A settled sub-family for the orphan tree: The phylogenetic position of the endemic Colombian genus *Orphanodendron* in the Leguminosae

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Abstract. *Orphanodendron* is a taxonomically and geographically isolated South American genus of two species. When first described by Barneby and Grimes in 1990, the genus was placed in Leguminosae subfamily Caesalpinioideae, but that placement was doubted and the name *Orphanodendron* (Gr. orphanos, orphan + dendron, tree) was chosen to reflect the uncertain subfamilial relationship of the genus. In this study, nucleotide sequence data from five *Orphanodendron* specimens were added to 662 other, previously sampled, Leguminosae taxa representing all three currently recognized subfamilies (Caesalpinioideae, Mimosoideae and Papilionoideae) in a *matK* maximum parsimony analysis that resolved *Orphanodendron* as a member of the genistoid s.l. clade of subfamily Papilionoideae. Two additional Bayesian phylogenetic analyses with reduced taxon sampling of plastid (*matK* combined with *trnL-F*) and nuclear (ITS) loci strongly support the monophyly of *Orphanodendron* and unambiguously establish *Orphanodendron* as a member of the genistoid sensu lato clade. Although our plastid phylogenetic analysis finds relatively low support for a sister-group relationship with the African genus *Camoensia*, the nuclear-encoded ITS resolves *Orphanodendron* as sister to the Bowdichia clade with strong support and *Camoensia* as sister to other core genistoids. The phylogenetic resolution of *Orphanodendron* as a member of the genistoid s.l. legumes based on nuclear and plastid sequences will undoubtedly advance future evolutionary investigations of this Colombian endemic tropical tree genus.

Keywords: Colombia, Fabaceae, genistoid clade, *matK*, Papilionoideae, phylogenetic analysis.

Resumen. *Orphanodendron* es un género taxonómicamente y geográficamente aislado de Sur América. Cuando se describió por primera por Barneby y Grimes en 1990, el género, fue ubicado dentro de las Leguminosae subfamilia Caesalpinioideae, pero su posición fue incierta y el nombre *Orphanodendron* (del griego orphanos, huérfano + dendron, árbol) fue elegido para reflejar la afinidad incierta relación del género dentro de la subfamilia. En este estudio, datos de la secuencia nucleotidica de cinco especímenes de *Orphanodendron* fueron adicionados a otros 662 taxa de leguminosas previamente muestreados que representan las tres subfamilias actualmente reconocidas (Caesalpinioideae, Mimosoideae y Papilionoideae) en un análisis de máxima parsimonia en *matK* que resolvió a *Orphanodendron* como un miembro del clado genistoide s.l. de la subfamilia Papilionoideae. Dos análisis filogenéticos Bayesiano adicionales con un reducido número de taxones muestreados por loci de plastidio

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(*matK* combinado con *trnL-F*) y nuclear (ITS) soportaron fuertemente la monofila de *Orphanodendron* y una estabilidad sin ambigüedades de *Orphanodendron* como miembro del clado genistoides *sensu lato*. Aunque nuestro análisis filogenético del plastidio encontró un soporte relativamente bajo para una relación de grupo–hermano con el género africano *Camoensia*, la decodificación nuclear ITS resolvió a *Orphanodendron* como hermano del clado Bowdichia con un soporte fuerte y a *Camoensia* como hermano para otro core genistoide. La resolución filogenética basada en plastídio y núcleo de *Orphanodendron* como miembro las leguminosas genistoides s.l. indudablemente será un avance para las futuras investigaciones evolutivas de este género de árbol tropical endémico en Colombia.

Orphanodendron, a genus of Leguminosae endemic to Colombia, was first described by Barneby and Grimes (1990). Currently the genus comprises two species: *Orphanodendron bernalii* Barneby & J. W. Grimes and *O. grandiflorum* C. Cast. & G. P. Lewis. *Orphanodendron bernalii* (Fig. 1-A) is restricted to the Pacific region, in the Departments of Antioquia and Chocó, from sea level up to 400 m above sea level (Lopez–Camacho et al., 2007), whereas *O. grandiflorum* (Fig. 1-B, C) is endemic to the Andean region, in the Departments of Santander and Boyacá, between 600 and 800 m above sea level (Castellanos et al., 2015). The two species of *Orphanodendron* usually grow in riparian forest on low hills or in seasonally flooded savanna, where dense stands may form. Both species are highly valued by the local timber industry. Local inhabitants of these areas use *Orphanodendron* wood to make canes and other handicraft items, to build boundary fences, and timber for framing houses (Lopez–Camacho et al., 2007; Castellanos & Lewis, 2012; Castellanos et al., 2015).

