

BRAZILIAN SPECIES OF *VIGUIERA* (*ASTERACEAE*) EXHIBIT LOW LEVELS OF ITS SEQUENCE VARIATION

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The internal transcribed spacer (ITS) region of the nuclear ribosomal DNA was sequenced from 21 accessions of *Viguiera* from South America, including 14 from Brazil. Overall there was only a small amount of sequence variation, less than 3.4% among South American samples and less than 4.5% between the South American samples and the Mexican *V. flava*. Phylogenetic analysis revealed little divergence among the Brazilian samples of *Viguiera*. The low level of ITS sequence variation was in striking contrast to the taxonomic diversity and chemical variability that have been reported for *Viguiera* in Brazil. The low level of sequence variation suggests that, despite its clear morphological and phytochemical adaptations to the cerrado, *Viguiera* is probably a relatively recent addition (less than 3 million years) to the flora of this area.

Keywords. Asteraceae, cerrado, ITS, molecular phylogeny, *Viguiera*.

A região do espaçador ribossômico interno (ITS) do DNA ribossômico nuclear foi seqüenciada a partir de 21 amostras de *Viguiera* provenientes da América do Sul, incluindo 14 do Brasil. No total, houve apenas uma pequena variação de seqüência, sendo menor que 3.4% entre as amostras da América do Sul e menor que 4.5% entre as amostras da América do Sul e a mexicana *V. flava*. As análises filogenéticas revelaram pequena divergência entre as amostras brasileiras de *Viguiera*. O baixo nível de variação da seqüência do ITS mostrou que há um interessante contraste com relação à diversidade taxonômica e à variabilidade química que tem sido descritos para espécies de *Viguiera* do Brasil. O baixo nível de variação de seqüência sugere que, embora suas adaptações morfológicas e fitoquímicas ao cerrado sejam claras, *Viguiera* é provavelmente uma adição relativamente recente (menos que 3 milhões de anos) à flora desta área.

Palavras-chave. Asteraceae, cerrado, filogenia molecular, ITS, *Viguiera*.

INTRODUCTION

Contributions to understanding the biogeographic history and adaptations of plants of the biologically distinctive Brazilian cerrado regions can be made through detailed phylogenetic analyses of individual taxonomic groups. In this study, a survey is made of variation for ITS (nuclear ribosomal internal transcribed spacer region) DNA

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sequences in a group of cerrado plants belonging to the *Asteraceae* genus *Viguiera* Kunth.

The cerrado is a seasonal savanna woodland formation that occupies about 20% of the land area of Brazil, and is mostly restricted to this country (Eiten, 1972, 1984; Harley, 1995; Eiten & Sambuichi, 1996). The cerrado typically includes an upper layer of scattered trees and a ground layer of shrubs and herbs. Plants of the cerrado exhibit a variety of characteristic adaptations to the seasonally dry, nutrient-poor soils. Although cerrado forms an almost continuous belt across the Brazilian planalto, it is rapidly undergoing massive conversion to farmland, to the severe detriment of the native vegetation (Ratter *et al.*, 1997). Thus there is considerable urgency to catalogue, record, and study the plants of cerrado areas. Despite its somewhat barren general appearance, the cerrado has a high level of biodiversity, ranking second in Brazil just behind the Amazon region (Castro *et al.*, 1999). It has been hypothesized that cerrado vegetation has been present for an extremely long time, perhaps dating to the Cretaceous (Ratter & Dargie, 1992), although it is also clear that there have been shifts in the locations of various vegetation types throughout recent geological history (Prado & Gibbs, 1993).

Viguiera, with about 200 species, is the paraphyletic core genus of subtribe *Helianthinae*. As currently circumscribed, it occurs from south-western North America to southern South America. The largest concentration of species is found in Mexico, and there are secondary centres of diversity in the Andes Mountains and in upland areas of Brazil. Of 72 species of *Viguiera* recognized by Blake (1918) from South America, 34 occur in Brazil, and most are restricted to the country.

Many of the Brazilian species of *Viguiera* occur in cerrado areas. Their species-level taxonomy is, however, still very poorly known, and there are many problems to be resolved. Many of the species are known only from a single type collection, sometimes without specific locality data. It is not clear how many species of *Viguiera* occur in Brazil, how they are to be distinguished, and how the names should be applied. In fact, the application of species names in this paper should be considered to be provisional pending further taxonomic study.

