 11, 16, 18$, as well as some intraspecific polyploids (later, this range was extended with new counts of $n = 10, 22, 36, 54$; see Shan *et al*. 2006). James (1981) argued, however, that species and genera of Rutaceae with $n = 18$ were diploids and suggested that the base number of the family was $x = 18$, with the lower numbers representing descending dysploidies. Weston *et al*. (1984) constructed a cladogram for the species based on 32 morphological traits of 37 species of *Boronia*, with their known chromosome numbers superimposed, and concluded that $x = 9$ explained the chromosome number variation observed in this genus more parsimoniously than $x = 18$. Stace *et al*. (1993) reevaluated that cladogram and noted a progressive reduction from $n = 18$ in the most basal lineage (*Boronia* section *Cyanothamnus*) to $n = 11, 9, 8$, and 7 in the most derived ones, thus supporting $x = 18$ for the genus. Additionally, $n = 18$ and $n = 36$ were largely dominant among the most basal representatives of the family, suggesting $x = 18$ as the ancestral chromosome number for the family as a whole (Stace *et al*. 1993). The assumption that haploid numbers lower than 18 originated through descending dysploidy was also supported by a comparative analysis of karyotype symmetry in *Boronia* (Shan *et al*. 2003a; 2006).

The concept of base (or ancestral) chromosome numbers is currently understood as the haploid number observed in a monophyletic group that most parsimoniously explains the chromosomal variability in that clade, and that shows a clear relationship with the base numbers of the most closely related taxa (Guerra 2000). The correct identification of the base number of a taxon is fundamental to understanding the karyotype variation occurring in that taxon as well as the implications of that variability to the evolution of the group. The search for that number usually demands a careful and critical evaluation of the cytological literature that will eliminate erroneous data and secure robust phylogenetic support (Guerra 2000). Alternatively, the base number can be inferred using probabilistic models (*e.g.*, Freyman & Höhna 2017), although such methods must be considered with caution as chromosome numbers are controlled by natural selection (Levin 2002). Several important taxonomical rearrangements have been undertaken with Rutaceae, making it necessary to reevaluate the base number of the family in light of current classifications. The most recent classifications of Rutaceae (Groppo *et al*. 2008, 2012; Morton & Telmer 2014) recognized a small subfamily, Cneoroideae, as the sister group of all other Rutaceae. The only species of Cneoroideae karyologically know are *Cneorum pulverulentum* and *C. tricocum*, both with $2n = 36$ (Chromosome Count Database, Rice *et al*. 2015; http://ccdb.tau.ac.il/), reinforcing the hypothesis of $x = 18$ for the family.

Karyotype characteristics other than chromosome number, such as genome size, heterochromatic bands, and rDNA chromosomal sites can also be used to better understand karyotype variations and evolution (reviewed by Weiss-Schneeweiss & Schneeweiss 2013). Heterochromatic bands and several DNA sequence sites have recently been examined in attempts to better understand the chromosome evolution of the former subfamily Aurantioideae (Wu *et al*. 2018; Yuan *et al*. 2019; Costa Silva *et al*. 2019; He *et al*. 2020; Mendes *et al*. 2020), which is now included in the subfamily Rutoideae as the tribe Aurantieae – although very little is currently known about other Rutaceae (Shan *et al*. 2003b).

We present here a detailed karyotypic analysis of *Dictyoloma vandellianum*, the only representative of the genus (Groppo 2010) formerly placed in the Rutaceae subfamily Dictyolomatoideae but now recognized for the subfamily Cneoroideae. We analyzed its chromosome number and size, the chromosomal locations of heterochromatin and rDNA sites, as well as genome size – and the implications of these characters to understanding the base number of the family are discussed.

## Materials and methods

### Plant material

Two young individuals of *Dictyoloma vandellianum* A. Juss. were obtained from a seedling nursery in Tambaú, São Paulo, Brazil, and cultivated in the Jardim Experimental do Departamento de Botânica da Universidade Federal de Pernambuco, Recife, Brazil. A voucher was deposited in the Prof. Jayme Coelho de Moraes herbarium (at the Universidade Federal da Paraíba, Brazil, voucher EAN 29425).

