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THE ITS REGION OF NUCLEAR RIBOSOMAL DNA: A VALUABLE SOURCE OF EVIDENCE ON ANGIOSPERM PHYLOGENY[1]

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ABSTRACT

The internal transcribed spacer (ITS) region of 18S–26S nuclear ribosomal DNA (nrDNA) has proven to be a useful source of characters for phylogenetic studies in many angiosperm families. The two spacers of this region, ITS-1 and ITS-2 (each <300 bp), can be readily amplified by PCR and sequenced using universal primers, even from DNAs of herbarium specimens. Despite high copy numbers of both spacers, the near uniformity of ITS paralogues, attributed to rapid concerted evolution, allows direct sequencing of pooled PCR products in many species. Divergent paralogues, where detected, require cloning, but may offer a means of obtaining multiple estimates of organismal relationships and of determining placement of the root in a phylogeny independent of outgroup considerations. In reported studies, variation between ITS sequences is mostly attributable to point mutations. A relatively minor proportion of sites is affected by insertions or deletions (indels) among sequences that are similar enough to have retained sufficient signal for phylogenetic analysis. Within these limits, sequence alignment is generally unambiguous except in small regions of apparently lower structural constraint. Phylogenetic analyses of combined data sets from both spacers, where examined, yield trees with greater resolution and internal support than analyses based on either spacer alone. This beneficial effect of simultaneous analysis is not surprising based on the low number of useful characters in each spacer. This effect also suggests high complementarity of spacer data, in accord with similarity in size, sequence variability, and G + C content of ITS-1 and ITS-2 in most investigated groups of closely related angiosperms. Non-independent evolution of ITS sites involved in intraspacer RNA base-pairing may occur, given possible functional constraints, but preliminary secondary structure analyses of ITS-2 in *Calycadenia* (Asteraceae) show no definite evidence of compensatory spacer mutations. As expected, levels of ITS sequence variation suitable for phylogenetic analysis are found at various taxonomic levels within families, depending on the lineage. The apparent rates of ITS molecular evolution are roughly correlated with plant life-form, as with chloroplast DNA (cpDNA) data, but reasons for this observation are unclear. ITS characters have improved our understanding of angiosperm phylogeny in several groups by (1) corroborating earlier unexpected findings, (2) resolving conflicts between other data sets, (3) improving resolution of species relationships, or (4) providing direct evidence of reticulate evolution. Hybridization or sorting of ancestral polymorphism in a lineage can complicate interpretation of trees based on any type of evolutionary evidence, including ITS or cpDNA sequences, particularly in the absence of at least one independent phylogenetic data set from the same organisms. The need for phylogenetic markers from the nuclear genome, to complement the rapidly growing body of cpDNA data, makes the ITS region a particularly valuable resource for plant systematists.

Until recently, most plant systematists reserved DNA sequencing for phylogenetic analyses of taxa with sequences too divergent to be easily interpreted by restriction site mapping. Consequently, only moderately to slowly evolving DNA sequences have been used widely in plant phylogenetics (e.g., *rbc*L, see Chase et al., 1993; 18S and 26S nuclear ribosomal DNA, see Bult et al., 1995; Nickrent & Soltis, 1995). With the recent advent of polymerase chain reaction (PCR) technology, however, DNA

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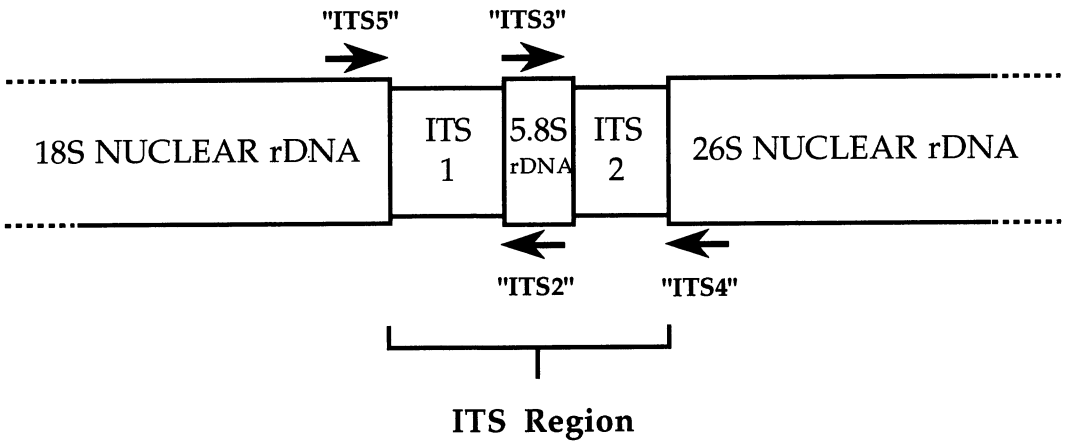


FIGURE 1. Organization of the ITS region. Arrows indicate orientation and approximate position of primer sites. Primer names (in quotation marks) and sequences are from White et al. (1990). Reprinted with permission from Baldwin (1993a).

sequencing is now sufficiently inexpensive and easy to use for phylogenetic studies at all taxonomic levels. This sequencing option offers increased precision by permitting better homology assessment of molecular characters and character-states than is possible by restriction site mapping.

The primary challenge to using nucleotide characters for lower-level phylogenetic studies is the identification of easily amplifiable and relatively rapidly evolving, but unambiguously alignable, DNA regions that can provide sufficient, suitable variation within a short sequence segment. There is an especially great need for such data from the nuclear genome, to supplement the wealth of chloroplast DNA (cpDNA) restriction site information that has accumulated over the past decade. This need is made particularly evident by an increasing number of reports of (1) strong conflict between cpDNA trees and relationships based on other lines of evidence, often explained by interspecific hybridization (reviewed in Rieseberg & Soltis, 1991; Rieseberg & Brunsfeld, 1992; Rieseberg & Wendel, 1993) or lineage sorting (see Pamilo & Nei, 1988; Doyle, 1992), and (2) insufficient phylogenetic resolution in cpDNA trees, with information often concentrated along a limited subset of internal tree branches. These findings do not detract from the value of cpDNA phylogenetic data but merely demonstrate the potential inadequacies of any estimate of organismal phylogeny based on a single organellar genome or nuclear gene (see Doyle, 1992).

In this paper we evaluate the phylogenetic utility of a genomic region that has attracted increased attention among those interested in applying nuclear-DNA sequencing analysis to lower-level phylogenetic questions in angiosperms (and other eukaryotes): the internal transcribed spacer (ITS) region of 18S–26S nuclear ribosomal DNA (nrDNA). This region includes three components as defined here (Fig. 1): the 5.8S subunit, an evolutionarily highly conserved sequence, and, most importantly from the perspective of this paper, two spacers designated ITS-1 and ITS-2. As testimony to the recent high level of interest in the ITS region, ongoing plant phylogenetic studies using ITS-1 and ITS-2 vastly outnumber those few that are published in detail. We therefore have relied largely on our own published and unpublished work with these sequences for most of the examples and comparisons presented here.

FUNCTIONAL CONSIDERATIONS

The ITS region is part of the transcriptional unit of nrDNA, but the spacer segments of the transcript are not incorporated into mature ribosomes. Instead, ITS-1 and ITS-2 regions of the nrDNA transcript appear to function, at least in part, in the maturation of nrRNAs. In vivo mutational analyses in yeast (*Saccharomyces cerevisiae*) indicate that deletions of certain regions within ITS-1 can inhibit production of mature small and large subunit rRNAs (Musters et al., 1990; van Nues et al., 1994), whereas certain deletions or point mutations in ITS-2 prevent or reduce processing of large-subunit rRNAs (van der Sande et al., 1992). Intrastrand Watson-Crick base pairing within spacers and perhaps between spacer and precursor nrRNA sequences may bring the subunit boundaries into close proximity within a processing domain (Gonzalez et al., 1990a; Thweatt & Lee, 1990; Venkateswarlu & Nazar, 1991; Melekhovets et al.,

1994). Given this hypothesized role, much like that of self-splicing group I introns, it seems probable that ITS-1 and ITS-2 are under some evolutionary constraint in structure and sequence, as suggested by size and G + C (guanine + cytosine) content comparisons among angiosperms (see Table 1 and below).

Evolutionary Considerations for Phylogeny Reconstruction

PRELIMINARY INSIGHTS

Early attempts at alignment of ITS sequences across angiosperm families indicated that plant ITS-1 and ITS-2 sequences have diverged more at the nucleotide level than have the nrDNA subunits (Yokota et al., 1989), with the exception of subregions in expansion segments or divergent domains of the large (26S) subunit (Hassouna et al., 1984; Cullings & Bruns, 1992; C. Bult, pers. comm.). In addition, nrDNA restriction site studies of closely related plant species have shown consistently that a high proportion of variable sites map to the ITS region, as well as to the intergenic spacer (IGS) and external transcribed spacer (ETS) (e.g., Appels & Dvořák, 1982; Sytsma & Schaal, 1985; Kim & Mabry, 1991). Such studies, however, have only rarely detected within-genome length variation in the ITS region (but see Sytsma & Schaal, 1985, 1990; and Jorgensen & Cluster, 1988), in contrast to repeated documentation of such length variants in the IGS (e.g., Rogers & Bendich, 1987; Schaal & Learn, 1988). These preliminary indications of ITS length conservation and high ITS nucleotide sequence variability suggested that DNA sequences of these spacers might be readily alignable across closely related taxa, yet sufficiently variable to allow resolution of lower-level phylogenetic questions in angiosperms. Such properties were evident in ITS sequences from Oomycetes (Lee & Taylor, 1992) and hominoids (Gonzalez et al., 1990b) prior to attempts by angiosperm systematists to utilize these spacers. These considerations were a major stimulus to initial exploration of the ITS region for use in plant phylogenetic studies (Baldwin, 1992).

FAVORABLE PROPERTIES

Several general features of the ITS region promote its use for phylogenetic analyses of angiosperms. First, along with the other components of the nrDNA multigene family, the ITS region is highly repeated in the plant nuclear genome. The entire nrDNA repeat unit is present in up to many thousands of copies arranged in tandem repeats at a chromosomal locus or at multiple loci (Rogers & Bendich, 1987; reviewed in Hamby & Zimmer, 1992). This high copy number promotes detection, amplification, cloning, and sequencing of nrDNA.

Second, and most importantly from the standpoint of phylogeny reconstruction, this gene family undergoes rapid concerted evolution (e.g., Arnheim et al., 1980; Appels & Dvořák, 1982; Arnheim, 1983; Hillis et al., 1991; and see Zimmer et al., 1980), via unequal crossing-over and gene conversion. This property promotes intragenomic uniformity of repeat-units, in some cases even between nrDNA loci on non-homologous chromosomes (e.g., Arnheim et al., 1980; Arnheim, 1983; Hillis et al., 1991; Wendel et al., 1995), and, in general, promotes accurate reconstruction of species relationships from these sequences (Hamby & Zimmer, 1992; Sanderson & Doyle, 1992). As a result, direct sequencing of pooled nrDNA PCR products can be used to extract phylogenetic information in many species. In addition, concerted evolution and sexual recombination may promote nrDNA uniformity within interbreeding populations and thereby minimize the importance of intrapopulational sampling in phylogenetic studies, e.g., as in *Lomatium grayi* Coult. & Rose and *L. laevigatum* Coult. & Rose (Soltis & Kuzoff, 1993), and *Limonium* (M. Hamilton, pers. comm.).

Third, the small size of the ITS region (<700 bp in angiosperms) and the presence of highly conserved sequences flanking each of the two spacers make this region easy to amplify, even from herbarium material, using universal eukaryotic primers designed by White et al. (1990) (Fig. 1). In many cases, these primers can be used to generate single-stranded DNA for sequencing directly from genomic DNA, bypassing a separate double-stranded DNA amplification procedure (Baldwin, 1992, 1993a; Wojciechowski et al., 1993). We have used this approach, and even more streamlined double-stranded sequencing methods (e.g., cycle-sequencing), to generate high-quality ITS sequences in several angiosperm groups (see below), generally without evidence of divergent repeat-types within individuals.

DIVERGENT PARALOGUES

If concerted evolution fails to homogenize ITS paralogues (e.g., those at different chromosomal loci) through a succession of speciation events, the possibility of unknowingly sampling sequences with different evolutionary histories is a real danger to phylogenetic analysis (Fitch, 1970; Sanderson & Doyle, 1992). On the other hand, if such paralogues are retained in most or all members of a

TABLE 1. Sizes and percent G + C content of ITS-1 and ITS-2 (see Fig. 1) of angiosperm taxa. ITS-1 and ITS-2 boundaries chosen by various authors may differ by up to a few base pairs. NA = data not available.

