TAXONOMY AND PHYLOGENY OF A GULF COAST DISJUNCT GROUP OF *SPIGELIA* (LOGANIACEAE SENSU LATO)

Katherine R. Gould[1] and Robert K. Jansen

Section of Integrative Biology and Institute of Cellular and Molecular Biology,
University of Texas at Austin, Austin, Texas 78712
[1]Present address: Brooklyn Botanic Garden, 1000 Washington Ave.,
Brooklyn, New York 11225

Abstract. We reconstructed phylogenies of populations of three closely related species of *Spigelia* (Loganiaceae sensu lato) to test hypotheses about relationships and taxonomic boundaries. The species studied occur in the southeastern U.S. with a disjunction in their distribution along the Gulf Coastal plain between Florida and Texas. Two sets of data, restriction sites of cpDNA and ITS sequences, were analyzed. We found three well-supported clades of cpDNA types that correspond to previous species delimitations, and the cpDNA clades representing the two Texas species were most closely related. This pattern of relationships was unexpected based on strong morphological similarities between the coastal plain Texas taxon, *S. texana*, and the Florida taxon, *S. loganioides*. Phylogenetic hypotheses based on ITS sequences were not strongly supported because there were few informative changes among the sequences, and therefore a combined analysis recovered similar topologies as with the cpDNA data alone. The distributional pattern in this group of *Spigelia* was compared to that of other groups of organisms in the Gulf Coast region in an effort to find similarities that may indicate a shared geological history.

Keywords: *Spigelia*, taxonomy, biogeography, southeastern U.S., cpDNA, ITS.

Spigelia L. is a mostly neotropical genus of herbs and small shrubs whose range extends into the warm-temperate zones of North and South America. This study focuses on three closely-related north-temperate taxa of *Spigelia* that were once considered to constitute a separate genus (*Coelostylis* Torr. & A. Gray) because of their two-flowered cymose inflorescences in contrast to the multi-flowered scorpioid cymes typical of most other *Spigelia* species.

The former *Coelostylis* taxa (hereafter referred to as the *Coelostylis* taxa) include *Spigelia loganioides* (Torr. & A. Gray) A. DC., *S. texana* (Torr. & A. Gray) A. DC., and *S. hedyotidea* A. DC. (= *S. lindheimeri* A. Gray). They are all small, rhizomatous, perennial herbs with small (<1.5 cm long), whitish, trumpet-shaped flowers. The monophyly of this group has been demonstrated by a molecular phylogenetic study of *Spigelia* (Gould, 1997) based on sequences of the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA), which included the other north-temperate species of *Spigelia* (*S. marilandica* L. and *S. gentianoides* Chapm. ex A. DC.) and a sampling of tropical North and South American species.

Distributed in north-central Florida, Texas and Mexico, the *Coelostylis* group has a striking disjunction along the Gulf Coastal plain between Texas and Florida (Fig. 1). Many other southeastern plant groups are known to have disjunct northern Florida populations or species (e.g., *Berlandiera* (Pinkava, 1967); *Leitneria floridana* (Day, 1975); *Callirhoe papaver*; *Carya texana* and *C. floridana*; *Schoenocaulon*), and vicariance has often been invoked to explain disjunct eastern and western populations in both plant and animal groups on the Gulf coastal

plain (*Lygodesmia* (Tomb, 1980); several species of freshwater fish and snakes (Wiley & Mayden, 1985); *Vitis* (Moore, 1990); *Ulmus crassifolia* (Sherman-Broyles et al., 1992)). The main vicariance event in this region is presumably dated to the early Pliocene, when high water levels created isolated western and eastern refugia of Gulf Coast populations.

During the Pliocene interglacial, the sea stood 50–80 m above present-day sea level for approximately one million years (Webb, 1990; Greenlee & Moore, 1988; Bermingham & Avise, 1986; Swift et al., 1985). Extinction of surrounding plant populations and genetic differentiation of surviving populations should have been the main effects of the Pliocene high sea level stand (Bermingham & Avise, 1986). In addition, Pleistocene climatic changes are believed to have caused southerly displacements of the biota in the eastern U.S. (e.g., Deevey, 1949; Dressler, 1954; Blair, 1965).

The taxonomy of the *Coelostylis* group is problematic because there are few consistent morphological characters to tell them apart. The taxa are defined in part by their distributions and ecological preferences. *Spigelia loganioides* has been characterized by its endemic occurrence in three counties of northwestern to central Florida, where it can be found in calcareous hammocks and rises in river bottoms (Kral, 1983). *Spigelia texana* is endemic to the bottomland hardwood forests along the east Texas coastal plain, in sandy or clayey soil. *Spigelia hedyotidea* occurs primarily in upland well-drained soil on dry, open prairies, limestone bluffs, and gravelly stream beds from central to southern Texas and in Mexico, where there are isolated populations in Chihuahua, Coahuila, San Luis Potosí and Hidalgo.