Barneby and Grimes (1990) tentatively placed *Orphanodendron* in subfamily Caesalpinioideae sensu Polhill and Vidal (1981), but they could only speculate as to its generic affinities based on morphology. The genus name *Orphanodendron* aptly described the apparent unsettled position of the genus and Barneby and Grimes commented that: “Attempts to trace the genus through Bentham's Conspectus of the Leguminosae (1865) or through keys to tribes and genera provided by Hutchinson (1964), Cowan (1981), and Polhill and Vidal (1981) quickly run into a blind alley." The authors noted that the species occupied a "precarious place" within the Caesalpinioideae.

Polhill (1994) accepted *Orphanodendron* as a genus of tribe Caesalpinieae and placed it in its own informal Orphanodendron Group. Lewis (2005) only partially retained the informal groups of tribe Caesalpinieae proposed by Polhill and Vidal (1981) and Polhill (1994), and because *Orphanodendron* had yet to be included in any molecular analyses, he included the genus as one of five unplaced genera in tribe Caesalpinieae.

The lack of freshly collected leaf material from which to extract good quality DNA has meant that *Orphanodendron* has remained unanalyzed in any molecular study until now. Due to a lack of available material, Bruneau et al. (2008) did not include *Orphanodendron* in their molecular phylogenetic studies of the Caesalpinioideae based on plastid sequences, nor did Manzanilla and Bruneau (2012) in their study of the Caesalpinieae grade based on duplicated copies of the sucrose synthase gene. In 2013, the LPWG (2013) included *Orphanodendron* in their list of legume genera that lacked any sequence data deposited in GenBank. Castellanos et al. (2015) described a second species of *Orphanodendron* and, based on unpublished molecular analyses, proposed that *Orphanodendron* is best considered a member of subfamily Papilionoideae rather than of subfamily Caesalpinioideae.

This study presents, for the first time, sequence data from both *Orphanodendron* species represented by five different accessions. We first conduct a maximum parsimony phylogenetic analysis with exhaustive taxonomic sampling of plastid *matK* sequences from 674 taxa. Subsequently, Bayesian phylogenetic analyses with more restricted taxon sampling of plastid (*matK* combined with *trnL-F*) and nuclear (ITS) loci were conducted to investigate the lower-level relationships of *Orphanodendron* species within Papilionoideae.

Fig. 1. *Orphanodendron* and *Camoensia*. **A**. Isotype of *Orphanodendron bernalii*, Atrato River basin, Antioquia (*Bernal* et al. *1482,* K; © Royal Botanic Gardens, Kew). **B**. Fruits of *Orphanodendron grandiflorum*, Magdalena River basin, Santander (photo: C. Castellanos). **C**. Flowers of *O. grandiflorum,* Magdalena River basin, Boyacá (photo: William Ariza). **D**. *Camoensia scandens*, cultivated in the Rio de Janeiro Botanic Gardens, (photo: G. P. Lewis).

Methods

Molecular methods

Whole genomic DNA was extracted from 15–20 mg of desiccated leaf tissue from both species of *Orphanodendron* and from two species of *Camoensia*, a putative close relative of *Orphanodendron.* DNA was extracted according to the procedure of Doyle and Doyle (1987). A minor adjustment of the protocol was made at the nucleic acid precipitation step where DNA was precipitated onto paramagnetic beads using a 0.6x ratio of MagNA solution (see supplementary notes of Rohland and Reich, 2012), washed twice with 80% ethanol and eluted in low TE buffer. Two plastid loci, the *matK* exon and the *trnL-F* intron and spacer region, and the nuclear ITS

locus were amplified in 20 μL reaction volumes containing 4.0 μg of BSA, 1x Phusion HF reaction buffer, 200 μM of each DNTP, 0.4 μM of forward and reverse primer, 0.4 units of Phusion Hot Start II DNA polymerase and 1 μL of genomic DNA extract. The PCR reaction protocol consisted of an initial denaturation for 1 min at 98°C; 35 cycles of 10s at 98°C, 25s at 57–60°C, 30s at 72°C; and a final extension for 2 min at 72°C. Oligonucleotide primer sequences and annealing temperatures used for PCR amplification are presented in Table I. Amplification products were visually inspected using 1% agarose gel stained with GelRed and successful amplifications were sequenced at the Genome Quebec Innovation Centre on an Applied Biosystems 3730xl DNA Analyzer.