Taxon	ITS-1		ITS-2	
	Size (bp)	% G + C	Size (bp)	% G + C
Adoxaceae, *Viburnum*, 28 spp. (Donoghue & Baldwin, 1993)	224–231	59–69	215–227	59–69
Apiaceae, *Daucus*, 1 sp. (Yokota et al., 1989)	215	49	224	52
Apiaceae, Apioideae, 42 spp. (S. Downie, pers. comm.)	204–221	49–58	215–226	43–61
Asteraceae, Madiinae, 8 gen., 45 spp. (Baldwin, 1992, 1993a; Baldwin & Robichaux, 1995)	254–261	48–51	216–223	50–53
Asteraceae, Lactuceae, 6 gen., 11 spp. (Kim & Jansen, 1994)	246–253	52–54	220–222	53–58
Betulaceae, *Alnus*, 3 spp. (Savard et al., 1993)	215–216	58–64	228–229	53–65
Betulaceae, *Betula*, 3 spp. (Savard et al., 1993)	214–219	62–63	226–231	63–65
Betulaceae, *Ostrya*, 1 sp. (Savard et al., 1993)	215	61	227	62
Brassicaceae, *Arabidopsis*, 1 sp. (Unfried & Gruendler, 1990)	268	57	187	55
Brassicaceae, *Sinapis*, 1 sp. (Rathgeber & Capesius, 1989)	265	51	188	54
Canellaceae, *Canella*, 1 sp. (Suh et al., 1992)	272	63	209	63
Cucurbitaceae, *Cucumis*, 1 sp. (Kavanagh & Timmis, 1988)	216	56	237	60
Cucurbitaceae, *Cucurbita*, 2 sp. (Torres et al., 1990)	187–229	51–60	245–252	54–66
Fabaceae, Galegeae, 4 gen., 31 spp. (Wojciechowski et al., 1993)	221–231	55–60	207–217	50–54
Fabaceae, *Vicia*, 1 sp. (Yokota et al., 1989)	235	52	208	50
Fabaceae, *Vigna*, 1 sp. (Schiebel & Hemleben, 1989)	205	60	220	59
Onagraceae, *Epilobium*, 22 spp. (Baum et al., 1994)	240–244	53–62	211–216	54–60
Malvaceae, *Gossypium*, 1 sp. (Mukhamedov, 1992)	287	58	229	60
Malvaceae, 7 gen. (Ray, 1994a, pers. comm.)	277–298	49–53	240	57
Poaceae, Pooideae, 6 gen., 10 spp. (Hsiao et al., 1994)	217–223	55–64	213–221	59–67
Poaceae, *Sorghum*, 1 sp. (Hsiao et al., 1994)	207	56	217	67
Poaceae, *Oryza*, 1 sp. (Takaiwa et al., 1985)	194	73	233	77
Polemoniaceae, 18 gen., 38 spp. (Porter, 1993b)	242–262	42–65	187–195	48–62
Ranunculaceae, 7 gen., 27 spp. (Hodges & Arnold, 1994)	214–246	NA	198–216	NA
Rosaceae, *Fragaria*, 1 sp. (Simovic et al., 1990)	249	68	207	65
Rosaceae, Maloideae, 20 gen., 20 spp. (Campbell et al., 1993a)	208–221	65–72	211–224	67–72

TABLE 1. Continued.

Taxon	ITS-1		ITS-2	
	Size (bp)	% G + C	Size (bp)	% G + C
Rosaceae, *Prunus*, 1 sp. (Campbell et al., 1993a)	242	62	209	69
Rosaceae, *Rosa*, 1 sp. (Campbell et al., 1993a)	249	60	207	57
Rosaceae, *Spiraea*, 1 sp. (Campbell et al., 1993a)	251	65	230	70
Salicaceae, *Populus*, 1 sp. (D'Ovidio, 1992)	214	67	207	70
Saxifragaceae, 9 gen., 28 spp. (Soltis & Kuzoff, in press)	256–267	NA	224–238	NA
Scrophulariaceae, *Mimulus*, 8 spp. (Ritland et al., 1993)	189–214	44–49	203–225	45–48
Solanaceae, *Lycopersicon*, 1 sp. (Kiss et al., 1988)	217	68	217	71
Solanaceae, *Nicotiana*, 1 sp. (Venkateswarlu & Nazar, 1991)	216	69	217	65
Styracaceae, *Styrax*, 19 spp. (Fritsch, 1994a)	255–264	NA	208–221	NA
Viscaceae, *Arceuthobium*, 22 spp. (Nickrent et al., 1994)	208 (mean)	ca. 31–36	226 (mean)	ca. 30–37
Winteraceae, 7 gen., 11 spp. (Suh et al., 1993)	235–252	NA	213–226	NA

species lineage, thorough sampling of these sequences can offer independent estimates of organismal phylogeny (Suh et al., 1993; see Sanderson & Doyle, 1992) and a means of rooting that portion of the tree in the absence of outgroup data (Iwabe et al., 1989). In other words, non-homogenized paralogues can present opportunities along with some potential danger. Cloning and sequencing of individual repeat-types may be necessary, however, to recover any useful information from such nrDNAs (see Ritland et al., 1993).

A direct sequencing approach can sometimes aid in detection of intragenomic repeat-type variants, which can be tentatively diagnosed by two or more nucleotide states at a site, implying superimposition of two or more sequence patterns. Persistence of the same apparent site-polymorphism(s) in ITS sequences of both complementary DNA strands reduces the likelihood that the multiple bands are attributable to a sequencing artifact, e.g., self-pairing of single-stranded sequencing products.

ITS variants within individuals that differ by an insertion or deletion (indel) of one or more nucleotides can be more readily detected from direct sequences than variants that differ by point mutations. At and above (i.e., in the direction away from the sequencing primer) an indel on a sequencing gel, a high proportion of sites will show two different states (bands). Sequence of the complementary strand will show the reverse pattern, i.e., will be unambiguous through the region that showed extensive polymorphism in the opposing sequence up to the other boundary of the indel, above which extensive site-polymorphism will be observed. Aside from identification of length-variant repeat-types, direct sequencing of both complementary strands can provide unambiguous sequence of the shortest repeat-type without cloning, assuming that the ITS variants differ by only one indel between the sequencing-primer sites.

It is still arguable whether sequencing of individual PCR clones or of pooled PCR products is the better approach to phylogenetic analysis of the ITS region, or of nrDNA in general. Routine sequencing of large numbers of individual PCR clones from each genomic DNA is ideal, but may be prohibitively expensive and labor-intensive for large-scale phylogenetic studies. Sequencing of only one PCR clone per reaction risks sampling of misreplicated products and, of course, undersampling of any repeat-type variants. In contrast, the small error rate of *Taq* polymerase dictates that the predominant signal in direct sequences of pooled PCR products is likely to represent DNAs that were accurately replicated (Saiki et al., 1988; Gyllensten & Erlich, 1988; Bruns et al., 1990).

The relative merits of sequencing cloned versus pooled PCR products for initial detection of variable repeat-types are less clear. Sequencing of several PCR clones may be necessary to detect repeat-type variants of similar size that are readily apparent from direct sequences of pooled PCR products, e.g., in the polyploid *Raillardella scaposa* (A. Gray) A. Gray (Asteraceae) (Baldwin, Wojciechowski & Donoghue, unpublished). Conversely, sequencing of only two PCR clones per reaction revealed widely-divergent ITS repeat-types in four of six species of Winteraceae that potentially possessed these two sequence classes of similar-sized ITS variants (Suh et al., 1993). Direct ITS-1 and ITS-2 sequences from pooled PCR products of two of these four species (*Bubbia comptonii* (E. G. Baker) Dandy and *Zygogynum bicolor* V. Tieghem, genomic DNAs kindly provided by Y. Suh) each showed evidence of only one repeat-type (Baldwin & Donoghue, unpublished), which corresponded to the orthologous "*Bubbia* 2" and "*Zygogynum bicolor* 2" sequences (Suh et al., 1993). Use of different PCR primers and reaction conditions by Suh et al. (1993) and Baldwin & Donoghue (unpublished) may account for lack of evidence of the other pair of orthologues in the direct sequences, e.g., by sequence divergence between the two classes of repeat-types at a primer annealing-site used by Baldwin & Donoghue. If the relative concentration of repeat-types in pooled PCR products is even crudely reflected by sequencing-autoradiograph band intensities, then direct sequencing of pooled PCR products should indicate whether ITS variants are present in sufficient concentration to allow cloning without extensive screening of recombinants. Most angiosperm workers have routinely taken such an initial direct sequencing approach (but see Suh et al., 1993). Denaturing gradient gel electrophoresis (DGGE), which allows separation of PCR products that differ by as little as one nucleotide substitution, has been used successfully as an alternative method for detecting ITS variants (R. K. Jansen, pers. comm.) that could potentially be gel-isolated and sequenced directly.

At a more fundamental level, PCR "selection" and "drift" (Wagner et al., 1994) can potentially lead to a pool of PCR products of the ITS region that includes a biased representation of the distinctive paralogues present in a genome. To ameliorate the effects of these processes, Wagner et al. (1994) recommend reducing the amount of genomic DNA in PCR, reducing the number of PCR cycles, and pooling products of multiple PCRs. Artifacts from PCR "jumping," i.e., chimeric sequence products formed by incomplete PCR products acting as PCR primers on heterologous sequences (Saiki et al., 1988; Scharf, 1990), may also be reduced by application of these recommendations. The possibilities of PCR "drift" and PCR "jumping" provide increased impetus for sequencing both strands of the ITS region from products of different PCRs.

An additional, follow-up strategy for detection of paralogues that may affect phylogeny reconstruction is to screen genomic DNAs of the study species for presence or absence of any restriction sites that diagnose the major ITS clades indicated by phylogenetic analysis of direct or cloned sequences of PCR products. Such screening can be accomplished by Southern blot hybridization of electrophoresed genomic DNA digests using labeled, PCR-amplified nrDNA sequences from the study group or, in some cases, labeled clones of the entire nrDNA repeat unit. Additivity for restriction patterns that define different ITS clades in a single genomic DNA can provide a reevaluation of orthology/paralogy relationships of ITS sequences. This strategy was recently used by Wendel (1995) to determine whether both parental ITS repeat types occur in allopolyploid species of *Gossypium*.

Technical Considerations for ITS Sequencing

Methods

In general, we have followed the asymmetric-PCR and sequencing procedures detailed in Baldwin (1992), which typically yield excellent results with high-purity DNAs (see below). We have also successfully used symmetric-PCR (with primers in equimolar, 10mM, concentrations, otherwise as in Baldwin, 1992) and cycle-sequencing methods, using ^{33}P (dsDNA cycle sequencing system, BRL-Gibco, Gaitherburg, Maryland), ^{35}S (Cyclist, Stratagene, La Jolla, California), or fluorescent dyes (with automated sequencing) for labeling. For amplification of DNAs from old herbarium specimens (up to at least 80 years old) we have achieved much greater success with the PCR reaction buffer described in Pääbo (1990) than with standard buffers. The ease of ITS amplification from herbarium specimens makes sampling of extinct, rare, or inaccessible populations practical and can increase the feasibility of comprehensive species sampling within widespread plant groups (see Loockerman & Jansen, in press, for a review of DNA extraction protocols for herbarium material).

DNA PURITY

High purity of genomic DNAs or PCR products can be critical for successful ITS sequencing. In our experience, most ITS sequencing problems in plants that are not resolved by changes in sequencing-reaction or gel-electrophoresis conditions can be solved by further purification of genomic DNAs (e.g., on cesium chloride gradients) or PCR products (e.g., by the Geneclean procedure, Bio 101, La Jolla, California). Template purity appears to be more important for ITS sequencing than for *rbc*L sequencing, based on communications with other workers.

SECONDARY STRUCTURE FORMATION

Sequencing of the ITS region can be complicated by within-strand Watson-Crick base pairing that may interfere with *Taq* polymerase activity or may alter the electrophoretic mobility of DNA fragments in sequencing gels. Spacer segments with G + C richness or any substantial intrastrand complementarity may form secondary structures under some reaction conditions. Such intramolecular pairing is expected based on the probable functional behavior of transcripts of these sequences (see "Functional Considerations"). For this reason, high-temperature sequencing reactions with *Taq* polymerase (or another thermophilic DNA polymerase) and use of dGTP analogs, such as dITP or deaza-dGTP, in sequencing reactions (and even in PCR amplifications) are recommended. Use of DMSO (5–10%), formamide, or tergitol in PCRs and sequencing reactions can also be useful for overcoming such problems (Winship, 1989; Zhang et al., 1992; Bechmann et al., 1990; and see Kim & Jansen, 1994).

ITS Sequence Comparisons in Angiosperms

In this paper, we focus primarily, but not exclusively, on results from our own ITS phylogenetic studies within five dicotyledon families: Adoxaceae (*Viburnum*: Donoghue & Baldwin, 1993, unpublished), Asteraceae (Madiinae: Baldwin, 1991, 1992, 1993a, b, 1994, unpublished; Baldwin & Robichaux, 1995), Fabaceae (Galegeae: Wojciechowski et al., 1993; Sanderson & Wojciechowski, 1993, unpublished; Sanderson & Liston, in press), Polemoniaceae (Porter, 1993a, b, 1994, unpublished), and Rosaceae (Maloideae: Campbell et al., 1993a, b, in press). A number of other ITS studies have been published or are in press, including Baum et al. (1994: *Epilobium*, Onagraceae), Fritsch (1994a, see 1994b: *Styrax*, Styracaceae), Hodges & Arnold (1994: *Aquilegia*, Ranunculaceae), Hsiao et al. (1994: Pooideae, Poaceae), Kim & Jansen (1994: *Krigia*, Asteraceae), Miller (1994: *Silene*, Caryophyllaceae), Nickrent et al. (1994: *Arceuthobium*, Viscaceae), Ray (1994a, see Ray, 1994b: *Lavatera* and *Malva*, Malvaceae), Ritland et al. (1993: *Mimulus*, Scrophulariaceae), Savard et al. (1993: *Alnus* and *Betula*, Betulaceae), Sang et al. (1994b, 1995: *Dendroseris* and *Robinsonia*, Asteraceae), Soltis & Kuzoff (in press: *Heuchera* group, Saxifragaceae), Suh et al. (1993: Winteraceae), and Sun et al. (1994: *Sorghum*, Poaceae).

Many other ITS phylogenetic investigations of angiosperms are, however, in progress. Some of these studies are partially documented by published abstracts, including work on Apiaceae (Downie et al., 1994; Hardig & Soltis, 1994), Apocynaceae (Sper-Whitis et al., 1992), Asteraceae (Urbatsch & Baldwin, 1993; Bain & Jansen, 1994; Kim et al., 1994), Capparaceae (Hahn et al., 1994), Cornaceae (Murrell, 1994), Ericaceae (Hileman et al., 1994; Kron & King, 1994; Markos & Parker, 1994), Fouquieriaceae (Schultheis & Baldwin, 1994), Lemnaceae (Les et al., 1994), Liliaceae sensu lato (Bogler & Simpson, 1993, 1994; Bogler, 1994a, b; Bogler et al., 1994), Nothofagaceae (Manos, 1993), Onagraceae (Hahn & Sytsma, 1993), Poaceae (Kellogg et al., 1994; Mason et al., 1994), Paeoniaceae (Sang et al., 1994a), Polemoniaceae (Johnson et al., 1994), and Portulacaceae and related families (Hershkovitz & Zimmer, 1994). As these new studies are published our conclusions based on a relatively limited sample of angiosperms will no doubt require some adjustment. Nevertheless, sufficiently clear patterns have emerged to warrant some tentative generalizations.

