

## Generic limits in *Rhamnus* L. s.l. (Rhamnaceae) inferred from nuclear and chloroplast DNA sequence phylogenies

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This study tested the monophyly of the previously proposed genera *Alaternus*, *Frangula*, *Oreoherzogia*, and *Rhamnus* s.str., and the phylogenetic relations suggested by Grubov (1949), within the *Rhamnus* s.l. clade. Based on a global sample of 22 species, we derived phylogenetic hypotheses using parsimony analysis of variation in *trnL-F* (chloroplast) and ITS (nuclear) DNA regions. Both *Alaternus*, *Frangula*, and *Oreoherzogia* gained strong support, and our results further support recognition of *Frangula* as a monophyletic genus. The resolution between *Alaternus*, *Oreoherzogia*, and the rest of *Rhamnus* s.str. was less clear, and the mainly Mediterranean *Oreoherzogia* was strongly grouped with the American *R. crocea*. Therefore, we consider it as unjustified to split the rest of *Rhamnus* into smaller genera. Regarding Grubov's phylogenetic hypothesis, our study could only support the dichotomy between *Frangula* and the rest of *Rhamnus*.

**KEYWORDS:** *Alaternus*, *Frangula*, ITS, northern hemisphere, *Oreoherzogia*, parsimony, *trnL-F*.

### INTRODUCTION

*Rhamnus* L. (Rhamnaceae) comprises insect-pollinated and bird-disseminated dwarf-shrubs, shrubs, or trees, mainly inhabiting temperate and subtropical areas of the Northern hemisphere. A few species within *Rhamnus* are found in Africa, with *R. prionoides* reaching as far as South Africa, whereas the *Frangula* group has an area of diversification in the mountainous areas of the Neotropics with its southern limit in northern Argentina (Johnston & Johnston, 1978). Grubov (1949) suggested that *Rhamnus* consists of almost 200 species, but as the 25 species of Neotropical *Frangula* listed by him were reduced to 12 by Johnston & Johnston (1978), Grubov was criticized for elevating too many varieties to specific level. Grubov (1949) was well aware of this problem, however, as evidenced by his comments regarding section *Cervispina* (Grubov, 1949: 402) and within *Frangula* (Grubov, 1949: 381); the information in Mabberley (1997) listing 125 species within *Rhamnus* may therefore prove to be more accurate.

Recently, Richardson & al. (2000a, b) suggested that *Rhamnus* belongs to a well supported tribe within Rhamnaceae characterized by drupaceous fruits. This tribe also includes *Berchemia*, *Reynosia*, *Condalia*, *Karwinskia*, *Rhamnidium*, *Krugiodendron*, *Rhamnella*, *Sageretia*, and *Scutia*. As with all other species within Rhamnaceae, the flowers of the *Rhamnus* clade are char-

acterized by stamens positioned opposite to the petals, but a large number of species within *Rhamnus*, e.g., *R. cathartica* and *R. crocea*, have 4-merous flowers, differing from the general 5-merous floral pattern within Rhamnaceae.

According to Grubov (1949), there are several examples of parallel evolution from subtropical forests to colder and drier conditions within *Rhamnus*. Both the evolution of shrub and dwarf-shrub habits and changes of leaf types from large, naked, elliptic mesomorphic leaves to all stages of small, hairy, and stiff xeromorphic leaf types was considered evidence by Grubov (1949) for a thermohygrophilic ancestor within the pantropical flora of the Late Cretaceous to Early Eocene. Subsequent adaptations evolved in colder and drier habitats.

The intrageneric classification of the *Rhamnus* clade rests on studies of a limited number of species by Candolle (1825), Brongniart (1826), Boissier (1872), and Weberbauer (1895), who developed a basic classification based on presence/absence of winter bud scales and thorns, sexual system, type of inflorescence, fleshiness of fruits, and number of petals. Grubov (1949) and Suessenguth (1953) independently suggested an elaborate intrageneric division with numerous sections within *Rhamnus*, based on a much larger set of species than had previously been used. Except for the American species, which Grubov humbly declared (1949) he did not know very well, the monograph has since stood unchallenged

(Appendix 1 gives an overview of our sample in relation to Grubov's classification), and so have also the phylogenetic hypotheses in his far-reaching discussion of character evolution and biogeography within *Rhamnus*. Grubov's proposition and motivation for elevating *Frangula* to generic status, however, has not gained full acceptance (Brizicky, 1964; Johnston & Johnston, 1978; Kartesz & Gandhi, 1994; Youngblood, 2003; but see Tutin, 1972), perhaps due to his comparatively less thorough treatment of the American species. The revision by Johnston & Johnston (1978) of Neotropical species of *Rhamnus* differs from that of Grubov; the 21 species are all placed in *Frangula* except for *R. serrata*.