Morphologically, *Spigelia texana* and *S. loganioides*, the two coastal plain taxa, are most alike and have been treated as conspecific (Hurley, 1968; Henrickson, 1996) or simply as closely related (Rogers, 1986). Together they are usually distinguishable from *S. hedyotidea* by their taller growth habit, their larger and more membranaceous leaves, their tendency to be glabrous on the leaves and between the ribs of the stem, and their tendency to produce a whorl of four leaves below the inflorescences (Henrickson, 1996). However, some populations of *S. hedyotidea* in central Texas growing in moist areas (e.g., river banks) display *S. texana*-like characters, having larger, membranaceous leaves and little stem pubescence. Furthermore, *S. loganioides* regularly has larger corollas than *S. texana*, which may be an indication of genetic differentiation.

We have examined the *Coelostylis* taxa in a phylogenetic framework using two molecular data sets: chloroplast DNA (cpDNA) restriction sites and DNA sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA to test hypothesized relationships and species boundaries. If some or all of these taxa are conspecific, we should find evidence of interbreeding as patterns of reticulation in phylogenetic analyses of multiple populations. Furthermore, if *Spigelia texana* and *S. loganioides* are sister taxa, we expect to find evidence of common ancestry.

MATERIALS AND METHODS

POPULATION SAMPLING. Plants were collected from 16 populations from throughout the recorded ranges of each *Coelostylis* taxon (Table 1), including three populations of *Spigelia loganioides* from Florida, five populations of *S. texana* from eastern Texas, and eight populations of *S. hedyotidea* from central to western Texas. Between five and 21 individuals were collected per population, depending on population density. A taxon designation was assigned to the population based on a combination of morphology and ecological conditions. In Texas, where morphological characters were sometimes not useful for distinguishing taxa, plants found in prairie habitats or in sandy soil were assigned to *S. hedyotidea*, and plants found in clayey soil

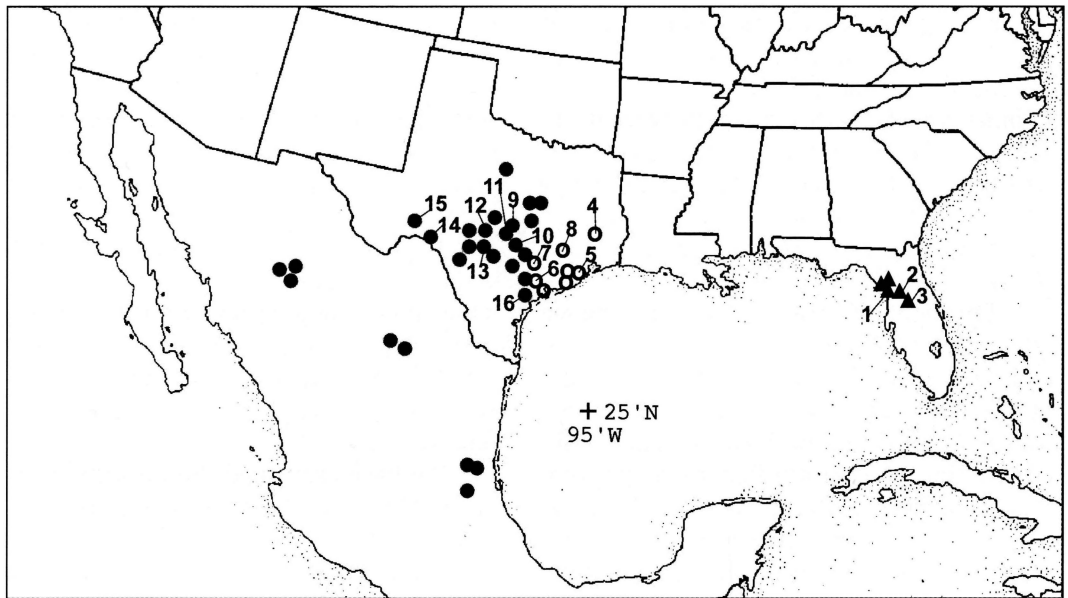FIG. 1. Documented distributions of *Spigelia loganioides* (triangles), *S. texana* (open circles), and *S. hedyotidea* (closed circles). Numbers represent actual samples collected for this study and correspond to locality names given in Table 1.

TABLE 1. *Spigelia* collection sites, corresponding numbers on distribution map (Fig. 1) and number of individuals collected at each site. Vouchers are deposited at TEX unless otherwise noted. Taxon abbreviations are as follows: hed=*S. hedyotidea*, log=*S. loganioides*, tex=*S. texana*, mar=*S. marilandica*, coel=*S. coelostylioides*.