LENGTH VARIATION

Length conservation of ITS-1 and ITS-2, as seen in earlier plant studies (see Baldwin, 1992), remains apparent from the expanded perspective of more recent investigations (Table 1). Sizes of ITS-1 and ITS-2 in these newly obtained sequences fall nearly within the narrow range reported previously for other angiosperms. In all flowering plants reported to date ITS-1 and ITS-2 are each less than 300 bp (ITS-1: 187 to 298 bp, ITS-2: 187 to 252 bp), in contrast to much longer spacers in some other eukaryotes, e.g., some vertebrates (Stewart et al., 1983; Goldman et al., 1983; Gonzalez et al., 1990b). Given that the 5.8S subunit is almost invariant in length in reported angiosperms (mostly 163 or 164 bp), the entire ITS region (Fig. 1) appears to be universally under 700 bp in flowering plants.

Relative sizes of the two spacers vary between

TABLE 2. Pairwise sequence divergence, potential phylogenetic information, and prevalence of gap-containing nucleotide positions in ITS-1 and ITS-2 sequences of angiosperms.

Taxa (number of species)	Percent pairwise sequence divergence				Potential informative sites/aligned sites (%)		% of aligned sites with >1 gap ITS-1 + 2
	ITS-1		ITS-2				
	Range	Mean (SD)	Range	Mean (SD)	ITS-1	ITS-2	
Hawaiian Madiinae, 3 gen. (25) (Baldwin & Robichaux, in press)	0–6.2	2.8 (± 1.2)	0–5.9	2.9 (± 1.5)	23/257 (9)	22/221 (10)	<1
Calycadenia/Osmadenia (12) (Baldwin, 1993a)	0–12.5	5.8 (± 3.5)	0–10.0	4.6 (± 2.6)	37/257 (14)	27/223 (12)	2
California/Hawaiian Madiinae, 7 gen. (11) (Baldwin, 1992)	0.4–21.0	12.1 (± 4.4)	0.5–14.1	8.7 (± 3.0)	46/259 (18)	23/206 (11)	5
Fouquieria (11) (Schultheis & Baldwin 1994)	0–7.8	4.0 (± 1.8)	0.4–7.1	3.5 (± 1.7)	14/259 (5)	15/227 (7)	3
Astragalus (26) (Wojciechowski et al., 1993)	0–10.2	3.7 (± 3.1)	0–8.8	3.7 (± 1.5)	22/238 (9)	22/215 (10)	7
Viburnum (28) (Donoghue & Baldwin, 1993)	0–13.6	7.3 (± 2.3)	0–11.9	6.1 (± 2.1)	43/239 (18)	36/229 (16)	5
Galegeae, 17 gen. (28) (Sanderson & Wojciechowski, 1993)	0–37.8	13.3 (± 8.3)	0–33.7	10.6 (± 8.5)	95/269 (35)	76/237 (32)	15
Gilia sect. *Gilliandra* (16) (Porter, 1993a, b)	0–13.9	5.7 (± 2.8)	0–12.5	5.4 (± 2.5)	30/256 (12)	28/188 (15)	6
Polemoniaceae, 19 gen., (28) (Porter, 1993a, b)	0–39.0	18.0 (± 7.3)	0–29.5	14.6 (± 5.5)	162/301 (54)	113/228 (50)	40
Maloideae, 20 gen. (20) (Campbell et al., 1993a, in press)	0–16.6	8.9 (± 3.1)	3.3–24.1	14.8 (± 3.7)	45/234 (19)	72/235 (31)	16

and, in at least some cases, within families, with little indication of a broad-scale phylogenetic pattern. ITS-1 is consistently longer that ITS-2 (or rarely equal in length) in all available sequences of Adoxaceae, Asteraceae, Brassicaceae, Canellaceae, Malvaceae, Onagraceae, Polemoniaceae, Ranunculaceae, Salicaceae, Saxifragaceae, Styracaceae, and Winteraceae. Both reported Brassicaceae sequences show a larger size disparity between ITS-1 and ITS-2 (77 bp in *Sinapis alba* L.; 81 bp in *Arabidopsis thaliana* (L.) Heynh.; Table 1) than in any other angiosperm sequences. Conversely, ITS-2 is larger than ITS-1 in all sequences of Betulaceae, Cucurbitaceae, Scrophulariaceae, and Viscaceae reported to date. The two spacers are nearly equal in length in both available sequences from Solanaceae. In the large families Fabaceae, Poaceae, and Rosaceae, ITS-1 may be longer or shorter than ITS-2.

SEQUENCE DIVERGENCE COMPARISONS

In most investigated groups of angiosperms, ITS-1 pairwise divergence values are similar on average to those of ITS-2 (e.g., Table 2). The greatest disparity in divergence comparisons of ITS-1 and ITS-2 is in Asteraceae. Distances between ITS-1 sequences of taxa in the Hawaiian silversword alliance (Madiinae) are generally similar to ITS-2 distances from the same species comparisons (Baldwin, unpublished). In *Krigia* (Lactuceae), however, ITS-1 pairwise divergence values are, on average, twice those of ITS-2 (Kim & Jansen, 1994). Other reported Asteraceae fall

largely between these extremes. Average pairwise distances between ITS-1 sequences exceed those between ITS-2 sequences by 1.3 to 1 in *Calycadenia* (Baldwin, 1993a) and 1.5 to 1 in a broader survey of Madiinae (Baldwin, 1992). Outside Asteraceae, *Epilobium* (Onagraceae, Baum et al., 1994) and *Gossypium* (Malvaceae, J. Wendel, pers. comm.), like *Krigia*, show much higher pairwise divergence between ITS-1 sequences than between sequences of ITS-2. Such comparisons in most other flowering plant families suggest more equitable distribution of variation between the spacers. In these families, interspecific distances are only slightly higher on average between ITS-1 sequences than between sequences of ITS-2 (1 : 1 to 1.4 : 1), or, in *Arceuthobium* (Viscaceae: Nickrent et al., 1994), the *Heuchera* group (Saxifragaceae; Soltis & Kuzoff, in press), Maloideae (Rosaceae; Campbell et al., 1993a, in press), and *Nothofagus* (Nothofagaceae; Manos, 1993, pers. comm.), somewhat greater divergence exists between ITS-2 sequences than between those of ITS-1.

It should be noted, however, that we do not consider comparison of average pairwise distance values to be an adequate measure of relative evolutionary rates. Relative rate tests (e.g., Muse & Weir, 1992) are better suited for this purpose. Average distances are based on a set of non-independent comparisons because they do not take phylogenetic relationships into account. This non-independence casts doubt on the statistical significance of differences in distance values. Furthermore, average distances mask variance in relative distances, which is considerable in these studies.

From a finer-scale perspective, likelihood ratio heterogeneity tests conducted by Gaut & Weir (1994) suggest that there have been differences in rates of nucleotide substitution between small (57 bp) subregions within ITS-1 and ITS-2 in species of Compositae sampled by Baldwin (1992). The existence of rate heterogeneity between small ITS sequence partitions in these species is further suggested by the somewhat irregular distribution of ITS substitutions calculated across parsimony trees of these taxa (Fig. 2). Patterns of conserved and variable ITS subregions consistently similar to those in Figure 2 are not obvious from examination of mutational distributions in other plant groups we have studied (data not shown).

POTENTIAL PHYLOGENETIC INFORMATION IN ITS-1 AND ITS-2

Comparison of the proportions of aligned sites that are potentially informative for phylogenetic analysis in ITS-1 and ITS-2 (e.g., in Table 2) suggests a distribution of variation between the spacers that is similar to that estimated from raw divergence values. Similar percentages of aligned nucleotide positions are potentially informative in ITS-1 and ITS-2 (0.8 : 1 to 1.3 : 1) in most reported studies of angiosperms. In two investigations of Asteraceae (Baldwin, 1992; Kim & Jansen, 1994), in *Epilobium* (Onagraceae; Baum et al., 1994), and in *Gossypium* (Malvaceae; Wendel, 1995), however, ITS-1 included 50 to 100% more potentially informative characters than ITS-2. In one of these studies (Baldwin, 1992), however, variation is somewhat underestimated in ITS-2 because 17 highly variable ITS-2 sites of ambiguous alignment were excluded from analysis, as opposed to only 9 such sites in ITS-1. In contrast to the results from Asteraceae, *Epilobium*, and *Gossypium*, ITS-1 contained only 61% as many potentially informative sites as ITS-2 in an analysis of Maloideae (Rosaceae; Campbell et al., 1993a, in press).

As expected, resolution in phylogenies based on data from ITS-1 or ITS-2 alone is generally well correlated with the extent of variation within each spacer suggested from divergence values and numbers of potentially informative sites. Strict consensus trees based on ITS-1 data generally showed more complete phylogenetic resolution than those based on ITS-2 sequences in groups wherein greater pairwise distances and higher numbers of potentially informative sites were found in ITS-1 than in ITS-2 (e.g., Baldwin, 1992, 1993a; Porter, 1993a, b). The reverse was generally true as well: strict consensus trees based on ITS-2 data showed better resolution than those based on ITS-1 data in groups wherein higher pairwise distances and higher numbers of potentially informative sites were found in ITS-2 than in ITS-1 (e.g., Manos, 1993, pers. comm.; Soltis & Kuzoff, in press). In all of these examples, however, trees based on either spacer alone provided at least some resolution of relationships that were unresolved or weakly supported in trees based on the other spacer.

In our studies, complementarity of ITS-1 and ITS-2 data sets was further indicated by more complete and robust resolution in trees based on combined spacer data than in trees based on either ITS-1 or ITS-2 alone. These results are not surprising given approximate doubling of the number of characters in the analyses, especially in light of the limited size of each spacer (see Huelsenbeck & Hillis, 1993) and the similarity in length, sequence variability, and G + C content of ITS-1 and ITS-2 in these plant groups. We therefore highly recommend that data from both ITS-1 and ITS-2 be combined for phylogenetic analyses of flowering plants unless strong incongruence be-

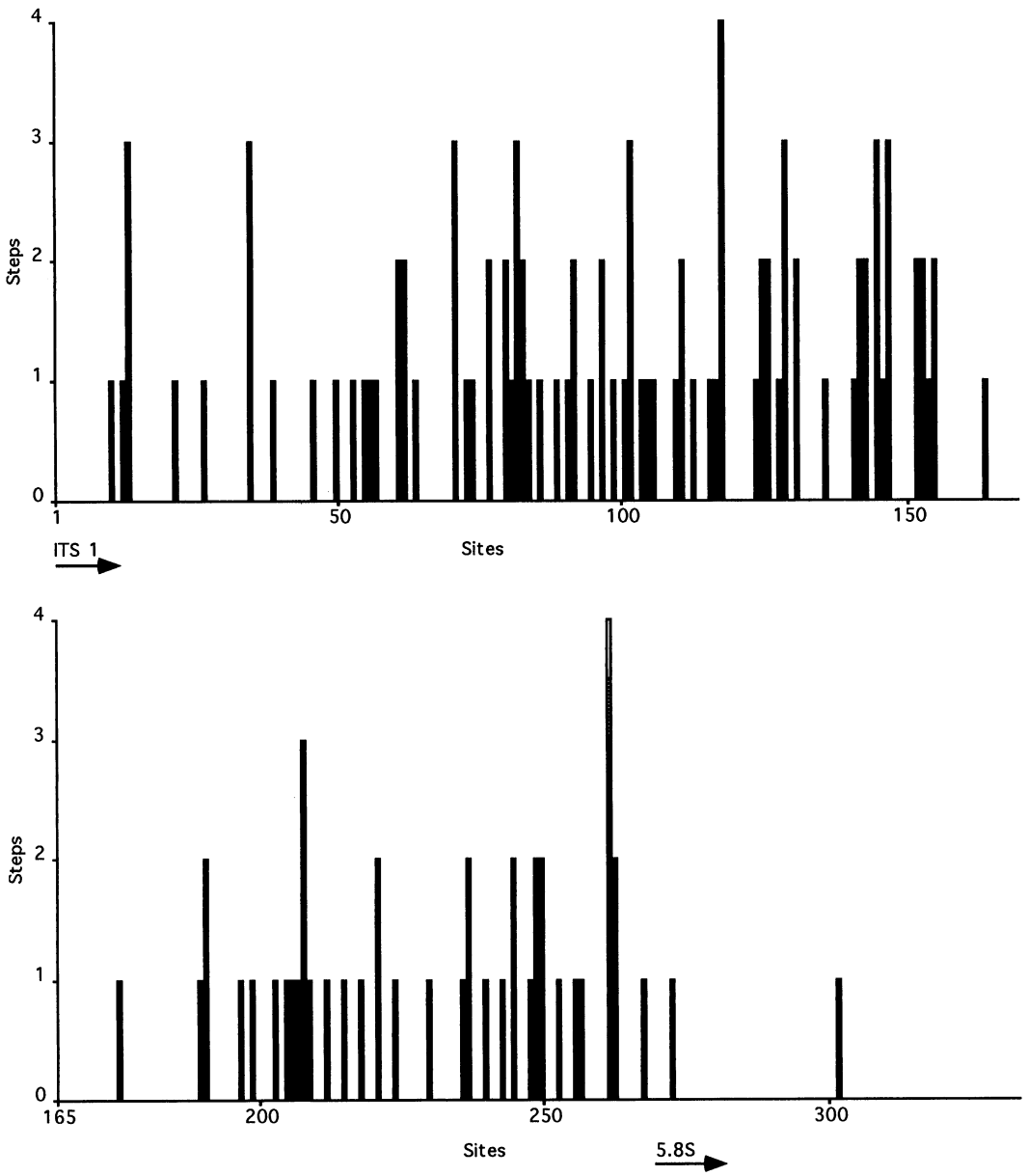


FIGURE 2 (pp. 256–257). Histogram of the number of changes at each aligned site of the ITS region in the 11 species of *Adenothamnus*, *Argyroxiphium*, *Dubautia*, *Madia*, *Raillardella*, *Raillardiopsis*, and *Wilkesia* (Asteraceae: Madiinae) discussed in Baldwin (1992). These changes were calculated across both minimum-length Fitch parsimony trees of these sequences using MacClade 3.0 (Maddison & Maddison, 1992). Numbers of changes at sites are the same in each of the two trees except at sites 261 and 532, for which the maximum numbers of changes are shown. Note interspersion of conserved and variable sites in both spacers and lack of variation in most of the 5.8S subunit. Aligned sequences are from Baldwin (1992), minus the five sites at which nucleotides were present only in outgroup species (which were not included in the McClade 3.0 analysis).

tween these spacers is indicated (see de Queiroz, 1993), such as by conflict between well-supported relationships in ITS-1 and ITS-2 trees. We have not observed such discordance between ITS-1 and ITS-2 data sets in angiosperms. To the contrary, these two spacers appear to be particularly good examples of "homogeneous" (Bull et al., 1993) sets of characters that have evolved similarly and are, therefore, best analyzed together for maximal phylogenetic resolution and support.

