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S.-C. Jeong · A. Liston · D. D. Myrold

Molecular phylogeny of the genus *Ceanothus* (Rhamnaceae) using *rbc*L and *ndh*F sequences

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Abstract Intrageneric phylogeny among ten representative *Ceanothus* species was investigated using DNA sequences of the chloroplast encoded *ndh*F and *rbc*L genes. Parsimony analysis of the *ndh*F sequences identified two main clades corresponding to two subgenera *Ceanothus* and *Cerastes*. The phylogenetic results suggest that three monophyletic clades within the subgenus *Ceanothus* can be delimited on the basis of (1) evergreen or (2) deciduous leaves and (3) thorn presence within the evergreen clade. The estimated divergence time based on *rbc*L sequences suggests that the two subgenera diverged 18–39 million years ago whereas species within each subgenus diverged more recently. Taken together, the results support the division of *Ceanothus* into two monophyletic subgenera and are consistent with the postulated recent divergence of many species within each subgenus.

Key words Intrageneric phylogeny · *Ceanothus* · *ndh*F · *rbc*L · Divergence time

Introduction

Ceanothus (Rhamnaceae), a genus of 55 species of shrubs or rarely small trees (McMinn 1942), is restricted to North America with a center of diversity in California. This genus is grouped into two subgenera (Schmidt 1993): *Ceanothus* (33 species) and *Cerastes* (22 species). Forty five species (over 80%) occur on the Pacific coast of North America; however, several other species occur widely in other areas of the continent (e.g., *C. americanus* and *C. fendleri*). Species of the subgenus *Ceanothus* and all of the species of the subgenus *Cerastes* are often important components of a broadleaf sclerophyllous vegetation.

Ceanothus is an important genus as cultivated ornamentals, forage plants, and plants for site amelioration and erosion prevention. Thirtyone species of the genus have been reported to be associated with nitrogen-fixing bacteria of the genus *Frankia* (Becking 1977). Host plants that form endosymbiotic nodules with *Frankia* are called actinorhizal plants (Torrey and Tjepkema 1979). The nitrogen-fixing ability of *Ceanothus* is a particularly important ecophysiological trait. *Ceanothus* species contribute substantially to the nitrogen status of soil in northwest North America (Conard et al. 1985).

The most recent comprehensive monograph of *Ceanothus* is that of McMinn (1942) and here we follow his taxonomic treatment. In the subgenus *Ceanothus*, the species have alternate leaves, hornless fruit, thin early-falling stipules, and stomata located on the lower surfaces of the leaves. Species of the subgenus *Cerastes* are characterized by the presence of opposite leaves, horned fruits, persistent corky stipules, and stomata located in sunken pits on the underside of the leaves. Among these characters, the thick leaves with sunken stomatal pits and persistent stipules exist in all members of the subgenus *Cerastes*. Most *Ceanothus* species have evergreen leaves with the exception of six species of the subgenus *Ceanothus*. Seven species within this subgenus have thorny twigs, which often occur in members of the Rhamnaceae.

Fossil records also support the distinctiveness of the two *Ceanothus* subgenera. *Ceanothus* has been present at least since the Oligocene (Chaney 1927; Raven and Axelrod 1978). The distinctive leaf forms of both subgenera, *Ceanothus* and *Cerastes*, are occasionally found in Miocene fossil floras, suggesting that genetic barriers

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S.-C. Jeong · D. D. Myrold (✉)
Plant Physiology Program and Department of Crop and Soil Science, Oregon State University, Corvallis, OR 97331, USA

A. Liston
Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA

between the two sections may already have been developed at this time (Nobs 1963).

