

Relationships in subtribe Diocleinae (Leguminosae; Papilionoideae) inferred from internal transcribed spacer sequences from nuclear ribosomal DNA

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Abstract

The complete sequences of nuclear ribosomal DNA (nrDNA) internal transcribed spacer regions (ITS/5.8S) were determined for species belonging to six genera from the subtribe Diocleinae as well as for the anomalous genera *Calopogonium* and *Pachyrhizus*. Phylogenetic trees constructed by distance matrix, maximum parsimony and maximum likelihood methods showed that *Calopogonium* and *Pachyrhizus* were outside the clade Diocleinae (*Canavalia*, *Camptosema*, *Cratylia*, *Dioclea*, *Cymbosema*, and *Galactia*). This finding supports previous morphological, phytochemical, and molecular evidence that *Calopogonium* and *Pachyrhizus* do not belong to the subtribe Diocleinae. Within the true Diocleinae clade, the clustering of genera and species were congruent with morphology-based classifications, suggesting that ITS/5.8S sequences can provide enough informative sites to allow resolution below the genus level. This is the first evidence of the phylogeny of subtribe Diocleinae based on nuclear DNA sequences.

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1. Introduction

The Leguminosae (Fabaceae) is the largest family of flowering plants after Compositae and Orchidaceae, with three subfamilies comprising some 650 genera and more than 18,000 species (Polhill and Raven, 1981). The tribe Phaseoleae (Papilionoideae) comprises the largest number of genera and it is the most economically important of any tribe of the Leguminosae. The tribe is currently divided into eight subtribes: Cajaninae, Phaseolinae, Clitoriinae, Ophrestinae, Kennediinae, Erythrinae, Diocleinae and Glycininae (Polhill and Raven, 1981; Polhill, 1994).

The Diocleinae are mostly from the New World and possess a number of primitive characteristics: the habit

is often woody or coarse, the frequently large flowers have prominent discs about the ovary, the hila are often long, and the non-protein amino acid canavanine is usually present (Lackey, 1981). Lackey recognized 13 genera of Diocleinae (*Dioclea*, *Cymbosema*, *Cleobulia*, *Canavalia*, *Pachyrhizus*, *Macropsychanthus*, *Luzonia*, *Camptosema*, *Cratylia*, *Collaea*, *Galactia*, *Calopogonium*, and *Herpyza*) and suggested that three, *Calopogonium*, *Herpyza* and *Pachyrhizus*, were likely to be improperly placed in the subtribe. Phylogenetic studies of Phaseoleae, based on chloroplast DNA (cpDNA) restriction-site mapping of the inverted repeated regions, showed that *Calopogonium* and *Pachyrhizus* arose within the Glycininae clade, outside Diocleinae (Doyle and Doyle, 1993). Based on these findings Polhill (1994) transferred *Calopogonium* and *Pachyrhizus* from the subtribe Diocleinae to Glycininae. More recently, a phylogenetic study of Glycininae using cpDNA rps16 intron sequences has also shown that Glycininae is monophyletic if

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Calopogonium and *Pachyrhizus* are included within Glycininae (Lee and Hymowitz, 2001). However, the study by Lee and Hymowitz did not include DNA sequence information from the true Diocleinae genera. On the other hand, it is known that phylogenetic hypotheses based on a single gene or character may not represent true organismal relationships. Therefore, it is crucial to evaluate additional genes for phylogeny reconstruction to test topologies based solely on chloroplast genes, to obtain additional resolution, and to elucidate relationships at a variety of taxonomic levels (Kuzoff et al., 1998). Although cpDNA sequences have been widely used in plant phylogeny, ribosomal RNA (rRNA) coding sequences (rDNA) have also been shown to be informative at distinct taxonomic levels, depending on the region investigated (Baldwin et al., 1995; Kuzoff et al., 1998). Thus, the nuclear ribosomal DNA (nrDNA) internal transcribed spacer region (ITS/5.8S) has been shown to provide informative sites for phylogenetic analysis in numerous plant families, including Leguminosae (Hu et al., 2002; Käss and Wink, 1997).

In the present work, we have sequenced the entire ITS/5.8S region of eight representative genera of Diocleinae sensu Lackey (1981). The goal of our work was to provide further evidence on the delimitation of the subtribe Diocleinae.

2. Results and discussion

The ITS region of nrDNA comprising both ITS sequences (ITS1 and ITS2), the 5.8S rDNA, the 3' end of 18S rDNA and the 5' end of 26S rDNA was amplified by PCR from 17 taxa belonging to *Canavalia*, *Camptosema*, *Cratylia*, *Dioclea*, *Cymbosema*, and *Galactia*; of the true Diocleinae as well as from the two pseudo Diocleinae genera, *Calopogonium* and *Pachyrhizus* (Table 1). The complete sequences of the PCR products generated in our laboratory were deposited in GenBank (accession numbers AY293832–AY293847, and AY343546). The DNA sequences from *Galactia striata* Urb. and *Canavalia brasiliensis* Mart. ex Benth. were retrieved from GenBank (accession numbers AF467049 and AF467034, respectively) and also included in the phylogenetic analyses. The length of the fully assembled ITS/5.8S region ranged from 613 bp (in *D. guianensis*) to 695 bp (in *C. caeruleum*), with an average length of 642 bp. ITS1 length varied from 229 bp (in *C. boliviana*) to 297 bp (in *C. caeruleum*), while ITS2 length ranged from 202 bp in *C. mucunoides* to 222 bp in *C. pedicellatum*. The above values agree with those reported for several plant species, including the Leguminosae (Baldwin et al., 1995). In all taxa examined, the 5.8S rDNA sequence had a constant length of 165 bp, which is similar to the values (162–164 bp) that have been

