Phylogeny and biogeography of Spathelioideae (Rutaceae)

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Chapter 4

Cneorum (Rutaceae) in Cuba? The solution to a 150 year old mystery.

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Published in: Taxon 59 (4), 2010: 1126-1134

Abstract

Cneorum trimerum (Urban) Chodat is only known from the type specimen collected in 1861 in eastern Cuba. The species has sometimes been regarded as a synonym of *C. tricoccon* L., which is otherwise confined to the Mediterranean. As no other *Cneorum* specimens are known from Cuba, the specimen is a mysterious finding with a disputed taxonomic rank. The goal of this study is to clarify the status of the Cuban specimen using molecular and wood anatomical data. We succeeded in extracting DNA out of the 150 year old type specimen in our ancient-DNA lab and amplified two chloroplast markers (*atpB*, *trnL-trnF*) and one nuclear marker (ITS). Comparison of the sequence data with several sequences from *C. tricoccon* clearly suggests inclusion of the Cuban specimen into the latter species; wood anatomical features confirm the molecular results. The transatlantic distribution of *C. tricoccon* is probably the result of an introduction in Cuba by humans.

Keywords: ancient DNA; *Cneorum*; Cuba; Rutaceae; transatlantic distribution; wood anatomy

Introduction

Cneorum L. is a genus of two or three species of flowering plants which has traditionally been placed in its own family, Cneoraceae, but is nowadays placed in Rutaceae (Sapindales) subfamily Spathelioideae based on molecular data (Chase *et al*., 1999; Groppo *et al*., 2008). The species grow as small shrubs, usually not exceeding 1.5 m, with simple and lanceolate leaves, and small, yellow flowers (Tutin, 1968; Bramwell & Bramwell, 1990). One species, *C. tricoccon* L., occurs in the western part of the Mediterranean and a second, *C. pulverulentum* Vent., is endemic to the Canary Islands (Bramwell & Bramwell, 1990; Traveset, 1995b). The two can be easily distinguished: *C. tricoccon* has trimerous flowers, nearly glabrous leaves, and tricolporate pollen, while *C. pulverulentum* is characterised by tetramerous flowers, densely pubescent leaves and 4–6-colporate pollen grains. Some authors (Van Tieghem, 1898; Erdtman, 1952) assign the two species to distinct genera because of the rather large differences, naming the Canary species *Chamaelea pulverulenta* Tiegh. or *Neochamaelea pulverulenta* (Vent.) Erdtman respectively.

A third species of *Cneorum* has been recognised based on a specimen collected in Cuba in 1861. It was first described as *Cubincola trimera* Urban (Euphorbiaceae) in 1918, and transferred to *Cneorum* as *C. trimerum* (Urban) Chodat in 1920 (Urban, 1918; Chodat, 1920). There are strong morphological similarities between the Mediterranean *C. tricoccon* and the Cuban *C. trimerum*. Lobreau-Callen & Jérémie (1986) compared macromorphological characteristics and the pollen morphology of the two species and proposed to merge them into a single species. However, wood anatomical characters seem to differ significantly between the two species and indicate stronger similarities of *C. tricoccon* to *C. pulverulentum* than to the Cuban *C. trimerum* (Carlquist, 1988).

The occurrence of *Cneorum* in the Mediterranean and Cuba has led to speculations about the historical biogeography of the genus. *Cneorum* is often regarded as a very old genus (Riera *et al*., 2002 and Traveset, 1995a,b assumed *C. tricoccon* to be of early Tertiary origin) and the transatlantic distribution was interpreted as the result of allopatric speciation caused by the divergence of the South American (and Caribbean) and African tectonic plates during the Jurassic or early Cretaceous (Melville, 1967; Lobreau-Callen, 1974; Straka *et al*., 1976; Borhidi, 1982, 1991; Lobreau-Callen & Jérémie, 1986). In contrast, Oviedo *et al*. (2009) assume that *C. trimerum* is a synonym of *C. tricoccon* (following Lobreau-Callen & Jérémie, 1986) and conclude a recent introduction of *Cneorum* by humans in Cuba.

During our studies we came across many misidentified herbarium specimens named *C. trimerum*; only one specimen - the type specimen - proved to be a *Cneorum*. As wood anatomical features are the only suggested discriminating characters between *C. tricoccon* and *C. trimerum*, we decided to reinvestigate the wood anatomy based on the type material. In this study, we combine the wood anatomical survey with a molecular phylogenetic study in order to decide on the taxonomic status of the Cuban specimen. Sequences of *atpB*, *trnL-trnF* and ITS obtained from the type specimen of *C. trimerum* were compared to sequences of five specimens of *C. tricoccon* using a Bayesian analysis and a maximum likelihood approach. *Cneorum pulverulentum* from the Canary Islands, the related *Harrisonia abyssinica* Oliv., and *Ruta graveolens* L. (Rutaceae) were chosen as outgroups.

The major questions of this study are: (1) Should *C. tricoccon* and *C. trimerum* be merged or do they represent two species? (2) Can the putative wood anatomical differences between *C. tricoccon* and *C. trimerum* be confirmed? (3) What are the true identities of the misidentified "*Cneorum trimerum*" specimens? (4) What are the biogeographical implications of the results?

