American Journal of Botany 84(4): 555–564. 1997.

# USING RESTRICTION-SITE VARIATION OF PCR-AMPLIFIED cpDNA GENES FOR PHYLOGENETIC ANALYSIS OF TRIBE CHELONEAE (SCROPHULARIACEAE)[1]

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Data from restriction-site variation of three PCR-amplified chloroplast genic regions (*trn*K, *rps*2, and *rbc*L) were used to assess the utility of PCR-based methodology for phylogenetic reconstruction. Seventeen genera from tribe Cheloneae s.l. (Scrophulariaceae), and one genus each from Solanaceae, Acanthaceae, and Bignoniaceae, representing 32 taxa, were sampled. Phylogenetic reconstruction, based on a combined data set of 138 variable restriction sites, revealed a monophyletic clade of North American Cheloneae, which were not inconsistent with a polyphyletic Scrophulariaceae. Separate analyses of individual genic regions were unable to completely resolve the phylogeny, but were adequate for resolving relationships of major clades among the taxa sampled. We suggest that analysis of PCR-product restriction-site variation is useful for phylogenetic reconstruction above the species level.

**Key words**: cleaved amplified polymorphic sequences (CAPS); *Chelone*; *Chionophila*; *Keckiella*; *Nothochelone*; polymerase chain reaction (PCR)-mapped restriction site polymorphisms (MRSP); *Penstemon*; restriction fragment length polymorphism (RFLP).

Chloroplast DNA (cpDNA) has proven to be a useful source of data for phylogenetic reconstruction at all taxonomic levels (Sytsma and Gottlieb, 1986; Coates and Cullis, 1987; Jansen and Palmer, 1988; Palmer et al., 1988; Lavin, Mathews, and Hughes, 1991; Chase et al., 1993; Brunsfeld et al., 1994; Downie and Palmer, 1994; Olmstead and Palmer, 1994; Steele and Vilgalys, 1994). Early studies employed restriction-site variation of cpDNA where restriction-site maps were produced via Southern hybridization of radioactively labeled cpDNA fragments (Sytsma and Gottlieb, 1986; Coates and Cullis, 1987; Jansen and Palmer, 1988; Lavin, Mathews, and Hughes, 1991; see also citations in Palmer et al., 1988). Recent investigations have used nucleotide sequencing of one or a few chloroplast genes (e.g., *rbc*L, *ndh*F, and *mat*K) for phylogenetic inference (Chase et al., 1993; Brunsfeld et al., 1994; Steele and Vilgalys, 1994; Olmstead and Reeves, 1995).

While there are some limitations, established methods in molecular systematics are effective for inferring phylogenies at many taxonomic levels (Olmstead and Palmer, 1994). However, gathering data for phylogenetic analysis via chloroplast genome restriction-site variation and/or sequencing is expensive, requires an extensive array of molecular biology skills and specialized equipment, and typically involves the use of radioisotopes.

Techniques using polymerase chain reaction (PCR) and restriction endonuclease digestion of PCR products, such as cleaved amplified polymorphic sequences (CAPS) and mapped restriction site polymorphisms (PCR-MRSP), offer an alternative method for gathering DNA-based data useful in phylogenetic analysis (reviewed in Wolfe and Liston, 1997). PCR-based assays have been employed effectively in population genetics (Karl and Avise, 1993; Simon, McIntosh, and Deniega, 1993), diagnostics (Chen and Hoy, 1993; Scholin et al., 1994), and to generate genetic markers (Arnold, Buckner, and Robinson, 1991; Liston, Rieseberg, and Hanson, 1992; Schwenk, 1993). However, relatively few investigations of phylogenetic relationships among plant taxa have been based on PCR-product restriction-site variation compared to traditional molecular methods (Liston, 1992; Rieseberg, Hanson, and Philbrick, 1992; Liston and Wheeler, 1994; Badeness and Parfitt, 1995; Ding et al., 1995; Schwarzbach and Kadereit, 1995; Jork and Kadereit, 1995; Tsumura et al., 1995). Representative examples of these investigations are discussed below.

In the Rieseberg, Hanson, and Philbrick (1992) study, six genera (*Datisca, Octomeles, Tetrameles, Begonia, Citrullus,* and *Cucumis*) from three angiosperm families (Datiscaceae, Begoniaceae, and Cucurbitaceae) were assayed for restriction-site variation of the PCR-amplified cpDNA region *rbc*L to ORF106 (3210-3320 bp). Fifty-three variable (38 phylogenetically informative) restriction sites were detected using 30 restriction enzymes (232 nucleotides sampled; L. H. Rieseberg, personal commu-

[1] Manuscript received 27 September 1995; revision accepted 3 October 1996.

ADW thanks the Oklahoma Biological Survey for the summer research support that facilitated the project and Shoichi Kawano, Linnea Hanson, Dick Edwards, Vince Carter, and the Berry Botanical Garden for their generous assistance in collecting seed or plant material. This work was supported by NSF grants DEB-9123080 (WJE and ADW), BSR-9110375 (LEW), and DEB-9120258 (CWD); University of Oklahoma Office of Research Administration; and University of Oklahoma Graduate College.

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nication, University of Indiana). The inferred phylogenetic tree was useful in assessing the evolution of androdioecy in the Datiscaceae.

Liston (1992) examined phylogenetic relationships among 14 species of *Astragalus* (Fabaceae) based on restriction-site variation of PCR-amplified *rpo*C1 to *rpo*C2 (4100 bp). Thirty-eight variable (nine phylogenetically informative) restriction sites were detected from 23 restriction enzyme digests (615 nucleotides sampled; A. Liston, personal communication, Oregon State University). A single most-parsimonious tree was obtained with clades among the ingroup taxa supported by single characters.

Liston and Wheeler (1994) used restriction-site variation of the *rpo*C1/C2 genic region to infer a molecular phylogeny for 51 species of *Astragalus* and genera from the legume tribes Galegeae, Millettieae, Trifolieae, and Vicieae. Eighty-nine variable (45 potentially informative) restriction sites were obtained using 19 restriction enzymes (556 nucleotides sampled; A. Liston, personal communication, Oregon State University). Weighted parsimony analysis yielded 288 equally parsimonious trees suggesting a polyphyletic relationship among species of *Astragalus.*

Major differences among the Rieseberg, Hanson, and Philbrick (1992), Liston (1992), and Liston and Wheeler (1994) studies include: taxonomic level targeted; number of nucleotides assayed; number of restriction enzymes used; and the amount of noncoding intergenic spacer region sampled. The results from these investigations based on PCR-product restriction-site variation suggest that resolution of intergeneric relationships is obtainable, but resolving interspecific relationships is more difficult. Additionally, the phylogenetic resolution was generally better as the number of bases sampled increased. Although phylogenetic reconstructions were obtained in previous studies, the usefulness of the technique for phylogenetic inference is not demonstrated fully because so few investigations have been conducted.