Phylogenetic analyses

Contigs were assembled and visually inspected using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA), aligned using the ClustalW algorithm (Thompson et al., 1994) in Bioedit (Hall, 1999) and adjusted manually. Insertion-deletion gaps were not coded for inclusion in phylogenetic analyses as they were mostly autapomorphic.

Since the phylogenetic placement of *Orphanodendron* species within the Leguminosae was uncertain, we initially generated a *matK* phylogeny using five newly collected *Orphanodendron* specimens along with 662 Leguminosae taxa representing all three currently recognized subfamilies (Caesalpinioideae, Mimosoideae, and Papilionoideae), as well as seven outgroup taxa from Polygalaceae, Rosaceae, Surianaceae, and Quillajaceae. This preliminary phylogenetic analysis was performed to establish the closest outgroup taxa, to facilitate subsequent nucleotide alignments, and to minimize processing time for the more computationally intensive Bayesian phylogenetic analyses. The maximum parsimony analysis was implemented in PAUPRat on the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal 2.0 (Miller et al., 2010).

We concatenated the *matK* and *trnL-F* intron alignments with SequenceMatrix 1.8 (Vaidya et al., 2011) as they are both on the same non-recombining plastid linkage group. Subsequently, we performed two separate Bayesian phylogenetic analyses of the concatenated plastid and ITS alignments that contained 79 and 63 taxa of Papilionoideae, respectively, including one sequence for each of the two *Orphanodendron* species. In addition, given that the objective of the present study was to evaluate the phylogenetic placement of *Orphanodendron*, we considered it best to not combine the plastid and nuclear data in a single analysis. Mrmodeltest2.3 (Nylander, 2004) was used to select the most suitable nucleotide substitution model for Bayesian analyses under the Akaike Information Criterion for each nucleotide alignment. The nucleotide substitution model GTR+G was selected for the *matK* and *trnL-F* regions, and GTR+I+G was selected for ITS. Individual Bayesian analyses were run for each alignment with the program MrBayes v3.2.2 (Ronquist & Huelsenbeck, 2003). Ten million generations were completed using 4 chains and 2 runs with trees sampled every 1000 generations. Posterior probabilities were estimated using a burn-in of 25,000 trees as log-likelihood values had stabilized after 2.5 million generations. TreeGraph2 (Stöver & Muller, 2010) was used to display and edit consensus trees. All sequences

Table I
Primer sequences and annealing temperatures used in the phylogenetic study of *Orphanodendron*.

Primer	Locus	Sequence 5'-3'	Reference	Annealing temp °C
trnK-685F	*matK*	GTATCGCACTATGTATCATTTGA	Lavin *et al.* (2000)	57
trnK-2RDet	*matK*	ACACGGCTTTCCCTATGTCTAC	Bruneau *et al.* (2008)	57
trnL-C	*trnL-F* intron	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)	57
trnL-F	*trnL-F* intron	ATTTGAACTGGTGACACGAG	Taberlet *et al.* (1991)	57
AB101-mod	ITS	TGAATGGTCCGGTGAAGTGT TCGG	This study	60
BEL-3	ITS	GACGCTTCTCCAGACTACAAT	Chiou *et al.* (2007)	60

generated in this study have been deposited in GenBank (Table II). Alignments, phylogenetic trees and related data have been accessioned in TreeBASE (study no 18227, http://purl.org/phylo/treebase/phylows/study/TB2:S18227).

Results

Maximum parsimony phylogenetic analyses

The preliminary *matK* parsimony analysis resolved both *Orphanodendron* species as nested within the genistoid s.l. clade sensu Cardoso et al. (2012), and members of this more inclusive clade were used for all subsequent Bayesian analyses (Suppl. Material 1).