### Preparation of slides

For mitotic analyses, young root tips were pretreated with 0.002 M 8-hydroxyquinoline at 10 ° C for 24 h, fixed in ethanol-acetic acid (3:1, v/v) for 2 h at room temperature, and stored at –20 °C. The fixed root tips were washed in distilled water, digested in a 2 % cellulase (Onozuka)-20 % pectinase

(Sigma) solution at 37 °C for one hour, and macerated in a drop of 45 % acetic acid. After coverslip removal in liquid nitrogen, the slides were air-dried and stained with a 2 µg/ml DAPI–glycerol (1:1) solution. The best preparations were then selected and subsequently destained in ethanol–acetic acid (3:1), air dried, and aged for 3 days at room temperature.

### *Chromosome staining with CMA/DAPI and FISH*

Chromosome double staining with the fluorochromes chromomycin A3 (CMA, Sigma) and 4',6-diamidino-2-phenylindole (DAPI, Sigma) was performed as described by Moraes *et al*. (2007). Briefly, the aged slides were stained with CMA (0.1 mg/ml) for 1 h followed by DAPI (1 µg/ml) for 30 min, and then mounted in 1:1 (v/v) McIlvaine's pH 7 buffer-glycerol. Images of the best cells were acquired using a Leica DMLB microscope equipped with a Cohu CCD video camera, and processed using QFISH Leica software.

The fluorescent *in situ* hybridization (FISH) procedure followed the protocol described by Moraes *et al*. (2007). The probes used for 5S and 35S rDNA sites were D2 from *Lotus japonicas* (Regel) K. Larsen and R2 from *Arabidopsis thaliana* (L.) Heynh., respectively. D2 was labeled with Cy3 dUTP, and the R2 probe was labeled with digoxigenin-11-dUTP (Roche), both by nick translation (Invitrogen). The hybridization mixture contained 60 % formamide (v/v), 5 % dextran sulfate (w/v), 2×SSC, and 2–5 ng/µl of the 35S rDNA probe. The 35S rDNA probe was detected with sheep anti-digoxygenin–FITC (Roche) antibody, and the signal amplified with FITC-conjugated anti-sheep secondary antibody (Serotec). All preparations were counterstained and mounted with 2 µg/ml DAPI in Vectashield (Vector). Images of cells previously stained with CMA/DAPI were captured as before, and processed for brightness and contrast using Adobe Photoshop CS6.

### *DNA content estimation and chromosome measurements*

DNA content estimations were performed using a CyFlow SL (Partec) cytometer and Flomax software (Partec), following Doležel *et al*. (2007). Briefly, small pieces of fresh young leaves from *Dictyoloma vandellianum* (and from *Glycine max* (L.) Merr. "Polanka" (2C = 2.55 pg), as internal standard), were mixed in a glass Petri dish with 0.6 mL ice-cold Otto buffer I (0.1 M citric acid + 0.5% Tween 20), co-chopped with a razor blade, and filtered through a 30 µm nylon mesh membrane into a cytometry tube. After addition of 0.6 mL Otto II buffer (0.4 M $Na_2HPO_4 12H_2O$) supplemented with RNAse (50 µg/mL) and propidium iodide (50 µg/mL), the samples were immediately analyzed by flow cytometry. Nuclear DNA content (2C value) was calculated as: (sample G1 peak mean/standard G1 peak mean) × 2C DNA content of standard (pg).

In order to evaluate chromosome size and morphology, five metaphases of *Dictyoloma vandellianum* were measured using Adobe Photoshop CS3 version 10.0 software. The chromosome arm ratio (AR = length of the long arm/length of the short arm) was used to classify the chromosomes as metacentric (AR = 1.0-1.4), submetacentric (AR = 1.5-2.9), or acrocentric (AR ≥ 3.0), according to Guerra (1986). Chromosome pairs were ordered from I to VI according to the sizes of their short arms.