In this study, we use PCR-MRSP for phylogenetic reconstruction of tribe Cheloneae (Scrophulariaceae), and we specifically address the utility of three chloroplast genic regions: *rbc*L, *rps*2 and *trn*K. Our study differs from previous investigations in that no intergenic spacer regions are included in the analyses, multiple genic regions are sampled, and an assessment of interspecific and intergeneric relationships is included.

***Taxa and background information***—Tribe Cheloneae (Scrophulariaceae) was first described by Bentham (1846), and was last treated as a whole by Wettstein (1891). The tribe is circumscribed by one character: possession of a cymose inflorescence. This broad definition of tribal affinity has resulted in the Cheloneae possessing a large amount of morphological heterogeneity (Pennell, 1935; Thieret, 1954, 1967; Burtt, 1965). Wettstein (1891) included 26 genera in the tribe (Table 1), and three additional genera have been included in recent years (Straw, 1966, 1967; Crosswhite and Kawano, 1970). Twenty-two genera have been transferred to other tribes of Scrophulariaceae or to other families (Table 1; Hallier, 1903; Bellini, 1907; Pennell, 1919, 1935; Urban, 1926; Campbell, 1930; Li, 1947; Monachino, 1949; van Steenis, 1949; Gleason, 1952; Burtt, 1965; Straw, 1966; Crosswhite and Kawano, 1970; Thieret, 1972; Armstrong, 1985; Barringer, 1993). Several genera have been particularly problematic (*Brandisia, Paulownia, Schlegelia* [*Dermatocalyx* in Table 1], *Synapsis,* and *Wightia*), and have been transferred repeatedly between Bignoniaceae and Scrophulariaceae, or have been designated as genera ''intermediate'' between the two families (Campbell, 1930; Li, 1947; Monachino, 1949; van Steenis, 1949; Thieret, 1967; Williams, 1970; Armstrong, 1985). Removal of South African genera from Cheloneae to form the tribes Bowkerieae, Freylineae, and Teedieae (Barringer, 1993) left only the New World members with *Brookea* and *Pennellianthus* representing the Old World.

Old World genera of Cheloneae sensu Wettstein (1891) are distributed primarily in southern Africa, Indonesia, and northeast Asia, whereas New World genera have the greatest diversity in North America (Table 1). Affinities between Old World and New World Cheloneae have rarely been proposed. However, the few phylogenetic hypotheses of New World/Old World relationships that do exist are between Indonesian or Asian and North American genera. For example, the Central American genus *Uroskinnera* is purportedly most closely related to either *Brookea* (Borneo; Burtt, 1965) or *Pennellianthus* (Japan and Kurile Islands; Straw, 1966). *Pennellianthus* was segregated from the North American genus *Penstemon* (Straw, 1966; Crosswhite and Kawano, 1970), and, in addition to *Uroskinnera,* is purportedly closely related to the North American genera *Penstemon, Chelone, Chionophila, Keckiella,* and *Nothochelone* (Straw, 1966; Crosswhite and Kawano, 1970; Hong, 1983).

## MATERIALS AND METHODS

***Taxa investigated***—Seventeen genera of Cheloneae s.l., *Thunbergia* (Acanthaceae), *Campsis* (Bignoniaceae), and *Nicotiana* (Solanaceae), representing 32 taxa (Table 2), were included in this study. *Nicotiana* was used as an outgroup taxon based on results from *rbc*L, *ndh*F, and *rps*2 sequencing (Olmstead et al,. 1993; Bremer et al., 1994; Olmstead and Reeves, 1995; dePamphilis, Young, and Wolfe, in press). *Thunbergia* and *Campsis* were included to assess their potential use as outgroup taxa to Cheloneae. All six subgenera of *Penstemon* (Table 2), the largest genus in Cheloneae, were sampled for this study in order to assess the usefulness of cpDNA PCR-MRSP for phylogenetic reconstruction at interspecific as well as higher taxonomic levels. Among the *Cheloneae* s.s., we were unable to obtain plant material for *Uroskinnera* and *Brookea,* but the Asian genus *Pennellianthus* and New World *Tetranema* were included. New World genera transferred from Cheloneae to other tribes (*Russelia* of Russelieae; *Collinsia* and *Tonella* of Collinsieae; Table 2) were also assayed because they were originally included in Wettstein's (1891) treatment.

*PCR protocols*—Total DNAs were isolated (Doyle and Doyle, 1987) from individual plants (Table 2). Three cpDNA genic regions were amplified for restriction-site analysis: (1) the photosynthetic gene *rbc*L (1382 bp; RH-1 5′-ATGTCACCACAAACAGAAACTAAAGC-3′, 1382R 5′-CTTCACAAGCAGCAGCTAGTTCAGGACTCC-3′); (2) an intron and maturase gene within a transfer RNA gene (Sugita, Shinozaki, and Sugiura, 1985)—*trn*K (2562 bp; 19 5′-CTCAACGGTAGAGTACTCG-3′, 20 5′-TCGAACCCGGAACTAGTCGGATG-3′); and (3) the ribosomal protein gene *rps*2 (656 bp; 1 5′-TCCCTCACAAATAGCGAATACCAA-3′, 4C 5′-TGGAAGAGATGATGGGGGC-3′). These genic regions were sampled because of the differences in substitution rates among them. For example, the synonymous substitution ($K_S$) rate for *mat*K (the gene encoding a maturase within the *trn*K intron) is 1.3 times faster than *rbc*L, whereas *rps*2 is 1.1 times faster than *rbc*L (Wolfe, 1991). The nonsynonymous ($K_A$) rates for *mat*K and *rps*2 are

TABLE 1. Taxonomic summary of tribe Cheloneae sensu lato. Approximate number of species in parentheses. Taxa in boldface type represent Cheloneae sensu stricto.