Various aspects of the chemistry of the Brazilian species of *Viguiera* have been investigated recently. The carbohydrate chemistry of underground storage structures is of particular interest, because fructose polymers are found in many herbaceous species of *Asteraceae* from the Brazilian cerrado, including *Viguiera* (Tertuliano & Figueiredo-Ribeiro, 1993). The physiology and biosynthesis of fructans from some species have been characterized (Isejima *et al.*, 1991; Tertuliano & Figueiredo-Ribeiro, 1993; Itaya *et al.*, 1997), as well as the developmental physiology of the plants (Ruggiero & Zaidan, 1997). Studies to characterize the sesquiterpene lactone chemistry have also been initiated (Da Costa *et al.*, 1996), and additional species are now being investigated (Schorr *et al.*, 1999; Torre *et al.*, 1999).

The present study represents a step toward resolving the taxonomic problems of Brazilian *Viguiera* by making an initial survey of molecular variation. The ITS region (Baldwin *et al.*, 1995) was chosen for sequence analysis because it has proven useful

in many groups of *Asteraceae* (Baldwin, 1992, 1993; Kim & Jansen, 1994; Sang *et al.*, 1994, 1995, Bain & Jansen, 1995; Susanna *et al.*, 1995; Kim *et al.*, 1996; Francisco-Ortega *et al.*, 1997; Koopman *et al.*, 1998; Kornkven *et al.*, 1998; Noyes & Rieseberg, 1999), including subtribe *Helianthinae* (Schilling & Panero 1996a; Schilling *et al.*, 1998). The results provided information on the level of variability present in ITS sequences, from which some conclusions regarding the systematics and timing of diversification of the species can be drawn.

MATERIALS AND METHODS

ITS sequences were obtained for a total of 21 accessions representing 15 species of *Viguiera* from South America, including 14 accessions representing 9 species from Brazil (Table 1). For most samples, DNA extractions were made following the general CTAB protocol of Doyle & Doyle (1987), using 0.3g of leaves that had been dried in silica gel. Leaves were crushed in liquid nitrogen prior to grinding. The crude DNA extracts of a few samples required further purification using the Wizard Kit protocol (Promega). ITS reactions were performed in 50 μ l reactions using 10–20ng of genomic DNA, 10X PCR buffer (Promega), 1.8–2.25mM MgCl₂, 0.2mM each dNTP, 1.25 units of *Taq* polymerase, and 0.2 μ M each primer. Primers used were 'ITS-4' (5'-TCCTCCGCTTATTGATATGC-3') and 'ITS-5' (5'-GGAAGTAAAAGTCGTAACAAGG-3'; White *et al.*, 1990). PCR was performed with the following protocol: 94°C for 2min; 25 cycles of 94°C for 1min, 52°C for 2min, and 74°C for 1min; a final extension of 72°C for 1min. PCR products were checked by agarose gel electrophoresis and purified by the Wizard Kit protocol. Sequencing was done at the University of Tennessee Automated Sequencing Facility, and utilized the ABI Prism Dye Terminator Cycle Sequencing reaction kit on an ABI 373 DNA sequencer (Perkin-Elmer Inc., Foster City, CA). Both the forward and reverse DNA strands were sequenced. The initial sequence data text files were edited following comparison with the same data displayed in four-colour electropherograms before they were analysed further.

Previously determined ITS sequences of species representing major clades of subtribe *Helianthinae* (Schilling & Panero, 1996a) were added to the data set for analysis. Because of the low level of sequence variation, it was possible to align sequences by eye.

Phylogenetic relationships were analysed using maximum parsimony, implemented with the computer program PAUP 4.0* (Swofford, 1998). Heuristic searches were performed with random sequence addition with replicates to increase the probability that all the shortest trees were found. Gaps were treated as missing data. Bootstrap analysis (Felsenstein, 1985) was performed with 10,000 replicates and the fast step option in PAUP*4.0. Pair-wise distances between accessions were calculated using the Kimura 2-parameter model. GenBank accession numbers (AF162286 to AF162329) are provided in Table 1.