Four different genera have been proposed within *Rhamnus* s.l.: *Rhamnus* s.str., *Alaternus* Mill., *Frangula* Mill., and *Oreohertzogia* Vent. *Rhamnus* (i.e., ράμνος = thorn) as a name dates back to Theophrastus (300 B.C.; reference in Grubov, 1949), referring to a low thorny thicket (probably *R. oleoides*) in the Greek archipelago. Since its first use as a generic name by Dodoens (1583) in the late 16<sup>th</sup> century, *Rhamnus* has been delimited in very different ways. After de Jussieu (1789), however, the main issue has been whether *Rhamnus* should be split into smaller genera or not. The *Alaternus* and *Frangula* groups have been more or less consistently recognized, and they may both represent monophyletic groups (Vent, 1962; Johnston & Johnston, 1978). *Frangula* is easily diagnosed by its 5-merous hermaphroditic flowers, its deciduous habit, and winter buds without scales, whereas *Alaternus* has 5-merous dioecious flowers, an ever-green habit, and leathery fruits. The rest of *Rhamnus* are generally 4-merous and dioecious. Regarding *Oreohertzogia*, the delimitation (Vent, 1962) corresponds to Grubov's (1949) section *Eurhamnus* Boiss., whereas it is only a part of Suessenguth's (1953) section *Costati*. The diagnostic features separating these taxa from other *Rhamnus* s.str. are primarily the presence of a dwarf thorn (phyllodium, sensu Vent, 1962) opposite to the leaf scar at the annual shoot bud, and further that the leaves within the shoot bud have conduplicate vernation compared to being involute in other *Rhamnus* (Vent, 1962).

Johnston & Johnston (1978: 30) commented "...it ill behoves us to indulge in capricious changes of hierarchic rank that are not based on clear evidence of polyphyletic", especially in groups that are well known among naturalists. A proper evaluation of these suggestions of intrageneric divisions has been hindered by the absence of an explicit phylogenetic treatment of the clade. Here we present a first step towards such a phylogenetic framework, challenging prevailing systematic opinions (Grubov, 1949; Vent, 1962; Brizicky, 1964; Tutin, 1972; Johnston & Johnston, 1978; Kartesz & Gandhi, 1994; Youngblood, 2003). Our phylogenetic

reconstruction is based on variation in the *trnL-trnF* region (*trnL-F*) of cpDNA and the nuclear ribosomal DNA internal transcribed spacer (ITS) regions. ITS and *trnL-F* have been useful sources of information for understanding phylogenetic relationships at low taxonomic levels (e.g., Baldwin & al., 1995; Soltis & Soltis, 1998). A combination of a chloroplast and a nuclear nucleotide sequence was further warranted in this study, as at least one species, *R. prinooides*, has a polyploid chromosome number (Brizicky, 1964).

The primary task of this study was to examine the appropriateness of previously suggested genera within *Rhamnus* s.l., i.e., *Alaternus*, *Frangula*, *Oreohertzogia*, and *Rhamnus* s.str., by testing if they represent monophyletic groups. Our sampling also allowed us to investigate the phylogenetic relationships suggested by Grubov (1949: 404).

## MATERIAL AND METHODS

**Nomenclature.** — Taxonomy and nomenclature at the species level follow Grubov (1949). Regarding names of genera and sections, we also follow Grubov except that we use the generic name *Oreohertzogia* (Vent, 1962). *Oreohertzogia* is equal to Grubov's (1949) section *Eurhamnus*. Thus, in the text *Rhamnus* equals the ingroup and *Rhamnus* s.str. the ingroup except for *Frangula*. Sometimes, for clarity, the ingroup is denoted *Rhamnus* s.l.

**Choice of taxa.** — 22 species were sampled to cover the global distribution of *Rhamnus* and the sections proposed by Grubov (1949) (see Appendix 1). One section, *Tetrrhamnus* containing two species from China, and one large subsection *Virgatiformes* within section *Cervispina*, were not sampled. Haploid chromosome numbers have been found to vary between 10–13 within *Rhamnus*, but very few species have been studied. One species that has been shown to be polyploid, *R. prinooides* L'Her. ( $2n = 34$ ; Brizicky, 1964: 448), was included in the study.

A previous molecular phylogenetic study by Richardson & al. (2000a) was used to select outgroup taxa. They concluded that *Rhamnus* belongs to a well supported monophyletic clade, the tribe Rhamneae, with nine other genera. The intergeneric phylogenetic relationships within this tribe were not resolved by Richardson & al. (2000a). Hence, we included as many as seven of these taxa in the outgroup. Voucher specimens, accession numbers, and references to sequences taken from the literature are given in Appendix 1 (see internet version of *Taxon*).

**DNA extraction, amplification, and sequencing.** — Total DNA was isolated from leaves of fresh, sil-

ica-dried, or herbarium specimens as described in Oxelman & al. (1997), and purified with a GeneClean Spin kit (TamroLab). J. E. Richardson and M. W. Chase (Royal Botanical Gardens, Kew, U.K.) kindly provided total DNA for outgroup taxa, and for *R. alpinus* and *R. purpurea*. For outgroup taxa and two ingroup taxa (*F. alnus* and *R. lycioides*), *trnL-F* sequences were available from GenBank. Sequencing failed for three taxa: *R. cathartica* (*trnL-F*), *Karwinskia* (ITS), and *F. polymorpha* (ITS).