| Cheloneae sensu Wettstein (1891) | Geographic distribution | Transfers from Cheloneae | References |
|---|---|---|---|
| *Anastrabe* (Benth.) E. Meyer (1) | South Africa | Bowkerieae (Scophulariaceae) | Barringer 1993 |
| *Berendtiella* Wetts & Harms (4) (*Berendtia*) | Central America | Mimuleae (Scrophulariaceae) | Burtt 1965 |
| | | Gratioleae (Scrophulariaceae) | Thieret 1967 |
| *Bowkeria* Harvey (5) | South Africa | Bowkerieae (Scrophulariaceae) | Barringer 1993 |
| *Brandisia* Hook.f. & Thomson (13) | Eastern Asia | Bignoniaceae | Li 1947 |
| | | Paulownieae (Scrophulariaceae) | Burtt 1965 |
| ***Brookea*** Benth. (3) | Borneo | Gesneriaceae | Hallier 1903 |
| | | Cheloneae? (Scrophulariaceae) | Burtt 1965 |
| ***Chelone*** L. (4) | Eastern North America | None | |
| ***Chionophila*** Benth. (2) | Western North America | None | |
| *Collinsia* Nutt. (17) | North America | Collinsieae (Scrophulariaceae) | Bellini 1907 |
| | | | Pennell 1935 |
| | | | Thieret 1967 |
| *Dermatocalyx* Oersted [*Schlegelia* Miq. (12)] | Central America | Schlegelieae (Bignoniaceae) | Monachino 1949 |
| | | Schlegelieae (Scrophulariaceae) | Armstrong 1985 |
| *Freylinia* Colla. (4) | South Africa | Freylinieae (Scrophulariaceae) | Barringer 1993 |
| *Halleria* L. (4) | South Africa Madagascar | Teedieae (Scrophulariaceae) | Barringer 1993 |
| *Hemichaena* Benth. (1) | Central America | Mimuleae (Scrophulariaceae) | Burtt 1965 |
| | | Gratioleae (Scrophulariaceae) | Thieret 1967 |
| *Ixianthes* Benth. (1) | South Africa | Bowkerieae (Scrophulariaceae) | Barringer 1993 |
| *Leucocarpus* D. Don (1) | Central America | Mimuleae (Scrophulariaceae) | Burtt 1965 |
| | South America | Gratioleae (Scrophulariaceae) | Thieret 1967 |
| *Paulownia* Siebold & Zucc. (6) | Asia | Paulownieae (Scrophulariaceae) | Pennell 1919 |
| | | | Gleason 1952 |
| | | Bignoniaceae | Campbell 1930 |
| | | | Li 1947 |
| | | Scrophulariaceae | Armstrong 1985 |
| ***Penstemon*** Mitchell (275) | North America | Several genera segregated [***Keckiella*** Straw (7), ***Nothochelone*** (A. Gray) Straw (1), ***Pennellianthus*** Crosswh. (1)] | Straw 1966 |
| *Phygelius* (Benth.) E. Meyer (2) | South Africa | Freylinieae (Scrophulariaceae) | Barringer 1993 |
| *Russelia* Jacq. (52) | Mexico | Russelieae (Scrophulariaceae) | Pennell 1919 |
| | Central America | | Thieret 1967 |
| | South America | | |
| *Scrofella* Maxim. (1) | China | Veroniceae? (Scrophulariaceae) | Burtt 1965 |
| *Scrophularia* L. (200) | Holarctic | Verbascaceae (Scrophulariaceae) | Straw 1966 |
| | | | Thieret 1967 |
| *Synapsis* Griseb. (1) | Cuba | Schlegelieae (Bignoniaceae) | Urban 1926 |
| | | Schlegelieae (Scrophulariaceae)? | Armstrong 1985 |
| *Teedia* Rudolphi (2) | South Africa | Teedieae (Scrophulariaceae) | Barringer 1993 |
| ***Tetranema*** (Lindley) Benth. (2) | Mexico Central America | None | |
| *Tonella* (Gray) Nutt. (2) | Western North America | Collinsieae (Scrophulariaceae) | Bellini 1907 |
| ***Uroskinnera*** Lindley (3) | Mexico | Gesneriaceae | Hallier 1903 |
| | Central America | Cheloneae? (Scrophulariaceae) | Burtt 1965 |
| | | Cheloneae (Scrophulariaceae) | Straw 1966 |
| | | Scrophulariaceae | Thieret 1967 |
| *Wightia* Wallich (3) | Indonesia Southeast Asia | Bignoniaceae | Hallier 1903 |
| | | | Campbell 1930 |
| | | | Li 1947 |
| | | Scrophulariaceae | Steenis 1949 |

6.3 and 2.7 times faster, respectively, than *rbc*L (Wolfe, 1991). In addition, the *trn*K amplification product includes ≈ 1000 bp of rapidly evolving intronic sequence distinct from the *mat*K gene. Our expectation was that the faster evolving genes would provide enough phylogenetically informative restriction sites to resolve relationships at the tips of the tree, whereas the more slowly evolving genes would facilitate resolution at the deeper nodes.

PCR amplifications used 0.126 μmol/L of each primer, 1X *Taq* DNA polymerase buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 9.0, 0.1% Triton X-100; Promega, Madison, WI), 0.8 mmol/L dNTPs, 3.6 mmol/L $MgCl_2$, 0.63 units of *Taq* DNA polymerase (Promega), and 1.0 μg DNA template in 50 μL total volume. The reaction mix was overlaid with mineral oil and subjected to 35 cycles of 40 s at 94°C, 1 min at 48°C, 2 min + 5 s/cycle at 72°C, following a 3-min denaturation at 94°C. A 5-min extension at 72°C followed the amplification cycle. The amount of PCR product needed for the study included an average of: 2.2 reactions totaling 165 μL for *rbc*L (for 17 restriction digests using 5 μL PCR product/digest) with a minimum of two reactions (150 μL) and a

TABLE 2. Taxa included in PCR-product restriction-site variation study of Cheloneae. Specimen vouchers deposited at OKL and VDU.