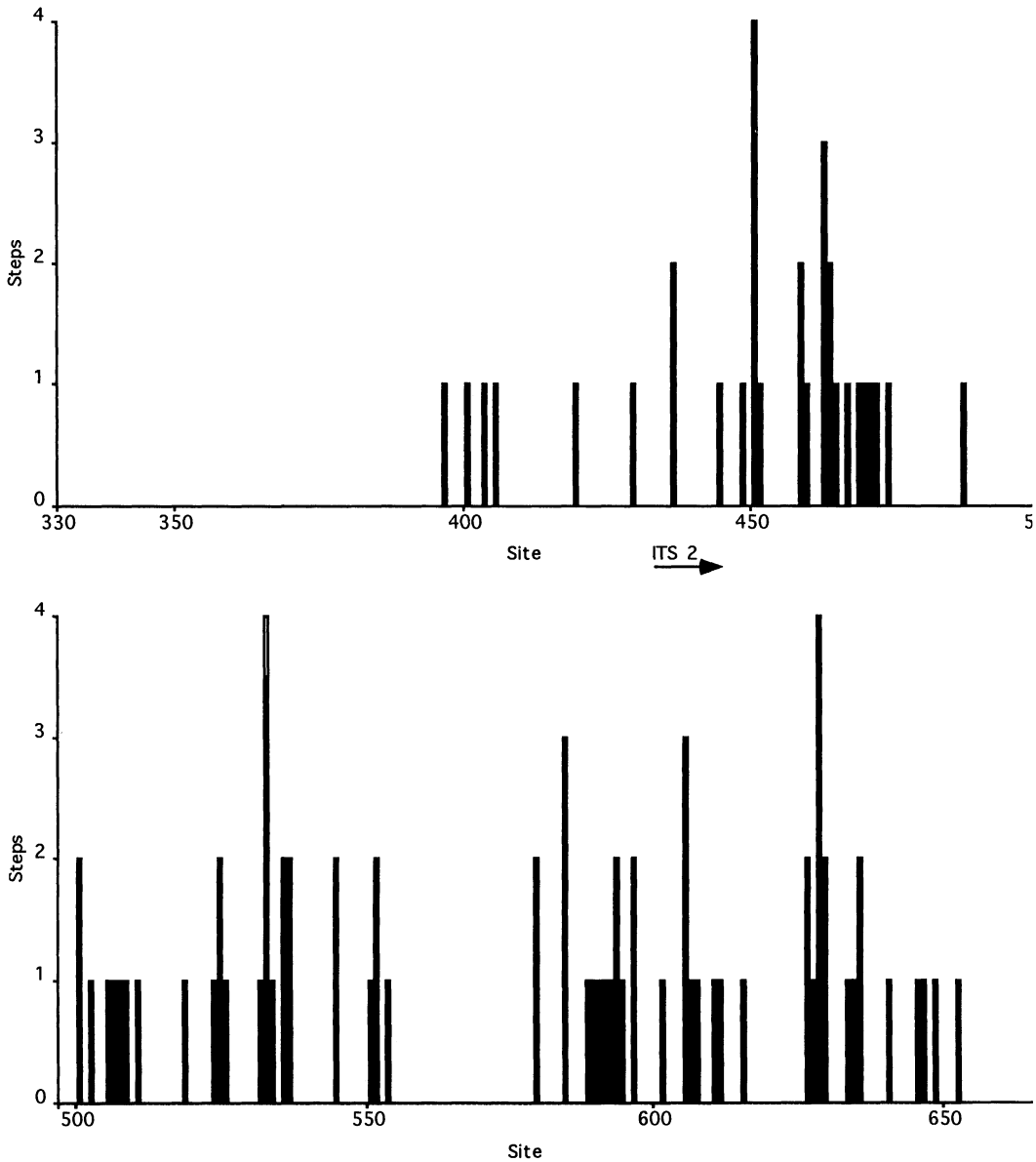


FIGURE 2. Continued.

G + C CONTENT VARIATION

Intraspecific G + C content in ITS-1 is roughly similar to that in ITS-2 in the same angiosperm sequences (Table 1), as seen in most other examined eukaryotes (Torres et al., 1990). This interspacer similarity probably reflects some degree of coevolution of ITS-1 and ITS-2 sequences, as suggested by evidence that both spacers are involved in maturation of large subunit rRNAs in *Saccharomyces cerevisiae* (van der Sande, 1992; van Nues et al., 1994). Overall ITS G + C content varies widely across angiosperm species, however, from ca. 50% in several groups to ca. 75% in *Oryza sativa* L. (Poaceae; Table 1). *Arceuthobium* (Viscaceae) falls outside this range, with unusually low ITS G + C content (ca. $\leq$36%; Nickrent et al., 1994; D. Nickrent, pers. comm.). Skewed G + C content may indicate a bias in substitution probabilities that could reduce detectability of homoplasy in such sequences compared to those with more equitable substitution expectations. A more practical concern about taxa with G + C rich ITS regions, however, is the difficulty of obtaining in-

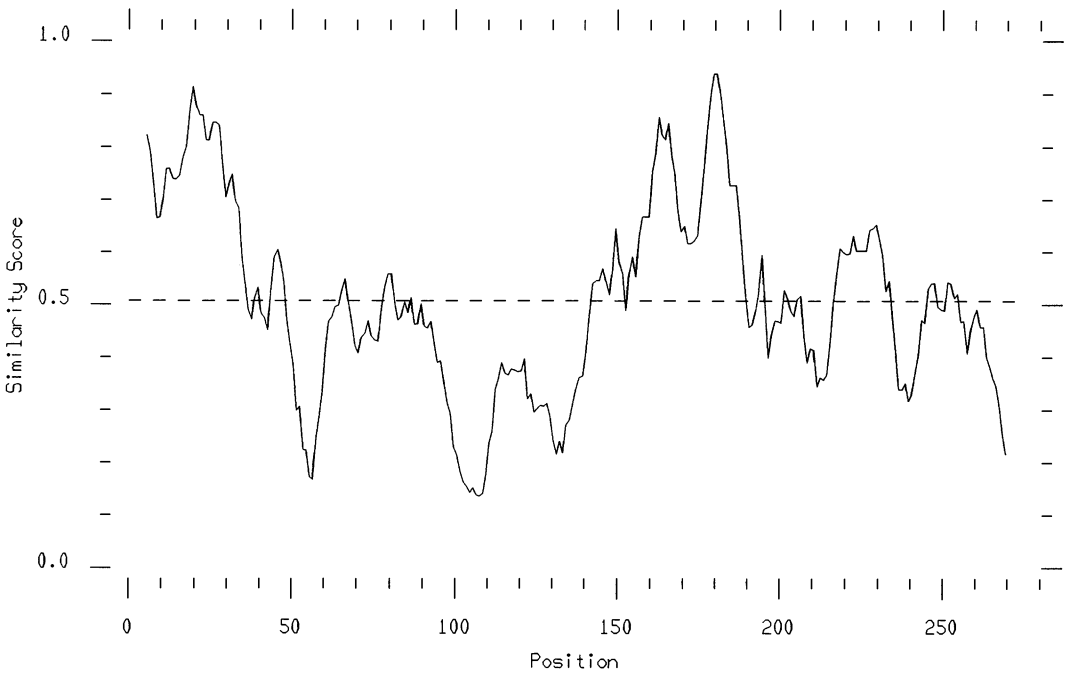


FIGURE 3 (pp. 258–259). Similarity of ITS sequences of 20 species (four per family) in Adoxaceae (*Viburnum*), Asteraceae (*Madia, Raillardiopsis*), Fabaceae (*Astragalus, Biserrula, Clianthus, Oxytropis*), Polemoniaceae (*Gilia, Ipomopsis, Loeseliastrum*), and Rosaceae (*Amelanchier, Cormus, Cydonia, Eriobotrya*) based on a PILEUP alignment (GCG, Genetics Computer Group, Madison, Wisconsin). Charted using PLOTSIMILARITY (GCG).—3A (this page). ITS-1.—3B (facing page). ITS-2.

terpretable DNA sequences because of the propensity for strong intrastrand Watson-Crick base pairing during sequencing reactions and sequencing-gel electrophoresis (see "Technical considerations for ITS sequencing").

INDELS: LIABILITIES AND OPPORTUNITIES

Most variation between angiosperm ITS sequences is attributable to point mutations rather than length mutations. Nevertheless, in contrast to most coding sequences, alignment of ITS sequences can be complicated by insertion/deletion (indel) mutations, which require insertion of gaps to preserve positional homologies of nucleotides. In our studies, sequence alignment required insertion of gaps in at least one sequence at only a minor to moderate proportion of sites, except in a family-wide analysis of Polemoniaceae that tested the outer limits of phylogenetic utility of the ITS region (Table 2). Placement of gaps in most indel regions proved unambiguous because of flanking conserved sites. In general, interspersion of conserved and variable positions through both spacers promotes sequence alignment in the ITS region (Fig. 2).

In most groups at least one region in both ITS-1 and ITS-2 appears to have experienced relaxed evolutionary constraint on length. Unambiguous alignment of sequences in such segments may be impossible because of independent and overlapping length mutations that have occurred in different taxa. Such regions have required, however, elimination of only a small proportion of total nucleotide positions from our phylogenetic analyses. Bruns et al. (1992) provided helpful examples of how character states within indel regions can be recoded to maximize extraction of phylogenetic information without overweighting characters or using questionably aligned sequence data.

Although alignments may be problematical in some ITS segments, the occurrence of indels can be seen as an opportunity rather than a liability. For example, in *Calycadenia* all but one of several potentially informative indels mapped without homoplasy onto trees based only on nucleotide site-substitutions (Baldwin, 1993a). Two of these indels provided phylogenetic markers for otherwise weakly supported lineages. Other angiosperm ITS studies have shown a similar lack of homoplasy in indels (e.g., Baum et al., 1994; Donoghue & Baldwin, 1993, unpublished; Baldwin & Robichaux, 1995). Based on these findings, it may be desirable to take advantage of information provided by unambigu-

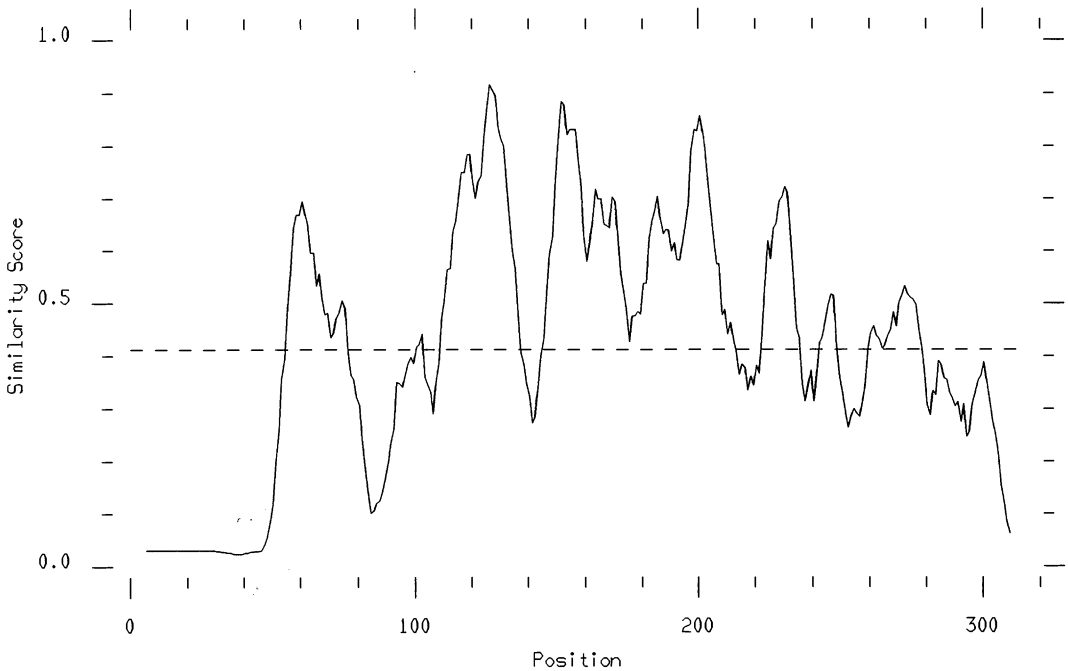


FIGURE 3. Continued.

ously placed indels rather than removing them entirely from phylogenetic analyses.

There are several ways of treating indel characters (see Swofford & Olsen, 1990; Bruns et al., 1992; Wojciechowski et al., 1993). For example, gaps introduced in indel regions can be treated as missing data or as a fifth character state. Treatment of gaps can be more problematical, however, in indels that span multiple nucleotide sites, even if gap placement is unambiguous (Wojciechowski et al., 1993). Overscoring of such indels is, of course, ensured if gaps are treated as a fifth character state at more than one nucleotide position. Alternatively, the indel may be recoded as an additional presence/absence character, with sequence gaps treated as missing data (Baum et al., 1994; Donoghue & Baldwin, 1993, unpublished). This last option, with or without recoding of the indel, requires that state-assignments to missing-data characters be examined following phylogenetic analysis to assess any effects on the resultant trees (assuming that potential phylogenetic information, aside from the indel, is present in the indel region). Although indels can introduce complications to phylogenetic analysis, the effects of different indel treatments (including exclusion) on resultant ITS tree topologies, where examined in angiosperms, have been minimal or absent (Wojciechowski et al., 1993; Baldwin, 1993a, unpublished; Baldwin & Robichaux, 1995; Baum et al., 1994; Donoghue & Baldwin, 1993, unpublished).