Although *Ceanothus* species can be conveniently resolved by morphological characters, karyological and hybridization studies indicate that species boundaries are not clear. Chromosome counts of 17 species and three varieties of the subgenus *Ceanothus* together with 17 species and five varieties of the subgenus *Cerastes* investigated by Nobs (1963) have a diploid number $2n = 24$. Hybrid plants were found to have the same number of chromosomes as their parents, with one exception that had 12 pairs of normal-size chromosomes, one very small pair, and three fragments. All reduction divisions examined appeared regular. Therefore, he suggested that no evident cytological barrier prevented hybridization. Field observation and hybridization studies (Nobs 1963) also indicated that species within each subgenus are interfertile, but the species of the two subgenera do not hybridize.

We are interested in elucidating the plant-*Frankia* symbiotic relationship. From experiences of our previous *Alnus*-infective *Frankia* studies (Crannell et al. 1994; Huss-Danell and Myrold 1994) we selected the genus *Ceanothus* as model plants because of their reticulate relationship among species, high species diversity within relatively small areas, and their ecophysiological importance. No rigorous phylogenetic analysis of *Ceanothus* has been reported using molecular markers. The objective of this study was to investigate the phylogenetic relationship among representative *Ceanothus* species (Table 1) using the chloroplast genes, *ndh*F and *rbc*L.

Several candidate chloroplast genes have been suggested for phylogenetic studies at lower taxonomic levels (Olmstead and Palmer 1994). *ndh*F has recently been demonstrated to be useful for phylogenetic studies at intrafamilial levels compared with the *rbc*L gene which has been widely used at higher taxonomic levels (Olmstead and Sweere 1994; Kim and Jansen 1995). The utility of sequence change in the *rbc*L gene has been recently evaluated for the estimation of divergence time between woody-taxon pairs (Albert et al. 1994). In the present study, we use *ndh*F sequences to reconstruct phylogenetic relationship and *rbc*L sequences to estimate divergence times.

Materials and methods

Plant materials

Ten species of *Ceanothus* (Tables 1 and 2) represent the taxonomic diversity of the plant genus, its geographical range, and a habitat gradient of environmental and soil conditions. *Rhamnus purshiana* (Rhamnaceae) was used as an outgroup (Table 2). *Nicotiana tabacum* (Solanaceae) and *Barnadesia caryophylla* (Asteraceae) were also included as distant outgroups. All collections by S.-C. Jeong, G. V. Johnson, B. C. Mullin, and N. J. Ritchie are deposited as vouchers in the herbarium of Oregon State University (OSC).

DNA sequencing

Total DNA was isolated from leaves following the protocol of Doyle and Doyle (1987). When needed, DNA was further purified by polyethylene glycol precipitation.

We utilized the polymerase chain reaction (PCR) to amplify *ndh*F and *rbc*L nucleotide sequences from all taxa listed in Table 2. DNA sequences for the two chloroplast genes were determined following similar procedures. DNA sequencing was carried out using a set of internal primers. The primers described by Olmstead and Sweere (1994) were used for the amplification and internal sequencing of *ndh*F, with the modification of five internal primers specified in Table 3. Primers for amplification of *rbc*L and the internal sequencing primers were provided by G. Zurawski (DNAX Research Institute, Palo Alto, Calif.), with the exception of C331F and C1022R (Table 3). The Z1 and C1022R primer combinations were used to amplify *rbc*L. The *ndh*F sequences of *Ceanothus* were

Table 1 Characteristics of representative *Ceanothus* species used in this study

Ceanothus species[a]	Distinguishing characteristics	
	Plant features[b]	Distribution
Subgenus *Ceanothus*		
C. americanus	Deciduous, W, leaves L and broadly ovate to oblong-ovate	Eastern U.S.
C. sanguineus	Deciduous, W, leaves L and broadly ovate but not orbicular	Pacific coast, northern Rocky Mountains
C. integerrimus	Semidecidous, W or B, leaves M/L and elliptical but never approaching orbicular	Pacific coast, southern Rocky Mountains
C. cordulatus	Evergreen, thorny, W, leaves M and not two-times longer than wide	Pacific coast
C. fendleri	Evergreen, thorny, W, leaves M and two-times longer than wide	Southern Rocky Mountains
C. thyrsiflorus	Evergreen, B, leaves M/L, branchlets flexible	Pacific coast
C. velutinus	Evergreen, W, leaves L and glutinous above	Pacific coast, northern Rocky Mountains
Subgenus *Cerastes*		
C. cuneatus	Erect, W, leaves S and typically entire	Pacific coast
C. prostratus	Prostrate, B, leaves S and usually broader	Pacific coast
C. pumilus	Prostrate, B, leaves M and narrowly oblanceolate	Pacific coast