Taxon	Locality	Map #	# Plants	Voucher	GenBank accession no.
log	Levy Co., FL	1	20	*K. R. Gould 149*	AF178000
log	Marion Co., FL	2	21	*K. R. Gould 153*	AF177998
log	Sumter Co., FL	3	2	*K. R. Gould 151*	AF177995
tex	Polk Co., TX	4	20	*K. R. Gould 159*	AF177997
tex	Brazoria Co., TX	5	20	*K. R. Gould 135*	AF177993
tex	DeWitt Co., TX	6	20	*K. R. Gould 154*	AF178006
tex	Gonzales Co., TX	7	21	*D. Lynch 7555*	AF177994
tex	Washington Co., TX	8	13	*K. R. Gould 156*	AF177996
hed	Travis Co., TX	9	5	*W. R. Carr 7513 (SMU)*	AF178008
hed	Hays Co., TX	10	6	*E. J. Palmer 12122*	AF178004
hed	Blanco Co., TX	11	10	*J. Reverchon 1330*	AF177999
hed	Gillespie Co., TX	12	14	*K. R. Gould 147*	AF178005
hed	Bandera Co., TX	13	20	*K. R. Gould 148*	AF178001
hed	Val Verde Co., TX	14	15	*K. R. Gould 111*	AF178007
hed	Terrell Co., TX	15	20	*K. R. Gould 158*	AF178003
hed	Goliad Co., TX	16	21	*W. R. Carr 11814*	AF178002
ma	Jackson Co., FL	N/A	1	*K. R. Gould 163*	AF177991
coel	Chiapas, Mexico	N/A	1	*K. R. Gould 139*	AF177992

were assigned to *S. texana*. Florida specimens were automatically assigned to *S. loganioides*. Vouchers were deposited in the University of Texas (TEX/LL) herbarium.

The small size of individual plants in this group necessitated using combined samples for the restriction enzyme analysis. Leaves were removed from individuals and combined to make a single population sample. The individuals (with some leaves still intact) were then either preserved frozen (in liquid nitrogen in the field, transferred to a -80° C freezer in the lab) or transplanted to pots and grown up in a greenhouse at the University of Texas. Individuals were preserved for resampling in order to determine the nature of intrapopulational variation in the event that polymorphisms were detected within populations. Intrapopulational variation has been found in cpDNA (Mason-Gamer et al., 1995; reviewed in Soltis et al., 1992) and can be detected on mixed-sample restriction digest gels (Bain & Jansen, 1996). Two North American *Spigelia* species (*S. marilandica* L. and *S. coelostylioides* K. R. Gould) were used as outgroups based on the results of an ITS-based phylogenetic study (Gould, 1997).

DNA EXTRACTION AND PURIFICATION. For the restriction site study, total DNA was extracted from all population samples using the CTAB method of Doyle and Doyle (1987) and purified by cesium chloride-ethidium bromide gradients (Sambrook et al., 1989). For ITS sequencing, one individual per population was extracted and sequenced for 12 of the 16 *Coelostylis* populations. Micro-extractions were performed on these using a modification of the CTAB method and grinding leaf material with sand in plastic microcentrifuge tubes. No additional purification was performed on the individual extractions. For the additional four populations (Travis, Val Verde, Hays, and Gillespie counties), the same mixed sample extraction was used for ITS sequencing as for the restriction digests.

RESTRICTION ENZYME ANALYSIS OF CPDNA. Samples were digested with 18 frequent-cutting restriction enzymes: 6-base cutters used were *Ase*I, *Ava*I, *Ava*II, *Ban*II, *Bgl*II, *Dra*I, *Eco*O109, *Eco*RI, *Nci*I, and *Xmn*I; one 5-base cutter used was *Bst*NI; four-base cutters used were *Bst*UI, *Hae*III, *Hha*I, *Hinf*I, *Msp*I, *Rsa*I, and *Taq*I. Fragments produced by enzymes with five or six base-pair recognition sequences were separated on 1.2% agarose gels and electrophoresed to a distance of 10 cm (as tracked by a bromphenol blue dye), while fragments produced by enzymes with four base-pair recognition sequences were separated on 1.4% gels and run to 15 cm. All gels were bidirectionally blotted (Smith & Summers, 1980) onto reusable nylon membranes (Zetabind, Cuno).

Membrane-bound DNA fragments were sequentially hybridized with ^{32}P-labeled (by nick translation), cloned fragments of the tobacco chloroplast genome (Olmstead and Palmer, 1992) and visualized by autoradiography. Hybridizations were carried out as described in Palmer (1986) and Jansen and Palmer (1987). Thirty-two fragments were used as probes (subclones 1-28 and 36–40 of Olmstead & Palmer, 1992), representing 83% of the chloroplast genome, and only excluding the inverted repeat region, which is the least variable region of the genome (Jansen & Palmer, 1987; Wolfe et al., 1987). Small adjacent probes were combined during hybridization.

Because of the low levels of sequence divergence and low incidence of length variation, it was possible to interpret all changes directly from the autoradiographs without constructing restriction site maps (Mason-Gamer et al., 1995). In cases where the exact nature of a difference could not be determined (e.g., when a site gain resulted in fragments too small to be detected), restriction site differences were scored by inferring the presence of small bands. When restriction site differences could not be distinguished from length changes by fragment patterns alone, multiple restriction digests of the same region of the genome were care-

fully examined to rule out the possibility of counting a single length difference more than once. Variable restriction sites were scored as present (1) or absent (0), and length differences were scored as the ancestral length (0) or derived length (1), using the outgroup method (Watrous & Wheeler, 1981). Chloroplast DNA haplotypes were thus determined for each population.