reported in other plant species (Yokota et al., 1989). The multiple aligned sequences of the entire ITS/5.8S region had 750 characters, with 309 sites (41.2%) being conserved, 406 sites (54.1%) were variables, and 95 sites (12.7%) were unique to individual taxa. Within the whole region, ITS segments are highly variable, with only 12.8 and 28% of sites being conserved in ITS1 and ITS2, respectively. The pairwise genetic distances based on the Tamura–Nei substitution model were calculated, and upon inspection of the matrix (data not shown), one could recognize two main groups, one comprising the true Diocleinae, which has an overall mean distance of ca. 0.146, and a second one composed by the *Calopogonium* and *Pachyrhizus* species, with an overall average distance of ca. 0.103. However, the mean distance between the *Calopogonium*–*Pachyrhizus* complex and the true Diocleinae is ca. 0.383, which is ca. 1.5-fold the distance between the most divergent sequences in the true Diocleinae group. Although the 5.8S sequence was highly conserved among the taxa studied, a more careful inspection of its multiple alignments (Fig. 1) allowed us to identify two sites that were diagnostic for these two groups. Therefore, all true Diocleinae 5.8S sequences showed an adenine (A) at nucleotide 55 whereas in the *Calopogonium*–*Pachyrhizus* complex sequences, there was a guanine (G) in the same site. In a second site (nucleotide 136), a thymine (T) was found in all true Diocleinae sequences whereas a cytosine (C) was observed for the *Calopogonium*–*Pachyrhizus* complex sequences. Indeed the topology of the neighbour-joining (NJ) tree obtained from the Tamura–Nei genetic distances (Fig. 2) shows that these two major clades (the true Diocleinae and the *Calopogonium*–*Pachyrhizus* complex) are very well supported as monophyletic. Within the true Diocleinae clade, two very well supported monophyletic subclades are: a clade containing the *Canavalia* and another containing the *Dioclea*. A third, moderately supported subclade within the true Diocleinae clade contains the *Camptosema*–*Cratylia*–*Galactia* complex. Within the second major clade, *Calopogonium* and *Pachyrhizus* are highly supported as monophyletic.

Three of the most parsimonious trees were generated by maximum parsimony (MP) analysis of the ITS/5.8S region sequences, and the bootstrap consensus tree is shown in Fig. 3. This analysis included 526 parsimony-informative sites and each generated tree has a length of 630 steps, a consistency index (CI) of 0.679, a retention index (RI) of 0.785, and a rescaled consistency index (RC) of 0.533. The three most parsimonious MP trees differed basically in the relative positions of *Canavalia* species belonging to subgenus *Canavalia*. The overall topology of the consensus MP tree (Fig. 3) is essentially similar to that produced by NJ analysis (Fig. 2). This same general topology was also observed in the maximum-likelihood (ML) phylogenetic tree (Fig. 4). Differences between NJ,

Table 1
Origin of plant samples used in the ITS/5.8S sequencing study

Taxon	Origin of samples	Voucher specimen numbers ^a
<i>Calopogonium caeruleum</i>	Parque Nacional de Ubajara, Ceará	EAC 28475
<i>Calopogonium mucunoides</i>	Campus do Pici-UFC, Fortaleza, Ceará	EAC 31697
<i>Camptosema pedicellatum</i>	Chapada do Araripe, Crato, Ceará	EAC 29770
<i>Canavalia boliviana</i>	Arquipélago de Anavilhasnas, Amazonas	EAC 24200
<i>Canavalia gladiata</i>	Campus do Pici-UFC, Fortaleza, Ceará	EAC 32586
<i>Canavalia grandiflora</i>	São Benedito, Ceará	EAC 25326
<i>Canavalia bonariensis</i>	Pelotas, Rio Grande do Sul	EAC 13393
<i>Cymbosema roseum</i>	Arquipélago de Anavilhasnas, Amazonas	EAC 24199
<i>Cratylia argentea</i>	Campus do Pici-UFC, Fortaleza, Ceará	EAC 27356
<i>Dioclea</i> sp.	Mucambo, Ceará	EAC 30896
<i>Dioclea guianensis</i>	Campus do Pici-UFC, Fortaleza, Ceará	EAC 30076
<i>Dioclea bicolor</i>	Chapada do Araripe, Crato, Ceará	EAC 28286
<i>Dioclea megacarpa</i>	Campus do Pici-UFC, Fortaleza, Ceará	EAC 29277
<i>Dioclea virgata</i>	Viçosa, Ceará	EAC 27783
<i>Galactia latisiliqua</i>	Campus da UENF, Campos, Rio de Janeiro	EAC 32587
<i>Pachyrhizus erosus</i>	INPA, Manaus, Amazonas	EAC 32588
<i>Pachyrhizus tuberosus</i>	INPA, Manaus, Amazonas	EAC 32589

^a Voucher specimens were deposited in the Herbarium Prisco Bezerra-UFC, Fortaleza-Ceará, Brasil.