Materials & Methods

Taxon sampling

Five specimens of *Cneorum tricoccon*, one of *C. pulverulentum*, one specimen of *Harrisonia abyssinica* (Rutaceae) and the type of *C. trimerum*, were used for molecular study (Appendix). A wood sample of the type specimen of *C. trimerum* (C. Wright s.n., GOET) was taken for wood anatomical observations and compared with the literature for *C. tricoccon* (Carlquist, 1988; Schweingruber, 1990) and *C. trimerum* (Carlquist, 1988). For *Cneorum pulverulentum*, *atpB* and *trnL-trnF* sequences were retrieved from GenBank (Accession numbers: EU853787, AF209567; www.ncbi.nlm .nih.gov). Sequences from *Ruta graveolens* (Rutaceae) as outgroup were also taken from GenBank (accession numbers: AF035913, EU853815, FJ434146).

Wood anatomical methods

Because the thickest available part of the stem from *C. trimerum* was only about 3 mm in diameter, sectioning in the traditional way was exceedingly difficult. We therefore embedded the material into LR white resin (London Resin Company Ltd., Reading, U.K.) following the company's instructions for plant material, and cut transverse, tangential and radial sections of 10 µm using a rotary microtome equipped with a glass knife (Leica 2065 Supercut), stained in 1% Toluidine Blue and mounted on gelatine-laminated slides in Canada-Balsam. Samples for macerations and for scanning electron microscopy were prepared and cut as described in Jansen *et al.* (1998). We followed the IAWA list of microscopic features for hardwood identification (Wheeler *et al.*, 1989) for our wood anatomical descriptions.

Molecular methods: DNA extraction, amplification and sequencing

All laboratory work on the 150-year-old type specimen of *Cneorum trimerum* was performed first, before analysing the other *Cneorum* specimens, to exclude contamination. Total DNA was extracted from the specimens mentioned in the Appendix except for *C. trimerum* using a standard CTAB protocol (Doyle & Doyle, 1990). DNA from the type specimen of *C. trimerum* was extracted in the Leiden Ancient DNA Facility (LAF) using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with following modifications: all steps were executed under a extractor hood; all pipette tips, buffers, racks and tubes were irradiated under UV-light before usage; and 0.6 mg Proteinase K (30 µl of 20 mg/ml) was added for the elongated (45 min) cell lysis step. The markers *atpB*, *trnL-trnF* and ITS were amplified using the primers designed by White *et al.* (1990), Taberlet *et al.* (1991), and Hoot *et al.* (1995). A total of five internal primer pairs had to be designed in addition to the existing primers (Hoot *et al.*, 1995) to obtain the complete *atpB* sequence of *C. trimerum* (Table 4-1). Primers were designed using Primer 3

(Rozen & Skaletsky, 2000).
PCRs of the DNA fragments were carried out in 25 µl total reaction volume containing 1 µl of template DNA, 2 mM MgCl2, 0.4 µM each of forward and reverse primer, 0.1 mM of each dNTP, 0.3 µg BSA (Promega, Madison, Wisconsin, U.S.A.) and 1 unit of Taq DNA polymerase (Qiagen, Hilden, Germany). Initial denaturation was 7 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 51°C–55°C, and extension for 30 s to 1.5 min (depending on the fragment length) at 72°C. A final extension for 7 min at 72°C was carried out. PCR products were checked for length and yield by gel electrophoresis on 1% agarose gels, cleaned using the Wizard® SV Gel and PCR Clean-Up kit (Promega, Madison, Wisconsin, U.S.A.) following the authors instructions and sent to Macrogen (www.macrogen.com) for sequencing. The obtained sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) under the accession numbers given in the Appendix.

Primer name	Sequences	Author
S2F	TATGAGAATCAATCCTACTACTTCT	Hoot & al. 1995
S322R	GCACGTTRAAAATTCGTCCT	Appelhans & al. (present study)
S280F	CACRGGAGCKCCTCTAAGTG	
S539R	CTGTTTTACCCACTCCMGCTC	
S492F	GGGGAGGAAAAATCGGACTA	
S825R	YGCTTGTACGAAACGRAARA	
S769F	GGCGGAATATTTCCGAGATG	
S1026R	AGTAGCATCTAAATGGGCAAATG	
S972F	TTCAAGCGGTTTATGTACCC	
S1263R	AATTTTKCGCGCTCTTGCTA	
S1218F	CTATCCTTGGGTTRGACGAA	
S1494R	TCAGTACACAAAGATTTAAGGTCAT	Hoot & al. 1995

Table 4-1. Location and base composition of the newly designed internal primers for *atpB*. The positions given in the primer name are based on the *atpB* sequence for Spinacia oleracea (U23082) on which the positions of the Hoot & al. (1995) primers are also based. The position of the reverse primers is in relation to the first base in 5´– 3´ direction.

Molecular methods: Sequence editing, alignment and phylogenetic analysis
Complementary strands were assembled and edited using SequencherTM (Gene Codes, Ann Arbor, Michigan, U.S.A.). The sequences for the three markers were aligned by hand using MacClade v.4.08 (Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.).
We concatenated the sequences for the three markers into one data matrix after checking for

significance with the incongruence length difference (ILD) test (Farris *et al*., 1995) as implemented in PAUP* v.4.0b10 (Swofford, 2002) and after running separate phylogenetic analyses for each marker in MrBayes (Ronquist & Huelsenbeck, 2003) using the settings described below. The ILD test and the tree topologies of the separate analyses revealed no conflict between the partitions.