Polymerase chain reactions were performed using an Eppendorf Mastercycler Thermal Gradient Cycler. Typically, 0.625 U Taq polymerase from Advanced Biotechnologies were used in 25 µl PCR reactions, with 2.5 mM Mg<sub>2</sub><sup>+</sup>, 200 µM of each dNTP, 0.5–1.0 µM of each primer, 0.01% bovine serum albumin (Boehringer Mannheim), and 0.5 µl total genomic DNA. The cycling program started with a denaturation step at 95°C for 2 min followed by 38 cycles of: 95°C 30 s, 55°C 1 min, 72°C 2 min. The program was terminated with a 72°C step for 15 min. PCR products were visualized on an agarose gel, and subsequently purified with the QIAquick PCR-purification Kit (Qiagen). Sequencing of the ITS region was performed with the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) according to the manufacturer's instructions, with the exception that the reaction volume was scaled down to 5 µl. The reactions were ethanol precipitated and visualized on an ABI PRISM 377 Sequencer (Perkin-Elmer). The *trnL-F* sequencing was done using the DYEnamic™ ET GEL termination cycle sequencing premix kit (Amersham Pharmacia Biotech), on a MegaBACE 1000 capillary machine (Amersham Pharmacia Biotech). The procedure followed the protocols provided by the manufacturer, except that the sequencing reactions were scaled down to 10 µl.

Primers used to amplify the nuclear ribosomal DNA internal transcribed spacer (ITS) region and the intergenic spacer of the cpDNA *trnL-trnF* (*trnL-F*) region, respectively, are listed in Table 1. All sequences used in the analyses have been deposited in GenBank. Accession numbers are provided in Appendix 1.

**Phylogenetic analysis.** — All sequences were aligned manually. Insertions and deletions larger than one position were coded as present/absent. Parsimony analyses were conducted with PAUP\* 4.0b10 (Swofford, 2002). All transformations were unordered and weighted equally in all datasets, and gaps were coded as missing values. For each analysis, 100 replicates of random addition were conducted in a heuristic search, with tree-bisection-reconnection (TBR) branch-swapping, collapse of zero branch lengths, and the MULTREES option on. Data were analyzed both as separate ITS and *trnL-F* sets, and as a combined dataset.

We evaluated the support for individual clades by parsimony bootstrapping (Felsenstein, 1985) as implemented in PAUP\* (Swofford, 2002). One bootstrap replicate was run using the same settings as described in the parsimony analyses, except that in those analyses replicates of random addition were performed. The strict consensus from this analysis was saved, and the procedure was replicated 500 times. A majority rule consensus tree was calculated from the 500 consensus trees. Thus, only groups which are unambiguously supported in the bootstrap pseudo-replicates contribute to the bootstrap frequency. Note that this differs from, and is generally more conservative than, the way PAUP\* calculates bootstrap frequencies.

## RESULTS

Tree statistics for each analysis are given in Table 2. Clades are referred to throughout the text by the outermost species (top : bottom) of the clade, as they are found in the corresponding figures.

***trnL-F*.** — The strict consensus tree is shown in Fig. 1A. Both the *Alaternus* (*R. alaternus* : *R. glandulosa*) and the *Frangula* (*F. alnus* : *F. purshiana*) clades gained strong bootstrap support. Our sample from the mainly Mediterranean genus *Oreohertzogia* (*R. pumilus* : *R. alpinus*) was weakly supported and grouped with the American *R. crocea* with 99% bootstrap frequency. Further, excluding *R. crocea*, *Alaternus* and *Oreohertzogia*, the rest of *Rhamnus* s.l. (*R. prinoides* : *R. saxatilis*) received 90% support. Grubov's (1949) large section within *Rhamnus*, *Cervispina* (*R. staddo* : *R. saxatilis*), also received good (89%) support.

**ITS.** — Strict consensus from the eight most parsimonious trees is shown in Fig. 1B. The ingroup received strong support (96%) from the analysis of ITS data set. Once again, the *Frangula* (*F. alnus* : *F. purshiana*) and *Alaternus* (*R. alaternus* : *R. glandulosa*) groups were strongly supported, and in addition, the ITS dataset gave strong support (98%) for *Oreohertzogia* (*R. pumilus* : *R. alpinus*). We also found good support (85%) for *Cervispina* (*R. staddo* : *R. saxatilis*).

**Comparison of *trnL-F* and ITS results.** — Even though several subclades (e.g., *R. staddo* : *R. saxatilis*, *F. alnus* : *F. purshiana*, *R. alaternus* : *R. glandulosa*) appear in and receive similar support levels from both datasets, some important incongruences can be seen. First, *R. prinoides* was strongly supported as a member of the *Rhamnus* s.str. clade by *trnL-F* data, but was sister taxon to the rest of *Rhamnus* s.l. in the ITS analysis. Second, *Alaternus* (*R. alaternus* : *R. glandulosa*) and *Oreohertzogia* (*R. pumilus* : *R. alpinus*) interchanged positions between the separate analyses, but with only

Table 1. List of primers used for amplification and sequencing.