MP and ML trees concerned mainly the bootstrap values at some nodes, which showed slight variations from tree to tree. For instance, the clade comprising the *Camptosema–Cratylia–Galactia* complex is supported by bootstrap values of 81, 83 and 93 in the NJ, MP and ML trees, respectively (Figs. 2–4). Felsenstein (1985) suggested that a bootstrap $\geq 95\%$ should be taken as robust support for monophyletic groupings. However, Hillis and Bull (1993) found, using empirical data and computer simulations, that almost every internal branch with a bootstrap value 80% defined a true clade. Therefore, taking into account these findings, the *Camptosema–Cratylia–Galactia* cluster is equally supported by the three methods used for the phylogenetic inference.

According to Lackey (1981), although Diocleinae is presumably natural, the subtribe is difficult to define except in a negative way. He noted that most characters do not give clear-cut indication of internal structure or some generic limits. However, one could recognize a general trend, from the primitive *Dioclea* and allies, which show a woody or coarse habit, through a series of intermediate genera terminating with *Galactia*, that has a more delicate habit, with smaller seeds with short hila. The NJ, MP and ML trees (Figs. 2–4) constructed using the ITS/5.8S region sequences of most Diocleinae genera (six out of ten) shows that species belonging to the same genus are consistently clustered together. Therefore, the ITS/5.8S sequences have enough informative sites to be useful in defining intrageneric limits of Diocleinae.

For *Dioclea* and *Canavalia*, which were both represented by species belonging to distinct subgenera, the ITS/5.8S sequences also provided resolution at the

infrageneric level (i.e., below the genus level). Maxwell (1969) had recognized three subgenera for *Dioclea*: *Pachylobium*, *Plathylobium* and *Dioclea*, based on characteristics of inflorescence, seeds and habitat. In our analyses, we have representatives for these three subgenera as follows: *D. megacarpa* (subgenus *Pachylobium*), *D. bicolor* (subgenus *Plathylobium*) and *D. guianensis* and *D. virgata* (subgenus *Dioclea*). In the NJ, MP and ML trees (Figs. 2–4) the clustering of *Dioclea* ITS/5.8S region sequences are congruent with the morphologically defined subgenera (Maxwell, 1969), being well supported by high bootstrap values. In addition, *Dioclea* sp. clustered together with *D. megacarpa* (subgenus *Pachylobium*), which is in complete agreement with the seed morphology showed by the species without identification.

In the case of *Canavalia*, Sauer (1964) grouped the species in four subgenera: *Canavalia*, *Catadonia*, *Mauualoa*, and *Wenderothia*. In the present work, species belonging to three of these subgenera were included: *C. bonariensis* (subgenus *Catadonia*), *C. grandiflora* (subgenus *Wenderothia*), and *C. brasiliensis*, *C. gladiata*, *C. boliviana* (subgenus *Canavalia*). As observed for *Dioclea*, the clustering of *Canavalia* ITS/5.8S sequences in the NJ, MP and ML trees (Figs. 2–4) correlates very well with the morphologically recognized subgenera, and these topologies are supported. The basal position of *C. grandiflora* (*Wenderothia*) in the *Canavalia*'s subclade (Figs. 2–4) is supported and agrees with the assumption that it represents the most primitive subgenus of *Canavalia* (Sauer, 1964). On the other hand, Piper (1925) had considered subgenus *Wenderothia* as generically distinct, an assumption that is not supported by the ITS/5.8S data. The ITS/5.8S molecular results