A Bayesian phylogenetic analysis was performed using MrBayes v.3.1.2. (Ronquist & Huelsenbeck, 2003). The models of sequence evolution were determined using MrModeltest v.2.2. (Nylander, 2004b) and set for the partitioned data matrix as follows: *atpB*—GTR model using gamma distribution rate variation among sites; *trnL-trnF*; and ITS—GTR model using inverse gamma distribution rate variation among sites. The temperature parameter value was set to 0.02. The Markov chain Monte Carlo was run in two independent runs with one cold chain and three hot chains each until stationarity was reached.

One tree every 100 generations was sampled. The first 25% of the trees were discarded as burn-in and all other trees were used to calculate a 50% majority-rule consensus tree.

The maximum likelihood (ML) analysis was executed using PAUP* v.4.0b10 (Swofford, 2002). All characters were unordered and equally weighted. A heuristic search using stepwise-addition was carried out on the combined dataset of *atpB*, *trnL-trnF*, and ITS sequences using the GTR + G model. Bootstrap support values were obtained from 500 replicates and a 50% majority-rule consensus tree was calculated.

Results

Identity of the misidentified "Cneorum trimerum" specimens

The only specimen observed named "*Cneorum trimerum*" and belonging to *Cneorum* is the type specimen (Fig. 4-1A). Other specimens examined were sterile collections from 1979 (*J. Bisse, H. Dietrich, D. Duany, J. Gutiérrez, E. Köhler, L. Lepper HFC40296*; B) and 1922 (*E.L. Ekman 14433*; K; det. by Urban), which were clearly misidentifications and do not belong to *Cneorum*. With the help of R. Oviedo (pers. comm.) we were able to identify the specimen *HFC40296* (Fig. 4-1B) which is *Hypericum fasciculatum* Lam. (Hypericaceae). Oviedo *et al.* (2009) studied several specimens named *Cneorum trimerum* and correctly identified them as *Schoepfia stenophylla* Urban (Schoepfiaceae). The specimen shown in Fig. 4-1C (*E.L. Ekman 14433*) also belongs to *S. stenophylla* (own observation).

The material of *C. trimerum* studied by Carlquist (1988) is based on a wood sample deposited in the Oxford University Herbaria (FHOw 10768; S. Harris pers. comm.). The - in all probability (Oviedo *et al.*, 2009) - associated herbarium voucher (*G.C. Bucher 168*) belonging to the wood specimen is deposited in the University of Madison and at the Instituto de Ecología y Sistemática at Havana (Oviedo *et al*., 2009; own observations). Oviedo *et al.* (2009) concluded that the specimen (*G.C. Bucher 168*) studied by Carlquist (1988) must belong to *C. tricoccon*. However, during a visit in Havana (HAC), the first author and R. Oviedo examined the specimen *G.C. Bucher 168* and identified it instead as *Schoepfia stenophylla*. Since Oviedo *et al.* (2009) report that the wood sample FHOw 10768 belongs to that herbarium specimen, it is likely that the material studied by Carlquist (1988) in fact belongs to *Schoepfia* and not to *Cneorum*.

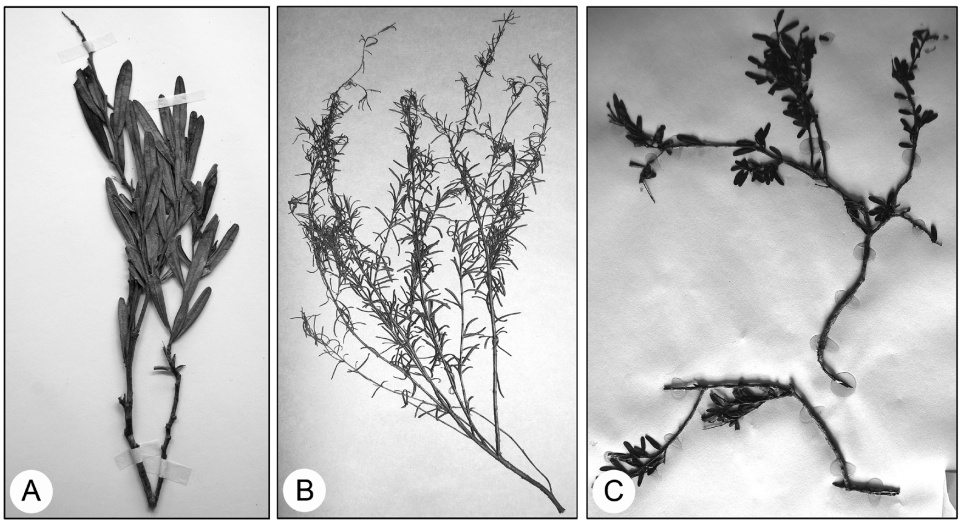

Fig. 4-1. Herbarium specimens named *Cneorum trimerum* (Urb.) Chodat. **A**, type specimen of *C. trimerum* (*C. Wright, s.n.*, GOET); **B**, *Hypericum fasciculatum* Lam. misidentified as *C. trimerum* (*J. Bisse, H. Dietrich, D. Duany, J. Gutiérrez, E. Köhler, L. Lepper, HFC40296*, B); **C**, *Schoepfia stenophylla* Urban misidentified as *C. trimerum* (*E.L. Ekman, 14433*, K).