[a] *Ceanothus* is divided into two subgenera: *Ceanothus* and *Cerastes*
[b] W – white flower, B – blue flower; S – leaves less than 10 mm long or broad, M – leaves 10 to 25 mm long or broad, L – leaves more than 25 mm long or broad

Table 2 Sources of taxa sampled for DNA sequences and previously published sequences

Taxon	DNA source/voucher	Genbank accession number	
		*rbc*L	*ndh*F
C. americanus L.	Tennessee, Anderson Co., 3 miles from Oak Ridge/B. C. Mullin 01		U78893
C. cordulatus Kell	Oregon, Douglas Co., 100 yards from Toketee Ranger Station/N. J. Ritchie 004	U78904	U78894
C. fendleri Gray	New Mexico/G. V. Johnson 01		U78895
C. integerrimus H. & A.	Oregon, Lane Co., 0.5 mile west of Blue River, Hwy. 126 /N. J. Ritchie 003		U78896
C. sanguineus Pursh	Oregon, Lane Co., H.J. Andrews Experimental Forest, Unit L/N. J. Ritchie 002	U06795 (Morgan et al. 1994)	U78897
C. thyrsiflorus Esch.	Oregon, Douglas Co., Bear Creek Recreational area, Hwy. 42/N. J. Ritchie 005		U78898
C. velutinus Dougl.	Oregon, Benton Co., 5.8 miles from Hwy. 34 on Marys Peak Rd./N. J. Ritchie 001		U78899
C. cuneatus (Hook.) Nutt.	Oregon, Benton Co., 0.6 miles north of Adair Village, Hwy. 99 W/N. J. Ritchie 008		U78900
C. prostratus Benth.	Oregon, Douglas Co., 6.5 miles west of Diamond Lake/N. J. Ritchie 006		U78901
C. pumilus Greene	Oregon, Coos Co., Powers Ranger District: FS 5325, S. FS 530/N. J. Ritchie 007	U78905	U78902
R. purshiana DC.	Oregon, Benton Co., Jackson-Frazier Wetland of Corvallis/S.-C. Jeong 001		U78903
R. carthartica L.		G13189 (Chase et al. 1993)	
N. tabacum L.			L14953 (Olmstead and Sweere 1994)
B. caryophylla Blake			L39394 (Kim and Jansen 1995)

Table 3 Oligonucleotides designed for PCR amplification and DNA sequencing

Gene	Primer	5′-3′ nucleotide sequence
*ndh*F	C538F	GTAACTAATCGTGTAGGGGATT
*ndh*F	C538R	AATCCCCTACACGATTAGTTAC
*ndh*F	C803F	CTATGGTAGCAGCCGGAATTTTTC
*ndh*F	C803R	GAAAAATTCCGGCTGCTACCATAG
*ndh*F	C1601F	TATCCGCAGGAATCGGACAATACTAT
*rbc*L	C331F	TCTACGTAGTAAATCAACAAAGCCTAAA
*rbc*L	C1022R	ATCACGTAGTAATAAATCAACAAAGCCT-AAA

amplified in two overlapping segments, using primers 1F-1318R and C803F-2110R. For *R. purshiana*, two different primer combinations were used (C538F-1318R and C803F-2110R) because of problems with 1F, the 5′ forward primer.