ITS SEQUENCES. Primers ITS5 and ITS4 of White et al. (1990) or modified primer ITS5 (Downie & Katz-Downie, 1996) or "ITS7A" (unpubl.) were used for double-stranded DNA amplification of the ITS region by polymerase chain reaction (PCR) following the protocol of Kim and Jansen (1994), with the following exceptions: 50 μl PCR reactions were performed using *Tfl* DNA polymerase (Epicentre Tech.) with *Tfl* polymerase reaction buffer, the thermal cycler (Perkin-Elmer) was programmed to perform an initial 3 min denaturation at 94° C followed by 35 rounds of the standard step-cycle, and the annealing temperature was lowered to 48° C. PCR products were purified from unused amplifying primers and deoxynucleotide triphosphates either directly, using the QIAquick™ PCR Purification Kit (Qiagen), or by agarose gel (1%) electrophoresis to remove non-specific amplification products and extracted with the QIAquick™ Gel Extraction Kit (Qiagen) or Wizard™ PCR Preps Purification System (Promega).

For all samples, cycle-sequencing reactions were carried out and sequencing gels run by technicians at the Institute of Cellular and Molecular Biology at the University of Texas. Complementary DNA strands were sequenced for each sample. The ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (Perkin Elmer Corp.) was used for cycle-sequencing reactions, samples were loaded into an acrylamide gel on an ABI 377 DNA sequencer (Applied Biosystems), and data were collected on a Macintosh platform. Resulting chromatographs were manually edited using the ABI Prism™

DNA Sequencing Software version 2.1.0 (Perkin Elmer Corp.). Published sequences of nrDNA of *Krigia biflora* (Asteraceae) (Kim & Jansen, 1994) and other Asteraceae (Baldwin, 1992) were used to determine the boundaries of the ITS 1 and 2 regions. All sequences were aligned manually using SeqApp version 1.9a169 (Gilbert, 1992).

DATA ANALYSES. Phylogenetic analyses on the cpDNA and combined data were performed in PAUP version 3.1.1 (Swofford, 1993) and on the ITS data in PAUP* version 4.0.0d64 (Swofford, 1997) using the branch-and-bound search algorithm (Hendy & Penny, 1982). Settings in PAUP were initial upper bound computed via stepwise, MULPARS, and addition sequence furthest. All characters were unweighted. For the ITS data, all gaps in aligned sequences were treated as missing data, and sequence polymorphisms were treated as uncertain.

Support for monophyletic groups was evaluated with 100 bootstrap replicates (Felsenstein, 1985). These were performed in PAUP with the ACCTRAN, MULPARS and TBR options in effect on heuristic searches with the simple addition sequence of taxa. Decay analyses (Bremer, 1988, Donoghue et al., 1992) were performed in PAUP on both data sets to determine the strength of support for clades. Trees longer than the most parsimonious trees were examined at one-step increments, by keeping near-minimal trees in heuristic searches with random addition sequence of taxa (10 replicates) and the steepest descent option in effect to increase the chance of finding trees from different islands of shortest trees.

RESULTS

RESTRICTION ENZYME ANALYSIS OF cpDNA. The cpDNA data set included 84 characters (Gould, 1997). Eighty-one variable characters were detected, including 74 restriction site changes and seven length changes, 42 of which were potentially informative, out of an estimated 918 sites (4,878 nucleotides) sampled. No polymorphisms

were detected within population samples. The list of character changes and the data matrix are available in Gould (1997). Among the ingroup taxa, 39 variable characters, including four length changes, were detected, 29 of which were informative, and 14 distinct cpDNA haplotypes were found (*Spigelia texana* populations from Polk and Brazoria Co. were identical, and *S. hedyotidea* populations from Gillespie and Terrell Co. were identical). Different amounts of interpopulational variation were detected within each taxon. Within *S. hedyotidea*, nine variable characters (five informative), and seven distinct haplotypes were detected; within *S. texana*, six variable characters (three informative) and four haplotypes were found; and within *S. loganioides*, four variable characters (one informative) and three haplotypes were found.

Unweighted parsimony analysis of the cpDNA data, including uninformative changes, identified four shortest trees of 82 steps each. Supportive index values are given in Figure 2. In each tree, as in the strict consensus tree, three well-supported cpDNA clades (bootstrap values of 83, 100, and 100%; Fig. 2) are found which correspond to the three taxa. Additionally, the clades corresponding to *Spigelia hedyotidea* and *S. texana* are strongly supported sister clades (defined by three synapomorphies, a decay value of 3, and a 98% bootstrap value; Fig. 2). The *S. loganioides* clade is sister to the Texas clade and separated from it by 13 changes. Relationships among the populations of *S. loganioides* are fully resolved, while interpopulational relationships within *S. hedyotidea* and *S. texana* are only partially resolved. The four trees obtained in the analysis differ only in the resolution of relationships among the *S. hedyotidea* populations.