| Taxon | Collection data |
|---|---|
| *Nicotiana tabacum* L. | dePamphilis s.n. |
| *Thunbergia alata* (Sim) Bojer | dePamphilis 90.71 |
| *Campsis radicans* (L.) Seem. | Wolfe s.n. |
| *Phygelius capensis* (Benth.) E. Meyer | dePamphilis, Morden and Palmer #844 |
| *Anastrabe integerrima* (Benth.) E. Meyer | Steiner 1902 |
| *Halleria lucida* L. | dePamphilis 90.23 |
| *Dermatobotrys saundersii* H. Bolus | dePamphilis, Morden and Palmer #885 |
| *Teedia lucida* Rudolphi | Steiner s.n. |
| *Paulownia tomentosa* (Thunberg) Steudel | Hortus Botanicus Hauniensis 3480-129; Wolfe s.n. |
| *Tetranema mexicanum* (Lindl.) Benth. | Berlin Botanical Garden 040-26-74-74; Wolfe s.n. |
| *Russelia equisetiformis* Schlecht. and Cham. | dePamphilis 90.70 |
| *Pennellianthus frutescens* Crosswh. | Hokkaido University 13615; Wolfe s.n. |
| *Scrophularia peregrina* L. | Hortus Botanicus Nationalis Beligii, Wolfe s.n. |
| *Scrophularia alpestris* L. | Botanischer Garten München-Nymphenburg; Wolfe s.n. |
| *Collinsia grandiflora* (Lindl.) Dougl. | Botanischer Garten München-Nymphenburg; Wolfe s.n. |
| *Collinsia heterophylla* (Grah.) Buist | Botanischer Garten München-Nymphenburg; Wolfe s.n. |
| *Tonella floribunda* Gray | dePamphilis SS# 23 |
| *Chelone lyonii* Pursh | Nelson 131 |
| *Chelone obliqua* L. | Wolfe 586 |
| *Nothochelone nemorosa* (Dougl.) Straw | Wolfe 468 |
| *Chionophila jamesii* Benth. | Wolfe 473 |
| *Keckiella corymbosa* (Benth.) Straw | Wolfe 437 |
| *Keckiella lemmonii* (Gray) Straw | Wolfe 436 |
| *Penstemon barrettiae* Gray | Berry Botanic Garden SB84-112; Wolfe s.n. |
| *Penstemon davidsonii* var. *menziesii* Keck | Hortus Botanicus Nationalis Beligii; Wolfe s.n. |
| *Penstemon serrulatus* (Smith) Menz. | Hortus Botanicus Hauniensis 3423-S1966-1135-259; Wolfe s.n. |
| *Penstemon dissectus* Elliot | Wolfe s.n. |
| *Penstemon cardinalis* W. & S. | Botanischer Garten München-Nymphenburg; Wolfe s.n. |
| *Penstemon strictus* Benth. | Hortus Botanicus Hauniensis 3427-3909-60-259; Wolfe s.n. |
| *Penstemon hirsutus* (L.) Willd. | Hortus Botanicus Hauniensis 3420-3909-12-259; Wolfe s.n. |
| *Penstemon spectabilis* (Gray) Thurb. | Wolfe 212 |
| *Penstemon personatus* Keck | Edwards and Carter s.n. |

maximum of four reactions (300 μL); 4.6 reactions totaling 350 μL for *trn*K (for 18 restriction digests using 5 μL PCR product/digest) with a minimum of two reactions (145 μL) and a maximum of 12 reactions (915 μL); 3.2 reactions totaling 277 μL for *rps*2 (for 15 restriction digests using 10 μL PCR product/digest) with a minimum of three reactions (250 μL) and a maximum of six reactions (600 μL). In general, it was more difficult to optimize the reactions for *trn*K compared to the other two genes. The total *Taq* DNA polymerase usage for the entire study was 489.9 units.

Seventeen restriction enzymes were used to cut *rbc*L PCR products according to the manufacturer's instructions: *Asp*700, *Ban*II, *Bcl*I, *Cfo*I, *Hae*III, *Msp*I, *Taq*I, and *Xba*I (Boehringer Mannheim, Indianapolis, IN); *Ava*II, *Bst*NI, *Bst*YI, *Dde*I, *Fok*I, *Mse*I, *Nla*IV, and *Nsi*I (New England Biolabs, Beverly, MA); and *Hin*fI (American Allied Biochemical, Aurora, CO). Fifteen restriction enzymes were used to cut *rps*2 PCR products: *Alu*I, *Hin*fI, and *Sau*3AI (American Allied Biochemical), *Ban*II, *Bcl*I, *Cfo*I, *Eco*RI, *Hae*III, *Hind*III, and *Msp*I (Boehringer Mannheim), *Ase*I, *Nla*IV, *Rsa*I, and *Sau*96I (New England Biolabs); and *Hin*cII (United States Biochemical, Cleveland, OH). Eighteen restriction enzymes were used to cut *trn*K PCR products: *Asp*700, *Ban*II, *Bcl*I, *Dra*I, *Eco*RI, *Eco*RV, and *Msp*I (Boehringer Mannheim); *Ase*I, *Ava*II, *Bst*NI, *Bst*YI, *Dde*I, *Fok*I, *Nla*IV, *Nsi*I, *Rsa*I and *Sau*96I (New England Biolabs); and *Bst*EII (Promega). *Ase*I, *Asp*700, *Ban*II, *Bcl*I, *Bst*EII, *Bst*YI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Nsi*I, and *Xba*I each recognize six base pairs (bp); *Ava*II, *Bst*NI, and *Fok*I are five-bp cutters; and *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Mse*I, *Msp*I, *Nla*IV, *Rsa*I, *Sau*3AI, *Sau*96I, and *Taq*I have four-bp recognition sites. Each restriction digest used one unit of enzyme per taxon. Enzyme choice was based on an analysis of restriction sites in the three genic regions from the tobacco chloroplast genome (Shinozaki et al., 1986). Some enzymes had recognition sites that overlapped. For example, *Hae*III (GGCC) recognizes some sites that are also recognized by *Nla*IV (GGNNCC). Duplicate restriction sites were counted only once in the phylogenetic analyses.

DNA fragments were separated either on 1.0% agarose or 3.5% NuSieve (FMC Bioproducts, Rockland, ME) agarose in the presence of ethidium bromide (EtBr). Restriction-site positions were inferred using the known DNA sequences and restriction sites of *Nicotiana* as a template for *trn*K, from *Nicotiana, Chelone, Collinsia, Paulownia, Scrophularia,* and *Thunbergia* for *rbc*L; and from *Nicotiana, Chelone, Halleria,* and *Tetranema,* for *rps*2. Restriction-site maps were constructed and the presence/absence of a site coded as the character state for each mutation (character) for phylogenetic analysis.