## Results

The two plants investigated had $2n = 18$, with metacentric to acrocentric chromosomes, four of them with a terminal secondary constriction and a small satellite (only visible in prometaphase). The CMA/DAPI stained chromosomes displayed two pairs of terminal $CMA^+$ bands (Fig. 1A-C); weak terminal $DAPI^+$ bands were sometimes observed in prometaphase chromosomes. The weak $DAPI^+$ bands became very well contrasted after *in situ* hybridization, showing a single terminal $DAPI^+$ band per chromosome (except with the second largest chromosome pair, which had a $DAPI^+$ band at both termini) (Fig. 1D). One or two pairs of interstitial dot-like bands were also observed (arrows in Fig. 1D). The rDNA probes revealed two pairs of terminal 35S rDNA sites co-localized with the $CMA^+$ bands (Fig. 1E). The only 5S rDNA site observed was adjacent to the 35S rDNA site of the largest chromosome pair, in a subterminal position (Fig. 1F).

The DNA content estimation made using young leaves was 1C = 1.30 pg, and the average haploid karyotype length was 32.6 µm, with individual metaphase chromosomes varying in size from 2.5 to 5.2 µm. Figure 1G presents a karyogram based on Figure 1A. The number below each chromosome pair indicates its average length (obtained from five well-spread metaphases). The chromosomes were ordered according to their sizes. They were recognized by the following combination of markers: I – the largest pair with $CMA^+$ bands, 5S and 35S rDNA; II – largest submetacentric pair with a $DAPI^+$ band at both chromosome termini; III – largest acrocentric; IV – the smallest chromosome pair bearing $CMA^+$ bands and 35S rDNA sites; V – chromosomes with two closely located $DAPI^+$ bands, observed after FISH; VI, second largest acrocentric; VII, VIII, and IX – chromosomes without bands, distinguished only by small differences in their sizes.

## Discussion

### *Comparing the karyotype of Dictyoloma vandellianum with those of Aurantieae*

There is very few information available concerning the karyotypes of other non-Aurantieae Rutaceae having $2n = 18$ beyond their chromosome numbers. The species of *Boronia* with $2n = 18$ had chromosomes smaller than *Dictyoloma*

*vandellianum*, and there were only meta- to submetacentric chromosomes, except for a single acrocentric pair in *B. denticulata* (Shan *et al*. 2003a). That latter species had two pairs of 35S rDNA sites, but no 5S rDNA sites (Shan *et al*. 2003b). No other structural chromosome information for species with n= 9 is available, besides for Aurantieae. In relation to Aurantieae, the karyotype of *Dictyoloma vandellianum* differed in the following aspects: 1. It had acro/telocentric chromosomes that are absent in Aurantoideae species (Guerra 1993; Costa Silva *et al*. 2019); 2. Apart from the CMA$^+$ heterochromatic bands associated with 35S rDNA sites, *Dictyoloma vandelianum* had no other CMA$^+$ band, whereas most Aurantieae species have several other CMA$^+$ bands associated with different repetitive sequences (Barros e Silva *et al*. 2010; Deng *et al*. 2019; He *et al*. 2020); 3. DAPI$^+$ heterochromatic bands, as observed in *Dictyoloma vandellianum,* were absent in Aurantieae (Guerra *et al*. 2000). Faint DAPI$^+$ bands were observed after direct CMA/DAPI staining in *Dictyoloma* but they became more evident after FISH, due to the differential DNA extraction of the FISH procedure (Barros e Silva & Guerra 2010); 4. The average chromosome size of *Dictyoloma vandellianum* was nearly