Marker	Name	Sequence	Reference	
ITS	forward	P17	5'-CTACCGATTGAATGGTCCGGTGAA-3'	(Popp & Oxelman, 2001)
	reverse	26S-82R	5'-TCCCGGTTTCGCTCGCCGTTACTA-3'	
	forward	P16	5'-TCACTGAACCTTATCATTAGAGGA-3'	(Popp & Oxelman, 2001)
	reverse	P25	5'-GGGTAGTCCCGCCTGACCTG-3'	
<i>trnL-F</i>	forward	c	5'-CGAAATCGGTAGACGCTACG-3'	(Taberlet & al., 1991)
	reverse	d	5'-GGGGATAGAGGGACTTGAAC-3'	(Taberlet & al., 1991)
	forward	e	5'-GGTTCAAGTCCCTCTATCCC-3'	(Taberlet & al., 1991)
	reverse	f	5'-ATTTGAACCTGGTGACACGAG-3'	(Taberlet & al., 1991)
	forward	<i>trnL</i> BOC	5'-GGCGRAATYGGTAGACGCTACG-3'	
	reverse	<i>trnL</i> BOF	5'-CCAGATTGAACTGGTGACACGAG-3'	

poor (<50%) bootstrap support for this. Finally, *Oreohertzogia* (*R. pumilus* : *R. alpinus*) strongly grouped with *R. crocea* in the *trnL-F* analysis, while the ITS data gave weak support for *R. purpurea* and *R. crocea* as an ascending grade to (*R. pumilus* : *R. alpinus*).

**Combined datasets.** — When analysing the combined (*trnL-F* + ITS) data matrix, the only incongruence between results from separate analyses was the divergent position of *R. prinoides*. This is the only species in our sample known to have a divergent chromosome number (Brizicky, 1964). Based on chromosome number and divergent phylogenetic positions derived from chloroplast (maternal) and nuclear DNA, we decided to perform additional analyses of the combined data, excluding *R. prinoides*.

Fig. 2 shows the strict consensus tree from the combined analysis in which *R. prinoides* was excluded. This tree topology was congruent with the analysis including *R. prinoides*, with one exception: the collapsed node (*R. purpurea* : *R. crocea*, Fig. 2) was resolved in the analysis when *R. prinoides* was included, with *R. pumilus* : *R. crocea* as sister to *R. purpurea* : *R. glandulosa*. Apart from this incongruence, exclusion of *R. prinoides* generally resulted in higher bootstrap support. As shown in Fig. 2, *Rhamnus* s.l. (*R. purpurea* : *F. purshiana*), *Frangula* (*F. alnus* : *F. purshiana*), *Rhamnus* s.str. (*R. purpurea* : *R. crocea*), *Alaternus* (*R. alaternus* : *R. glandulosa*), the clade (*R. pumilus* : *R. crocea*), *Oreohertzogia* (*R. pumilus* : *R. alpinus*), and Grubov's (1949) section *Cervispina* (*R. staddo* : *R. saxatilis*), all received strong support. *Rhamnus prinoides* itself, which we suspect to be a hybrid, occurred in a collapsed node together with *R. purpurea* and the clade *R. staddo* : *R. saxatilis* in the combined analysis.

## DISCUSSION

**Systematics.** — Various opinions have been expressed as to whether *Rhamnus* should be split into smaller genera (Grubov, 1949; Vent, 1962; Brizicky,

1964; Johnston & Johnston, 1978; Kartesz & Gandhi, 1994). Grubov (1949) found morphological variation within *Rhamnus* s.str. to be so large that any diagnostic feature common for the group was difficult to find.

The present study is the first to address this problem by explicitly examining phylogenetic relationships within this ingroup. Previously, four different genera have been suggested: *Alaternus*, *Frangula*, *Oreohertzogia*, *Rhamnus* s.str. That *Frangula* and *Alaternus* are natural groups has not been questioned previously, but the great morphological variation within *Rhamnus* s.str. has made it difficult to determine whether *Alaternus* and *Frangula* should be included within *Rhamnus*. Monophyly of *Rhamnus* s.str. has been considerably less clear, i.e., is *Rhamnus* s.str. paraphyletic when groups clearly diagnosed by synapomorphies are recognized at the generic level? The discussion of raising taxa to generic level has been further complicated by Vent's (1962) suggestion of recognizing *Oreohertzogia* (= section *Eurhamnus*) as a distinct genus. Johnston & Johnston (1978) cautioned against unnecessary splitting of a well known genus. As both *Alaternus* and *Oreohertzogia* are species-poor groups, and as the rest of the species within *Rhamnus* s.l. and their relationships are not well understood, Johnston & Johnston (1978) argued that several equal-sized and well supported subclades will probably be discovered in future studies. Considering our results, there still may be several taxa within *Rhamnus* s.str. that were not or poorly sampled by us, within *Rhamnus* s.str., e.g., Grubov's (1949) sections *Pseudofrangula*, *Tetraharmnus*, and *Pseudoceanothus*. Until these are resolved it would be premature, therefore, to recognize other genera within the *Rhamnus* clade.