TABLE 1. Species of *Viguiera* from South America that were analysed for ITS sequence data. Collectors: FBC, F. B. Da Costa; JLP, J. L. Panero; DS, D. Spooner; EES, E. E. Schilling. Vouchers for samples from Brazil are deposited at SPFR, with duplicates at TENN; other vouchers are at TENN

Species	Sample number	Country	GenBank number	
			ITS-1	ITS-2
<i>V.</i> subgen. <i>Viguiera</i> sect. <i>Paradosa</i> S. F. Blake				
<i>V.</i> ser. <i>Tenuifoliae</i> S. F. Blake				
<i>V. aspilioides</i> Baker	FBC 89	Brazil	AF62287	AF162309
<i>V.</i> ser. <i>Revolutae</i> S. F. Blake				
<i>V. revoluta</i> (Meyen) S. F. Blake	DS 4475	Chile	AF162300	AF162322
<i>V.</i> ser. <i>Grandiflorae</i> S. F. Blake				
<i>V. missionensis</i> A. A. Sáenz	EES 90–23	Argentina	AF162293	AF162315
<i>V. obtusifolia</i> Baker	FBC 91	Brazil	AF162295	AF162317
<i>V. squalida</i> S. Moore	FBC 88	Brazil	AF162305	AF162327
<i>V.</i> ser. <i>Aureae</i> S. F. Blake				
<i>V. discoidea</i> (Griseb.) S. F. Blake	EES 90–6	Argentina	AF162288	AF162310
<i>V. mollis</i> Griseb.	EES 90–1	Argentina	AF162294	AF162316
<i>V. salicifolia</i> Hassler	EES 1978	Argentina	AF162303	AF162325
	EES 1979	Argentina	AF162304	AF162326
<i>V. szyszyłowiczii</i> Hieron.	JLP 863	Peru	AF162306	AF162328
<i>V.</i> ser. <i>Bracteatae</i> S. F. Blake				
<i>V. arenaria</i> Baker	FBC 7	Brazil	AF162286	AF162308
<i>V. gardneri</i> Baker	FBC 72	Brazil	AF162290	AF162312
	FBC 81	Brazil	AF162291	AF162313
<i>V. quinqueremis</i> S. F. Blake	FBC 65	Brazil	AF162298	AF162320
	FBC 63	Brazil	AF162296	AF162318
	FBC 64	Brazil	AF162297	AF162319
	FBC 67	Brazil	AF162299	AF162321
<i>V. robusta</i> Gardner	FBC 66	Brazil	AF162302	AF162324
	FBC 61	Brazil	AF162301	AF162323
<i>V.</i> sect. <i>Trichophylla</i> S. F. Blake				
<i>V. trichophylla</i> Dusén	FBC 70	Brazil	AF162307	AF162329
<i>V.</i> subgen. <i>Yerbalesia</i> S. F. Blake				
<i>V. macrorhiza</i> Baker	FBC 24	Brazil	AF162292	AF162314

RESULTS

The organization and relative lengths of the ITS-1, ITS-2, and 5.8S regions from the Brazilian samples of *Viguiera* were consistent with those previously reported for other *Helianthinae* (Schilling & Panero, 1996a) and varied little between samples. The entire ITS region among Brazilian samples varied from 645 to 649bp. The ITS-1 region was 258–262bp, the ITS-2 region was 222–224bp, and the 5.8S region was uniformly 164bp. Because it was almost completely uniform, the 5.8S region was not included in subsequent phylogenetic analyses. Alignment of sequences among

Brazilian and other South American samples of *Viguiera* required the insertion of 10 indels, all but one of which was a single bp in length, with six in the ITS-1 region and four in ITS-2. Alignment of the sequences of the South American samples to those of Mexican samples, including *V. flava*, *V. pinnatilobata* and *V. grammatoglossa*, required no additional indels.

The aligned character matrix for the ITS-1 and ITS-2 regions of South American *Viguiera* was 493 characters in length, of which 61 (12%) were variable. There were 26 parsimony informative characters for the 21 samples. Pair-wise distance values (based on the Kimura 2-parameter model) for samples ranged from identity for several pairs to 3.4%. Pair-wise distance values between the South American samples and the Mexican *V. flava* ranged from 1.9% to 4.5%.

Parsimony analysis that included representatives of major lineages of subtribe *Helianthinae* (Schilling & Panero, 1996a) produced a total of 27,343 minimum length trees of length 377, the strict consensus of which is shown in Fig. 1. The overall topology of the tree was consistent with the results obtained in previous studies (Schilling & Panero, 1996a; Schilling *et al.*, 1998). The basally diverging clades included groups from Mexico classified within *Viguiera* or *Heliomeris*. The South American samples of *Viguiera*, including all of the Brazilian samples, were placed in a relatively derived clade that also included *V. flava* (*V. subgen. Amphilepis*) from Mexico. This clade had only moderate (64%) bootstrap support. Within this clade, there was a basal polytomy, with a well supported (84%) clade containing most of the South American samples separated from three of the South American samples (*V. mollis*, *V. discoidea* and *V. revoluta*) and *V. flava*, each of which was an individual branch. All of the Brazilian species occurred within the exclusively South American clade, together with two species from Argentina (*V. missionensis* and *V. salicifolia*) and the Peruvian *V. szyszlowiczii*. There was little resolution within this clade, and none of the subclades corresponded to previously recognized supraspecific groups (sections or series).