[1]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATGCCC	CAACATC--A	----AANTGG	CGGTGAGA-A	GGGTGAATGT	550	TGGCTTCCCTG
[2]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATGCCC	CAACATC--A	----AANTGG	CGGTGAGA-A	GGGTGAATGT	TGGCTTCCCTG	
[3]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATACCC-	-AATGC--A	----AANTGG	TGTTGAGA-A	GGGTGAATGT	TGGCTTCCCTG	
[4]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TGCACCA	ACACCTTTGA	----AANTGG	TGTTGAGC-A	GGGTGAACGC	TGGCTTCCCTG	
[5]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TGCACCA	ACGCCTTTGA	----AANTGG	TGTCGAGC-A	GGGTGAACGC	TGGCTTCCCTG	
[6]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TGCTCCA	ACACCTTTGA	----AGTTGG	CGTTGAGC-A	GGGTGAATTT	TGGCTTCCCTG	
[7]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATGCCA	ATGCTTTTGA	-ATAATC-GG	TGTTGAGC-A	AGGTGAATGT	TGGCTTCCCTG	
[8]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATGCCA	ATTTCTTTTGA	-ATTATC-GG	TGTTGAGC-A	GGGGGAATGT	TGGCTTCCCTG	
[9]	CGAAGCCATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ATAATGTTTTC	CC-TATACCT	ATGCTTTTTC	CA--ATAAAG	TATTTTGT-G	GGGTGAAGGT	TGGCTTCCCTG	
[10]	CGAAGCCATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATACCT	ATGCTTTTTC	CA--ATAAAG	TATTTTGT-G	GGGTGAAGGT	TGGCTTCCCTG	
[11]	CGAAGCCATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATACCT	ATGCTTTTTC	CA--ATAAAG	TGTTTTGT-A	GGGTGATGT	TGGCTTCCCTG	
[12]	CGAAGCCATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-TATACCT	ATACCTTTTTC	TATAATAAAG	TATCTTTTGT	GGTFAAATGT	TGGCTTCCCTG	
[13]	TGAAACCAATT	AGGTTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACAATGTTTTC	CC-AACGCCA	ACGCCGTTTTC	CACGATC-GG	CGTTTWTAGC	GGGGGAAAGT	TGGCTTCCCTG	
[14]	CAAAGCCATT	AGGCTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACATCGTTTTC	CC-TCCACAC	GCACACACAC	-----TG	TGCAGGAGGA	GGGTGATGT	TGGCTTCCCTG	
[15]	CAAAGCCATT	AGGCTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACATCGTTTTC	CC-TCCACAC	GCACACACAC	-----TG	TGCAGGAGGA	GGGTGATGT	TGGCTTCCCTG	
[16]	CAAAGCCATT	AGGCTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACATCGTTTTC	CC-TC-ACGC	AAAC-TGTGC	-----CG	TGCTG----	-GGTGGAGCG	TGACTTCCCTG	
[17]	CAAAGCCATT	AGGCTGAGGG	CACGCCCTGCC	TGGGTGTCCAC	ACATCGTTTTC	CC-TC-ACGC	AAAAACGTTGC	-----CG	TGCTG----	-GGTGGAGCG	TGACTTCCCTG	
	** ** *	** ** *	*****	*****	*****	**	*	*	**	*	**	*****
[1]	CGAGC-ATGG	TCTCGTGGTT	GGTTGAAAAA	TAAGTCCCTCG	GCGGAATGCT	CCATGATAA-	ATGGTAGTTG	AGTAA--TGC	TCGAGAGCCA-	ATCATGTGTG	660	GCTTCCCT-C-
[2]	CGAGC-ATGG	TCTCGTGGTT	GGTTGAAAAA	TAAGTCCCTCG	GCGGAATGCT	CCATGATAA-	ATGGTAGTTG	AGTAA--TGC	TCGAGAGCCA-	ATCATGTGTG	GCTTCCCT-C-	
[3]	CGAGC-ACGG	TCTCGTGGTT	GGTTGAAAAA	TGAGTCCCTCG	GCGGAATGCT	TCAATGATAA-	ATGGTAGTTG	AGTGA--TGC	TCGAGAGCCA-	ATCATGTGTG	GCTTCCCT-C-	
[4]	CGAGC-GTGG	CCTCGCGGTT	GGTTGAAAAA	TGAGTCCCTCG	GTGGAATGCT	CCATGACCA-	ATGGTAGTTG	AGTGA--TGC	TCGAGAGCCA-	ATCATGGTGT	GCTTCCCT-T-	
[5]	CGAGC-GTGG	CCTCGCGGTT	GGTTGAAAAA	TGAGTCCCTCG	GTGGAATGCT	CCATGACCA-	ATGGTAGTTG	AGTGA--TGC	TCGAGAGCCA-	ATCATGGTGT	GCTTCCCT-T-	
[6]	CAAGC-ATGG	TCTTGTGGTT	GGTTGAAAAA	TGAGTCCCTTG	GTGGAATGCT	CCATGATGA-	ATGGTAGTTG	AGTGA--TGC	TCGAGAGCCA-	ATCATGTGTG	GCTTCCCTAT-	
[7]	CGAGC-ATCG	TCTCGTGGTT	GGTTGAAAAA	TGAGTCCCTCG	ATGGAATGCT	TCAATGATAA-	ATGGTAGTTG	AGTCA--TGC	TCGAGAGCCA-	AAATGTGTG	GCTTCCCTTC-	
[8]	CGAGC-ATCG	TCTCGTGGTT	GGTTGAAAAA	TGAGTCCCTCG	GCGGAATGCT	CCATGATAAG	ATGGTGTGTTG	AGTGA--TGC	TCGAGATCT-	ATCATGTGTG	GCGTCCCTGG	
[9]	TAAGC-ATTG	CCTTGTGGTT	GGTTGAAAAA	TGAGTCCCTTG	GTGGAATGCT	CCATGATAA-	ATGGTAGTTG	AGTGA--TCC	TCGAGAGCCA-	ATCATGCATG	GTTTCCCTCT-	
[10]	TAAGC-ATTG	CCTTGTGGTT	GGTTGAAAAA	TGAGTCCCTTG	GTGGAATGCT	CCATGATAA-	ATGGTAGTTG	AGTGA--TCC	TCGAGAGCCA-	ATCATGCATG	GTTTCCCTCT-	
[11]	CAAGC-ATTG	TCTTGTGGTT	GGTTGAAAAA	TGAGTCCCTTG	GTGGAATGCT	CCATGATAA-	ATGGTAGTTG	AGTGA--TGC	TCGAGAGCCA-	ATCATGCATG	GCTTCCCTCT-	
[12]	CAAAGTTGTTG	TCTTGTGGTT	GGCTGAAAAA	TGAGTCCCTTG	GTGGAATGCT	CCATGATAA-	ATGGTAGTTG	AGTGA--TCC	TCGAGAGCCA-	ATCATGCATG	GCTTCCCTAT-	
[13]	CGAGC-TCGT	TCCCGTGGTT	GGTTGAAAAA	TGAGTCCCTCG	GTGGAATGCT	CCATGAGAA-	ATGGTGTGTTG	AGTGA--TGC	TCGAGAGCCA-	AAATGCCTG	GCTTCCCTCC-	
[14]	CGAGC-ACCG	ACTCGCGGTT	GGTTGAAAAA	CGAGTTCGCG	ACAGTCTGCG	TCCGTGATAA	ATGGTGGATG	GGCAACATG	TCGAGAGCCG-	ATCACACGGG	ACATGTC----	
[15]	CGAGC-ACCG	ACTCGCGGTT	GGTTGAAAAA	CGAGTTCGCG	ACAGTCTGCG	TCCGTGATAA	ATGGTGGATG	GGCAACATG	TCGAGAGCCG-	ATCACACGGG	ACATGTC----	
[16]	TGAGC-ACCG	ACTCGCGGTT	GGTTGAAAAA	TTAGTCCACG	GCCGTCTGCG	TCCGTGATCAA	ACGGTGGATG	GGCAACACG	TCGAGAGCCA-	ATCACACGGG	ACGGCC----	
[17]	CGAGC-ACCG	ACTCGCGGTT	GGTTGAAAAA	TTAGTCCACG	GCTGTCTGCG	TCCGTGATCAA	ATGGTGGATG	GGCAACATG	TCGAGAGCCG	ATCACACGGG	ACACGTC----	
	** ** *	** ** *	*****	*****	*****	**	*	*	**	*	**	*****