Our study supports generic recognition of *Frangula* (Tutin, 1972; Grubov, 1974; Kartesz & Gandhi, 1994; PLANTS, 2003). The global sample of *Frangula* used in this study represents a well supported monophyletic sister clade to the rest of *Rhamnus* in its widest sense. Considering revisions by Johnston & Johnston (1978) and Grubov (1949), there is good delimitation of *Frangula* based on diagnostic characters (Grubov, 1974).

Table 2. Values and statistics from PAUP\* analyses of separate and combined data sets.

Matrix	<i>trnL-trnF</i> cpDNA	ITS nr DNA	Combined data	Combined data, <i>R. prinooides</i> excluded
No. characters in matrix (no. indels)	1040 (10)	703 (6)	1743 (16)	1743 (16)
No. variable characters	122	234	352	335
No. phylogenetically informative characters	55	124	177	171
No. shortest trees	24	8	48	48
Length of shortest trees	147	411	565	528
Consistency Index (excl. uninformative characters)	0.76	0.58	0.59	0.61
Retention Index	0.92	0.74	0.78	0.80
No. clades in bootstrap consensus with >85% support	7	7	10	10

Some easily recognizable morphological differences in the character complex separating *Frangula* from *Rhamnus* are, according to Grubov (1949; for English translation see Johnston & Johnston, 1978): (1) bud scales absent, (2) 5-merous hermaphroditic flowers, (3) erect and fleshy sepals, (4) well-developed and short-clawed petals, (5) unexserted and unbranched style, and (6) almost straight, pinnate leaf nerves. Another characteristic of *Frangula*, which in addition to bud scales being absent is probably the most important synapomorphy for *Frangula*, is (7) the protruding ‘beak’ of the seed, i.e., a testa with a thickening “that protrudes from the opening of the endocarp in the shape of a beak-like rostellum” (Johnston & Johnston, 1978: 5).

Support for not recognizing *Frangula* as a genus could be taken from the ITS phylogeny (Fig. 1B), where *R. prinooides* was found as sister taxon to the rest of the ingroup, leaving *Frangula* as a monophyletic subclade within a paraphyletic *Rhamnus* clade. In this case, *R. prinooides* ended up within a monophyletic *Rhamnus* s.str. clade in the combined analysis (results not shown), but the divergent positions of *R. prinooides* between separate *trnL-F* and ITS analyses could indicate its hybrid origin. This argument for not recognizing *Frangula*, therefore, is believed weak.

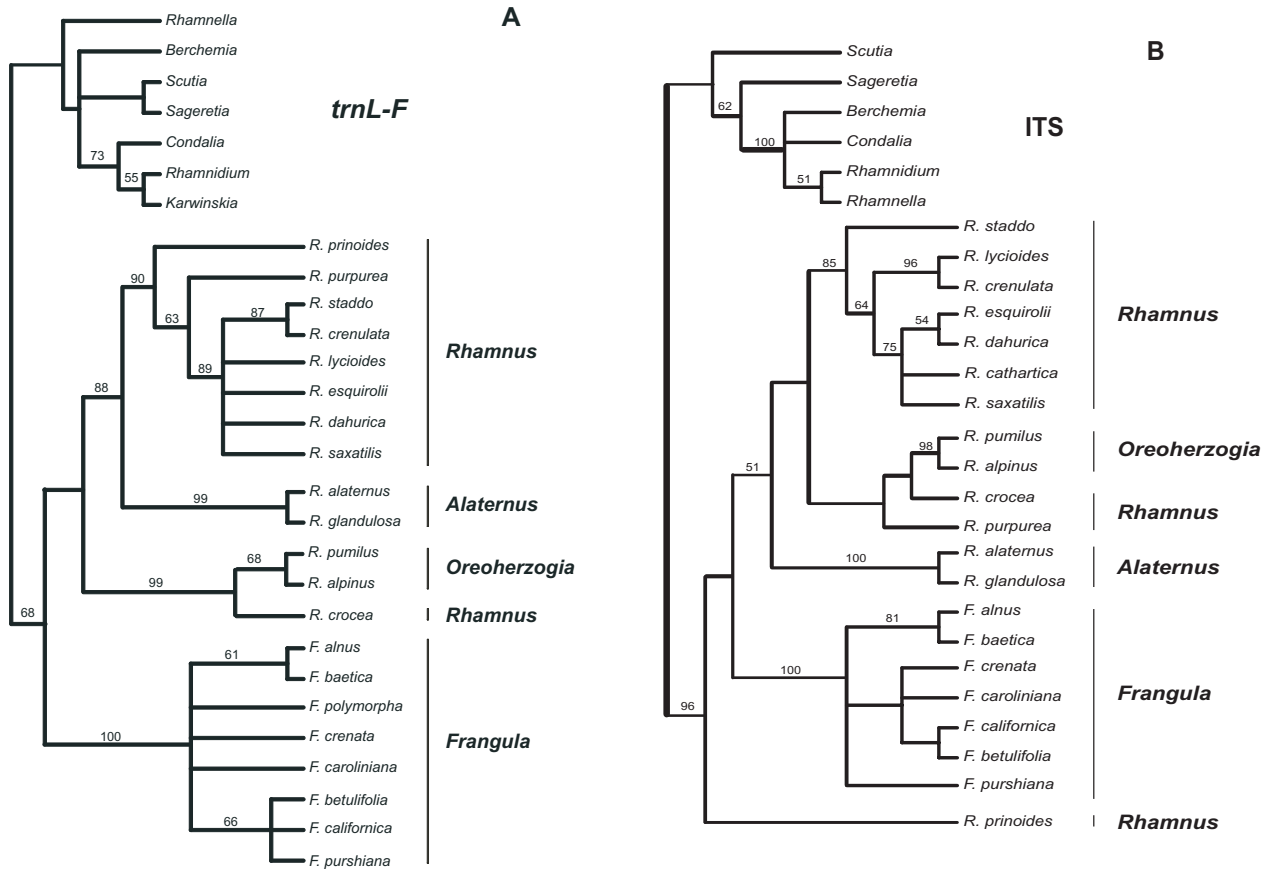
Regarding suggested genera within *Rhamnus* s.str., i.e., *Alaternus* and *Oreohertzogia*, we believe that more extensive sampling must be done before conclusions can be drawn. According to our results, recognition of *Alaternus* as a monophyletic genus requires recognition also of *Oreohertzogia*, which is problematic, however, because *Oreohertzogia* strongly groups with *R. crocea* of section *Pseudalaternus*. This problem raises two issues. First, the relationships between *Oreohertzogia* and *Pseudalaternus* need to be better resolved. Grubov (1949) did not suggest a sister clade relationship (indicated by our study) between sections *Eurhamnus* Boiss. (= *Oreohertzogia*) and *Pseudalaternus*. On the other hand, Heppeler (1928) included *R. serrata*, *R. lanceolata* (both part of Grubov’s section *Pseudalaternus*), and all *Eurhamnus* species, in his section *Costati*, based on