***Data analysis***—The computer program PAUP 3.1.1 (Swofford, 1993) was used to analyze cpDNA PCR-MRSP. Wagner parsimony was used in a heuristic search using 100 random stepwise addition sequences. The TBR with STEEPEST DESCENT and MULPARS options were used during the searches, and ACCTRAN was used to optimize branch lengths. The data were analyzed by two methods: individually by genic region and combined into one large data set. This approach was taken to evaluate the information content of each genic region and to determine if combined and consensus methods would result in similar tree topologies. For both methods, bootstrap (Felsenstein, 1985) values were calculated using 100 replicates (RANDOM ADDITION with ten replicates). Decay analysis (Bremer, 1988; Donoghue et al., 1992) on the combined data set was done as follows: all trees up to three steps longer were found in heuristic searches (RANDOM ADDITION with ten replicates). After three extra steps, there was insufficient computer memory to save all of the trees generated, so an abbreviated search via an ''inverted constraints'' method (Johnson and Soltis, 1994) was conducted to estimate the number of steps required to decay the remaining branch nodes.

TABLE 3. Restriction-site information content of PCR-amplified products.

| | Cheloneae survey | | | | *Astragalus*[a] | *Datisca*[b] | Galegeae[c] |
|---|---|---|---|---|---|---|---|
| | *trn*K | *rbc*L | *rps*2 | Total | *rpo*C1/C2 | *rbc*L/ORF106 | *rpo*C1/C2 |
| Size of PCR-product | 2562 bp | 1382 bp | 654 bp | 4598 bp | 4100 bp | 3210–3320 bp | 4100–4150 |
| Total nucleotide sites sampled[d] | 447 | 280 | 118 | 845 | 615 | 232 | 556 |
| No. of variable sites | 80 | 37 | 21 | 138 | 38 | 53 | 89 |
| No. of informative sites | 49 | 25 | 12 | 86 | 9 | 38 | 45 |
| Informative sites/variable sites | 0.613 | 0.676 | 0.571 | 0.623 | 0.263 | 0.717 | 0.506 |
| Informative sites/fragment size | 0.019 | 0.018 | 0.018 | 0.019 | 0.002 | 0.012 | 0.011 |
| Sites sampled/fragment size | 0.175 | 0.203 | 0.180 | 0.184 | 0.150 | 0.072–0.070 | 0.136–0.134 |

[a] From Liston, 1992.
[b] From Rieseberg, Hanson, and Philbrick, 1992.
[c] From Liston and Wheeler, 1994.
[d] Includes invariant sites not used in phylogenetic analyses.

Restriction-site differences and sequence divergence estimates were calculated using SDE 1.2 (Wolfe and Wolfe, 1993), which implements Eqs. 5.3, 5.38, and 5.41 in Nei (1987), and 3.19, 3.32, and 3.33 in Li and Graur (1991) and the Jukes and Cantor (1969) correction for multiple hits. Average restriction-site length was used in the calculations for each sequence divergence estimate (SDE) of the combined data set taxa pairs.

## RESULTS

***Restriction-site variation***—A total of 180 restriction sites was found in this survey and was partitioned as follows: 89 sites for *trn*K PCR products (nine invariable, 80 variable, 49 informative sites); 63 sites for *rbc*L PCR products (26 invariable, 37 variable, 25 informative sites); and 29 sites for *rps*2 PCR products (eight invariable, 21 variable, 12 informative sites). Original map data and the data matrix are available from the senior author. No insertion or deletions (indels) were detected. At first glance the number of variable and informative restriction sites for each PCR-amplified gene appears to be associated with the size of the PCR product. For example, *trn*K produces the largest fragment and the most variable and informative restriction sites, whereas *rps*2 produces the smallest fragment and fewest variable and informative sites. However, the information content measured in the ratios of informative to variable sites (0.571-0.676) and informative sites to fragment size (0.018-0.019) is similar for each PCR product (Table 3).

The number of restriction-site differences ranged from zero (within species of *Chelone, Keckiella,* and *Penstemon*) to 69 (between *Nicotiana* and *Tonella*; SDE = 8.71). Among genera within the Cheloneae s.l., the number of differences ranged from one (*Nothochelone* and *Chionophila*; SDE = 0.11) to 46 (*Scrophularia* and *Tonella*; SDE = 0.540). The number of restriction-site differences among species of *Penstemon* ranged from zero to ten (SDE = 0.00-0.93).

The sequence divergences estimated from these data have lower values than estimates obtained from total chloroplast DNA restriction-site variation for species within *Penstemon* (Wolfe and Elisens, 1995). For example, the SDE for *P. spectabilis* and *P. barrettiae* was 0.69 based on eight restriction-site differences of cpDNA PCR products, but was 0.95 based on 25 site differences in a total cpDNA survey (Wolfe and Elisens, 1995). We interpret this to mean that PCR-based methods provide a more limited estimate of sequence divergence, which may result in an underestimate of the amount of divergence when compared to the entire chloroplast genome.

***Phylogenetic analyses***—The heuristic search of the combined molecular data sets yielded two most-parsimonious trees (Fig. 1) of 228 steps in length, with a consistency index (CI) of 0.605 (0.554 without autapomorphies) and a retention index (RI) of 0.792. The two trees differ primarily in the topology of genera in clade A.

The individual and strict consensus trees depict *Thunbergia* (Acanthaceae) and *Campsis* (Bignoniaceae) nested within the Scrophulariaceae of clade A (Fig. 1). These results are not inconsistent with a polyphyletic Scrophulariaceae. The polyphyletic nature of the Scrophulariaceae is also supported by *rbc*L and *ndh*F sequence data with genera from other families intercalated between clades of Scrophulariaceae genera (Olmstead and Reeves, 1995; Nickrent et al., 1997), and by *rps*2 and *rbc*L sequence data including a larger sample of Scrophulariaceae genera (dePamphilis, Young, and Wolfe, in press). Our results differ from Olmstead and Reeves (1995) in that our only representative of Bignoniaceae was nested within Clade A (Fig. 1), whereas the Bignoniaceae branched early in the *rbc*L and *ndh*F sequence trees. The placement of *Thunbergia* is similar in both studies based on the relationships among genera of Scrophulariaceae inferred from *rps*2 and *rbc*L sequence data (Nickrent et al., 1997).

Several monophyletic clades are well supported (*Scrophularia, Chelone, Collinsia, Keckiella,* and *Penstemon* subg. *Dasanthera* and *Habroanthus*; the sister taxa *Collinsia* and *Tonella*; and the North American genera; Fig. 1). The South African genera do not form monophyletic clades, in agreement with recent tribal reassignments proposed by Barringer (1993).