Wood anatomy

The wood anatomical characters of the type specimen of *Cneorum trimerum* are in strong agreement with the characters of *C. tricoccon*, but strikingly contradict previous information on *C. trimerum* (Carlquist, 1988). The wood of the type specimen of *C. trimerum* shows growth rings and may be regarded as semi ring-porous. Vessels are arranged in diagonal aggregations and show a dendritic pattern (Fig. 4-2A) which is not as distinctive as that published for *C. tricoccon*. Perforation plates are simple. Helical thickenings are very distinctive and occur throughout the body of all vessel elements (Fig. 4-2B). The mean length of the vessel elements is 340 µm (SD: 49 µm) with a mean diameter of 35 µm (SD: 5 µm). Intervessel pits are alternate and loosely arranged (Fig. 4-2C). The diameter of the pit borders range from 6 to 8 µm. Vascular tracheids are present in a vasicentric position and show distinctive helical thickenings. Fibres are thick-walled (Fig. 4-2D), non-septate, and have a mean length of 595 µm (SD: 91 µm). The minutely bordered pits occur in radial and tangential walls but are more common in radial walls. Parenchyma is scanty paratracheal, and in one-cell-layered discontinuous marginal bands (Fig. 4-2D). Rays are mostly uniseriate (Fig. 4-2D) but a small percentage of biseriate rays occurs. The ray height does not exceed 500 µm and the ray cells appear upright to squarish in a radial view (Fig. 4-2E). There were no storied structures, secretory elements or crystals observed.

Molecular phylogeny

The *atpB* (1405 bp alignment) and *trnL-trnF* (944 bp alignment) sequences of the type specimen of *C. trimerum* and the five specimens of *C. tricoccon* examined were completely identi-

cal, except for one site each, and some bases which could not be determined. In both cases, a single base of one of the five *C. tricoccon* specimens (*M. Appelhans MA236*) was different from *C. trimerum* and the other four specimens of *C. tricoccon*. The ITS sequences showed a little more variation: a total of three bases within the 746 bp alignment were variable within *C. tricoccon* and *C. trimerum* and a total number of 14 gaps occurred. The gaps were randomly distributed throughout the taxa and consisted of only one or two base pairs. Among the three variable bases were one autapomorphy for one of the *C. tricoccon* specimens (*M. Appelhans MA236*) and one autapomorphy for the *C. trimerum* type specimen. The third variable base pair grouped *C. trimerum* with three *C. tricoccon* specimens (*J.H. Wieffering 17265, E.F. Galiano & B. Valdés 999.71, M. Appelhans MA449*). The variability of the *C. tricoccon*/*C. trimerum* sequences towards those of *C. pulverulentum*, *Harrisonia abyssinica* and *Ruta graveolens* was significantly greater in the ITS alignment than it was for *atpB* and *trnL-trnF*.

The 50% majority-rule consensus trees of the Bayesian analyses based on *trnL-trnF* and *atpB* (not shown) alone show *C. trimerum* and *C. tricoccon* as an unresolved polytomy according to the nearly 100% identity of their sequences. Sister taxon to the polytomy was *C. pulverulentum* supported by a posterior probability of 1.00.