Ten microliters of each PCR reaction was run on a 0.8% agarose gel to check the quality of amplification. To purify the resulting DNA, 40 µl of each PCR reaction was run on a 1.0% TBE agarose gel. Agarose blocks containing the DNA were excised from the gel with a scalpel over UV light, and purified with the GeneClean II gel-extraction kit according to the manufacturer's protocol (BIO 101, Vista, Calif.). Final pellets were dissolved in 22 µl of water and DNA amounts measured using a fluorometer. The purified double-stranded DNA products were sequenced directly by automatic sequencing with dye terminator extension in the Center for Gene Research and Biotechnology, Oregon State University.

Eleven *ndh*F sequences, corresponding to ten taxa and one outgroup, and two *rbc*L sequences were generated. Sequence alignment was performed using the PILEUP program of GCG (Genetics Computer Group, Madison, Wis.). No gaps (indels) were observed in the *ndh*F sequences of *Ceanothus* species. Two gaps (indels) in the *ndh*F sequence of *R. purshiana* were observed. There were no missing cells in the data matrix except for the gaps and missing sequences caused by outgroups. In all cases where sequences were ambiguous, due to compression or otherwise, samples were sequenced in both directions. All sequences determined as part of this study have been submitted to GenBank (Table 2).

Phylogenetic analysis

The *rbc*L data were used to calculate divergence times (Li and Graur 1991). Maximum-parsimony analyses of the *ndh*F data were conducted using PAUP 3.0s + 4 (beta) (Swofford 1991), with all changes weighted equally. Gaps caused by outgroups were treated as missing data. We used the branch-and-bound algorithm with the delayed transformation option. Analyses of the complete data set were performed rooting the tree with *R. purshiana*, *B. caryophylla*, and *N. tabacum*. To evaluate relative levels of support for individual clades, the bootstrap method was used with 100 replicates of a heuristic search.

Results

A total of 2122 bp of *ndh*F from ten *Ceanothus* species and 993 bp of *rbc*L from two *Ceanothus* species were sequenced. As an outgroup, 1616 bp of the *ndh*F of *R. purshiana* was sequenced due to difficulty in PCR-amplification of the gene. No length variation was observed for *rbc*L sequences. No length variation was

Table 4 Pairwise distances of *ndh*F sequences among ten *Ceanothus* species[a]

	1	2	3	4	5	6	7	8	9	10
1. *C. cuneatus*	–	0.000	0.001	0.011	0.010	0.010	0.010	0.010	0.011	0.011
2. *C. pumilus*	1	–	0.001	0.010	0.009	0.009	0.010	0.010	0.010	0.011
3. *C. prostratus*	3	2	–	0.011	0.010	0.010	0.011	0.011	0.011	0.012
4. *C. americanus*	23	22	24	–	0.002	0.004	0.004	0.004	0.005	0.005
5. *C. sanguineus*	21	20	22	4	–	0.003	0.003	0.003	0.004	0.004
6. *C. integerrimus*	21	20	22	8	6	–	0.000	0.000	0.002	0.002
7. *C. thyrsiflorus*	22	21	23	9	7	1	–	0.001	0.002	0.003
8. *C. velutinus*	22	21	23	9	7	1	2	–	0.002	0.003
9. *C. fendleri*	23	22	24	10	8	4	5	5	–	0.002
10. *C. cordulatus*	24	23	25	11	9	5	6	6	5	–

[a] Numbers above the diagonal indicate percent distance and below the diagonal is the number of base differences between species

observed for *ndh*F nucleotide sequences among *Ceanothus* species. In the sequenced 1616 bp of *ndh*F from *R. purshiana*, we found one deletion (9 bp) and one insertion (39 bp), relative to published sequences (Olmstead and Sweere 1994; Clark et al. 1995; Kim and Jansen 1995). Interestingly, primer pair 1F and 803R for the amplification of *ndh*F nucleotides of *R. purshiana* produced a single PCR product of approximately 800 bp. However, the sequencing of the PCR product gave a partial *ndh*H nucleotide sequence, suggesting that both primers have high homology to the *ndh*H nucleotide sequence.