CONSIDERATION OF SECONDARY STRUCTURE

Alignments of ITS-1 sequences across five plant families show insufficient retention of sequence similarity to allow unambiguous alignment except near the highly conserved 18S end of the spacer, and, to a lesser extent, near the center of the spacer (Fig. 3A). Reconstructions of ITS-1 secondary structures across these families, using FOLD in GCG (Genetics Computer Group, Madison, Wisconsin), also exhibit a lack of similarity. In contrast to ITS-1, ITS-2 displays somewhat more sequence similarity across families in the central region of the spacer (Fig. 3B).

Reconstructed secondary structures of ITS-2 sequences in species of *Calycadenia* (Asteraceae), *Gilia* (Polemoniaceae), and *Viburnum* (Adoxaceae) (using FOLD in GCG) show great similarity, with a common three-stem/loop structure, like that of *C. oppositifolia* (E. Greene) E. Greene (Fig. 4). These conformational similarities may reflect stronger structural constraints on ITS-2 than on ITS-1 in these taxa (all in Asteridae sensu lato), which also show less sequence variation in ITS-2 than in ITS-1 (Table 2). It should be noted, however, that only minor differences in free energy exist between

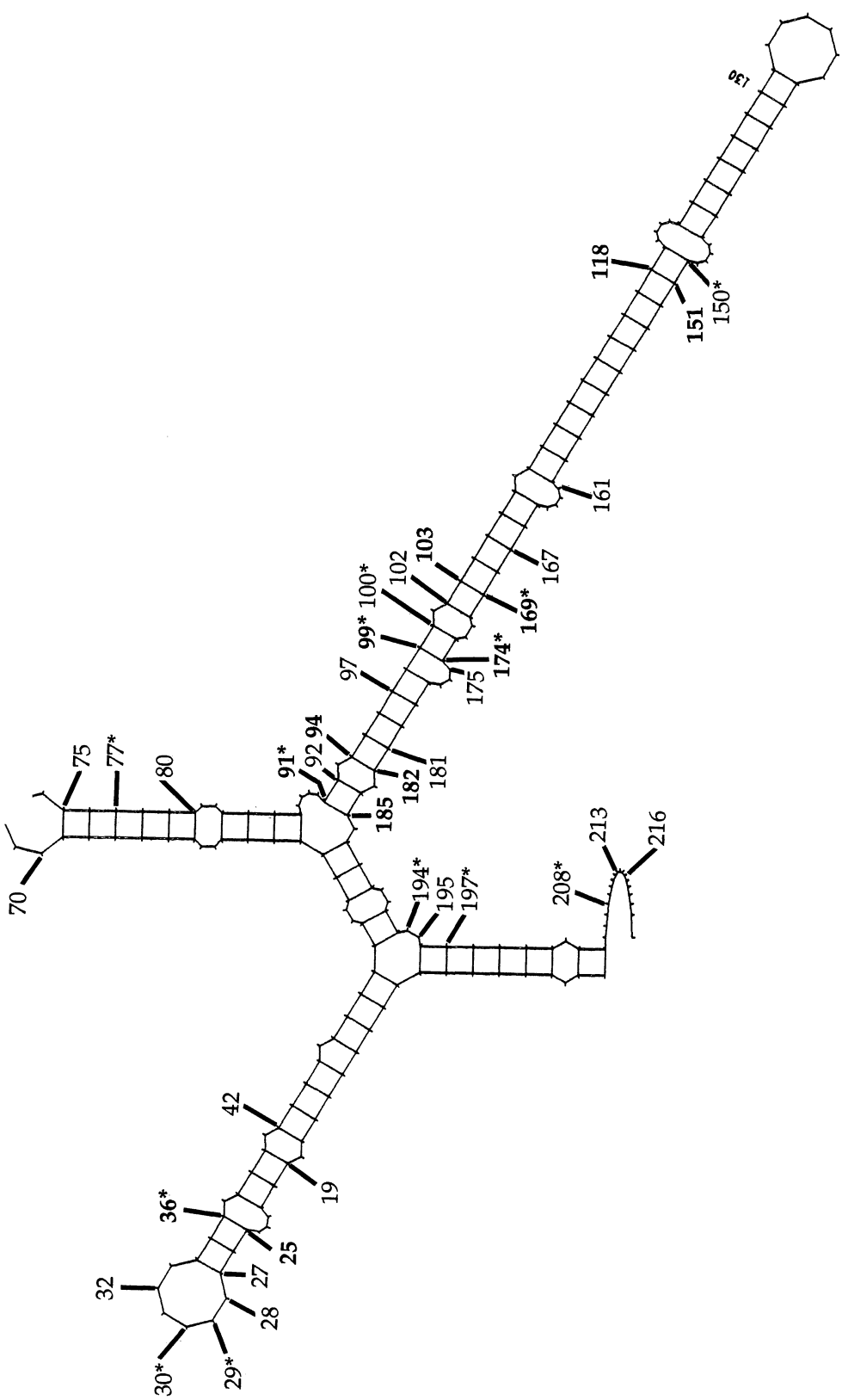


FIGURE 4. Hypothetical secondary structure of ITS-2 RNA of *Calycadenia oppositifolia* (Asteraceae) based on a minimum free-energy reconstruction using FOLD and SQUIGGLES in GCG (Genetics Computer Group, Madison, Wisconsin). Numbered sites marked by lines are positions where nucleotide variation was detected among species of *Calycadenia*. Asterisks indicate positions at which more than one evolutionary change possibly occurred in *Calycadenia*, based on MacClade 3.0 (Maddison & Maddison, 1992) character-evolution reconstructions on one of the 11 maximally parsimonious trees found by Baldwin (1993a). Bold numbers indicate variable sites in direct opposition. All changes at these directly opposing variable sites occurred along different branches of the phylogeny and therefore appear to be evolutionarily independent, based on this provisional secondary structure.

the most parsimonious structures reconstructed by FOLD and other conformations that can be radically different in shape. In addition, minimum free-energy ITS-2 conformations in some species of *Calycadenia*, *Gilia*, and *Viburnum* were quite different from that depicted in Figure 4. The ITS-2 reconstruction presented here (Fig. 4) should, therefore, be interpreted as a highly provisional estimate. Perhaps as algorithms for reconstructing secondary structures become more sophisticated it will be possible to use such conformational features as additional characters in phylogenetic analyses, much as complex morphological characters are used presently.

Potential non-independence of characters complicates phylogenetic analysis of DNA sequences, such as those of the ITS region, that produce transcripts with seemingly functional secondary structures. Mutations at ITS positions involved in stem formation (via intrastrand nrRNA pairing) may necessitate compensatory mutations at directly opposing sites to maintain structural integrity and proper functioning of the molecule. Differing estimates of the prevalence of such linked changes in nrDNA have led to various character-weighting recommendations, ranging from 50% weighting of all stem characters relative to loop sites in 5S and 5.8S rDNA (Wheeler & Honeycutt, 1988) to ≥80% weighting of stem sites compared to loop sites in large subunit (26S) rDNA (Dixon & Hillis, 1993). Given that Dixon & Hillis (1993) found <40% as many compensatory changes in 26S rDNA as expected under a model of 100% compensation in stem regions, the extent of such non-independence in ITS sequences needs to be determined empirically.

A preliminary search for compensatory changes in ITS-2 sequences of *Calycadenia* failed to reveal any such mutations. The evolutionary history of ITS-2 sequences in *Calycadenia* was first inferred from examining all possible character changes along one of the 11 maximally parsimonious ITS trees (Baldwin, 1993a) that appeared most compatible with cytology and morphology. These mutations, reconstructed using MacClade 3.0 (Maddison & Maddison, 1992), were mapped onto minimum free-energy ITS-2 structures of most *Calycadenia* species, one of which is shown in Figure 4. This approach revealed two to six pairs of changes that occurred at directly opposing positions along stems. Opposing mutations of a pair, however, represented independent events that occurred in different lineages, i.e., the changes were apparently unlinked evolutionarily. Simultaneous changes in a single lineage (along the same branch of the tree) did occur within two to three stem regions where clusters of changes were found. None of these mutations were directly opposing changes, but their close proximity may signal cryptic non-independence. Slight readjustments of intrastrand RNA pairing through slippage could obscure evidence of compensation in such instances. This possibility needs to be explored by considering secondary structures of ancestral sequences that existed at the time of each change, i.e., structures inferred for each node in the tree. In addition, improved reconstructions of ITS secondary structure may reveal evidence of more compensatory change. Alternatively, selection for compensatory mutations in this spacer may be weaker than in the nrDNA subunits. If so, concerns about non-independence of ITS mutations would be alleviated.

Limits of Phylogenetic Utility

Taxonomic levels of utility

Available data indicate that ITS sequences are phylogenetically useful at various intrafamilial levels in angiosperms, depending on the lineage, but are unlikely to retain sufficient evolutionary signal or alignability for examining relationships among species in different plant families (but see Hershkovitz & Zimmer, 1994). Of course, this generalization holds only to the extent that family rank signifies an ancient origin, which is certainly not true in all cases. In addition, low levels of ITS variation in some ancient plant groups (see below) raise the possibility that the ITS region may prove useful for assessing relationships between old families that have experienced exceptionally low rates of spacer evolution. The arbitrariness of taxonomic ranks is a major impediment to any general statements about taxonomic limits on the phylogenetic utility of ITS sequences.

Divergence between ITS sequences has apparently been sufficient to render family-wide phylogenetic studies untenable in Asteraceae (Baldwin, 1991, 1992; Kim & Jansen, 1994), Fabaceae (Sanderson & Wojciechowski, unpublished), Rosaceae (Campbell, Donoghue, Baldwin & Wojciechowski, unpublished), Saxifragaceae (D. Soltis & Kuzoff, in press), Viscaceae (D. Nickrent, pers. comm.), and, to a lesser extent, Polemoniaceae (Porter, 1993a, b). In these groups, ITS sequences have proven most valuable for examining relationships within genera and among the more closely related genera. ITS sequences do, however, appear suitably conserved for family-wide phylogenetic studies in Fouquieriaceae (Schultheis & Baldwin,

1994), Nothofagaceae (Manos, 1993), and Winteraceae (Suh et al., 1993).

Within species, ITS sequences show some promise for investigations of relationships among allopatric or disjunct populations. For example, up to 3.7% ITS sequence divergence was found between individuals from conspecific, allopatric populations in *Calycadenia* (Baldwin, 1993a). Some of this variation was potentially informative for resolving relationships among subspecies.

In view of the relatively large number of DNA sequences identified for higher-level, as opposed to intrageneric or other lower-level, phylogenetic analyses in flowering plants, we feel that ITS sequences present special opportunities for evolutionary studies. The small number of nucleotide positions available for analysis in both spacers is, in several groups, compensated for by the high levels of variation found in ITS-1 and ITS-2. In fact, in several groups ITS sequences are much more variable than cpDNA from the same total DNA accessions, as estimated from the plastome as a whole based on cpDNA restriction site data [e.g., Madiinae (Baldwin, 1989, 1992), *Astragalus* (Sanderson & Doyle, 1993; Wojciechowski et al., 1993), Rudbeckiinae (Urbatsch & Jansen, 1992; Urbatsch & Baldwin, 1993, unpublished), and *Viburnum* (Donoghue & Sytsma, 1993; Donoghue & Baldwin, 1993, unpublished)].

LIFE HISTORY AND EVOLUTIONARY RATES

ITS sequences appear to have evolved more slowly in some ancient woody groups than in herbaceous, primarily annual groups of comparatively recent origin. Similar levels of ITS sequence divergence were found between taxa in lineages that diverged in the early Tertiary or Cretaceous, e.g., sections of *Viburnum* (Donoghue & Baldwin, 1993, unpublished), genera of Winteraceae (Suh et al., 1993), or clades within *Nothofagus* (Manos, 1993), and between taxa in herbaceous lineages that presumably diverged in the Pliocene/Pleistocene (e.g., genera of Madiinae; Baldwin, 1992).

Such correlations between plant life-form and apparent rates of molecular evolution have been noted from cpDNA data as well (e.g., Schilling & Jansen, 1989; Wilson et al., 1990; Gaut et al., 1992, 1993; Brunsfeld et al., 1994), although the basis for this pattern is unclear. A generation-time explanation for differences in molecular evolutionary rates (with longer generation times resulting in slower molecular evolution), although roughly consistent with observed patterns and perhaps compelling for some animals, is problematical in plants, wherein open development might allow fixation of mutations in vegetative meristems as readily as in reproductive cells that arise from such meristems (Klekowski, 1988). Generational turnover is also a more complex issue in plants than in animals because of the potentially stabilizing effect of seed banks and clonal reproduction on evolutionary rates in herbs. This is an area that needs more theoretical and empirical attention by plant molecular evolutionists.

IMPORTANCE OF PHYLOGENETIC RESULTS FROM THE ITS REGION

Independent lines of phylogenetic evidence can be of great value for identifying and resolving conflicting hypotheses of relationship and for providing enhanced resolution within lineages. The ITS region has proven repeatedly to be of such benefit in angiosperm studies. Below, we provide examples that demonstrate how ITS characters have aided understanding of plant phylogeny by providing (1) corroboration of unexpected findings, (2) resolution of conflict between data sets, (3) improved resolution of species relationships, or (4) direct resolution of reticulate evolution. These four categories are, to some extent, overlapping and some of the results discussed under one heading are actually exemplary of two or more of these types of findings.

CORROBORATION OF UNEXPECTED FINDINGS

A cpDNA restriction site study of the Madiinae (Compositae) indicated a previously unpredicted relationship between the Hawaiian silversword alliance and a subgroup of California tarweeds (Baldwin, 1989, 1992; Baldwin et al., 1991) (Fig. 5A). The cpDNA trees strongly indicate that the Hawaiian lineage (*Argyroxiphium, Dubautia,* and *Wilkesia*) is nested within Californian *Madia/Raillardiopsis.* An ITS phylogeny of these same species provides robust corroboration of this finding (Baldwin, 1992) (Fig. 5B), thereby reinforcing and extending Carlquist's (1959) hypothesis that the Hawaiian silversword alliance originated from American tarweeds.