Fig. 1 (continued)

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[1]	CTAAACGTGG	GCC-----TT	GACCCTTGCA	TTTTCT--TA	AGAATGCTCA	TAT
[2]	CTAAACGTGG	GCC-----TT	GACCCTTGCA	TTTTCT--TA	AGA-TGCTCA	TAT
[3]	CTAAATGTGG	GCT-----TT	GACCCTTGCG	TTTTCT--TG	AGAACGCTCA	TAT
[4]	CCAAAGGAGG	GCC-----TT	GACCCTTGCG	TTTTTTG-CG	AAAACGCTCA	TAT
[5]	CTAAAGGAGG	GCC-----T-	GACACTTGCG	TTTTTT--CG	AAAACGCTCA	TAT
[6]	CTAAATGTGG	GCC-----TT	GACCCTTACG	TTTTCT--TG	AAAACGTTCa	TAT
[7]	-TAAATGTGG	GAC---CTTT	GACCCTTGCG	TTCTCTTAC-	AAC--GCTCA	CAA
[8]	CTGTATCCGG	GTCGCTCTTT	ACCGCCCGCG	TTCTCCTAC-	AATCTGTTCA	CTA
[9]	-CACATTTGG	ACC---CTG	ATCCCTTGAG	TTTTCTCT--	AGAACACTCA	TAA
[10]	-CACATTTGG	ACC---TTG	ACCCCTTGAG	TTTTCTTT--	AGA-CACTCA	TAA
[11]	-TACAATTGG	ACC---TTG	ACCCTGTGTT	CTCTCTTTTCG	AGAACACTCA	CAA
[12]	-TAAATGTGG	ACT---TTT	GACCCTTGCG	TTTT-----	AGAACACTCG	ATA
[13]	--TAAGGTGG	ACG---TTCG	ACCCCTTGCG	TCCTAAAAC-	-GACGCTCA	CAA
[14]	--GATTTCCG	ACT---CTTT	GATCCCTGTG	CGTCT----G	TGGACGCTCT	TCA
[15]	--GATTTCCG	ACT---CTTT	GATCCCTGTG	CGTCT----G	TGGACGCTCT	CA-
[16]	--GCTCCTGG	ACT---TGCT	GATCCCCACG	CGTCTT--TG	TGCACGCTCT	CAA
[17]	--GTTTCCGG	ACT---CACT	GATCCCCACG	CGTCTT--TG	TGCACGCTCT	CAA

* * * * *

Fig. 1 (continued)

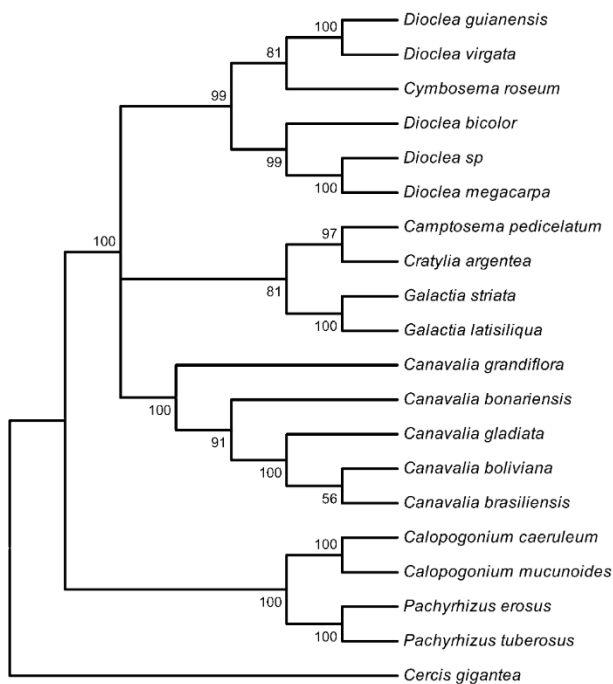


Fig. 2. Neighbour-joining (NJ) tree based on the Tamura–Nei distance algorithm from ITS/5.8S sequences from Diocleinae and the anomalous *Calopogonium* and *Pachyrhizus* genera. *Cercis gigantea* (Caesalpinioideae) was used as the outgroup. Bootstrap values (500 replicates) are shown at each node.