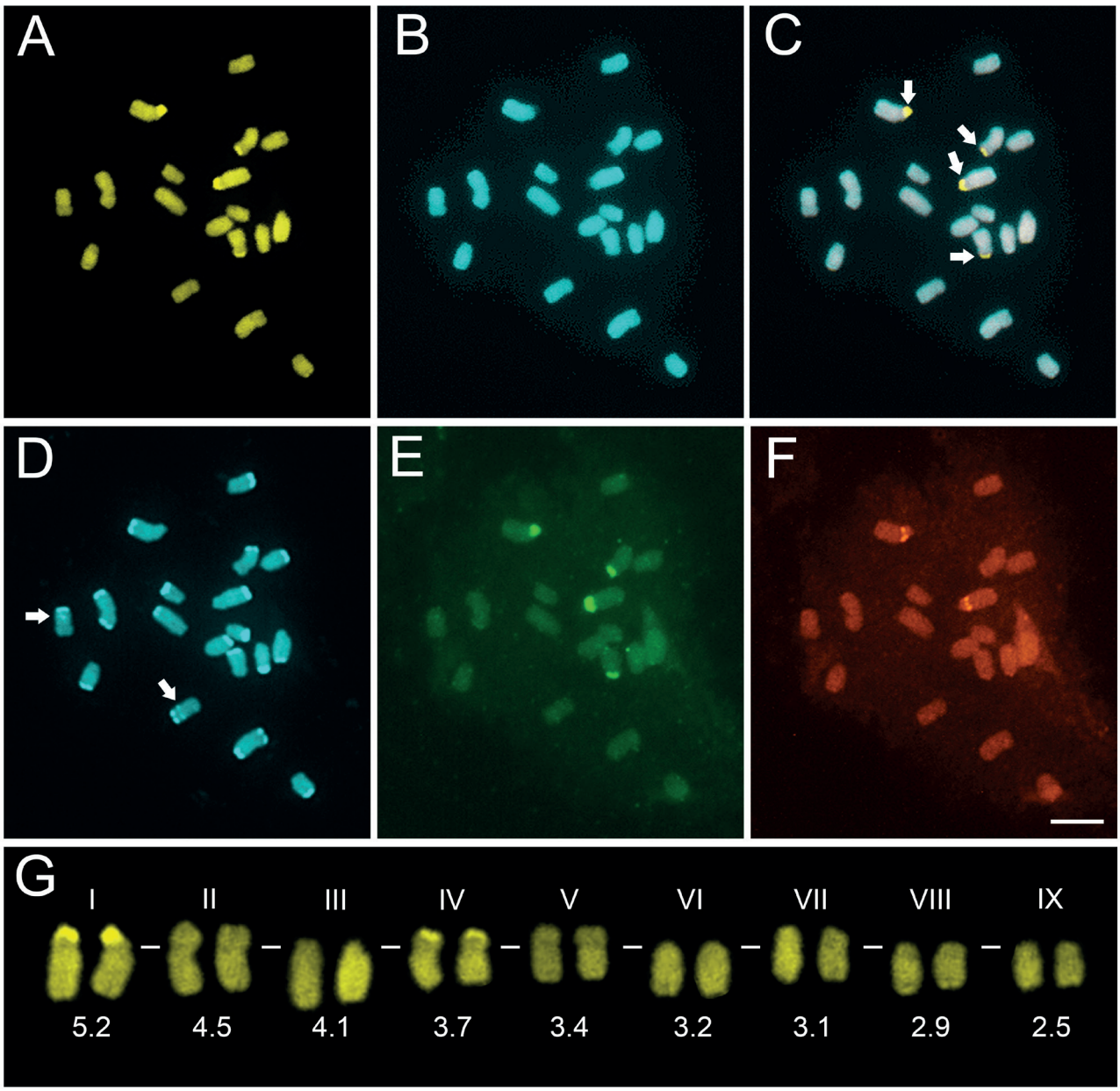


**Figure 1.** Metaphase of *Dictyoloma vandellianum* stained in different ways. **A-C.** CMA (**A**), DAPI (**B**), and merged CMA-DAPI image (**C**). Arrows in **C** highlight CMA$^+$ bands. **E-F.** The same cell after FISH showing heterochromatic bands stained with DAPI (**D**) and 35S (**E**) and 5S rDNA (**F**) sites. Arrows in **D** point to subterminal bands. **G.** Karyogram showing chromosome pairs ordered from the largest to the smallest and nominated in roman numbers (above). The average chromosome size obtained from five metaphases is indicated below each chromosome pair. Horizontal lines indicate centromeres. Bar in **F** corresponds to 5 µm.