DISCUSSION

The most striking aspect of the ITS sequence data for the Brazilian species of *Viguiera* was the lack of variation, both among the species and also with other South American species. The low levels of variation were further reflected in the results of phylogenetic analysis, which suggested that the Brazilian species of *Viguiera* are relatively derived within the subtribe, and probably of only recent origin. These results were, however, in striking contrast to the diversity based on morphological characters and chemical variability that have been reported for *Viguiera* in Brazil.

The maximum level of divergence among South American samples of *Viguiera* was 3.4%, and between the South American samples and the Mexican species *V. flava* only 4.5%. This is low compared with levels of divergence reported for ITS sequences for other groups of *Asteraceae*. For example, levels of divergence in North American *Eupatorieae* between *Eupatoriadelphus* (Joe Pye Weeds) and *Eupatorium*

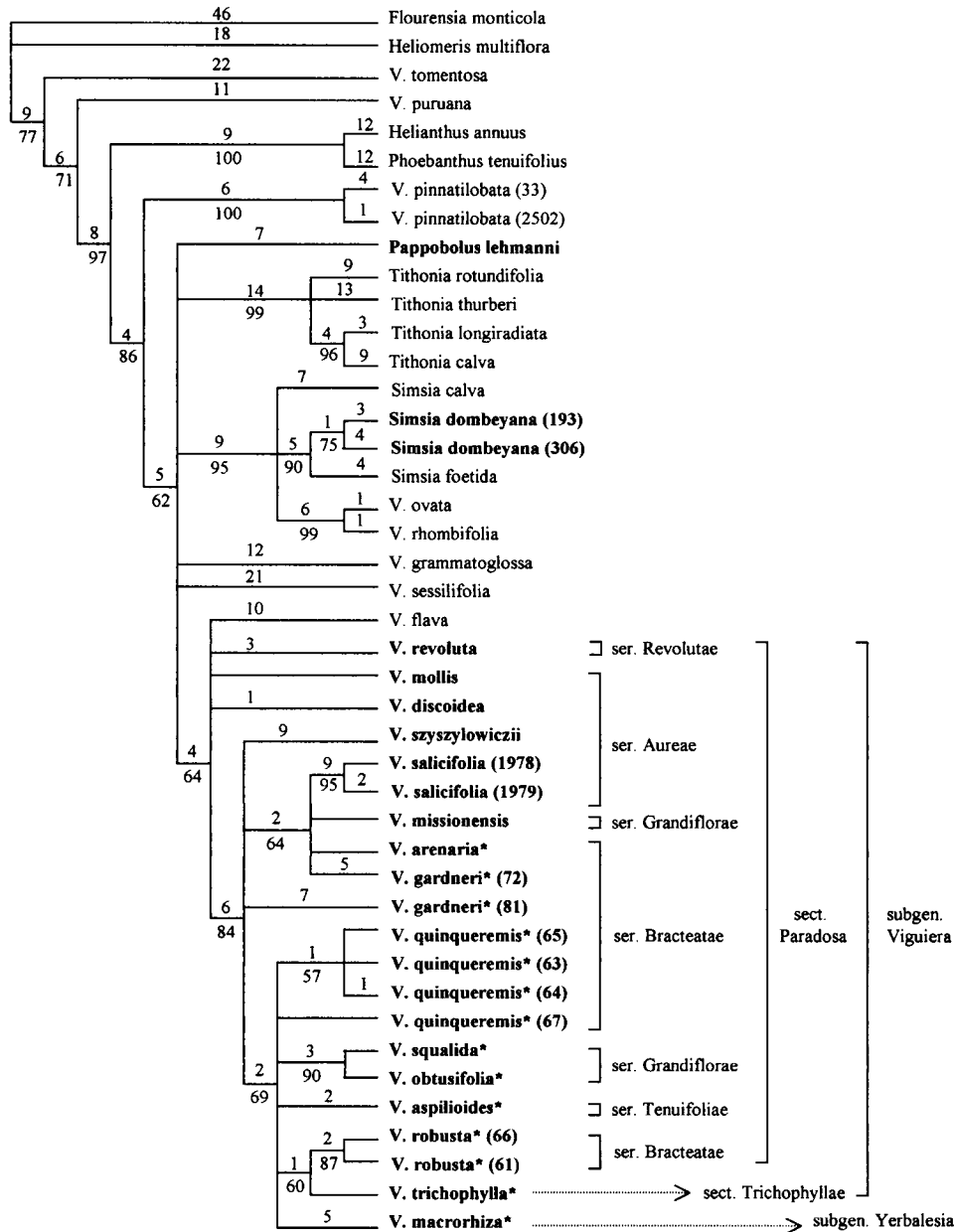


FIG. 1. Strict consensus tree generated by maximum parsimony analysis of ITS sequence data showing the placement of South American samples of *Viguiera* relative to the outgroup, *Flourensia*, and other members of subtribe *Helianthinae*. Character changes (using accelerated transformation option) shown above branches; bootstrap support (based on 10,000 replicates) shown below branches. Boldface, South American samples (infrageneric classification indicated following Blake, 1918); *, samples from Brazilian cerrado areas.

s.s. are 8–16% and between Eurasian and North American species of *Eupatorium* are 3–7% (Schmidt, 1998). Based on this level of sequence divergence, a Pliocene age (3.5 ± 1.2 mya) was estimated for the North American-Eurasian split in *Eupatorium* (Schmidt and Schilling, 2000). Similarly, Baldwin & Robichaux (1995) report divergence values of up to 4.5% among Hawaiian silverswords, and basal branching within this lineage is estimated to have occurred at about 5.8 million years ago (Baldwin & Sanderson, 1998). Thus, unless the rate of ITS sequence divergence differs dramatically for South American *Viguiera* compared with other *Asteraceae*, it can be hypothesized that divergence among South American species dates to less than about 3 million years.

The South American species of *Viguiera* were all placed in a single, relatively derived position in the ITS phylogeny, although there were no synapomorphies for ITS sequences that united them as a single monophyletic group. This is in contrast to the cpDNA results, for which all South American samples analysed share a minimum of 5 synapomorphic restriction site changes (Schilling *et al.*, 1994; Schilling & Panero 1996b). There was, however, a one bp gap in ITS-2 relative to all other *Helianthinae* that was shared by all but one (*V. szyszyłowiczii*) of the South American samples of *Viguiera*, and would provide additional supporting evidence that they are collectively monophyletic.