ITS SEQUENCES. The aligned ITS data set contained 618 characters including the 5.8S rRNA gene (Gould, 1997). The ITS 1 region ranged from 222 to 227 base-pairs long, and the ITS 2 region ranged from 225 to 226 base-pairs long. The entire data set contained a total of 66 variable characters, 22 of which were potentially informative. Within the ingroup, there were 31 variable characters, 13 of which were informative. Eight of the informative characters in the ingroup were in ITS 1, and five were in ITS 2. Brazoria and Washington Co. populations of *Spigelia texana* had identical sequences, and Sumter and Levy Co. populations of *S. loganioides* had identical populations. Intrataxon variation in *S. texana* and *S. loganioides* rivaled that found in the cpDNA data, with *S. texana* containing 11 variable characters (one informative) and *S. loganioides* containing five variable characters (none informative). Intrataxon variation was much greater, however, within *S. hedyotidea*, with 23 variable sites (10 informative). Of the 13 informative characters, seven were coded as polymorphic in some of the accessions (Table 2).

Twenty-seven shortest trees of length 71 were found in the parsimony analysis (uninformative characters removed; supportive indices are given in Fig. 2). Intertaxon relationships were mainly unresolved. Unlike in the cpDNA trees, groupings of ITS types did not correspond to taxa, except in the case of *Spigelia texana* (supported by a bootstrap value of 77%, Fig. 2). A clade containing all *S. texana* populations, all *S. loganioides* populations, and five of the eight *S. hedyotidea* populations was found (clade A in the ITS tree, Fig. 2). These five *S. hedyotidea* populations were monophyletic (bootstrap of 64%). The other three *S. hedyotidea* populations (Blanco, Terrell and Gillespie Co.) were outside this main clade and formed a sister clade to it (clade B in the ITS tree, Fig. 2) in nine of the 27 trees. Overall, the ITS data did not resolve relationships among populations well, and those that were found were weakly supported due to the overall low number of informative changes in the ITS sequences.

ITS POLYMORPHISMS. In the ITS data set, polymorphisms were interpreted to be present in some accessions at seven nucleotide positions (Table 2). These poly-
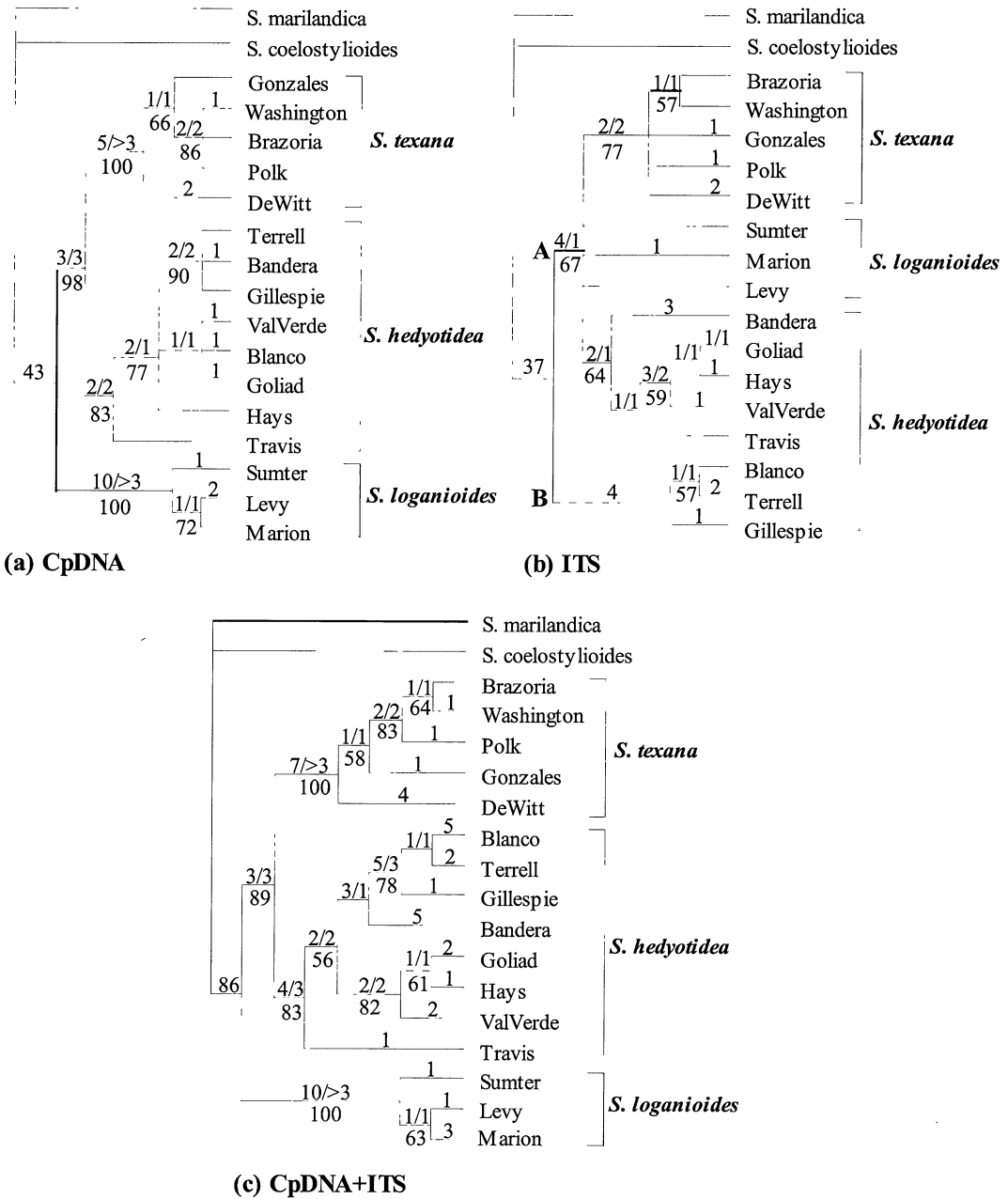
FIG. 2. Results of parsimony analyses of independent data sets for cpDNA restriction sites and ITS sequences, and for the combined data analysis. Terminal nodes for the *Coelostylis* taxa are labeled by county names where collected. (a) One of four shortest cpDNA trees (length = 82, CI excluding uninformative characters = 0.977, RI = 0.990, RC = 0.978). (b) One of 27 shortest ITS trees (length = 71, CI excluding uninformative = 0.930, RI = 0.881, RC = 0.819). (c) One of two shortest trees from the combined data analysis (length = 160, CI excluding uninformative = 0.827, RI = 0.910, RC = 0.829). For all trees, numbers above the lines indicate branch length followed by decay value, numbers below the branches are bootstrap values greater than 50%, and dashed lines indicate branches that collapse in the strict consensus tree.