twice that of Aurantieae (*e.g.*, Costa Silva *et al*. 2019); 5. The genome size (1C = 1.30 pg) of *Dictyoloma vandellianum* was more than twice the average value for Aurantieae species (1C = 0.53 pg) (The Plant DNA C-values database, release 7.1; https://cvalues.science.kew.org/. Pellicer & Leitch 2020). *Dictyoloma vandellianum* has the highest known 1C value among diploid species of Rutaceae, and its average DNA content per chromosome (0.14 pg) is one of the highest for the family (Guerra 1984).

Curiously, *Dictyoloma vandellianum* displayed a single pair of 5S rDNA sites adjacent to one of its 35S rDNA sites, a characteristic observed in nine out of 10 other Aurantieae genera investigated (Barros e Silva *et al*. 2013). Adjacent 5S/35S rDNA sites have been observed in many angiosperm species, but in no other suprageneric taxon have they been so extensively conserved as in Aurantieae (Roa & Guerra 2015). It would be interesting to know to what extent this rather instable characteristic is conserved in other Rutaceae. The positions of 35S rDNA sites in other Rutaceae species have only been reported for some *Boronia* species (Shan *et al*. 2003b), but without information concerning 5S rDNA sites.

## *The base number of Cneoroideae and its implication to the base number of Rutaceae*

The finding of $2n = 18$ in *Dictyoloma vandellianum* (together with the known $2n = 36$ in the closely related species of *Cneorum*) suggest that the base number of the subfamily Cneoroideae is $x = 9$. Assuming Cneoroideae as the sister group to the remaining Rutaceae (Rutoideae or "core Rutaceae"; Groppo *et al*. 2008; 2012), it can be seen that $n = 9$ and $n = 18$ are represented in both clades. The core Rutaceae is split into two branches: the tribes Aurantieae and Ruteae (except *Dictamnus*) plus the genus *Chloroxylon* and the "RTF Clade", composed of representatives of the former subfamilies Rutoideae (except the genera *Ruta* and *Chloroxylon*) and Toddalioideae plus the genus *Flindersia* (Groppo *et al*. 2012). Figure 2 indicates the phylogenetic relationships among those main taxa. The haploid numbers $n = 9$ and $n = 18$ occur in at least one genus of each of these clades, indicating the hypothesis of $x = 18$ as unsustainable, as it would imply that a descending dysploid series from $n = 18$ to $n = 9$ occurred independently in Aurantieae, Cneoroideae, Ruteae, and in the RTF clade.

Within the entire Rutaceae family, the only descending dysploid series known from $n = 18$ to $n = 9$ (with intermediate numbers $n = 10, 11, 12, 16$) is that reported by Smith-White (1954) and complemented by Shan *et al*. (2006) in the genus *Boronia* (included in the RTF clade). However, the species of the former *Boronia* section *Cyanothamnus,* most of them with $n = 18$, are now segregated into the genus *Cyanothamnus* (Duretto *et al*. 2020). As $n = 9$ appears in most sections of *Boronia* and $n = 18$ is now absent, the base number of the genus should be $x = 9$ (Weston *et al*. 1984). The remaining chromosome numbers of *Boronia* probably arose through dysploidy, polyploidy, and by interspecific hybridizations, as experimentally demonstrated by Astarini *et al*. (1999).