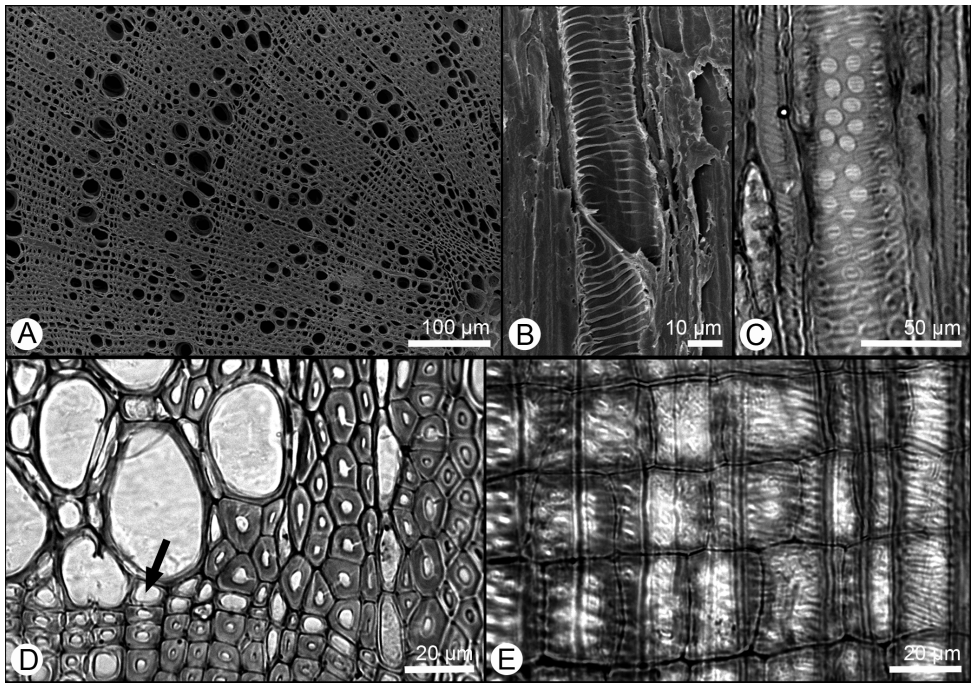

Fig. 4-2. Wood anatomical features of *Cneorum trimerum* (Urb.) Chodat (*C. Wright, s.n.*, GOET). **A**, transverse section showing weakly dendritic pattern of vessel elements (SEM photo); **B**, helical thickenings in vessel elements (SEM photo); **C**, alternate intervessel pits loosely arranged, tangential section; **D**, detail of a transverse section showing a one-cell-layered discontinuous marginal band of parenchymatic cells (arrow); **E**, square to upright ray cells in a radial section.

The 50% majority-rule consensus trees (Bayesian analysis) of the combined data matrix (Fig. 4-3) and ITS alone (not shown) provide slightly more resolution. *Cneorum* is monophyletic with a posterior probability of 1.00 and reveals *C. pulverulentum* as the sister group to *C. tricoccon* and *C. trimerum* also with a posterior probability of 1.00. The *C. trimerum* type specimen clusters together in a polytomy (posterior probability 0.90) with three specimens of *C. tricoccon* (*E.F. Galiano & B. Valdés 999.71, J.H. Wieffering 17265, M. Appelhans MA449*). This group forms a polytomy with the two other specimens (*M. Appelhans MA236, P. Heukels 193*) of *C. tricoccon* supported by 1.00 posterior probability.

The topology of the bootstrap 50% majority-rule consensus tree of the ML analysis shows exactly the same topology as the consensus trees from the Bayesian analyses based on ITS alone and the combined dataset. The monophyly of *Cneorum* and sister group relationship between *C. pulverulentum* and *C. tricoccon*/*C. trimerum* is supported by bootstrap values of 100. The five specimens of *C. tricoccon* and the type specimen of *C. trimerum* are grouped in a polytomy and, as in the Bayesian analyses, *C. trimerum* clusters together with three specimens of *C. tricoccon* (*E.F. Galiano & B. Valdés 999.71, J.H. Wieffering 17265, M. Appelhans MA449*) although this is weakly supported by a low bootstrap support of 55.

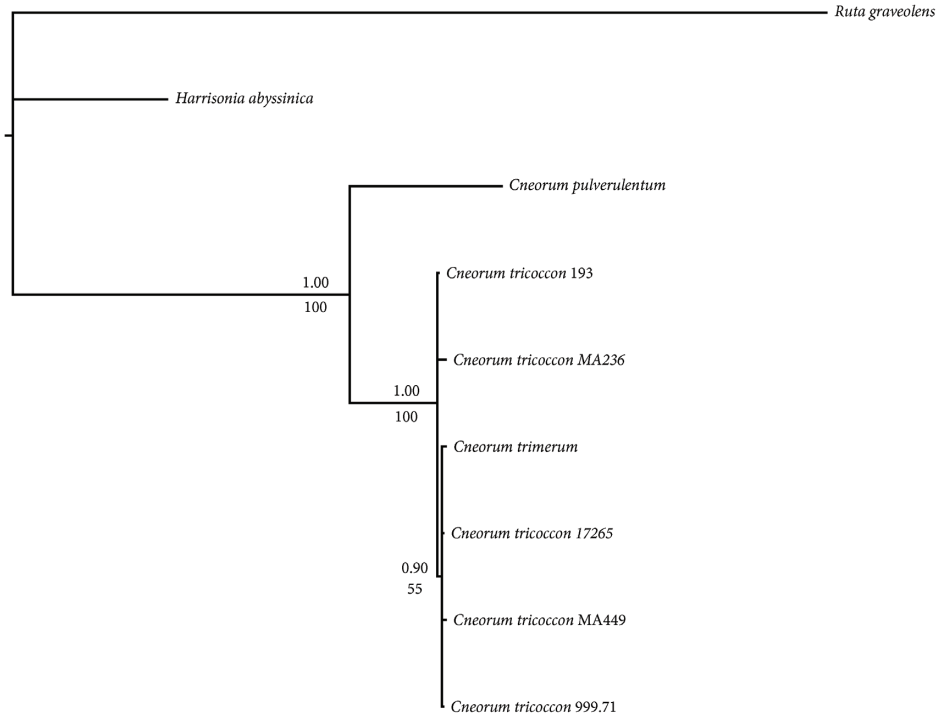

Fig. 4-3. 50% majority-rule consensus tree of the combined data matrix (*atpB*, *trnL-trnF*, ITS) analysis. Posterior probability values of the branches are given above the branches and the voucher numbers of the five *Cneorum tricoccon* specimens (see Appendix) are listed next to the species names. The bootstrap values of the maximum likelihood analysis are shown below the branches.

Discussion

Wood anatomy and molecular phylogeny

Both wood anatomy and molecular phylogeny clearly demonstrate that *Cneorum trimerum* is not a species on its own, and has to be included into *C. tricoccon*.