The *rbc*L sequences allowed us to estimate divergence times between the *Ceanothus* subgenera *Ceanothus* and *Cerastes* and between *Rhamnus* and *Ceanothus* pairs using the mean calculated divergence rate estimated by Albert et al. (1994). Based on patristic distances between woody taxon pairs from Search II of Chase et al. (1993) and the divergence time assumption, they obtained a mean divergence rate of approximately $2.05 \pm 0.72 \times 10^{-10}$ total substitutions per site per year. Estimated divergence time of the two *Ceanothus* subgenera is 18–39 million years ago; for *Rhamnus* and *Ceanothus* it is 147–317 million years ago. The *rbc*L sequences of *C. cordulatus* and *C. sanguineus*, which belong to the same subgenus, showed 100% similarity.

Pairwise distances between taxa are shown in Table 4. There were 1–3 base differences within the subgenus *Cerastes* (0.0–0.1%) and 1–11 base differences within the subgenus *Ceanothus* (0.0–0.5%). A total of 20–25 (0.9–1.1%) base differences were observed between the two subgenera. In the data set 30 of the 2122 bp were variable, 11 of which represented third-codon-position substitutions. Seven were due to first-position substitutions, whereas the remaining 12 were second-position substitutions. One percent of the nucleotides were potentially phylogenetically informative.

Parsimony analyses of the 13 *ndh*F sequences by the branch-and-bound algorithm yielded one most-parsimonious tree (Fig. 1), with a length of 567 steps, a consistency index (CI) of 0.947, homoplasy (HI) of 0.053, a CI excluding uninformative characters of 0.878, a HI excluding uninformative characters of 0.122, a retention index of 0.888, and a re-scaled CI of 0.841. Bootstrap analysis of the nucleotide-sequence data yielded a topology that was exactly congruent with the branch-and-bound analysis.

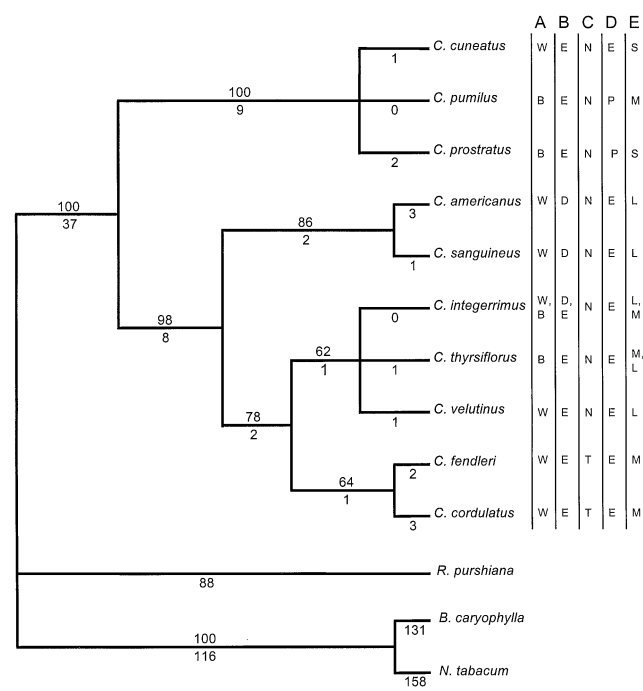

Fig. 1 Single most-parsimonious tree constructed by using the branch-and-bound algorithm with maximum parsimony of 13 *ndh*F sequences of *Ceanothus* species plus outgroup taxa. Percentages based on 100 bootstrap replicates are shown above the branches; branch lengths are shown below. Columns A–E are a key to the distribution of selected morphological characters of *Ceanothus* species: *A*-flower color (*W*-white, *B*-blue); *B*-leaf type (*D*-deciduous, *E*-evergreen); *C*-thorny twigs (*T*-thorny, *N*-non-thorny); *D*-growth form (*P*-prostrate, *E*-erect); *E*-leaf size (*S*- < 10 mm long or broad, *M*-10 to 25 mm long or broad, *L*- > 25 mm long or broad)