Bayesian phylogenetic analyses

A *matK* sequence was obtained from each of the five *Orphanodendron* specimens and one *trnL-F* sequence was obtained from each *Orphanodendron* species. The concatenated plastid nucleotide alignment consisted of 79 taxa and 2393 characters (1611 from *matK* and 782 from *trnL-F*). The majority-rule consensus tree derived from the combined plastid Bayesian analysis strongly supported the monophyly of *Orphanodendron* and found relatively weak support (PP = 0.60) for a sister relationship with *Camoensia* (Fig. 2-A), and this analysis also found weak support for these two genera as sister to the core genistoids (PP = 0.58).

Bi-directional ITS sequences were obtained from one specimen of each *Orphanodendron* species. The resulting DNA nucleotide alignment consisted of 94 taxa and 773 characters. The majority-rule consensus tree derived from the ITS Bayesian analysis also strongly supported the monophyly of *Orphanodendron* and placed it as sister to the Bowdichia clade with strong support (PP = 1.0; Fig. 2-B).

Discussion

Orphanodendron is a genus endemic to the Andean region of Colombia and, until recently, it was tentatively placed in the Caesalpinioideae (Barneby & Grimes, 1990). It has not been sampled in any previous molecular phylogenetic studies (e.g., Wojciechowski et al., 2004; Bruneau et al., 2008; Simon et al., 2009; Cardoso et al., 2012; Manzanilla & Bruneau, 2012). Contrary to its provisional placement based on morphology, all of our molecular phylogenetic analyses place the genus in the genistoid s.l. clade sensu Cardoso et al. (2012) of the Papilionoideae. The genistoid s.l. clade circumscribed by Wojciechowski et al. (2004) included the tribes Brongniartieae, Crotalarieae, Genisteae, Euchresteae, Liparieae, Podalyrieae, Thermopsideae and some members of Sophoreae, and appeared to group almost exclusively taxa that produced quinolizidine alkaloids. Cardoso et al. (2012) expanded the taxon sampling to include genera such as *Camoensia* for the first time in molecular phylogenetic analyses. The result was a clear definition, for the first time, of a genistoid s.l. clade that included a core genistoid clade [as defined by Crisp et al. (2000), with *Camoensia* sister to this group], a Bowdichia clade, a Brongniartieae clade, the

Table II
GenBank accession numbers for three loci of five specimens of *Orphanodendron* and three specimens of *Camoensia* sequenced for this study. N/A, sequence data not available.

Species	Collector and #	GenBank Accession #'s		
		matK	trnL	ITS
Orphanodendron bernalii Barneby & J. W. Grimes	*Castellanos 701*	KT718814	N/A	N/A
Orphanodendron bernalii	*Castellanos 703*	KT718815	N/A	N/A
Orphanodendron bernalii	*Cogollo et al. 12796*	KT718816	KT718813	KT718823
Orphanodendron grandiflorum C. Cast. & G. P. Lewis	*Castellanos & Marino-Z 451*	KT718817	N/A	N/A
Orphanodendron grandiflorum	*Castellanos & Cepeda 750*	KT718818	KT718819	KT718824
Camoensia brevicalyx Benth.	*5205*	N/A	N/A	KT718820
Camoensia scandens (Welw.) J. B. Gillett	*459*	N/A	N/A	KT718821
Camoensia scandens	*17109*	N/A	N/A	KT718822