The wood anatomical features of the type specimen of *C. trimerum* show some minor differences with those of *C. tricoccon*. The dendritic pattern of the vessels is not as pronounced in *C. trimerum* as it is in *C. tricoccon*. Uniseriate with a low percentage of biseriate rays occur in *C. trimerum*, while uni-, bi-, and triseriate rays are of equal frequency in *C. tricoccon*. Ray cells in *C. trimerum* are upright to squarish but are mostly procumbent in *C. tricoccon* (Carlquist, 1988). All these differences may be explained by the small diameter/ immaturity of the stem of *C. trimerum*. The only differences that may not be explained by the age factor are the diameter of the intervessel pits, which is significantly bigger in *C. trimerum* (6–8 μm; this study) compared to *C. tricoccon* (3 μm; Carlquist, 1988)[9], and the rhomboid crystals that are present in some ray cells in *C. tricoccon* (Carlquist, 1988) but not in *C. trimerum*.

Our wood anatomical results surprisingly contradict the anatomical description of *C. trimerum* published by Carlquist (1988). Carlquist described the wood of *C. trimerum* as diffuse porous with vessels in small clusters or short radial multiples. He did not observe vascular (and vasicentric) tracheids and he mentions the presence of aliform or aliform-confluent axial parenchyma, which are not seen in the type material of *C. trimerum* (own observation) and the other *Cneorum* species (Carlquist, 1988; Schweingruber, 1990). Furthermore, no helical thickenings were present in Carlquist's material and multiseriate rays were more common than uniseriate ones. Storying is described for "vessels, axial parenchyma, and a few wider libriform fibres adjacent to axial parenchyma" (Carlquist, 1988: 12). These differences can by no means be explained by the low diameter/immaturity of the type material of *C. trimerum*, nor can climatic or ecological factors offer an explanation.

The material Carlquist studied (FHOw 10768) most likely belongs to a herbarium specimen (*Bucher 168*) that has been identified as *Schoepfia stenophylla* by Ramona Oviedo and the first author. Comparing the wood anatomical characters of Carlquist's material with the genus *Schoepfia* reveals a strong similarity. The wood of *Schoepfia* is diffuse porous and is characterized by aliform and/or confluent parenchyma, short and numerous rays and a lack of vascular tracheids (Metcalfe & Chalk, 1957; own observations). Additionally, the "helical grooves interconnect[ing] pit apertures in many vessels" (Carlquist, 1988: 12) are present in *Schoepfia* (own observation). The differences in wood anatomy between the type of *Cneorum trimerum* and the material studied by Carlquist, the strong similarity in wood anatomy between *Schoepfia* and Carlquist's sample, and the strong hint that Carlquist's material belongs to the herbarium specimen *Bucher 168* leads us to conclude that the *Cneorum trimerum* sample in Carlquist's (1988) study was based on misidentified material of *Schoepfia stenophylla*.

The wood anatomy of *C. pulverulentum* (Carlquist, 1988) is very close to that of *C. tricoccon*

[9] After the publication of this chapter, we measured the intervessel pits in Carlquist´s figure (1988) and Schweingruber´s material (1990; s.n., Mallorca, 3 slides) and found that their diameter is indeed also 6-8 μm in *Cneorum tricoccon*.

and the type specimen of *C. trimerum*. Similarities include the non-storied structure of the wood, the axial parenchyma arrangement and the presence of vascular tracheids (although less abundant in *C. pulverulentum*). Differences include the radially grouped vessels, grooved vessel walls instead of helical thickenings, the predominantly uniseriate rays and the absence of crystals in ray cells in *C. pulverulentum* (Carlquist, 1988). However, the latter two differences may be not diagnostic as we found mostly uniseriate rays in the type of *C. trimerum* and we did not observe crystals in the ray cells.

The wood anatomical results corroborate the macromorphological and palynological results by Lobreau-Callen & Jérémie (1978) and Lobreau-Callen *et al.* (1986), showing that there are no morphological and anatomical differences between *C. tricoccon* and *C. trimerum*. Our molecular phylogeny confirms this view as *C. trimerum* is clustered together in a polytomy with the *C. tricoccon* specimens, and because the monophyly of this group is beyond question. The genetic variation between the Cuban specimen and the five specimens of *C. tricoccon* is minimal. The three markers we chose are frequently used in reconstructing Rutaceae phylogenies, and especially *trnL-trnF* and ITS have proven to give good resolution at species level (Chase *et al.*, 1999; Morton *et al.*, 2003; Mole *et al.*, 2004; Poon *et al.*, 2007; Groppo *et al.*, 2008; Bayer *et al.*, 2009). Moreover, our selection of molecular markers covers one nuclear, one coding chloroplast, and one non-coding chloroplast marker, confirming that the low genetic variation is not biased due to the selection of markers.

Biogeographic implications

Based on the low genetic variation, a separation of the Mediterranean and the Cuban populations during tectonic movements in the Jurassic and Cretaceous, as it was assumed previously (Melville, 1967; Lobreau-Callen, 1974; Borhidi, 1982, 1991; Lobreau-Callen & Jérémie, 1986), can be definitely excluded as the cause of the present distribution of the genus. Our view is supported by molecular dating studies on Rutaceae (Pfeil & Crisp, 2008), where the age of Rutaceae is inferred to be between 53.3 to 72.7 Ma.