Discussion

The results presented here suggest that *Ceanothus* is well divided into two subgenera, in accord with the traditional classification system of the genus. McMinn (1942) divided the subgenus *Ceanothus* into two groups and the subgenus *Cerastes* into three groups on the basis of flower color. Our phylogenetic analysis indicates that flower color varies within putatively monophyletic clades. In contrast, our analysis hypothesizes three monophyletic clades corresponding to vegetative characters within the subgenus *Ceanothus* (Fig. 1). The three monophyletic clades correspond to (1) evergreen or (2) deciduous leaves and (3) thorn presence within the evergreen clade. An exception is *Ceanothus integerrimus*, which has semideciduous leaves (Table1). The cladogram topology suggests that deciduous leaves in this species are a derived feature (Fig. 1). This polymorphism for leaf persistence is apparently a response to water availability, and not a result of hybridization (McMinn 1942).

The phylogenetic tree (Fig. 1) is consistent with low levels of genetic differentiation (Table 4) among representative western North America *Ceanothus* species. *C. integerrimus*, *C. thyrsiflorus*, and *C. velutinus*, which belong to the same clade, are sympatric in southern Oregon and northern California (Conard et al. 1985). *C. cuneatus*, *C. pumilus*, and *C. prostratus*, which belong to another clade, are distributed together in the southern Cascade Range and in the Sierra Nevada of southern Oregon and northern California. Although *C. cordulatus* and *C. fendleri*, which do not have a similar distribution pattern, belong to the same clade, they show substantial *ndh*F sequence divergence.

Albert et al. (1994) suggested that the *rbc*L sequence is quasi-ultrametric in terms of a clock assumption, implying that the extent of nucleotide substitution in a given taxon should roughly reflect the underlying cladogenetic time. Divergence time calculated from the *rbc*L nucleotide sequence suggested that the two *Ceanothus* subgenera separated in a comparable time to woody taxon pairs (see Albert et al. 1994), but species within a subgenus originated more recently. Low sequence divergency of *ndh*F and complete identity of the *C. sanguineus* and *C. cordulatus* partial *rbc*L nucleotide sequences correspond with previous hybridization and karyological studies (Nobs 1963), indicating a high genetic similarity of many *Ceanothus* species.

We used the sequence information to make oligonucleotides for sequencing the *ndh*F gene. However, we had to modify several internal primers proposed by Olmstead and Sweere (1994) on the basis of the partial sequences obtained because of sequence differences between *Ceaonthus* and tobacco (Table 3). Therefore, our revised primer information will be useful for sequencing plant species related to *Ceanothus*. However, we succeeded in sequencing only three-fourths of the *ndh*F sequence of *R. purshiana*. This difficulty may come from the lack of homology of primer 1F. The fact that the PCR product from the 1F and C803R primer pair is a part of the *ndh*H nucleotide sequence (unpublished data) points out the necessity for a new design of primer 1F for extensive use of the *ndh*F gene as phylogenetic data. Olmstead and Palmer (1994) suggested several candidate genes to study plant phylogenetic relationships on the species or genus level. One advantage of the *ndh*F gene is that it is long enough to resolve phylogenetic relationships at the species level. Recently, several groups have reported intrageneric analyses using *mat*K and ITS nucleotide sequences (e.g., Soltis et al. 1996; Yuan et al. 1996). Our report demonstrates the utility of the *ndh*F nucleotide sequence to estimate phylogenetic relationship at the intrageneric level.