morphic characters were detected as either two fluorescent peaks superimposed on one another or as a lower peak superimposed on a higher peak, and in most cases were found on both complementary strands. Polymorphisms were present mainly in *Spigelia hedyotidea* accessions, but also appeared less frequently at the same sites in the other two taxa and once in the outgroups (Table 2).

The incidence of polymorphisms did not completely coincide with the mixed-individual DNA extractions, so most of these must have represented multiple ITS types within individuals. Polymorphisms shared between different taxa coincided with geographic proximity only in the case of populations from DeWitt Co. (*Spigelia texana*) and Goliad Co. (*S. hedyotidea*), which shared two polymorphisms, at positions 123 and 129. Other cases of shared polymorphisms between taxa occurred in populations that were not from adjacent counties and often separated by great distances within Texas (e.g., the *S. texana* population from Gonzales Co. in eastern Texas and the *S. hedyotidea* population from Val Verde Co. in western Texas shared a polymorphism at position 225) and between Texas and Florida.

COMBINED ANALYSIS. The cpDNA and ITS analyses resulted in incongruent topologies with respect to the relationships of *Spigelia hedyotidea* populations (Fig. 2a, b). However, the support for these relationships in the ITS analysis is weak as shown by the bootstrap and decay values (Fig. 2b). It has been discerned that if conflict in results from separate data sets exists only in portions of the resulting trees where character support is weak, then stochastic processes are as likely to be the cause as factors that may violate the assumptions of phylogenetic analysis, such as character nonindependence (Olmstead & Sweere, 1994). Combining the data in this case is appropriate. Furthermore, the results from the two analyses are not in conflict regarding relationships among populations of *S. texana* and *S. loganioides.* Since the main questions of this study concern *S. texana* and *S. loganioides* and whether they are distinct taxonomically and whether they are sister taxa, we wanted to know what support a combined data analysis would have for the results found in the cpDNA analysis.

When the data were combined, two shortest trees of length 160 were found (Fig. 2c). The overall structure of the trees is like that in the cpDNA analysis, with three clades corresponding to the three taxa and a sister-group relationship between the *Spigelia texana* clade and the *S. hedyotidea* clade. Interpopulational relationships within *S. texana* are completely resolved and not incompatible with the trees from the separate data sets. *Spigelia loganioides* interpopulational relationships are also resolved as in the cpDNA trees. The relationships among *S. hedyotidea* populations in the combined analysis, on the other hand, resemble those found in the ITS trees and have better bootstrap support, with a clade containing Goliad, Hays and Val Verde counties (bootstrap value 82% vs. 59% in the ITS analysis) and a clade containing Blanco, Terrell and Gillespie counties (bootstrap value 78% vs. 30%; this node also collapses in the strict consensus tree of the ITS analysis).