A more recent introduction of *Cneorum* to Cuba must have taken place instead. The fact that lizards are probably the only natural dispersers of *Cneorum* fruits (the introduced pine martens and genets also disperse the fruits; Traveset, 1995a,b; Riera *et al.*, 2002), as opposed to birds that would be capable of such long-distance dispersal, enhances the probability of an introduction of *Cneorum* to Cuba by humans. The introduction of *Cneorum* by men is discussed and favoured by Oviedo *et al.* (2009), who theorise that the genus could have been introduced by French colonists. *Cneorum tricoccon* is used as an ornamental plant in the Mediterranean (Straka *et al.*, 1976) and is also used in traditional medicine to treat ulcers and as a purgative (Duhamel de Monceau, 1755) which could have been the reasons for introducing it to Cuba. An introduction by humans would also explain why *Cneorum* has only been found once. Using this scenario, *Cneorum* would not have established in the warmer and wetter climate of Cuba and became extinct soon after its introduction on the island, explaining why only one Cuban specimen was found.

A second explanation is that it could be the result of a mix-up of specimens during mounting or labelling. This is unlikely because the plant has been collected as the host of the parasitic *Eremolepis wrightii* Griseb. which is endemic to Cuba (Urban, 1918).

Summing up, "one of the most intriguing geographical disjunctions among vascular plants"

(Lorenzo *et al.*, 2003: 953) is not a natural one and *Cneorum* must be abandoned in discussions about transatlantic genera.

Taxonomic aspects

Our analysis shows *Cneorum pulverulentum* as the sister taxon to *C. tricoccon*/*C. trimerum*. The most recently proposed name of this species is *Neochamaelea pulverulenta* (Vent.) Erdtman but this has been ignored by most recent authors (e.g. Caris *et al*., 2006; Appelhans *et al*., 2008; Groppo *et al.,* 2008) as well as by the APG (Stevens, 2001 onwards).

Neochamaelea pulverulenta was first described in 1802 (Ventenat, 1802) under the name *Cneorum pulverulentum* Vent. and was transferred to a new genus *Chamaelea* (*Chamaelea pulverulenta* (Vent.) Van Tieghem) in 1898 (Van Tieghem, 1898). Engler (1931) returned the species to *Cneorum*, but placed in a subgenus of its own, *Neochamaelea* Engl. Erdtman (1952) restored the species to generic rank under the name *Neochamaelea pulverulenta* (Vent.) Erdtman. Erdtman adopted *Neochamaelea* from the epithet of the subgenus recognised by Engler (1931) because *Chamaelea* Van Tieghem is a later homonym of *Chamaelea* Duhamel (1755) a superfluous name for *Cneorum* L. and first used for *Cneorum tricoccon* by pre-Linnaean botanists (e.g., Bauhin, Tournefort) and by French contemporaries of Linnaeus like Adanson, Gagnebin, and Lamarck.

The main characters that led to the separation of *Neochamaelea* from *Cneorum* were: type of indumentum, flower merosity, and pollen morphology (Van Tieghem, 1898; Erdtman, 1952). The indumentum of *N. pulverulenta* is strikingly different from that of *Cneorum tricoccon*. *Neochamaelea pulverulenta* has thick, T-shaped hairs which densely cover the leaves, the young shoots, and the gynophore (Lobreau-Callen *et al*., 1978). These hairs add a greyish to pale-green colour to the plant and account for the epithethon "*pulverulenta*/*pulverulentum*". The flowers of *N. pulverulenta* are tetramerous whereas trimerous flowers normally occur in *C. tricoccon*. This difference led Van Tieghem to separate them into two genera (Van Tieghem, 1898). However, this character is by no means stable as tetramerous flowers may sometimes also be observed in *C. tricoccon* (Traveset, 1995a).

Pollen morphological characters vary greatly between *N. pulverulenta* and *C. tricoccon*. Pollen grains of *N. pulverulenta* are 4–6-colporate, have a verrucose ornamentation, and are considerably larger than the tricolporate, striate-reticulate ornamented pollen grains of *C. tricoccon*. Based on the pollen morphological characters, Erdtman separated the species into two genera (Erdtman, 1952; Lobreau-Callen *et al.*, 1978). Erdtman (1952: 115) gives a rather vague citation of a voucher specimen mentioning only "(Canary Islands 1949!)". There is one specimen of *Neochamaelea pulverulenta* collected in 1948 in the herbarium (S) of the Swedish Museum of Natural History (*Sventenius s.n.*, A. Anderberg pers. comm.) which may be the source of the material that Erdtman studied. Considering this, there is a possibility that the study was also based on misidentified material. We therefore checked the pollen grains of one of our specimens (*T. Becker MA291*) by light microscopy and they match the descriptions by Erdtman (1952) exactly.