Recent work on the phylogeny of nitrogen-fixing plants suggests a single evolutionary origin (Soltis et al. 1995), which is in contrast with conventional taxonomy. Actinorhizal plants are found in three of the four subclades that contain nitrogen-fixing symbioses. At this higher taxonomic level there is general congruence between host plant and *Frankia* phylogenies (Hönerlage et al. 1995; Normand et al. 1996), although *Frankia* that nodulate members of the Rhamnaceae are poorly represented in these trees. Two recent studies have grouped *Ceanothus*-infective *Frankia* either with those that nodulate members of the Rosaceae (Benson et al. 1996) or the Elaeagnaceae (Murry et al. 1997). Both of these plant families are in the same subclade of nitrogen-fixing plants; however, *Ceanothus* is much more closely related to members of the Elaeagnaceae (Soltis et al. 1995).

At lower taxonomic levels there is considerably less specificity between plant host and microbial symbiont (Myrold 1994), although the study of Rouvier et al. (1996) showed some degree of specificity between *Frankia* strains and species within the Casuarinaceae. Whether such specificity exists with *Ceanothus*-infective *Frankia* is unknown, although Baker and Mullin (1994) detected no plant population or geographic patterns of *Frankia* within *C. americanus* nodules. We have collected nodules from the same *Ceanothus* populations as the present plant-collection populations and analyzed them by PCR-RFLP (restriction fragment length polymorphism) and DNA sequencing. The preliminary data show that we have uncovered several putative new *Frankia* strains from nine *Ceanothus* species (Ritchie and Myrold, unpublished data). When these data are combined with our present results, they may give an insight into the elucidation of *Frankia*-host plant relationship. Therefore, the present molecular phylogenetic study of *Ceanothus* species provides important data to reveal how *Frankia* and *Ceanothus* have co-evolved on the intrageneric level as well as clarifing systematic relationships among *Ceanothus* species.

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References

Albert VA, Backlund A, Bremer K, Chase MW, Manhart JR, Mishler BD, Nixon KC (1994) Functional constraints and *rbc*L evidence for land plant phylogeny. Ann Mo Bot Gard 81: 534–567

Baker DD, Mullin BC (1994) Diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus* as assessed by RFLP patterns from single nodule lobes. Soil Biol Biochem 26: 547–552

Becking JH (1977) Dinitrogen-fixing associations in higher plants other than legumes. In Hardy RWF, Silver WS (eds) A treatise on dinitrogen fixation Section 3, Biology. Wiley, New York, pp 185–275

Benson DR, Stephens DW, Clawson ML, Silvester WB (1996) Amplification of 16s rRNA genes from *Frankia* strains in root nodules of *Ceanothus griseus*, *Coriaria arborea*, *Coriaria plumosa*, *Discaria toumatou*, and *Purshia tridentata*. Appl Environ Microbiol 62: 2904–2909

Chaney RW (1927) Geology and paleontology of the Crooked River Basin, with special reference to the Bridge Creek flora. Carnegie Inst Wash Publ 346: 45–138

Chase MW, Soltis DE, Olmstead RG, Morgan D, Les DH, Mishler BD, Duvall MR, Price RA, Hills HG, Qui Y-L, Kron KA, Rettig JH, Conti E, Palmer JD, Manhart JR, Sytsma KJ, Michaels HJ, Kress WJ, Karol KG, Clark WD, Hedrén M, Gaut BS, Jansen RK, Kim K-J, Wimpee CF, Smith JF, Furnier GR, Strauss SH, Xiang G-Y, Plunkett GM, Soltis PS, Swensen SM, Williams SE, Gadek PA, Quinn CJ, Eguiarte LE, Golenberg E, Learn Jr GH, Graham SW, Barrett SCH, Dayanandan S, Albert VA (1993) Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. Ann Mo Bot Gard 80: 528–580