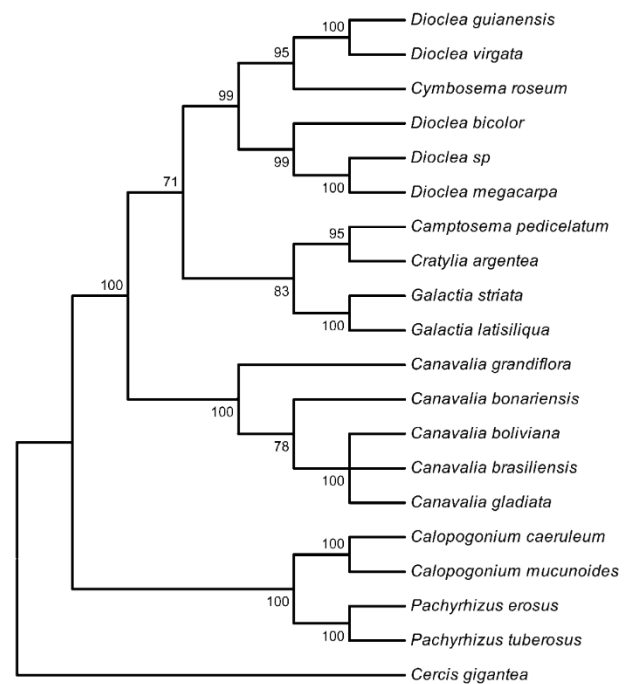


Fig. 3. Consensus maximum parsimony (MP) tree derived from ITS/5.8S sequences from Diocleinae members and the anomalous *Calopogonium* and *Pachyrhizus* genera. *Cercis gigantea* (Caesalpinioideae) was used as the outgroup. Bootstrap values (500 replicates) are shown at each node.

support previous leaflet anatomy studies on Phaseoleae that showed that all *Canavalia* (including *Wenderothia*) consistently share the presence of epidermal crystals which are distributed in a distinctive paired arrangement (Lackey, 1978). This particular anatomic trait has been considered a unifying trend for this genus (Lackey, 1981).

Lackey (1977a) had questioned the maintenance of the monotypic Brazilian genus *Cymbosema* as a distinct genus from *Dioclea*. In his words, “*Cymbosema* differs

from *Dioclea* by the free vexillary stamen”. Pollen morphology analyses placed *Cymbosema* with the *Dioclea* subgenus (Kavanagh and Ferguson, 1981). In the NJ, MP and ML trees obtained using ITS/5.8S sequences (Figs. 2–4), *Cymbosema* arose as a sister taxon of subgenus *Dioclea*, inside the *Dioclea* clade. This shows that *Dioclea* is not monophyletic. Therefore to maintain the monophyly of *Dioclea*, *C. roseum* should be treated as a *Dioclea* species. To our best knowledge, this is the first molecular sequence that shows the close relationship

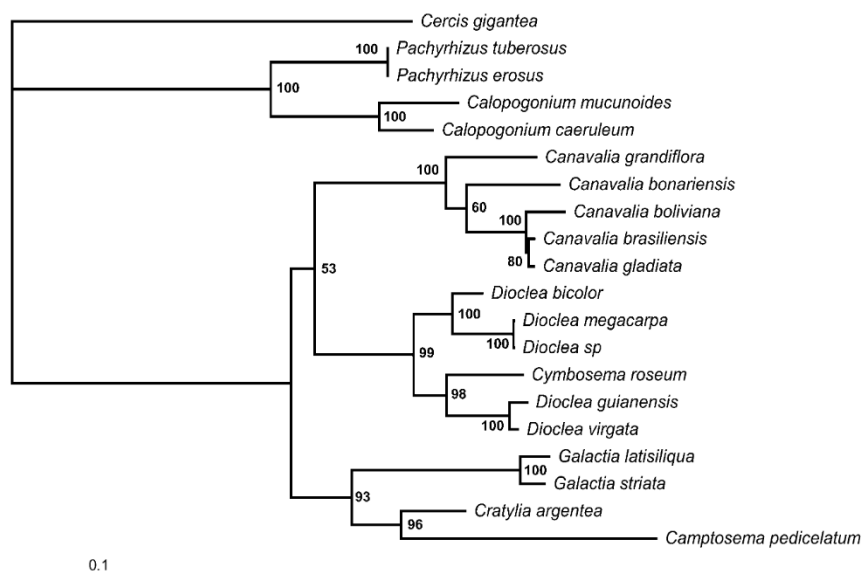


Fig. 4. Maximum likelihood (ML) tree derived from ITS/5.8S sequences from Diocleinae members and the anomalous *Calopogonium* and *Pachyrhizus* genera. *Cercis gigantea* (Caesalpinioideae) was used as the outgroup. Bootstrap values (100 replicates) are shown at each node. The scale bar represents the branch lengths (0.1 nucleotide substitutions per site).

between *Cymbosema* and *Dioclea*, providing additional support for the previous morphological studies (Lackey, 1977a; Kavanagh and Ferguson, 1981). It is apparent that a revision about the status of *Cymbosema roseum* is needed in the light of these data.