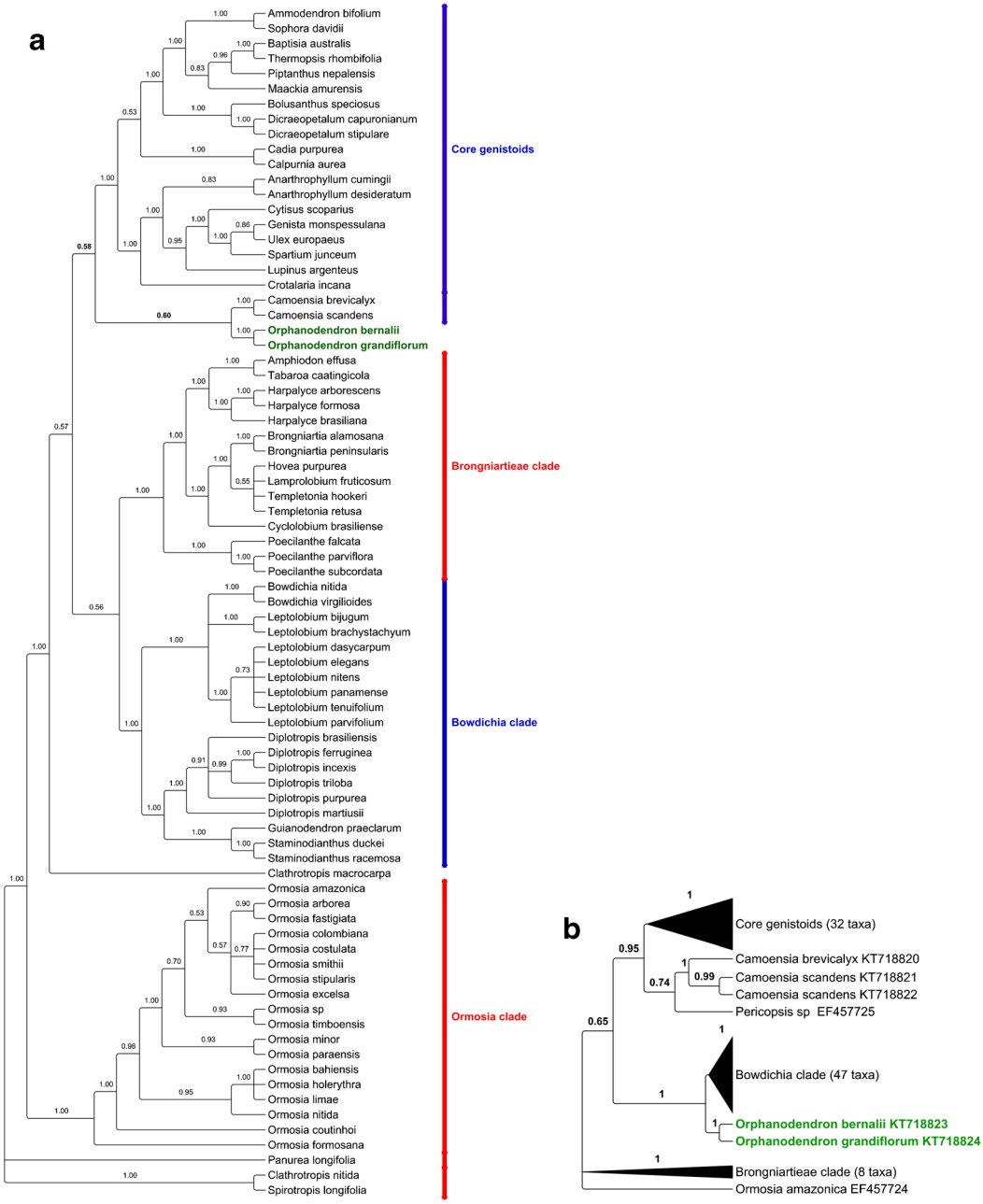

Fig. 2. **A**. Majority-rule ultrametric phylogram derived from combined *matK* and *trnL-F* Bayesian analysis of genistoid s.l. papilionoid legumes. Support values of two nodes with weak support subtending *Orphanodendron* are in bold. **B**. Majority-rule ultrametric phylogram from ITS analysis of 94 taxa. Major clades have been collapsed into single terminals with branch widths proportional to the number of taxa. Posterior probabilities are labeled above branches.

newly recircumscribed genus *Clathrotropis*, and a clade that includes the genus *Ormosia* and other close relatives that produce quinolizidine alkaloids. However, they found poor resolution amongst these diverse sublineages based upon their *trnL* and *matK* data.

Our *matK* parsimony analysis adds to our understanding of this early branching papilionoid

lineage by unambiguously resolving *Orphanodendron* as a member of the genistoid s.l. clade. Our combined *matK* and *trnL-F* Bayesian analyses further resolves that the genus diverged after the Ormosia clade and finds weak support for a sister-group relationship with *Camoensia* spp. and the core genistoids. In contrast to the plastid loci, the nuclear ITS analysis places *Orphanodendron* as sister to the Bowdichia clade. This incongruence between the plastid and nuclear analyses with respect to the lower level relationships of *Camoensia* and *Orphanodendron* could be due to differences in taxon sampling between the analyses, differences in informational content of loci, or differing evolutionary histories of the nuclear and plastid genomes due to incomplete lineage sorting or hybridization. A single nucleotide polymorphism (snp) of *matK* supports the *Camoensia-Orphanodendron* clade (site 1439) and one other snp (site 127) supports the grouping of *Orphanodendron* within the core genistoids. The exclusion of these two positions from the Bayesian analyses results in *Orphanodendron* being placed in a polytomy among the Bowdichia, Brongniartieae and core genistoid clades (tree not shown) and analysis of *trnL-F* on its own (tree not shown) results in the same polytomy. The incongruence of these trees with respect to the placement of *Orphanodendron* within the genistoids s.l. prohibits the deduction of lower-level relationships. It is evident that additional analyses using more informative plastid and nuclear markers are required to confidently establish the lower-level phylogenetic relationships in the genistoid s.l. clade, including *Orphanodendron*.