The base number $x = 9$ of Rutaceae appears to be unique within the order Sapindales (Raven 1975), although most other families have been less investigated cytologically. It is currently accepted that all sequenced eudicot genomes are derived from a paleohexaploid ancestor originated through a Whole Genome Triplication that occurred before the split of the rosids and asterids (Jiao *et al*. 2012; Murat *et al*. 2017). That was the last polyploidy event before the origin of *Citrus* and other Aurantieae (Leebens-Mack *et al*. 2019), and the origin of several triplicated chromosome segments present in the genomes of those species (Xu *et al*. 2013; Yuan *et al*. 2019).

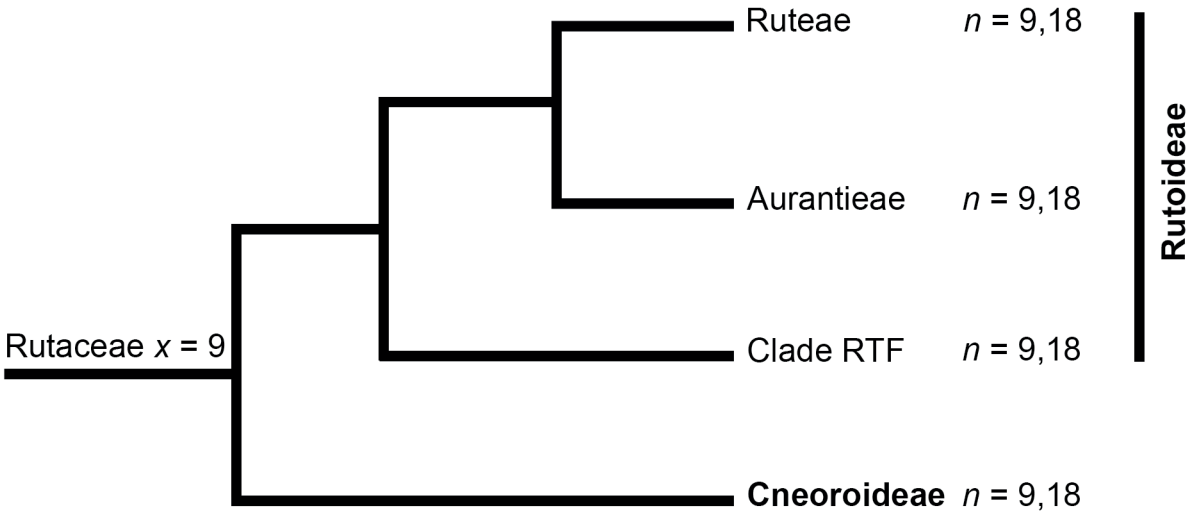


**Figure 2.** Phylogenetic relationships among Rutaceae main clades. Note the occurrence of chromosome numbers $n = 9$ and $n = 18$ in all these clades. Subfamilies highlighted in bold. Cladogram modified from Groppo *et al*. (2012).

Taken together, our data suggest that the ancestral chromosome number of Rutaceae, $x = 9$, has been conserved in some evolutionary lineages since the family originated at 94 Mya and then intensified diversification at 80 Mya (Muellner-Riehl *et al*. 2016). Nevertheless, structural aspects of the ancestral karyotype (such as chromosome size and morphology, the presence/absence of heterochromatic bands, AT/GC content, nuclear DNA content, among others) are certainly quite distinct among extant $n = 9$ lineages. Parallel to the few taxa that conserved $n = 9$, other lineages with $n = 18$ generated the large number of tetraploid genera found throughout the geographic distribution of the family, such as *Zieria, Zanthoxylum, Clausena, Dictamnus, Melicope, Phellodendron, Calodendrum, Esenbeckia,* and *Flindersia* (Stace *et al*. 1993). Additional genome sequencing of other distantly related non-Aurantieae species with $n = 9$, such as *Dictyoloma vandellianum* and some species *of Haplophyllum, Thamnosma,* and *Boronia*, would be expected to shed more light on the karyological evolution of Rutaceae.

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