A further difference between the two species/genera is seen in their reproductive biology. Both *N. pulverulenta* and *C. tricoccon* have been described as andromonoecious (Tébar & Llorens, 1997) but *N. pulverulenta* might be (functionally) androdioecious (Lorenzo *et al*., 2003). Additionally, septal cavities in the ovules were found in *C. tricoccon* but are absent in *N.*

pulverulenta (Schmid, 1985; Caris *et al.*, 2006). Apart from these characters, the two species are very much alike. Both are small shrubs that usually reach about 1 m in height and do not exceed 2 m. They are characterised by simple, lanceolate, and estipulate leaves with an entire margin, similar small yellow flowers (except for the number of sepals and petals) and coccoid drupaceous fruits that fall apart into three to four drupelets at maturity. Further characters that unite the two species are the number of chromosomes (Goldblatt, 1976, 1979), the seed anatomy (Boesewinkel, 1984), and the propagation of the seeds by lizards (Valido & Nogales, 1994; Traveset, 1995a,b; Riera *et al.*, 2002; Rigueiro *et al.*, 2009).

Taxonomic conclusions

Based on our molecular and wood anatomical data, as well as the macromorphological and palynological data of Lobreau-Callen & Jérémie (1986), we propose the following synonymy:

Cneorum tricoccon L., Sp. Pl. 1: 34. 1753 ≡ *Chamaelea tricoccos* (L.) Lam. in Fl. Franç. 2: 682. 1779 – Lectotype (designated by Lobreau-Callen & Jérémie, 1986: 156): *Burser s.n.*, Herbarium-BURSER XXIV: 38 (UPS!).
= *Cubincola trimera* Urb. in Ber. Deutsch. Bot. Ges. 36: 502. 1918 ≡ *Cneorum trimerum* (Urb.) Chodat in Bull. Soc. Bot. Genéve 2: 23. 1920 – Type: *C. Wright s.n.*, 1861, in Cuba orient. (GOET!).

We propose to treat *Neochamaelea* as a synonym of *Cneorum* because the most important character (flower merosity) that discriminates between the two genera/species is variable, there is a large overall resemblance in habit and morphology, and the differences of the two are captured by the variety within a single genus. Also most recent authors ignored the name *Neochamaelea*, although they did not formally propose synonomy for it.

Cneorum pulverulentum Vent. in Descr. Pl. Nouv.: tab. 77. 1802 ≡ *Chamaelea pulverulenta* (Vent.) Tiegh. in Bull. Mus. Hist. Nat. (Paris) 4: 244. 1898 ≡ *Neochamaelea pulverulenta* (Vent.) Erdtman, Pollen Morph. & Pl. Taxon., Angiosp.: 115. 1952 – Lectotype (designated here): *W. Broussonnet s.n.*, in Tenerife, Herbarier de Ventenat G!; isolectotype: B-W! (IDC microfiche no. 7440).

Acknowledgements

The authors thank J. Heinrichs and S.R. Gradstein (GOET) for their permission to take samples from the unique specimen of *Cneorum trimerum*; M.C.M. Eurlings and B.J. van Heuven (L) for their help and assistance in the ancient-DNA lab and anatomical lab respectively; R. Oviedo (HAC) for the help in identifying the material of *Hypericum fasciculatum* and for the access to the *Schoepfia stenophylla* collections; J.F. Veldkamp (L) and L. Gautier (G) for helping to find the information on the type of *Cneorum pulverulentum*; A. Anderberg (S) for the

information on the pollen material of *Cneorum pulverulentum*; S. Harris (OXF) for information on the wood sample of *Cneorum trimerum*; the herbaria K and B for loans and access to their collections of *Cneorum*; the National Botanic Garden of Belgium in Meise for material of *Harrisonia abyssinica*; N. Webster (NCB Naturalis, Leiden) for checking the English and F. Lens (K.U. Leuven), J. McNeill (E), as well as three anonymous reviewers for very useful comments on the manuscript.

Appendix

Voucher specimens: species, collector and collection number (herbarium), country/region of collection, year of collection; GenBank accession numbers for *atpB*, *trnL-trnF*, ITS.

Cneorum pulverulentum Vent.: *T. Becker MA291* (L), Tenerife (Canary Islands, Spain), 2006; AF209567, EU853787, GU178979. *Cneorum tricoccon* L.: *E.F. Galiano & B. Valdés 999.71* (L), Spain, 1971; GU178991, GU178984, GU178975. *Cneorum tricoccon* L.: *J.H. Wieffering 17265* (L), France, 1969; GU178990, GU178983, GU178974. *Cneorum tricoccon* L.: *P. Heukels 193* (L), France, 1969; GU178989, GU178982, GU178973. *Cneorum tricoccon* L.: *M. Appelhans MA236* (L), Mallorca (Spain), 2005; GU178994, GU178988, GU178978. *Cneorum tricoccon* L.: *M. Appelhans MA449* ((L), Cultivated in Hortus botanicus Leiden, 2009; GU178995, GU178987, GU178981. *Cneorum trimerum* (Urb.) Chodat: *C. Wright s.n.* (GOET), Cuba, 1861; GU178992, GU178985, GU178976 and GU178977 (two parts of *trnL-trnF*). *Harrisonia abyssinica* Oliv.: *M. Appelhans MA313* (L), Cultivated in National Botanic Garden of Belgium (Meise), 2008; GU178993, GU178986, GU178980. *Ruta graveolens* L.: Sequences obtained from GenBank; AF035913, EU853815, FJ434146.