the occurrence of the chemical emodin. This system was accepted by Suessenguth (1953), who also followed Heppeler (1928) in putting *R. crocea* in a monotypic section, based on its densely veined leaves and its evergreen habit. On the other hand, Johnston & Johnston (1978) confirmed Grubov’s (1949) conclusions that the New World taxa (*R. lanceolata*–*R. smithii*–*R. serrata*)–(*R. crocea*–*R. insularis*) are closely related, but they were not able to connect this group to any Old World species. The present analyses create such a biogeographic link between the Eastern European section *Eurhamnus* and the American species of section *Pseudalaternus*.

Second, the basal polytomy within *Rhamnus* s.str. found in our study (i.e., *R. purpurea* : *R. saxatilis* – *R. alaternus* : *R. glandulosa*) – (*R. pumilus* : *R. crocea*), needs to be investigated further. The topology may change considerably if more samples are included.

Even if the sections *Cervispina*, comprising most of *Rhamnus* s.str., *Alaternus*, and *Eurhamnus* gained strong support in the present study, additional sampling, not only of the mainly Mediterranean sections (*Alaternus*, *Pseudalaternus*, and *Eurhamnus*), but also of sections *Pseudofrangula*, *Tetraharmnus*, and *Pseudoceanothus*, is needed before *Alaternus* and *Oreohertzogia* as genera can be accepted. Considering our results and the arguments by Johnston & Johnston (1978), we believe there is no reason to split *Rhamnus* s.str. into smaller genera.

**Evolution.** — Apart from taxonomic issues, we can also contrast the phylogenetic relationships derived here with those suggested by Grubov (1949), who developed an elaborate hypothesis for character evolution within *Rhamnus* s.l. He claimed that the general habit of the plants in the clade within *Rhamnus* s.l. has evolved from a thermohydrophilic ancestor in Late Cretaceous or Early Eocene. Many *Frangula* species have retained the ancestral features, but within *Rhamnus* s.l., our phylogenetic hypothesis suggests several independent adaptations to a colder and drier climate, such as xeromorphic leaves in *Pseudalaternus*, *Eurhamnus*, *Alaternus*, *Cervispina*, and some *Frangula* species. Richardson & al. (2001) suggested a similar evolution in *Phyllica*, a South African



**Fig. 1.** Strict consensus of the most parsimonious trees from *trnL-F* (A) and ITS (B) datasets. Bootstrap support (if above 50%) is given above branches. Genera to the right in both figures are previously suggested genera within *Rhamnaceae* s.l.

genus of *Rhamnaceae*. A proper analysis of character evolution within *Rhamnaceae* s.l. would require a larger sample and a detailed quantification of leaf traits.

*Rhamnaceae* have been deduced to be 94–96 Myr old by later studies (Richardson & al., 2000a), or at least originating in the Upper Cretaceous (Collinson & al., 1993). Using nucleotide variation, Wikström & al. (2001) derived the split between *Rhamnaceae* and its closest relatives to approximately 62 Myr BP, and the age of the tribe *Rhamneae* 55 Myr. Despite huge differences, the results by Collinson & al. (1993) and Wikström & al. (2001) both suggest that *Rhamnaceae* s.l. originated in a period characterized by a warm and moist climate and with a much denser and more stratified vegetation compared to now (Potts & Behrensmeier, 1992: 423). The evolution and geographic spread of the *Rhamnaceae* s.l. clade has occurred in a successively cooler and drier environment from the Upper Eocene onwards, thus corroborating Grubov’s ideas. The datings of this clade also suggest that it radiated during a period when South America was disconnected from Africa and Laurasia.