An ITS phylogeny of subfamily Maloideae (Rosaceae) places *Vauquelinia,* a capsular-fruited species usually referred to tribe Quillajeae of Spirioideae, within the pome-fruited Maloideae (Fig. 6) (Campbell et al., in press). This result supports the floral morphological findings of Sterling (1966) and the conclusion of Goldblatt (1976) based on chromosomal and morphological evidence. cpDNA *rbc*L sequences place *Vauquelinia* and other Quillajeae

at the base of Maloideae (Morgan et al., 1994), in near agreement with the ITS results.

An ITS phylogeny of Polemoniaceae (Fig. 7) (Porter, 1993a, b) provides several examples of unexpected relationships that corroborate recent phylogenetic findings based on cpDNA *mat*K sequences (Steele & Vilgalys, 1994; Johnson & Soltis, 1995). For example, in both the ITS and cpDNA trees, the spiny-leaved genus *Navarettia* is placed within a clade that includes species of *Allophyllum*, *Collomia*, and *Gilia*, and outside a lineage that includes other spiny-bracted genera, such as *Eriastrum*, *Langloisia*, and *Loeseliastrum*, with which *Navarettia* has been traditionally associated. In addition, *Phlox* appears closely related to *Leptodactylon* and *Linanthus* in both the ITS and cpDNA trees, a result previously unpredicted but supported by the unusual occurrence of opposite leaves in these genera, a potential morphological synapomorphy.

An ITS phylogeny of Galegeae (Fabaceae) (Sanderson & Wojciechowski, unpublished; Sanderson & Liston, in press) corroborates cpDNA evidence (Sanderson & Liston, in press) that this tribe and *Astragalus* are paraphyletic. Another unanticipated finding from both lines of evidence is the existence of a strongly supported core clade of genera that comprises all of subtribe Coluteinae and the astragaloid part of subtribe Astragalinae. ITS sequences of members of this core clade show high similarity, but are highly divergent from those of other members of Galegaeae.

RESOLUTION OF CONFLICT BETWEEN DATA SETS

A striking example of conflict resolution by ITS data involves *Viburnum* (Adoxaceae). A morphological phylogeny of *Viburnum* (Fig. 8A) suggested that the purple-fruited species of section *Oreinotinus* from the New World and some members of section *Odontotinus* are the sister group of a predominantly Asian, red-fruited clade of section *Opulus* and the other members of section *Odontotinus* (Donoghue, 1983). A cpDNA phylogeny (Fig. 8B), however, indicated that one of the purple-fruited species, *V. acerifolium* L., is more closely related to certain Asian, red-fruited species than it is to other New World taxa (Donoghue & Sytsma, 1993, unpublished). Initially, these results raised concern that the cpDNA trees deviated from the species tree because of introgression or cpDNA lineage sorting. An ITS phylogeny (Fig. 8C), however, corroborates the cpDNA placement of *V. acerifolium* with the Asian, red-fruited group (Donoghue & Baldwin, 1993, unpublished). In this case, reexamination of morphology indicates that several characters that were not included in the previous analysis, including subtle differences in endocarp shape (Donoghue & Strauss, 1988), support the molecular results. Congruence among ITS, cpDNA, and endocarp data strongly indicates that the evolution of fruit color has been more complex than implied by the original morphological analysis.

In the Hawaiian silversword alliance, cytogenetic (Carr & Kyhos, 1986, pers. comm.) and cpDNA data (Baldwin, 1989; Baldwin et al., 1990) conflict about relationships of several species of *Dubautia* from Kaua'i that possess either the D1 or D3 chromosomal arrangements. The cpDNA trees strongly support species groupings that indicate multiple origins of one or both of these chromosomal arrangements (Fig. 9A). The ITS data, however, robustly support monophyly of the D1 species and paraphyly of the D3 species, relationships compatible with single origins of both genomic arrangements (Fig. 9B) (Baldwin, unpublished). Further, ITS data, unlike the cpDNA results, are highly congruent with an ordered evolutionary series of three chromosomal arrangements (including the two in question), with adjacent members of this series separated by single reciprocal translocations (see Fig. 9). These data, together with other evidence, indicate that the cpDNA results are, in part, incongruent with the nuclear DNA component of species relationships, probably as a result of introgressive hybridization (Baldwin, unpublished).

In the *Heuchera* group (Saxifragaceae), cpDNA trees strongly suggest that northern and southern populations of *Tellima grandiflora* (Pursh.) Dougl. are not closely related, a result attributed to introgressive hybridization between a species of *Mitella* and *T. grandiflora* that led to chloroplast capture of *Mitella* cpDNA by *Tellima* (Soltis et al., 1991a, b). ITS trees of the *Heuchera* group strengthen this introgression hypothesis by strongly corroborating morphological and isozymic evidence that the northern and southern populations of *T. grandiflora* are conspecific (Soltis & Kuzoff, in press).

IMPROVED RESOLUTION OF SPECIES RELATIONSHIPS

An ITS phylogeny of *Astragalus* (Fig. 10) (Wojciechowski et al., 1993) provides strong support for a lineage previously unpredicted from morphological evidence but congruent with biogeographic and cytological data. All of the aneuploid species of the New World sampled, with chromosome numbers ranging from $n = 11$ to $n = 15$, form a strongly supported monophyletic group in

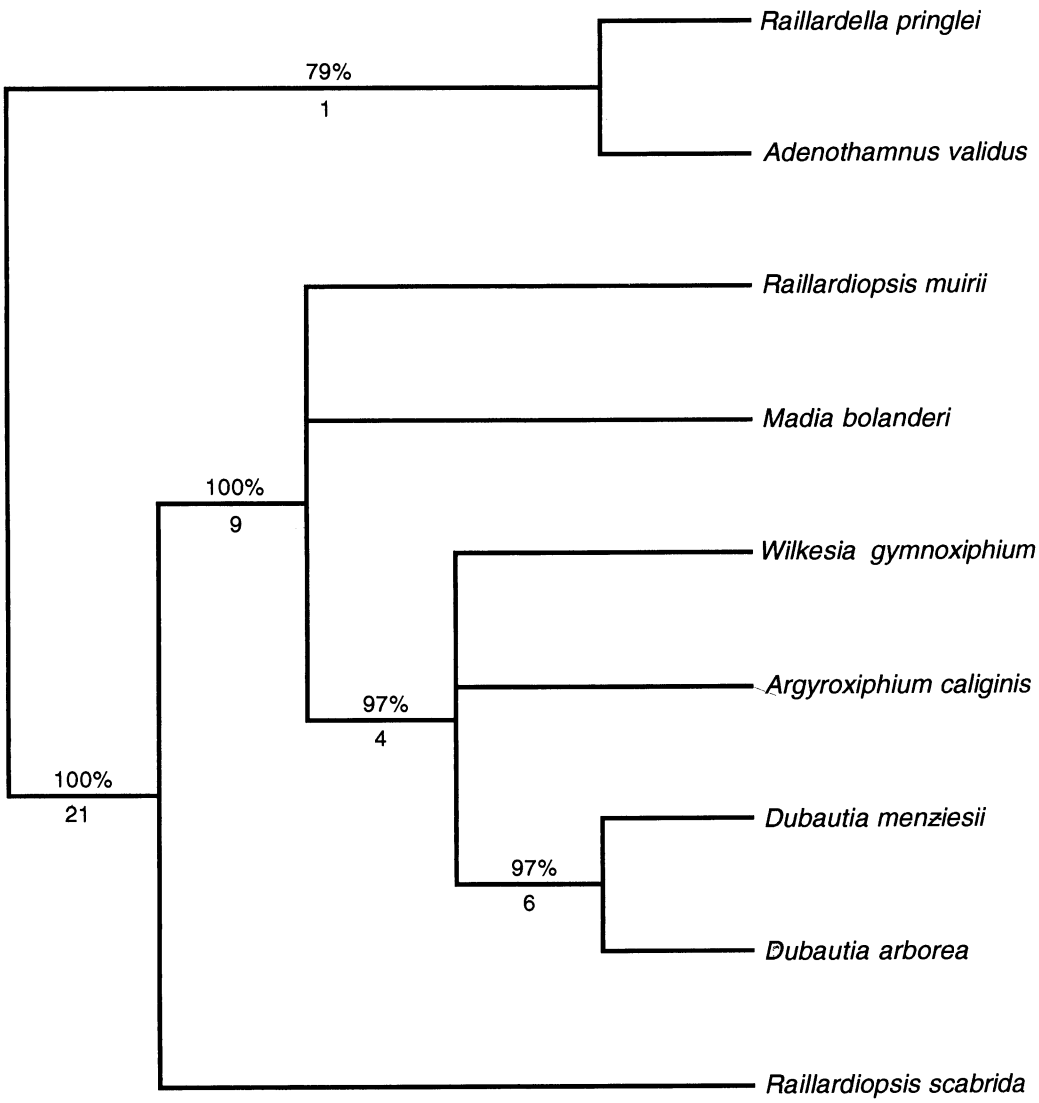


FIGURE 5 (pp. 264–265). Strict consenses of maximally parsimonious DNA trees of Californian and Hawaiian Madiinae (Asteraceae).—5A (this page). cpDNA restriction site tree (reprinted with permission from Baldwin, 1992).—5B (facing page). ITS sequence tree (reprinted with permission from Baldwin, 1992). Bootstrap percentages are presented above the branches. Decay index values appear below the branches. Asterisks denote outgroup species outside Madiinae. Monophyly of the large Hawaiian silversword alliance (*Argyroxiphium, Dubautia,* and *Wilkesia*) is strongly supported by more inclusive parsimony analyses of cpDNA (Baldwin, 1989; Baldwin et al., 1990, 1991) and ITS (Baldwin & Robichaux, 1995) data. Note that the Hawaiian species are nested within *Madia/Raillardiopsis* in trees A and B.

the ITS trees despite the lack of a single known morphological character that could thus unite these taxa. Monophyly of this aneuploid species assemblage is congruent with cpDNA restriction site data from a less extensive species sampling (Sanderson & Doyle, 1993). This large, morphologically cryptic lineage is of sufficient biogeographic and cytogenetic importance to warrant taxonomic recognition (Wojciechowski et al., 1993).

In the Hawaiian silversword alliance, cpDNA restriction site studies failed to discern a single restriction site mutation that could potentially unite the species of *Argyroxiphium,* the true silverswords and greenswords, as a monophyletic group (Baldwin, 1989; Baldwin et al., 1990). In sharp contrast, one of the two longest branches in the ITS tree supports monophyly of *Argyroxiphium* (Baldwin & Robichaux, 1995; Baldwin, unpub-

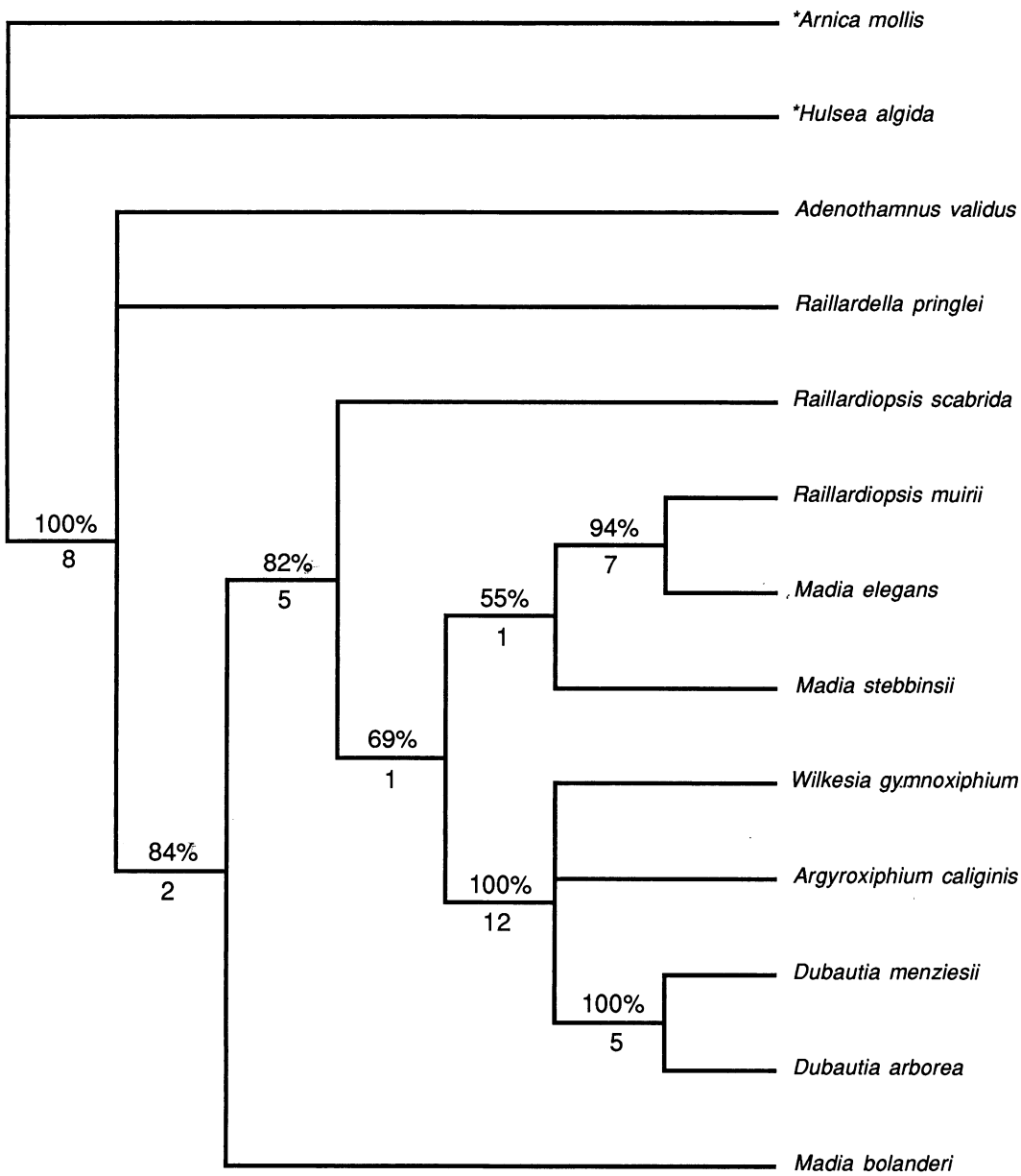


FIGURE 5. Continued.

lished). This provides a striking example of how inequity in relative rates of ITS as opposed to cpDNA evolution can affect phylogenetic resolution.