DECAY ANALYSES. The decay analysis of the cpDNA data found 35, 140, and 944 trees at one, two, and three steps longer than the most parsimonious trees, respectively. The decay analysis of the ITS data found 777 and 12,498 trees at one and two steps longer than the most-parsimonious trees (at this point all nodes in the trees collapsed). For the combined data set, 21, 108, and 444 trees were found at one, two and three steps longer than the optimal trees. Decay indices are given for all analyses in Figure 2. There is currently no widely agreed-upon way to test for the statistical significance of a decay value, but values for conflicting nodes resolved by different data sets can be compared. In the cpDNA analyses, the decay index for the node making *Spigelia loganioides* sister to the Texas clade was three. In the ITS analyses, the

TABLE 2. Polymorphisms detected in ITS automated sequence chromatograms for each population sample. Nucleotide position corresponds to the aligned ITS sequences in Gould (1997). Abbreviations are as in Table 1.

	Locality	\<Nucleotide position\>						
		93	95	123	129	160	225	560
hed clade A	Bandera Co.	A/T	T/C	A/T	C	G/A	T	A
	Goliad Co.	A/T	T/C	A/T	G/C	G/A	A	G/A
	Hays Co.	A/T	T/C	A/T	G/C	G/A	A	G/A
	Travis Co.	T	T/A/C	A/T	G	G	A/T	G
	ValVerde Co.	T	T/A	A/T	G	G/A	A/T	G
hed clade B	Blanco Co.	A/T	T/C	A/T	C	G/A	T	A
	Gillespie Co.	A/T	T/C	A/T	C	G/A	T	A
	Terrell Co.	A/T	T/C	A/T	C	G/A	A/T	G/A
log	Marion Co.	T	T	T	G	G/A	A	G
	Levy Co.	T	T	T	G	G/A	A	G
	Sumter Co.	T/C	T	T	G/C	G	A	G
tex	Brazoria Co.	T/C	T	T	G	G	A/C	G
	De Witt Co.	T/C	T/A	A/T	G/C	G	A	G
	Gonzales Co.	T/C	T	T	G	G/T	A/T	G
	Polk Co.	T	T	T	G	G	A	G
	Washington Co.	T/C	T	T	G	G	A/C	G
out	S. coelostylioides	T	T	A	C	G	A/T	A
	S. marilandica	T	T	A	C	G	A/T	A

decay index for the node causing *S. hedyotidea* to be paraphyletic was one.

DISCUSSION

The cpDNA restriction site data provide an extremely well-supported phylogeny, with high haplotype variation and no homoplasy within *Coelostylis*. Fourteen out of the 16 populations sampled had distinct haplotypes, while no intrapopulational variation was found. The two population pairs with identical haplotypes are not geographically closest to one another and are separated by great distances (Polk and Brazoria Co. sites are approximately 130 miles apart; Gillespie and Terrell Co. sites are approximately 169 miles apart). In fact, there seems to be no geographical correlation to the population-level relationships. At a broader (taxon) level, however, there is geographical structure, with eastern and most central-western Texas populations forming separate clades (*Spigelia hedyotidea* from Goliad Co. is actually in eastern Texas, adjacent to De Witt Co., but still groups with the other *S. hedyotidea* accessions) and the Florida populations forming a clade. It would appear from these data that each taxon contained a unique ancestral chloroplast genotype from which individual populations are presently diverging. Genetic isolation of populations, at least in the (presumably) maternally inherited genome must be strong. Seed dispersal would appear to be short-distance; these plants have small, unornamented seeds that fall close to the parent plant after capsular dehiscence. However, water is probably the most effective dispersal vector, since these plants often grow near or in stream beds or in periodically inundated woodlands.

We felt it was important to include a nuclear marker in this study for comparison with the chloroplast data, so we conducted the analysis of ITS sequences and present the phylogeny here even though the ITS data do not provide much resolution and contain many polymorphic sites. Determining the exact nature of the polymorphisms was beyond the scope of this paper, but may prove useful at some point to study population structure. The ITS findings could be indicative of lineage sorting of various ITS types which may have persisted because of the relatively recent divergence time of these taxa (presumably Pliocene-Pleistocene, see Introduction above). The polymorphisms may also represent different ITS paralogs resulting from a gene duplication event that preceded the differentiation of these taxa. They do not seem to point to introgression, since, as stated above, there is no obvious geographical structure to the shared polymorphisms.

ITS provided some resolution at the population level, but again these relationships do not correlate with geography. For example, in *Spigelia hedyotidea*, the two westernmost and adjacent county sites, Terrell and Val Verde, separated from the next nearest county site (Bandera) by approximately 117 miles, do not appear in the same clade. Also, Gillespie, Blanco, Travis and Hays Co. sites, all clustered in the center of the state, were expected to form a clade due to geographic proximity, but did not. The Florida populations do not form a monophyletic clade.