Thus, despite its many ancestral characteristics the main center of diversity of *Rhamnaceae* must be a secondary area of diversification, as Grubov (1949) already hypothesized.

In addition to the most important differences between *Rhamnaceae* and *Frangula* (see above), *Rhamnaceae* s.str. generally differs in a number of characteristics related to its dioecious breeding system. Suessenguth (1953) described all *Rhamnaceae* s.str. as dioecious, while Grubov (1949) noted that some species are polygamous. The information on breeding systems within different species is incomplete, and species critical for the phylogenetic resolution of this issue may be lacking in the present sample. Even if unisexual flowers seem to be a good synapomorphy for *Rhamnaceae* s.str., therefore, the question of how dioecy evolved from hermaphroditism, directly or via monoecy (Weller & Sakai, 1999; Weiblen & al., 2000), cannot be answered.

Since Candolle (1825) thorns have been used as the diagnostic feature of section *Cervispina*, which makes up most of *Rhamnaceae* s.str. This long-recognized group has

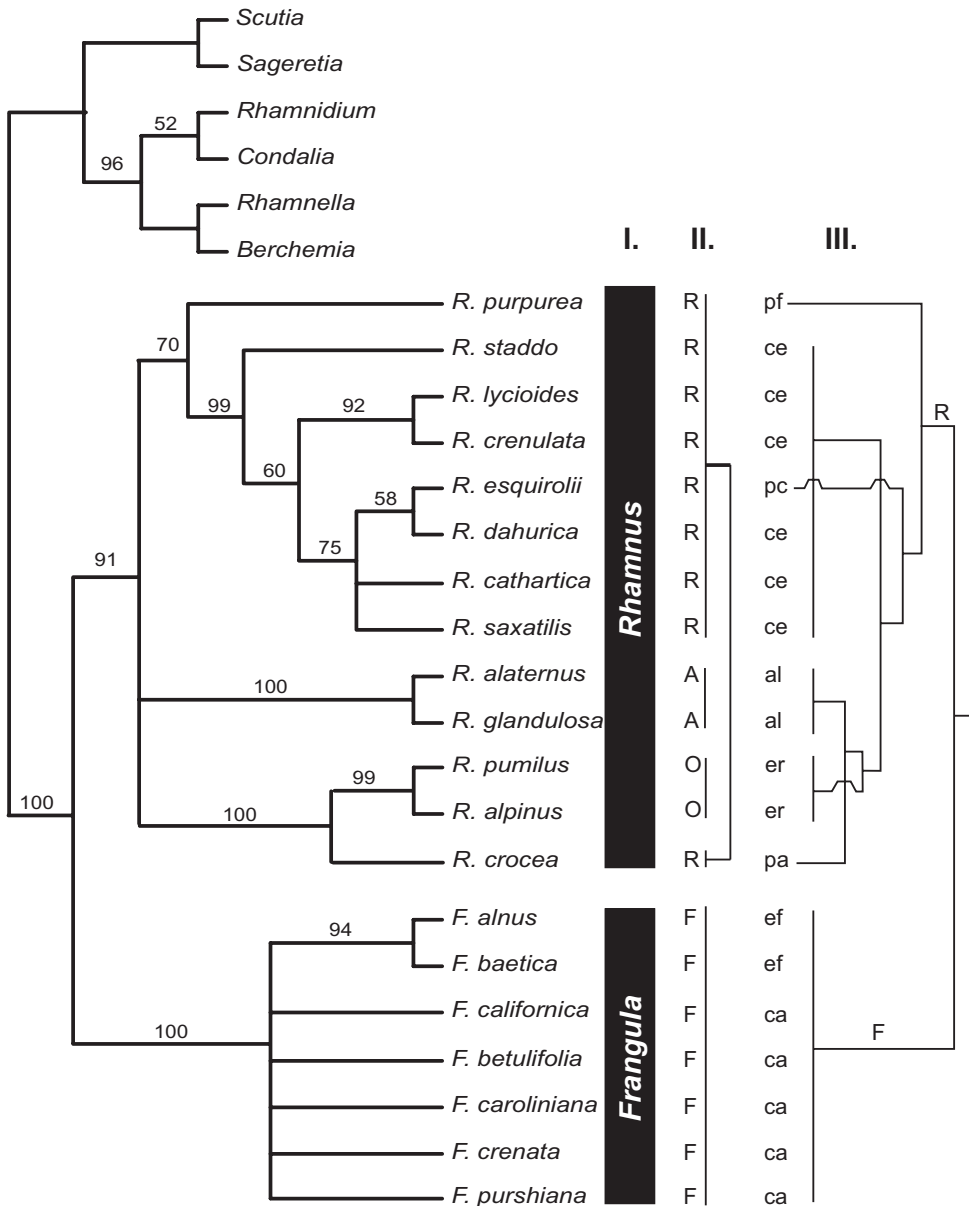


Fig. 2. Strict consensus of 48 most parsimonious trees from the combined (*trnL-F* + ITS) dataset excluding *R. prinoides*. Bootstrap support (if above 50%) shown above branches. The right part of the figure shows (I) generic limits supported in the present study, (II) previously suggested genera, and (III) Grubov's (1949) phylogenetic hypothesis. Genera: A = *Alaternus*, F = *Frangula*, O = *Oreohertzogia*, and R = *Rhamnus*. Sections: al = *Alaternus*, ca = *Cascara*, ce = *Cervispina*, ef = *Eufrangula*, er = *Eurhamnus*, pa = *Pseudalaternus*, pc = *Pseudoceanothus*, pf = *Pseudofrangula*.