Clark LG, Zhang W, Wendel JF (1995) A phylogeny of the grass family (Poaceae) based on *ndh*F sequence data. Systematic Bot 20: 436–460

Conard SG, Jaramillo AE, Cromack Jr K, Rose S (1985) The role of the genus *Ceanothus* in western forest ecosystems. USDA For Serv Gen Tech Rep PNW-182

Crannell WK, Tanaka Y, Myrold DD (1994) Calcium and pH interaction on root nodulation of nursery-grown red alder (*Alnus rubra* Bong.) seedlings by *Frankia*. Soil Biol Biochem 26: 607–614

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11–15

Hönerlage W, Hahn D, Zepp K, Zeyer J, Normand P (1995) A hypervariable 23s rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. System Appl Microbiol 17: 433–443

Huss-Danell K, Myrold DD (1994) Intrageneric variation in nodulation of *Alnus*: Consequences for quantifying *Frankia* nodulation units in soil. Soil Biol Biochem 26: 525–531

Kim K-J, Jansen RK (1995) *ndhF* sequence evolution and the major clades in the sunflower family. Proc Natl Acad Sci USA 92: 10379–10383

Li W-H, Graur D (1991) Fundamentals of molecular evolution. Sinauer, Sunderland, Massachusetts

McMinn HE (1942) *Ceanothus*: part II; a systematic study of the genus *Ceanothus*. Santa Barbara Botanic Garden, Santa Barbara, California pp 129–308

Morgan DR, Soltis DE, Robertson KR(1994) Systematic and evolutionary implications of *rbc*L sequence variation in Rosaceae. Am J Bot 81: 890–903

Murry MA, Konopka AS, Pratt SD, Vandergon TL (1997) The use of PCR-based typing methods to assess the diversity of *Frankia* nodule endophytes of the actinorhizal shrub *Ceanothus*. Physiol Plant (in press)

Myrold DD (1994) *Frankia* and the actinorhizal symbiosis. In: Weaver RW, Angle VS, Bottomley PJ (eds) Methods of soil analysis: part 2. Microbiological and biochemical properties. Soil Science Society of America, Madison, Wisconsin pp 291–328

Nobs MA (1963) Experimental studies on species relationships in *Ceanothus*. Carnegie Inst Wash Publ, Washington D.C.

Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, Dawson J, Evtushenko L, Misra AK (1996) Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family *Frankiaceae*. Int J System Bacteriol 46: 1–9

Olmstead RG, Palmer JD (1994) Chloroplast DNA systematics: a review of methods and data analysis. Am J Bot 81: 1205–1224

Olmstead RG, Sweere JA (1994) Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. Syst Biol 43: 467–481

Raven PH, Axelrod DI (1978) Origin and relationships of the California flora. Univ Calif Publ Bot 72: 1–134

Rouvier C, Prin Y, Reddell P, Normand P, Simonet P (1996) Genetic diversity among *Frankia* strains nodulating members of the family Casuarinaceae in Australia revealed by PCR and restriction fragment length polymorphism analysis with crushed root nodules. Appl Environ Microbiol 62: 979–985

Schmidt CL (1993) *Ceanothus*. In: Hickman JC (ed) The Jepson manual: higher plants of California. University of California Press, Berkeley, California, pp 932–938

Soltis DE, Kuzoff RK, Conti E, Gornall R, Ferguson K (1996) *mat* K and *rbc*L gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. Am J Bot 83: 371–382

Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc Natl Acad Sci USA 92: 2647–2651

Swofford DL (1991) PAUP: phylogenetic analysis using parsimony, version 3.0. Illinois National History Survey, Champaign, Illinois

Torrey JG, Tjepkema JD (1979) Preface. Bot Gaz 140(Suppl): i-ii

Yuan Y-M, Küpfer P, Doyle JJ (1996) Infrageneric phylogeny of the genus *Gentiana* (Gentianaceae) inferred from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. Am J Bot 83: 641–652