The African genus *Camoensia* (Fig. 1-D) comprises two species with a combined geographical distribution from west central Africa to Angola. Yakovlev (1972) suggested that *Camoensia* might belong to the Caesalpinioideae, whereas Polhill (1981), based on morphological floral characters, classified the genus in its own monogeneric group within tribe Sophoreae sensu lato. Cardoso et al. (2012), who found *Camoensia* to occur as sister to the core genistoids, noted that it is unusual morphologically in having large flowers with a long hypanthium and free, weakly differentiated petals, characteristics that are not unlike those found in *Orphanodendron* (Castellanos et al., 2015). Although *Orphanodendron* and *Camoensia* are morphologically similar in a number of other respects, such as in having petals that are marginally undulate, flowers that are markedly pedicellate, and dehiscent sub-woody legumes with valves twisting on dehiscence, they differ in other characteristics. For example, leaves of *Orphanodendron* are imparipinnate, whilst those of *Camoensia* are trifoliolate. It is noteworthy that there is a large-flowered species [*Camoensia scandens* (Fig. 1-D) and *Orphanodendron grandiflorum* (Fig. 1-B, C)] and a relatively small-flowered species [*Camoensia brevicalyx* and *Orphanodendron bernalii* (Fig. 1-A)] in each genus, although the flowers of both *Camoensia* species are larger than either of the *Orphanodendron* species, and *C. scandens* has one of the largest flowers in papilionoid legumes.

The inclusion of *Orphanodendron* within the genistoid s.l. clade is also supported by preliminary studies of its chemistry at the Royal Botanic Gardens, Kew (Kite et al., unpubl. data). Like other members of the genistoid s.l. clade that are widely known to accumulate quinolizidine alkaloids (van Wyk, 2003; Wink & Mohamed, 2003), these preliminary analyses reveal that *Orphanodendron* also accumulates quinolizidine alkaloids, and that it is not unlike the type found in *Camoensia* (Polhill, 1981; Waterman & Faulkner, 1982).

Orphanodendron bernalii is known from only two populations in the Atrato river basin, between the departments of Antioquia and Choco (Castellanos & Lewis, 2012) and it has accordingly been listed as "in danger of extinction" under the category: Vulnerable [VU B1ab(iii)] (Lopez–Camacho et al., 2007). The newly described second species, *O. grandiflorum*, is currently known from three populations in the Magdalena and Cauca river basins in the departments of Santander, Boyacá and Antioquia (Castellanos et al., 2015). It currently is not listed in the Red Book of Colombia, but Castellanos et al. (2015) reported excessive local use of this species, putting it under threat, and a conservation assessment of vulnerable seems appropriate.

Orphanodendron has been difficult to taxonomically align because, in part, it displays many general legume features, such as elastically dehiscent fruits and tricolporate pollen, which are widely shared among diverse members of the legume family. Such general features include a weak bilaterally symmetric flower morphology, which has evolved independently many times among legume subgroups including the subclades of Papilionoideae. Weak bilateral floral

symmetry, once considered taxonomically important, is prone to convergent evolution in legumes and is now generally considered a poor indicator of phylogenetic relationships in Papilionoideae (e.g., Pennington et al., 2000; Ramos et al., 2016).

Our phylogenetic analyses of the first plastid and nuclear DNA sequences of *Orphanodendron bernalii* and *O. grandiflorum* establish the monophyly of the genus and that its natural placement resides in the genistoid s.l. clade of papilionoid legumes, rather than in the caesalpinioids. Although this revised subfamily placement appears stable, the sister-group relationships with other genera of the genistoid s.l. papilionoids remains to be more thoroughly resolved. Our results advance the understanding of these endemic Colombian trees and will aid subsequent evolutionary investigations into basal branching lineages of papilionoid legumes.

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