There was little phylogenetic signal in ITS sequence data to distinguish among South American species of *Viguiera* (Fig. 1). The lack of phylogenetic signal was not surprising, given the relatively homogeneous nature of the sequences. The lack of variation for ITS sequence data is not reflective of the taxonomic treatment of the Brazilian species of *Viguiera* by Blake (1918). Blake (1918) recognized 34 species of *Viguiera* to occur in Brazil, and placed them into two different sections. There was no support for the sectional treatment, or for the division of sect. *Paradosa* into series, from the ITS-based phylogeny.

The low level of divergence for ITS sequences adds to other evidence to suggest that a reassessment is needed of the species-level taxonomy of *Viguiera* in Brazil. Where multiple samples of a species were analysed, there were few sequence differences between samples. For example, the ITS sequences for two different samples of *V. robusta* were identical, and the ITS sequences for four different samples of *V. quinqueremis* were either identical or differed by two or fewer positions. The distinction of *V. arenaria* from *V. robusta* (two morphologically similar species that may have been the source of some confusion in the chemical literature) was, however, supported, and this is in agreement with recent preliminary phytochemical analyses. The results of these analyses of sesquiterpene lactones showed that, although the major compound was found to be the same in both species, there were qualitative differences between those in minor concentrations, indicating the presence of different chemical compositions, and qualitative chemical differences have also been observed in the chemistry of diterpenoids (Da Costa, unpublished).

The reported physiological and phytochemical variation among Brazilian species of *Viguiera* is remarkable, given the apparent limited age of the group in Brazil. This

appears to provide an example of a group that has diversified rapidly upon reaching and successfully colonizing a novel habitat.

The relatively young age for Brazilian *Viguiera* contrasts with the postulated ancient age of the cerrado regions that form the primary habitat for these species. Thus, *Viguiera* appears to be a relatively recent addition to the cerrado flora. It has recently been emphasized that such floristic associations will change over time, and in the case of the cerrado, the likelihood that Pleistocene climatic changes have changed the extent and locations of cerrado areas would have provided opportunities for new species to be added to this formation.

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