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, Campsobema, Cratyliia, 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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Keywords: Subtribe Diocleinae; Leguminosae; Phylogenetic analysis; Nuclear ribosomal DNA; ITS/5.8S region

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, Clitorinae, Ophrestiinae, Kennedinae, Erythrininae, 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, Campsobema, Cratyliia, 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 Dicieinae 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 Dicieinae sensu Lackey (1981). The goal of our work was to provide further evidence on the delimitation of the subtribe Dicieinae.

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, Dioleca, Cymbosema, and Galactia; of the true Dicieinae as well as from the two pseudo Dicieinae 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. mucronoides 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 Dicieinae, 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 Dicieinae is ca. 0.383, which is ca. 1.5-fold the distance between the most divergent sequences in the true Dicieinae 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 Dicieinae 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 Dicieinae 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 Dicieinae and the Calopogonium–Pachyrhizus complex) are very well supported as monophyletic. Within the true Dicieinae clade, two very well supported monophyletic subclades are: a clade containing the Canavalia and another containing the Dioleca. A third, moderately supported subclade within the true Dicieinae 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 differ 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,
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 *Camptosoma*--*Cratyxila*--*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 *Camptosoma*--*Cratyxila*--*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 *Dioclea*.

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*: Pachyllobium, Plathyllobium and Dioclea, based on characteristics of inflorescences, seeds and habitat. In our analyses, we have representatives for these three subgenera as follows: *D. megacarpa* (subgenus Pachyllobium), *D. bicolor* (subgenus Plathyllobium) 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 Pachyllobium), 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, Mau-naloa, 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

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Origin of samples</th>
<th>Voucher specimen numbersa</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Calopogonium cereum</em></td>
<td>Parque Nacional de Ubajara, Ceará</td>
<td>EAC 28475</td>
</tr>
<tr>
<td><em>Calopogonium nucensides</em></td>
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<td>EAC 31697</td>
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<td><em>Camptosoma pedicellatum</em></td>
<td>Chapada do Arraípe, Crato, Ceará</td>
<td>EAC 29770</td>
</tr>
<tr>
<td><em>Canavalia boliviensis</em></td>
<td>Arquipélago de Anvilhasnas, Amazonas</td>
<td>EAC 24200</td>
</tr>
<tr>
<td><em>Canavalia gladiata</em></td>
<td>Campus do Pici-UFC, Fortaleza, Ceará</td>
<td>EAC 32586</td>
</tr>
<tr>
<td><em>Canavalia grandiflora</em></td>
<td>São Benedito, Ceará</td>
<td>EAC 25326</td>
</tr>
<tr>
<td><em>Canavalia bonariensis</em></td>
<td>Pelotas, Rio Grande do Sul</td>
<td>EAC 13393</td>
</tr>
<tr>
<td><em>Cymbosorea roseum</em></td>
<td>Arquipélago de Anvilhasnas, Amazonas</td>
<td>EAC 24199</td>
</tr>
<tr>
<td><em>Cratyxila argentea</em></td>
<td>Campus do Pici-UFC, Fortaleza, Ceará</td>
<td>EAC 27356</td>
</tr>
<tr>
<td><em>Dioclea sp.</em></td>
<td>Mucambo, Ceará</td>
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<tr>
<td><em>Dioclea guianensis</em></td>
<td>Campus do Pici-UFC, Fortaleza, Ceará</td>
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<td><em>Dioclea bicolor</em></td>
<td>Chapada do Arraípe, Crato, Ceará</td>
<td>EAC 28286</td>
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<tr>
<td><em>Dioclea megacarpa</em></td>
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<td>EAC 29277</td>
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<tr>
<td><em>Dioclea virgata</em></td>
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<td>EAC 27783</td>
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<td>EAC 32587</td>
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<td><em>Pachyrhizus erosus</em></td>
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<td>EAC 32588</td>
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<tr>
<td><em>Pachyrhizus tuberosus</em></td>
<td>INPA, Manaus, Amazonas</td>
<td>EAC 32589</td>
</tr>
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</table>

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*a* Voucher specimens were deposited in the Herbarium Prisco Bezerra-UFC, Fortaleza-Ceará, Brasil.
Fig. 1 (continued)
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
between *Cymbosoma* and *Dioeclea*, providing additional support for the previous morphological studies (Lackey, 1977a; Kavanagh and Ferguson, 1981). It is apparent that a revision about the status of *Cymbosoma 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 Dioeleinae was difficult to define except in a negative way. Although he had included *Calopogonium* and *Pachyrhizus* in Dioeleinae (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 Dioeleinae sensu Lackey (1981) by Polhill (1994). The segregation of *Calopogonium* and *Pachyrhizus* from Dioeleinae 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 Dioeleinae. 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 Dioeleinae. *Calopogonium* and *Pachyrhizus* produce complex isoflavonoids of various types that are also found in many representatives of the Phaseoleae subtribes Glycininae, Kennediinae 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 (*Amphicarpaea*, *Glycine* and *Puercaria*), 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* stronger support, ITS/5.8S sequences from the subtribe Phaselinae were also included in the ingroups (GenBank accession numbers AF115135, AF115137-AF115139,
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 Hems., C. mucunoides Desv., Campsotoma pedicellatum Benth., Canavalia gladiata DC., C. grandiflora Benth., C. bonariensis Lindl., C. boliviana Piper, Cratylia argentea (Desv.) Kuntze, Cymbosera roseum Benth., Dioclea sp., Dioclea bicolor Benth., D. guianensis Benth., D. megacarpa Rolfe, D. virgata (L.C.Rich.) Amshoff, Galactia laitisiliqua Desv., Pachyrhizus erosus (L.) Urb., and P. tuberosus Spreng. Voucher specimens are deposited in the Herbarium Prisco Bezerra, Universidade Federal de 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 trimethylammonium 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 (A260) 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 µl containing 500–800 ng of genomic DNA (template), 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 100 µM 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 µg/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.
primers used for amplification were ITS4 (TCCCTCCGCTATTGATATGC) and ITS5 (GCAAGTAAAAAGTCGATAACAAGG), 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 (Amer sham Biosciences, Upsala, 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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