gained additional support from our studies. Having thorns can be expected to be adaptive in an increasingly more open landscape. This seems to have evolved only once, followed by a comparatively rapid cladogenesis.

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**Appendix 1. Sources of sequences, areas of distribution, accession numbers in the GenBank sequence database, and relationships in the classification by Grubov (1949) for taxa of *Rhamnus* s.l. included in this study.**

Grubov classification	Sampled taxa	Area of distribution	Source of Sequence	Acc. no.	
				trnL-F	ITS
INGROUP					
<i>Rhamnus</i>					
sect. <i>Pseudofrangula</i>					
	<i>R. purpurea</i> Edgew.	E. Asia	*Chase, 8483 (K)	AY626418	AY626439
sect. <i>Tetrrhamnus</i>	-				
sect. <i>Pseudoceanothus</i>					
	<i>R. prinooides</i> L'Her.	Africa	*Bolmgren, #101 (S)	AY626413	AY626432
	<i>R. esquirolii</i> Léveillé	China	*Bell AA, 1706-80 (S)	AY626419	AY626440
sect. <i>Eurhamnus</i>					
	<i>R. alpinus</i> L.	Europe	*Chase, 8482 (K)	AY626417	AY626438
	<i>R. pumilus</i> Turra	Europe	*Bolmgren, #102 (S)	AY626414	AY626433
sect. <i>Pseudalaternus</i>					
	<i>R. crocea</i> Nutt.	N. America	*Keller, 12-IV-2001 (S)	AY626415	AY626434
sect. <i>Alaternus</i>					
	<i>R. glandulosa</i> Ait.	Canary Islands	Eriksson, March 1997 (S)	AY626425	AY626446
	<i>R. alaternus</i> L.	Europe	Eriksson, #788 (S)	AY626416	AY626435
sect. <i>Cervispina</i>					
	<i>R. dahurica</i> Pall.	China	*Bolmgren, #103 (S)	AY626420	AY626441
	<i>R. cathartica</i> L.	Europe	Bolmgren, #9 (S)	-	AY626436
	<i>R. saxatilis</i> Jacq.	Europe	*Bolmgren, #2 (S)	AY626426	AY626447
	<i>R. lycioides</i> L.	Europe	Eriksson, #784 (S)	AJ390374	AY626437
	<i>R. crenulata</i> Ait.	Canary Islands	Eriksson, March 1997 (S)	AY626428	AY626448
	<i>R. staddo</i> Rich.	Africa	Thulin & Warfa, #6053 (UPS)	AY626427	AY626449
<i>Frangula</i>					
sect. <i>Eufrangula</i>					
	<i>F. baetica</i> (rev. et Willk) Grub.	S. Spain	Hampe, 27-IX-1999 (S)	AY626429	AY626450
	<i>F. alnus</i> Mill.	W. Eurasia	Bolmgren, #104 (S)	AJ251691	AY626431
sect. <i>Cascara</i>					
	<i>F. crenata</i> Sieb. Et Zucc.	E. Asia	Nie Min Xiang 92169 (UPS)	AY626422	AY626443
	<i>F. purshiana</i> DC.	N. America	*# 97840 (JEPS)	AY626411	AY626430
	<i>F. caroliniana</i> (Walter) Gray	N. America	*Schmidt, #2559 (S)	AY626423	AY626444
	<i>F. betulifolia</i> Greene	N. America	Bolmgren, #105 (S)	AY626424	AY626445
	<i>F. californica</i> Esch.	N. America	*Holland, #114 (S)	AY626421	AY626442
sect. <i>Frangella</i>					
	<i>F. polymorpha</i> (Reiss.) Weberb.	S. America	Novara, N7121 (S)	AY626412	-
OUTGROUP					
	<i>Berchemia</i> Necker ex DC.		Richardson & al. (2000)	AJ225793	AY626455
	<i>Condalia</i> Cav.		Richardson & al. (2000)	AJ390334	AY626456
	<i>Karwinskia</i> Zucc.		Richardson & al. (2000)	AJ390333	-
	<i>Rhamnella</i> Miq.		Richardson & al. (2000)	AJ390330	AY626454
	<i>Rhamnidium</i> Reissek		Richardson & al. (2000)	AJ390332	AY626452
	<i>Sageretia</i> Brongn.		Richardson & al. (2000)	AJ225792	AY626453
	<i>Scutia</i> Comm. ex Brongn.		Richardson & al. (2000)	AJ390335	AY626451

\* specimen growing in botanical garden