In the NJ, MP and ML trees (Figs. 2–4), the two *Galactia* species form a cluster, which arises as a sister group of a subclade comprising *Camptosema* and *Cratylia*. The *Camptosema*–*Cratylia*–*Galactia* complex is also supported suggesting a common ancestor. Existing taxonomic evidence shows that *Camptosema*, *Cratylia* and most of the *Galactia* species have similar pollen types (Kavanagh and Ferguson, 1981). It is also noteworthy that some species that were described before as belonging to *Camptosema* have been transferred to either *Cratylia* or *Galactia* genera (Queiroz, 1999).

Lackey (1981) realized that the subtribe Diocleinae was difficult to define except in a negative way. Although he had included *Calopogonium* and *Pachyrhizus* in Diocleinae (Lackey, 1977b), he questioned whether these genera might be improperly placed in this subtribe on the basis of some anomalous morphological characters, such as the peculiar stigma-style structure of *Pachyrhizus* and the anomalous somatic chromosome number ($2n = 36$) of *Calopogonium*. In addition, because he could not recognize any distinctive features that could be used to place them elsewhere, they were therefore retained in the subtribe. The phylogeny derived from ITS/5.8S region sequences reported here does support the exclusion of *Calopogonium* and *Pachyrhizus* from Diocleinae sensu Lackey (1981) by Polhill (1994). The segregation of *Calopogonium* and *Pachyrhizus* from

Diocleinae in the ITS/5.8S phylogeny is congruent both with morphological and phytochemical data. Kavanagh and Ferguson (1981) examined the pollen morphology of the 13 genera included in the subtribe Diocleinae. They found that pollen type clearly separates the genera *Calopogonium* and *Pachyrhizus* from the rest of the subtribe. Ingham (1990) carried out a survey of phytoalexins within the tribe Phaseoleae, and reported that *Calopogonium* and *Pachyrhizus* produce phytoalexins sufficiently distinct from other Diocleinae. *Calopogonium* and *Pachyrhizus* produce complex isoflavonoids of various types that are also found in many representatives of the Phaseoleae subtribes Glycininae, Kennediiinae and Phaseolinae. Indeed the inclusion of *Calopogonium* and *Pachyrhizus* in subtribe Glycininae has been supported by a restriction-site mapping study of the cpDNA inverted repeat region (Doyle and Doyle, 1993) as well as by a phylogenetic study based on cpDNA rps 16 intron sequences (Lee and Hymowitz, 2001). To test this hypothesis using the nuclear sequence information, a MP tree was constructed including ITS/5.8S sequences from Glycininae species available in GenBank at the time of this study. Sequences of only three Glycininae genera were available (*Amphicarpea*, *Glycine* and *Pueraria*), and representative species for each genus were randomly chosen (GenBank accession numbers AF338215, AF417013, AF417017, GMU60551 and AF023444). To give the hypothesis on the phylogenetic affiliation of *Calopogonium* and *Pachyrhizus* a stronger support, ITS/5.8S sequences from the subtribe Phaseolinae were also included in the ingroups (GenBank accession numbers AF115135, AF115137–AF115139,

AF069120, AF069114–AF069117, AF115239, AF009125, AF069123, AF069122). Twenty-four most equally parsimonious trees were generated, and the bootstrap consensus tree is shown in Fig. 5. Each generated tree has a length of 806 steps, a CI of 0.481, a RI of 0.785, and a RC of 0.378. The main finding of this analysis is that the *Calopogonium*–*Pachyrhizus* complex arises as a sister group of the Glycininae genera, and the Glycininae clade (including *Calopogonium* and *Pachyrhizus*) is very well supported. Therefore, the phylogeny produced by the nuclear ITS/5.8S sequence (herein) is congruent to that obtained using a cpDNA gene sequence (Lee and Hymowitz, 2001).

In conclusion, the ITS/5.8S phylogeny reported in this work unambiguously supports the hypothesis that *Calopogonium* and *Pachyrhizus* do not belong to subtribe Diocleinae. The nuclear sequence data suggest that these genera are more closely related to Glycininae species. In addition, the ITS/5.8S sequences from the true Diocleinae also provide informative sites to resolve relationships below the subtribe level.

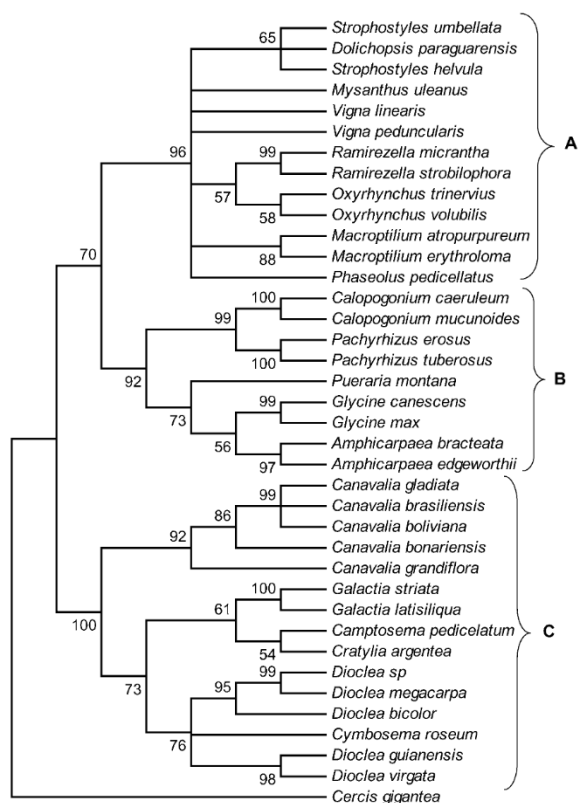


Fig. 5. Consensus maximum parsimony (MP) tree derived from ITS/5.8S sequences showing the close relationship of *Calopogonium* and *Pachyrhizus* genera with some Glycininae members (clade B). Sequences from subtribe Phaseolinae (clade A) were also included. The subtribe Diocleinae clade is indicated by C. *Cercis gigantea* (Caesalpinioideae) was used as the outgroup. Bootstrap values (500 replicates) are shown at each node.