The degree of support for particular nodes, as indicated by bootstrap and decay index values, do not always correspond (Fig. 1). Examples of agreement are found on nodes supported by 100% bootstrap values (d5-d18). However, d5 nodes have bootstraps ranging from 90 to 100% and d3 nodes have bootstrap values ranging from 76 to 96%.

The individual analyses from each genic region are inadequate to resolve phylogenetic relationships among all taxa investigated (Fig. 2a-c). There are 198 most-parsimonious *trn*K trees (135 steps; CI = 0.593 [0.538 without autapormorphies]; RI = 0.757), a single most-parsimonious tree for *rps*2 (24 steps; CI = 0.875 [0.850 with-

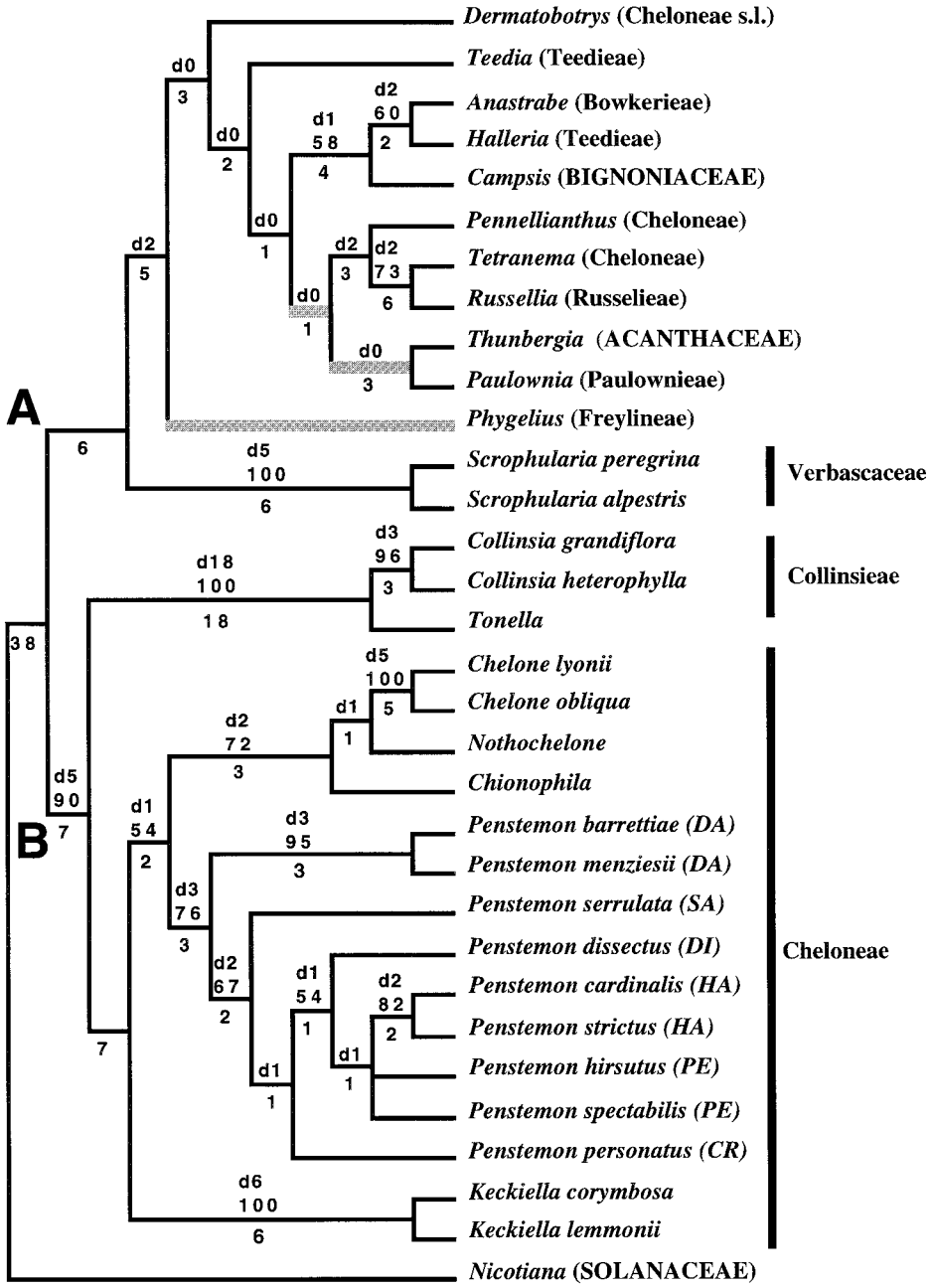


Fig. 1. One of two most-parsimonious trees (228 steps; CI = 0.605; RI = 0.792) based on combined restriction-site data for *trn*K, *rps*2, and *rbc*L. Number of synapomorphies supporting node indicated below; decay indices and bootstrap values above. Only bootstrap values above 50% shown. Branches shown in gray demarcate topological differences with the second most-parsimonious tree. Clade B represents North American genera. *DA* = *Penstemon* subg. *Dasanthera*; *SA* = *Penstemon* subg. *Saccanthera*; *DI* = *Penstemon* subg. *Dissecti*; *HA* = *Penstemon* subg. *Habroanthus*; *PE* = *Penstemon* subg. *Penstemon*; *CR* = *Penstemon* subg. *Cryptostemon.* Family names in caps except for Scrophulariaceae; tribal affiliations of taxa from Scrophulariaceae indicated.

out autapomorphies]; RI = 0.951), and 1560 trees (58 steps; CI = 0.638 [0.596 without autapormorphies]; RI = 0.855) for *rbc*L. However, the different amplification product data sets are able to resolve different areas of the tree. For example, data from *trn*K and *rps*2 can be used to assess New World relationships with *trn*K having greater resolution in examining *Penstemon* and *Collinsia*/*Tonella*, and results from *rps*2 and *rbc*L depict the monophyletic relationship of the North American genera. The combined treefiles of the individual analyses revealed major agreements among the data sets in that the three clades found in each analysis also appear in the strict consensus tree (Fig. 2d). The differences among the data sets are manifested in the formation of a ''phylogenetic rake'' when a strict consensus was performed on all of the individual trees from the separate analyses (Fig. 2d). Taken together, the relatively well-resolved tree of the combined analysis, the lack of resolution of phylogenetic