DIRECT EVIDENCE OF RETICULATE EVOLUTION

Unlike cpDNA, nrDNA data can provide direct evidence of reticulate evolution if concerted evolution fails to act across repeat units contributed by different parental species. Such lack of sequence homogenization may occur if, for example, (1) the hybridization event was recent, (2) nrDNA repeats are at different loci in the parental taxa (e.g., on different chromosomes) and interlocus gene conversion is inoperative in their hybrid, or (3) the hybrid is asexual. Parentage of suspected early generation hybrids may be resolved simply by screening for presence or absence of restriction sites diagnostic for ITS sequences of each of the

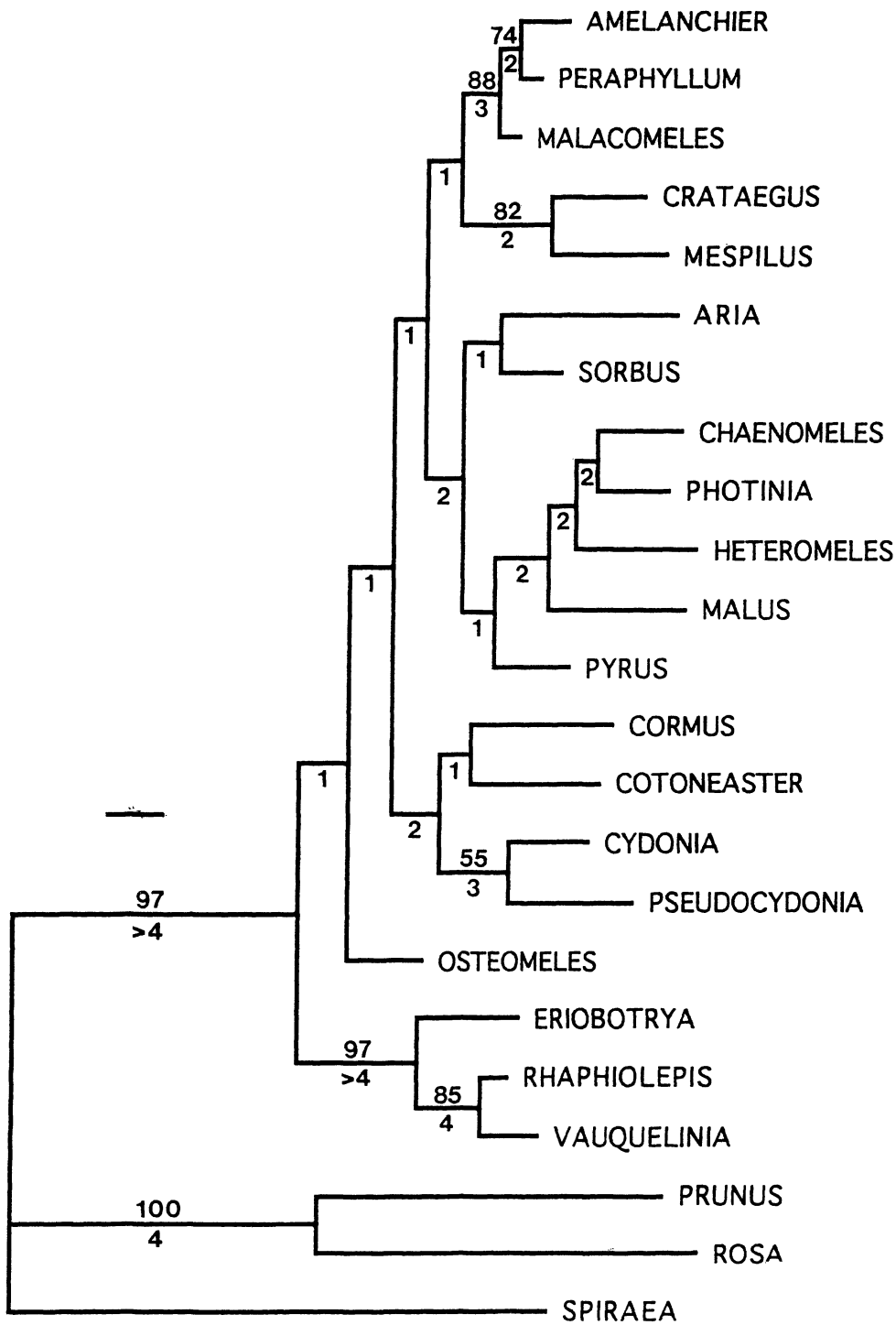
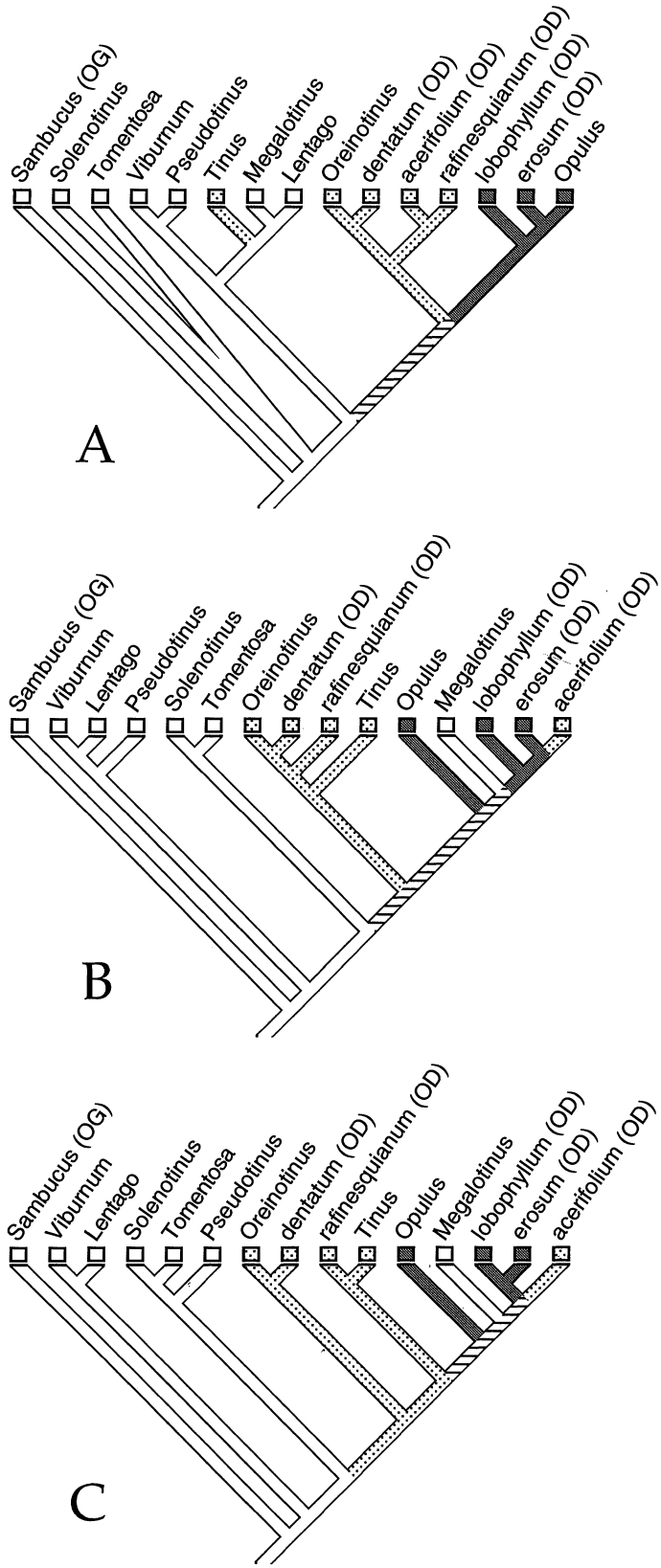


FIGURE 6. Phylogram of the single most-parsimonious tree based on ITS and partial 5.8S rDNA sequence variation in Maloideae (Rosaceae) (Campbell et al., 1993a; in press). The tree is rooted between *Prunus*, *Rosa*, and *Spiraea* and the 19 Maloideae genera plus *Vacquelinia*. Branch lengths are based on accelerated transformation (ACCTRAN) of characters. The separate bar indicates the branch length equivalent to 10 substitutions. Numbers above branches indicate bootstrap percentages for clades found in both this tree and the bootstrap majority rule consensus tree. Numbers below branches are decay index values. Note the well-supported position of *Vauquelinia* (Quillajeae) within Maloideae.

FIGURE 7. Strict consensus of maximally parsimonious trees based on ITS sequences of Polemoniaceae (Porter, 1993a, b). *Fouquieria* (not shown) is the outgroup. Branch lengths were calculated using the minimum-average-maximum reconstruction procedure of MacClade 3.0 (Maddison & Maddison, 1992), with the internal nodes placed at the maximum value. Bootstrap percentages are given below the branches. Decay index values appear above the branches. Nodes for which phylogenetic estimates are considered unreliable, based on jackknifing of taxa, and g_1 values of tree length distributions are identified with an asterisk. Tribal placement (Grant, 1959) of each species is noted as follows: open square = *Gilia* tribe, closed square = *Polemonium* tribe, open circle = *Cobaea* tribe, closed circle = *Bonplandia* tribe, closed triangle = *Cantua* tribe. The close relationships of (1) *Navarretia* to *Allophyllum*, *Collomia*, and part of *Gilia* and (2) *Leptodactylon*, *Linanthus*, and *Phlox* were unanticipated given the current tribal classification, but are supported by new cpDNA evidence (Steele & Vilgalys, 1994; Johnson & Soltis, 1995).


Sambucus (OG)
Solenotinus
Tomentosa
Viburnum
Pseudotinus
Tinus
Megalotinus
Lentago
Oreinotinus
dentatum (OD)
acerifolium (OD)
rafinesquianum (OD)
lobophyllum (OD)
erosum (OD)
Opulus
A
Sambucus (OG)
Viburnum
Lentago
Pseudotinus
Solenotinus
Tomentosa
Oreinotinus
dentatum (OD)
rafinesquianum (OD)
Tinus
Opulus
Megalotinus
lobophyllum (OD)
erosum (OD)
acerifolium (OD)
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Sambucus (OG)
Viburnum
Lentago
Solenotinus
Tomentosa
Pseudotinus
Oreinotinus
dentatum (OD)
rafinesquianum (OD)
Tinus
Opulus
Megalotinus
lobophyllum (OD)
erosum (OD)
acerifolium (OD)
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presumed parental species. In such cases, additivity for the parental restriction patterns can provide excellent evidence of hybridity. Resolution of ancient hybridization, however, requires more detailed characterization of ITS variation, e.g., by sequencing of ITS clones.

A striking example of maintenance of parental ITS sequences in a presumed hybrid has been found in *Amelanchier* (Rosaceae) (Campbell et al., 1993b). Direct ITS sequences of *A.* × *neglecta* (Eggelst.) Eggelst. show additivity of nucleotide states at each site where the presumed parental species, *A. bartramiana* (Tausch) Roemer and *A. laevis* Wieg. (Weber & Campbell, 1989), differ. These data support the hypothesis that *A. bartramiana* and *A. laevis* are the parental species of *A.* × *neglecta*, although sequences from clones of the divergent repeat types in this apomict are being obtained to confirm this reconstruction.

Kim & Jansen (1994) detected a similar phenomenon by direct ITS sequencing of pooled PCR products from hexaploid *Krigia montana* (Michaux) Nutt. (Compositae). Previous cpDNA and nrDNA restriction site data (Kim & Jansen, 1992; Kim & Mabry, 1991) indicated that diploid *K. biflora* Blake and tetraploid *K. montana* were the maternal and paternal parents, respectively, of allohexaploid *K. montana*. Direct ITS sequences of these taxa revealed additivity of the nucleotide states that differentiate *K. biflora* and tetraploid *K. montana* in hexaploid *K. montana*. As in the *Amelanchier* study, cloning of ITS repeat-types will be necessary to determine exact sequences of these repeat types and to detect any possible recombinants.

Fixation of maternal and paternal repeat types in different populations of a hybrid species can also allow direct identification of both ancestral parental lineages, if sufficient intraspecific sampling is undertaken, as in allopolyploids of *Gossypium* (Wendel, 1995). Evidence of such bidirectional ITS repeat-type fixation in *Gossypium* requires a note of caution for plant phylogeneticists, however. Misleading rDNA trees can result if lineages arise from populations of hybrid species that are fixed for nrDNA repeat-types of different parental origin. Species derived from such differentially fixed hybrid populations will not form a monophyletic group in the nrDNA trees but will group separately with the non-hybrid parent lineage that bears the orthologous nrDNA repeat-type. If, however, populations persist with individuals that maintain both parental ITS repeat-types, then inclusion of these sequences in the phylogenetic analysis (treated as separate OTUs) can resolve unambiguously the hybrid origin and biparentage of the hybrid lineage. It is important to note that with or without comprehensive sampling, reticulation events will always lead to evolutionary patterns that can be potentially misinterpreted in a dichotomous tree framework, regardless of the set of characters chosen for phylogenetic analysis.

Chimeric ITS repeat-types that combine distinctive motifs of ITS sequences from different lineages have been documented in species of *Gossypium* (Wendel, in press) and *Amelanchier* (Campbell, Wojciechowski, Baldwin & Donoghue, unpublished). These mosaic ITS sequences appear to have arisen by partial gene conversion or recombination between divergent paralogues brought together by hybridization. Formation of such "hybrid" sequences poses problems similar to those that can be expected in phylogenetic analysis of nuclear genes in general. Enhanced sampling of ITS repeat-types within individuals and across species and hybrids has improved resolution of the origin of chimeric ITS sequences in *Amelanchier* (Campbell, Wojciechowski, Baldwin & Donoghue, unpublished).