3. Experimental

3.1. Plant material

Mature seeds were harvested from plants growing wild in different localities of the states of Ceará, Amazonas and Rio Grande do Sul, in Brasil. Seeds were stored at 4 °C until used. The species were: *Calopogonium caeruleum* Hemsl., *C. mucunoides* Desv., *Camptosema pedicellatum* Benth., *Canavalia gladiata* DC., *C. grandiflora* Benth., *C. bonariensis* Lindl., *C. boliviana* Piper, *Cratylia argentea* (Desv.) Kuntze, *Cymbosema roseum* Benth., *Dioclea sp.*, *Dioclea bicolor* Benth., *D. guianensis* Benth., *D. megacarpa* Rolfe, *D. virgata* (L.C.Rich.) Amshoff, *Galactia latisiliqua* Desv., *Pachyrhizus erosus* (L.) Urb., and *P. tuberosus* Spreng. Voucher specimens are deposited in the Herbarium Prisco Bezerra, Universidade Federal do Ceará, Fortaleza-Ceará, Brasil. Voucher numbers are shown in Table 1.

3.2. DNA purification

Genomic DNA was purified from fresh leaves of 1 week-old seedlings grown from mature seeds. The protocol for DNA purification uses the CTAB (cetyl triethylammonium bromide) reagent, as described by Foster and Twell (1996). The concentration of DNA in the various samples was determined by measuring the absorbance at 260 nm (A_{260}) of a ten-fold dilution of each sample. The quality of all DNA preparations was checked by 0.8% agarose gel electrophoresis according to Sambrook et al. (1989).

3.3. PCR amplification and DNA sequencing

Amplification reactions were performed in a final volume of 25 μ l containing 500–800 ng of genomic DNA (template), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μ M of each dATP, dCTP, dGTP and dTTP (Amersham Biosciences, Sweden), 5 pmoles of each primer and 0.5 units of Taq DNA Polymerase (Amersham Biosciences, Sweden). PCR reactions were carried out in a MJ-Research Inc. (Watertown, MD) PTC-100 thermocycler programmed for an initial denaturation step (3 min at 94 °C) followed by 45 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The last cycle was followed by a final incubation of 10 min at 72 °C. The samples were then stored at 4 °C until used. Amplified fragments were analyzed by standard horizontal electrophoresis on 1.0% agarose gels in TBE buffer (10 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V. The DNA bands were stained with 0.5 μ g/ml ethidium bromide as described before (Sambrook et al., 1989). Control samples containing all reaction components except DNA were always used to test that no self-amplification or DNA contamination occurred. The

primers used for amplification were ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GCAAGTAAAAGTCGTAACAAGG), as suggested by Becerra and Venable (1999). These primers are complementary to the end of the 18S rDNA and to the beginning of the 26S rDNA, therefore they amplify a fragment of nrDNA containing ITS1, 5.8S rDNA and ITS2.

Sequences of PCR products were determined using the DYEnamic ET terminators sequencing kit (Amersham Biosciences, Uppsala, Sweden) following the protocol supplied by the manufacturer. Sequencing reactions were then analysed in a MegaBACE 1000 automatic sequencer (Amersham). Each PCR product was sequenced four times in both directions using the same primers (ITS4 and ITS5) previously described.

3.4. Sequence alignment and phylogenetic analyses

The quality of DNA sequences was checked and overlapping fragments were assembled using Phred/Phrap/Consed package (<http://www.phrap.org>). The sequence boundaries between the two spacers (ITS1 and ITS2) and the three coding regions (18S, 5.8S and 26S) of nrDNA were determined by comparison with a published sequence from *Vicia faba* (Yokota et al., 1989). Assembled sequences with high quality (phred > 20) were aligned using CLUSTALX (Thompson et al., 1997), with default gap penalties. The corresponding ITS/5.8S sequence from *Cercis gigantea* (Caesalpinioideae) was used as outgroup (GenBank accession number AF390194). The multiple alignments were then used to construct phylogenetic trees using distance matrix (neighbour-joining) and maximum parsimony methods programs of MEGA (Molecular Evolutionary Genetics Analysis) software (Kumar et al., 2001). In the neighbour-joining (NJ) analyses genetic distances were calculated based on the Tamura–Nei algorithm. The choice of the most suitable substitution model to be used for the data set in the NJ analyses was previously calculated using ModelTest program (Posada and Crandall, 1998). Clades stability in NJ and MP trees was assessed by bootstrap analysis (Felsenstein, 1985) with 500 replicates. Maximum likelihood analysis was done with the DNAML program of PHYLIP package (Felsenstein, 1993). A bootstrap analysis of these data was carried out using 100 resampled data sets, which were generated using the SEQBOOT program. PHYLIP's CONSENSE program was then implemented in order to construct a strict consensus tree. Trees generated using PHYLIP were visualized by TreeView (Page, 1996).

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