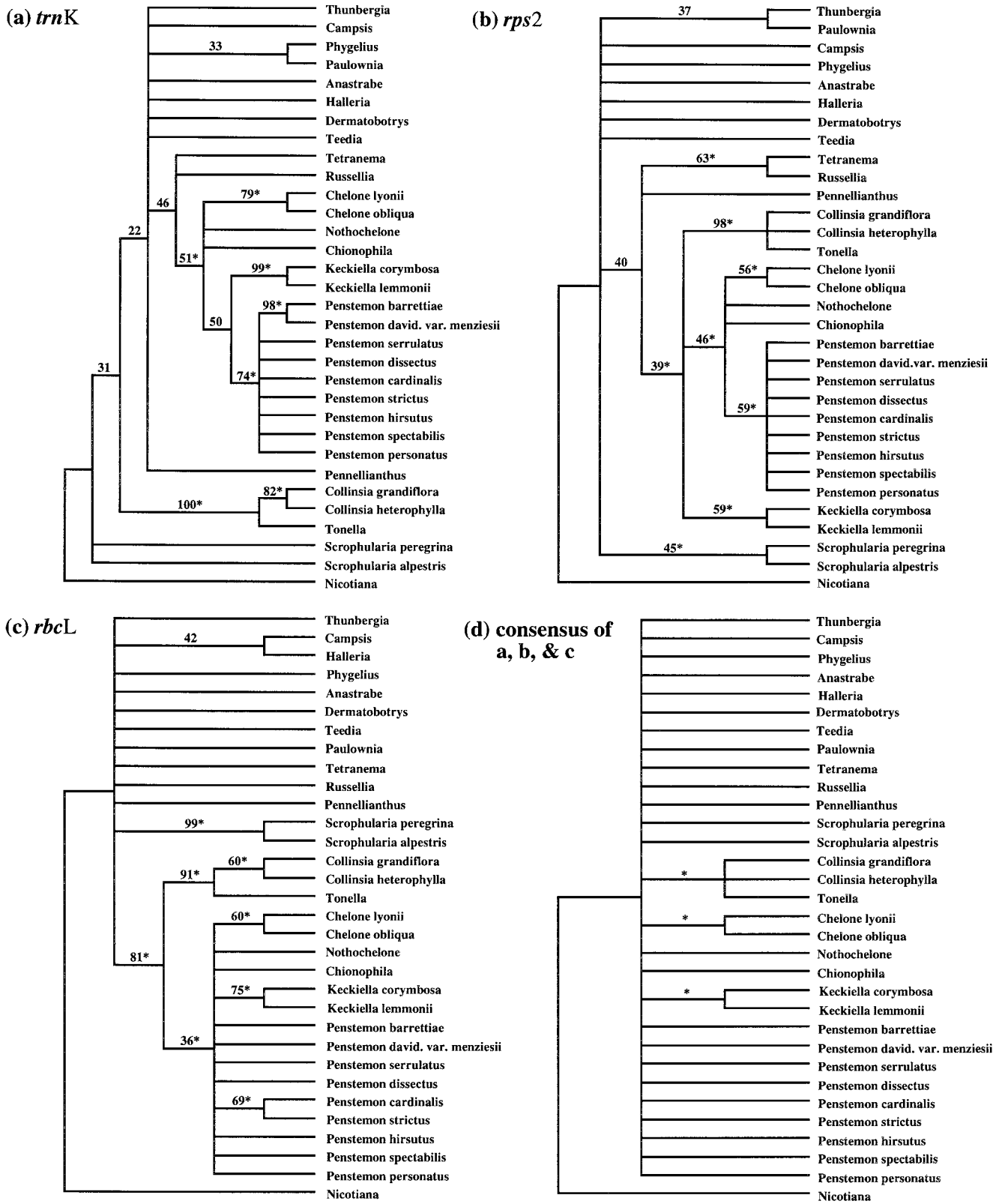


Fig. 2. Strict consensus trees from individual genic regions. Bootstrap values above nodes, except for (d). Clades in agreement with strict consensensus tree from combined data analysis depicted by *. All bootstrap values shown. (a) Strict consensus of 198 trees from *trn*K analysis; (b) Single most-parsimonious tree from *rps*2 analysis; (c) Strict consensus of 1560 trees from *rbc*L analysis; (d) Strict consensus of combined treefile representing 1759 trees from *trn*K, *rps*2, and *rbc*L individual analyses.

trees from individual genes, and the loss of information from a consensus approach suggest that increasing the number of characters using a combined data approach boosted the phylogenetic signal-to-noise ratio (de Queiroz, Donoghue, and Kim, 1995) in this study.

Given the small number of total nucleotide sites sampled for the *rps*2 gene compared to the *trn*K and *rbc*L genic regions (Table 3), it is surprising that the *rps*2 data set yielded the most resolved phylogenetic reconstruction. The number of nodes resolved for each data set were similar (Fig. 2). However, the *rps2* data set exhibited the least amount of homoplasy as measured by the consistency index for phylogenetic reconstruction compared to *rbc*L and *trn*K. The pattern of these results could be the consequence of stochastic factors in enzyme choice and/or number of nucleotides sampled. Alternatively, the pattern observed may reflect the molecular evolution of each genic region. For example, the proportion of variable positions at each codon position is distributed across all codon positions for *mat*K, whereas the major proportion of variation within the *rbc*L and *rps*2 genes are found at the third codon position (Steele and Vilgalys, 1994; C. dePamphilis and A. Wolfe, unpublished data). It is possible that the greater number of potential nucleotide sites with a faster rate of evolution for *mat*K compared to *rbc*L and *rps*2 facilitates nonhomologous restriction-site losses that increase the amount of homoplasy in the data set.