The relatively low bootstrap values on the ITS tree do not give us great confidence in the resolving power of the ITS data alone, whereas the cpDNA data resolved strongly supported nodes in trees with little homoplasy. Combining the data yields two trees with a combination of the two single-data set topologies, with an average bootstrap value (the mean for all nodes resolved in the strict consensus tree) in between those for the cpDNA and ITS analyses (cpDNA=85.8%, ITS=63.5%, combined=71.5%).

The resolution of interpopulational relationships is the most important result emerging from the combined data analysis. The *Spigelia texana* population branch order was fully resolved, with Brazoria and Washington Co. sites as sister terminals, as in the ITS tree. The *S. hedyotidea* populations were almost fully resolved, with the exception of the Goliad-Hays Co. clade which collapses in the combined data strict consensus tree. Bootstrap values decreased slightly within the *S. texana* and *S. loganioides* clades in the combined analysis. However, bootstrap values increased within the *S. hedyotidea* clade, even though there appeared to be no support for some of these relationships in the cpDNA trees (i.e., Goliad-Hays-Val Verde Co. clade and Blanco-Terrell-Gillespie Co. clade). Bandera, Blanco, Terrell and Gillespie Co. accessions formed a novel, combinatorial clade, although the bootstrap and decay support are not high (37%, 1). Finally, the Florida populations are monophyletic and resolved as in the cpDNA tree.

Furthermore, the combined data analysis, like the cpDNA analysis, resolved three well-supported clades that correspond to each *Coelostylis* taxon. *Spigelia texana* and *S. hedyotidea* are still resolved as sister taxa in the combined data analysis, although the bootstrap support for this finding decreased slightly (89% vs. 98% in the cpDNA trees; but the decay was the same at 3). There are also two strongly differentiated (13 cpDNA changes) clades representing plants on either side of the Gulf Coast disjunction. This eastern-western genotypic split in *Spigelia* coincides with geographical limits found in both inter- and intraspecific plant distributions and with distributional limits of species of freshwater fishes in the southeastern U.S. (see Introduction, above; Swift et al., 1985). One study in particular by Bermingham and Avise (1986) found major mtDNA genetic breaks in four species of the sunfish *Lepomis*, which they calculated to have occurred at the time of the Pliocene interglacial. These breaks occur between

groups of eastern and western populations of all species of *Lepomis* on the Gulf coastal plain between Louisiana and South Carolina. These distributional correlations implicate a strong role for historical biogeographic patterns in the Gulf Coast region and for concurrent episodes of fragmentation of floras and faunas.

The results presented here suggest that the geological history of the region has also influenced the phylogenetic history of this group of *Spigelia*. The eastern and western cpDNA genotypes in *Spigelia* could be due to a combination of events resulting from glaciation from the early Pliocene through the Pleistocene, including fluctuating sea levels causing widespread extinction and the creation of refugial areas, as well as cooling climates pushing ranges southward and causing population fragmentation in the Gulf Coast region. For example, a potential refuge area during the Pliocene high sea stand was the Ocala highlands region in north-central peninsular Florida, which contains two of the main present-day populations of *S. loganioides* (Bermingham & Avise, 1986; Swift et al., 1985; Greenlee & Moore, 1988). The fact that *S. texana* and *S. loganioides* are not sister taxa, but are morphologically quite similar may indicate a strong correlation between ecological factors (e.g., lowland adaptation to periodic inundation and saturated soils) and morphology.

Recognizing the three *Coelostylis* taxa as three species would be compatible with both a biological species concept (Mayr 1963) and an autapomorphy (Donoghue 1985; Mishler and Brandon 1987; de Queiroz and Donoghue 1988) or genealogical species concept (Baum and Shaw 1995). First, the cpDNA and the combined data sets strongly support the monophyly of all three taxa. Second, they seem to be acting as three discrete gene pools. The cpDNA data give no indication of gene flow among these three taxa. From the ITS data, one might expect to find a pattern in the polymorphisms that indicated gene flow if taxa were intercrossing. As discussed above, however, the shared polymorphisms do not coincide with geographic proximity. Furthermore, the *Spigelia texana* populations appear to be mostly homogenized for one ITS type, as is *S. loganioides*. Finally, ecological and geographical data used to designate taxa correlated with monophyletic groupings, and these data may be used as criteria for species recognition. Thus we conclude that these taxa are best maintained as three distinct species and provide a key below.

1. Plants usually multi-stemmed; leaves usually all opposite, narrowly lanceolate, slightly thickened; stems and leaves scabrous. Central Texas and Mexico; open prairies or dry woods on thin, rocky or sandy soils.
    S. hedyotidea
1. Plants 1-few-stemmed; uppermost leaves usually whorled; leaves ovate to lanceolate, membranaceous; stems and leaves glabrous to slightly papillate.   2.
    2. Corollas 8–13 mm long. Eastern Texas; riparian woodlands on clayey soils.   *S. texana*
    2. Corollas 10–17 mm long. North-central Florida; sandy soils.   *S. loganioides*

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