Conclusions

Available data from several angiosperm groups demonstrate that the ITS region can provide a valuable set of characters for addressing lower-

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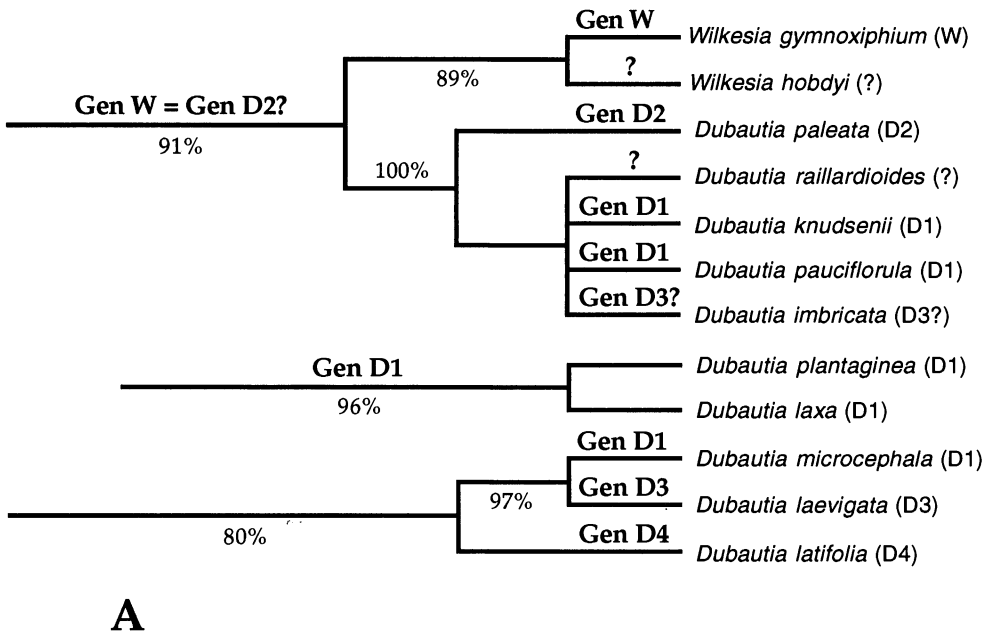
Figure 8. Phylogenies of *Viburnum* (Adoxaceae) based on different data sets, reduced to a common set of taxa to illustrate areas of incongruence and implications for the evolution of fruit color (drawn using MacClade; Maddison & Maddison, 1992).—A. Morphological tree (based on Donoghue, 1983).—B. cpDNA restriction site tree (based on Donoghue & Sytsma, 1993, unpublished).—C. ITS sequence tree (based on Donoghue & Baldwin, 1993, unpublished). In each tree, capitalized terminal taxa are sections of *Viburnum* (except *Sambucus*). The remaining five taxa (all lower case) are representative species of the large section *Odontotinus* (OD), which appears not to be monophyletic. Open boxes/branches represent taxa or lineages with fruits that undergo a prolonged red phase before turning purple at maturity; lightly stippled boxes/branches symbolize taxa or lineages with fruits in which the red phase is greatly reduced or missing, and the shift to purple color occurs rapidly and synchronously; dark boxes/branches represent taxa or lineages with fruits that are red at maturity; hatched lines indicate that there are equally parsimonious state assignments (Maddison & Maddison, 1992). Note that cpDNA and ITS data both imply that *V. acerifolium* is not directly related to other New World species with purple fruits, as suggested by morphology. Instead, it appears to be more closely related to Asian species with red fruits.



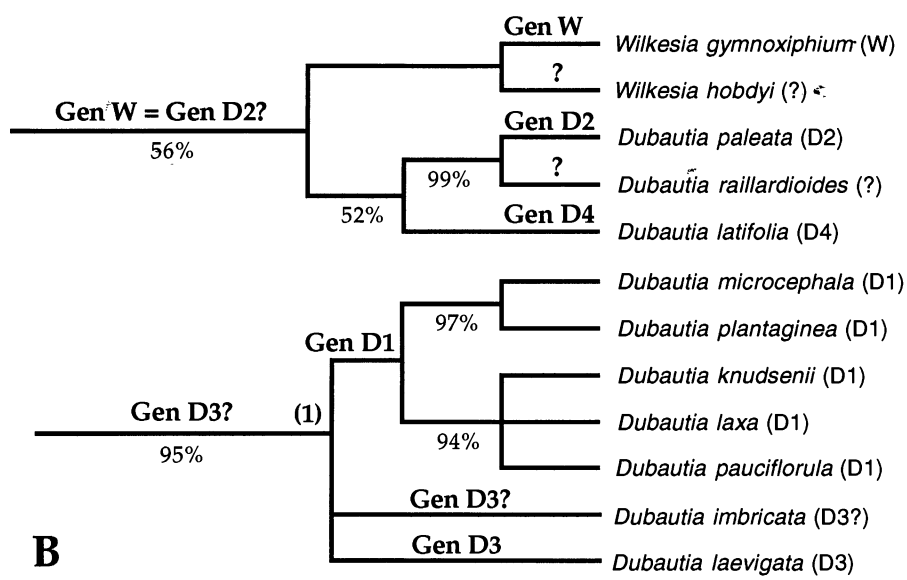


FIGURE 9. All Kaua'i lineages of the Hawaiian silversword alliance (Asteraceae: Madiinae) excised from phylogenies based on DNA evidence. Nuclear chromosomal arrangements (Carr & Kyhos, 1986, pers. comm.) are superimposed on the branches according to parsimony (if ambiguous, the earliest unambiguous possession of an arrangement is shown).—A. cpDNA restriction site tree (Baldwin, 1989; Baldwin et al., 1990).—B. ITS sequence tree (Baldwin & Robichaux, 1995; Baldwin, unpublished). Percentages below branches are bootstrap values. Genomic abbreviations (D1, D2, D3, D4, W) follow Carr & Kyhos (1986) with correction (Carr & Kyhos, pers. comm.). None of these chromosomal arrangements except D4 is known from other species of the silversword alliance. All species shown have the same chromosome number (n = 14; Carr, 1985; Carr & Kyhos, 1986). Question marks indicate an uncertain genomic arrangement. Available data are consistent with identity of genomes D2 and W, but confirmation from additional hybrids is needed (Carr & Kyhos, 1986, pers. comm.). Provisional assignment of D3 to *Dubautia imbricata* is based on high pollen stainability (99%) of a putative natural hybrid between this species and *D. laevigata* (D3) (Carr, 1985). If *D. imbricata* does possess the D3 chromosomal arrangement, then reconstruction of D3 as the

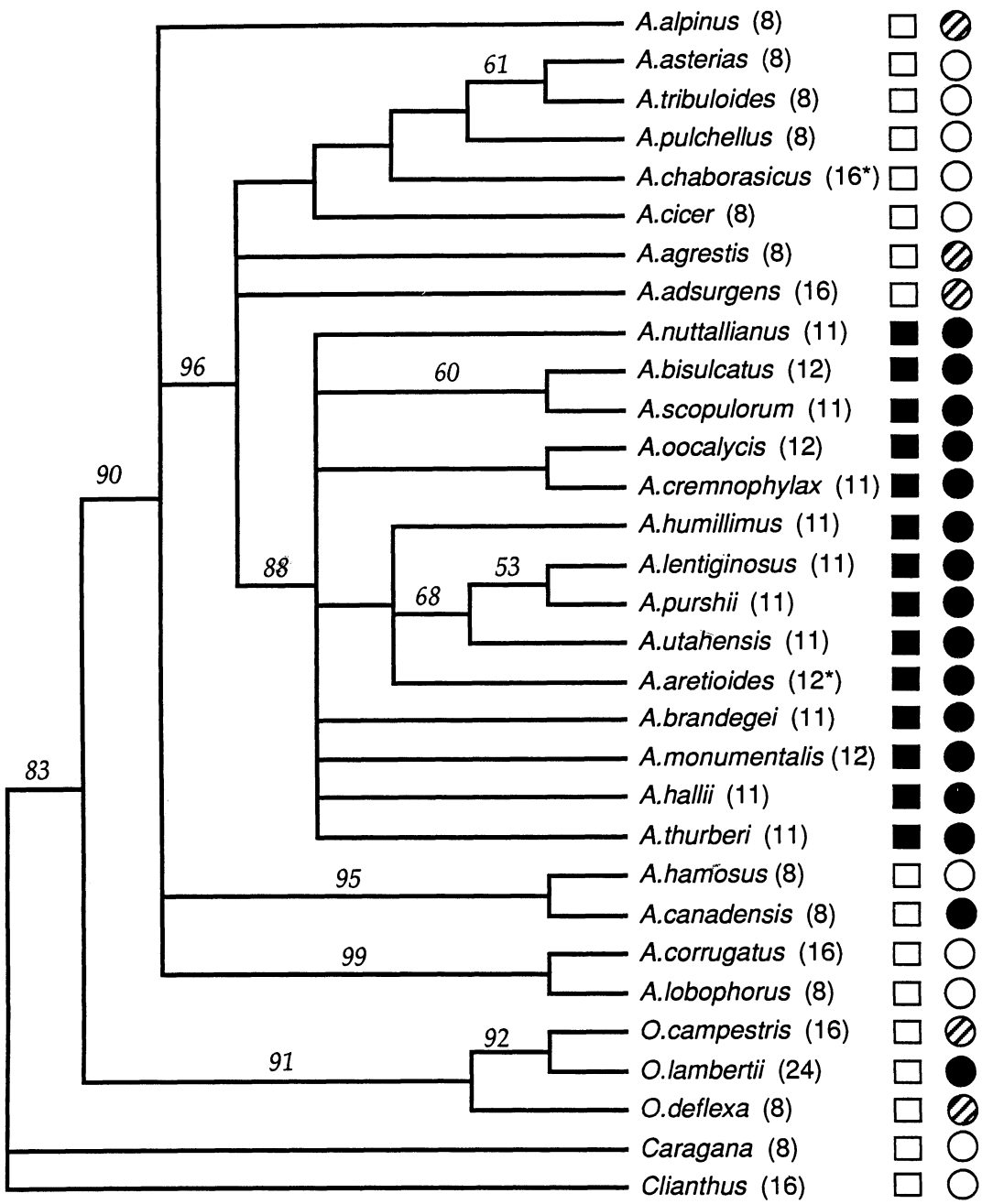


FIGURE 10. Strict consensus of the maximally parsimonious trees based on ITS sequences from 26 species of *Astragalus* and five outgroup taxa in *Caragana*, *Clianthus*, and *Oxytropis* (reprinted with permission from Wojciechowski et al., 1993). Bootstrap values are shown above the branches. Haploid chromosome numbers follow the taxon name. Asterisks indicate that the chromosome number of a species is unknown and that the number from other species in that section is given instead. Open squares indicate euploid species; black squares represent aneuploid species. Open circles indicate Old World taxa; hatched circles represent circumboreal species; black circles designate New World species. Note well-supported monophyly of the New World aneuploids, a morphologically cryptic clade that is corroborated by cpDNA results (Sanderson & Doyle, 1993).

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ancestral condition in the lower ITS lineage would be unambiguous. D3 differs from D1 and D5 by different single reciprocal translocations and bridges the two latter arrangements. D5 is the basal condition in the sister group (not shown) to the lower of the two ITS lineages. Note that the ITS relationships are consistent with single origins of D1 and D3, unlike the cpDNA resolution.

level phylogenetic questions in flowering plants. The lack of identified nuclear DNA regions that possess such favorable properties for phylogenetic studies of closely related plant species makes the ITS region a particularly important new resource for angiosperm systematists. ITS data should play an especially useful role in angiosperm studies by offering independent assessment of lower-level phylogenetic hypotheses based on morphology or cpDNA evidence.

One of the most attractive aspects of using the ITS region for plant molecular phylogenetic studies is the ease with which sequences of both spacers can be obtained and interpreted. The small size, highly conserved flanks, high copy number, rapid concerted evolution, and length conservation of angiosperm ITS sequences greatly aid their PCR amplification, sequencing, alignment, and phylogenetic analysis (see Baldwin, 1992). A minor sequencing effort can yield complete sequences of ITS-1 and ITS-2 from many taxa. In turn, these sequences may provide sufficient potentially informative variation for robust resolution of phylogenetic relationships, as now shown in several groups of closely related angiosperms (see above). Predominance of site-substitutions over indel mutations and interspersion of conserved and variable sites promote ITS sequence alignment and use of ITS indels as characters in phylogenetic analysis. Rapid homogenization of nrDNA repeats by concerted evolution can allow reconstruction of species relationships from direct sequences of the ITS region, whereas limited persistence of divergent ITS paralogues can provide direct evidence of hybridization, independent estimates of phylogeny, or a novel criterion for tree rooting.

The primary limitation of ITS data for phylogenetic studies in angiosperms is the small number of characters from these short spacers. Four-taxon simulations by Huelsenbeck & Hillis (1993) suggest that sequences of such short length are, under most conditions and types of analysis, less effective for accurate tree reconstruction than longer sequences. Useful variation must be more highly concentrated within a set of ITS-1 and ITS-2 sequences than in longer DNA regions in order to achieve the same level of phylogenetic resolution and support. Further constraints on the number of useful ITS characters can be imposed by the need to delete small indel regions from phylogenetic analysis because of uncertain sequence alignment. These limitations may require that data from other sources (e.g., morphology) be combined with ITS evidence to obtain enough characters for well-supported phylogenetic resolution (see Kluge, 1989; Barrett et al., 1991; Donoghue & Sanderson, 1992).

Where incongruence statistics have been applied (Campbell et al., in press; Kim & Jansen, 1994), ITS data have appeared substantially congruent with other independent data sets. Some relationships suggested by ITS trees have been seen as questionable based on apparent conflict with multiple lines of congruent evidence (e.g., Kim & Jansen, 1994; Soltis & Kuzoff, in press). In most of these cases, mere homoplasy in the ITS data sets may be responsible for the conflicts. As discussed above, differential fixation or partial conversion of non-homologous repeat-types in hybrids, or in a set of species derived from a polymorphic ancestor, could lead to a well-supported ITS phylogeny that does not accurately reflect species relationships, as in *Gossypium* (Wendel, 1995; in press). Hybridization or lineage sorting can, however, lead to deviation between *any* tree derived from a single gene or organellar genome and a species tree and are therefore not a special liability of ITS data. A confident assessment of organismal phylogeny can come only from examination of multiple lines of evidence, both in parallel and in combination.

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