## DISCUSSION

***Taxonomic conclusions: a redefinition of tribe Cheloneae***—The results from this investigation support a redefinition of tribe Cheloneae to include, at a minimum, the North American genera *Chelone, Nothochelone, Chionophila, Keckiella,* and *Penstemon.* It is clear from the polyphyletic nature of the taxa sampled in this investigation (Fig. 1) that the morphological character ''cymose inflorescence'' is not a synapomorphy for taxa historically included in tribe Cheloneae. Our results are compatible with maintaining the tribe Collinsieae, but are equally congruent with merging the Collinsieae and Cheloneae. Although several systematists have placed the east Asian genus *Pennellianthus* with the North American groups (Straw, 1966; Crosswhite and Kawano, 1970), our results do not consistently resolve relationships among New and Old World genera. Phylogenetic reconstruction based on *rps*2 sequences (C. dePamphilis et al., unpublished data) reveal *Tetranema* and *Chelone* as sister taxa. Our results from individual gene PCR-MRSP analyses (*trn*K and *rps*2; Fig. 2a, b) also place *Tetranema* and *Russelia* with the North American genera, and phylogenetic reconstruction from *rps*2 restriction-site data and the combined data set (Fig. 1) places *Pennellianthus* in the *Tetranema* clade (Fig. 2b). Because the southwest Asian genus *Brookea* and the central American genus *Uroskinnera* were not sampled for this study, we cannot define their tribal placements at this time. However, morphological data available for these genera do not exclude their consideration as members of Cheloneae (Burtt, 1965; Thieret, 1967). The delimitation of Cheloneae apparently includes the North American clade that contains *Chelone, Chionophila, Keckiella, Nothochelone,* and *Penstemon,* and may very well include what is now classified as tribe Collinsieae (*Collinsia* and *Tonella*). However, the relationships and/or inclusion of other New World genera of Cheloneae sensu lato and Old World *Pennellianthus* need to be examined with additional data before any firm taxonomic conclusions can be made as to the circumscription of tribe Cheloneae in its entirety.

***Usefulness of PCR-MRSP for phylogenetic reconstruction***—Most studies that have used cpDNA restriction-site variation sampled the entire chloroplast genome and used radioisotope labeling techniques. This approach has the distinct advantage of sampling many nucleotides, sometimes >3% of the chloroplast chromosome (i.e., >4500 bp of a genome of ≈ 150 kb; Olmstead and Palmer, 1994). Clearly, a whole-genome approach yields more data than a PCR-based approach. The advantages and constraints of chloroplast whole-genome restriction-site methods were reviewed by Olmstead and Palmer (1994).

The advantages of PCR-based methods compared to conventional Southern-blot-based methods for phylogenetic analysis highlighted by Olmstead and Palmer (1994) were: (1) less DNA is required for PCR-based methods, which is a plus where the amount of DNA or plant material is limiting, and (2) PCR-based methods may be preferable where many genome rearrangements make mapping difficult. In addition to these particular advantages of PCR methods compared to Southern-blot-based methods previously proposed we add: (3) elimination of radioisotope use via direct visualization of DNA fragments on EtBr-stained gels. Although a chloroplast whole-genome mapping study can be done with nonradioactive labeling protocols, only one (Wolfe and Elisens, 1995) of the seven chloroplast restriction-site studies published by *Systematic Botany* in 1995 used this approach (Urbatsch and Jansen, 1995; Haufler, Soltis, and Soltis, 1995; Bogler and Simpson, 1995; La Duke and Doebley, 1995; Wolfe and Elisens, 1995; Francisco-Ortega et al., 1995; Davis, 1995).

The advantages listed above also facilitate the use of PCR-product restriction-site variation for a feasibility study prior to a major chloroplast nucleotide sequencing effort. If there is low sequence divergence estimated among taxa for a particular genic region, it is doubtful that a major sequencing project will generate sufficient data points to adequately resolve relationships utilizing that area of the chromosome. Additionally, because of the large database of chloroplast DNA sequences available for many plant taxa, it is possible to map restriction sites in chloroplast genes easily by direct comparison of inferred sites to known restriction sites. The challenges associated with mapping restriction-site data in whole genome studies was discussed in Olmstead and Palmer (1994).

We would like to add one final note about whole-genome and PCR-based approaches to phylogenetic reconstruction. A major advantage of a whole-genome approach is the random nature of character sampling (Cummings, Otto, and Wakely, 1995). Phylogenetic reconstruction based on sequencing contiguous regions of DNA may not reveal the same pattern of relationships as a whole-genome tree. By sampling several different genes in the chloroplast genome, it may be possible to recover a similar tree topology as a whole-genome approach (Cummings, Otto, and Wakely, 1995).

Two factors are important in phylogenetic reconstruction using a PCR-based method: (1) the number of nucleotides sampled—the more, the better; and (2) the ratio of informative restriction sites to fragment size. By amplifying several genes with varying substitution rates vs. a single genic region, it is possible to increase the number of nucleotides sampled and potentially provide resolution at all levels of the tree. Eliminating intergenic regions reduces the variability incurred with indels, but makes mapping of restriction sites easier. However, indels may provide informative characters if homology can be assessed effectively. The ratio of informative sites to fragment size can be improved by careful selection of restriction enzymes. For example, four-bp cutters will yield more sites than five- and six-bp cutters. Additionally, by limiting a survey to nonoverlapping recognition sequences, the problem of duplicate restriction sites is eliminated.

Previous studies using PCR-product restriction-site variation examined intergeneric and/or interspecific relationships (Liston, 1992; Rieseberg, Hanson, and Philbrick, 1992; Liston and Wheeler, 1994). Although these studies used regions with low functional constraints and high rates of nucleotide substitution, there were few variable and informative restriction sites obtained at the interspecific level compared to the number of nucleotides sampled per fragment size (Table 3). In contrast, our investigation employed more slowly evolving genic regions and more restriction sites (characters) compared to most of the previous investigations (Table 3; Wolfe and Liston, 1997).

The phylogenetic reconstruction from our PCR-MRSP analysis was sufficient to reveal the polyphyletic nature of the Scrophulariaceae and monophyly of the North American genera of Cheloneae. Although separate analyses of individual gene-amplification products yielded little resolution in our study (Fig. 2), the combined molecular data set was useful in assessing intergeneric phylogenetic relationships (Fig. 1). However, there was a marked lack of resolution, as measured by bootstrap and decay analyses, within *Penstemon* (Fig. 1). Interspecific relationships were resolved at the subgeneric level within *Penstemon*, but not below that taxonomic designation (Fig. 1). Previous investigations (Liston, 1992; Rieseberg, Hanson, and Philbrick, 1992) were also unable to resolve interspecific relationships with much relative nodal support. Our results, added to results from previous studies, are a strong indication that PCR-product restriction-site variation is an appropriate tool for phylogenetic analysis above the species level. It may prove to be particularly useful in taxa that have highly rearranged chloroplast genomes (a challenge in constructing restriction-site maps) and for taxa for which fresh leaf material is unavailable.

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