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Gymnopodium toledense (*Polygonaceae*), a new species from Belize resolved by morphology and distance analyses of molecular data

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Abstract: *Gymnopodium floribundum* Rolfe (*Polygonaceae*) is a shrub or a small tree inhabiting the tropical dry forests of Mesoamerica. A detailed examination of herbarium specimens for the taxonomic treatment of the genus for *Flora Mesoamericana* allowed us to find a gathering from Toledo, Belize that showed a different set of morphological characters from those of *G. floribundum*. We then carried out a morphological and molecular study to test if the Toledo plant was a different taxon. Morphological characters such as glandular trichomes on leaves, inflorescences and flowers, as well as longer inflorescences and perianth segments, are unique for the Toledo plant. The delimitation between it and *G. floribundum* was also supported by molecular characters obtained from ITS and LFY nuclear markers. The Toledo plant is described here as a new species, *G. toledense* Ancona & Ortiz-Díaz, based on diagnostic characters of morphology and the ITS and LFY markers. It is endemic to southern Belize, in the biogeographic region of Eastern Central America, and is assessed according to IUCN categories and criteria as Critically Endangered. A morphological diagnosis and description, key, illustration and distribution map for *G. toledense* are included.

Key words: Belize, Central America, endemism, *Flora Mesoamericana*, *Gymnopodium*, ITS, LFY, new species, *Polygonaceae*

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Introduction

Gymnopodium floribundum Rolfe is an important component of the seasonal forests and savannahs of southern Mexico, Guatemala and Belize from sea level up to 1000 m (Miranda 1952; Flores & Espejel 1994; Goodwin & al. 2013). Along its distribution area, *G. floribundum* exhibits variation in shape, size and degree of pubescence of leaf blades, inflorescences, flowers and fruits, and hence two varieties have been described. However, Ortiz-Díaz (1994; unpublished data) recognized only one highly polymorphic species. Phylogenetic studies in the subfamily *Eriogonoideae* confirm the monophyly of *Gymnopodium* Rolfe and place it as a sister group of the tribe *Eriogoneae* (Burke & al. 2010; Burke & Sánchez 2011).

During the review of herbarium specimens for the taxonomic treatment of *Gymnopodium* for the *Flora Mesoamericana* project, a population from Toledo in the

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south of Belize, represented by a gathering made in 1997, showed a set of characters in leaf, inflorescence, flower and fruit different from those described for *G. floribundum*; however, only three specimens of the same population were available. Due to the limited collections, we wanted to look for new characters to support the morphological ones, so we carried out a molecular study using the nuclear markers *ITS1*, *5.8S* and *ITS2* as well as *lfy* (exon2 and intron 2). The *ITS1*, *5.8S* and *ITS2* markers have been used in the last two decades as excellent barcodes for several groups of angiosperms because of their short length and easy amplification (Hoot & Taylor 2001; Lee & al. 2002; Kress & al. 2005; Cowan & al. 2006; Pang & al. 2011; CPBOL 2011; Liu & al. 2014; Michel & al. 2016; Nithaniyal & Parani 2016). Moreover, the *lfy* marker has also been used in phylogenetic studies and species delimitation (Hoot & Taylor 2001), particularly in *Polygonaceae* (Schuster & al. 2011; Sánchez & Kron 2011). This work describes *G. toledense* Ancona & Ortiz-Díaz as a new species using an integrative approach to include diagnostic characters of morphology and multiple DNA loci.

Material and methods

Morphological studies — The present study was based on a morphological and biometrical analyses carried out on material collected by two of the authors and on material preserved in the herbaria BM, CHIP, CICY, HEM, MEXU, MO and UADY (herbarium codes according to Thiers 2018+). All the studied material was compared and measured to the nearest 0.1 mm using the Absolute Digimatic Mitutoyo digital calliper.

Plant selection for molecular studies — Based on the distribution and morphological variation, 29 individuals representing eight populations of *Gymnopodium* were collected by the first author in Mexico. Two further samples of *Gymnopodium* from Belize were obtained from herbarium collections (in MO) to complete the sampling. Unfortunately, we could not find any individuals of *G. toledense* during our field trip in 2016 to re-locate the Toledo population because the landscape there has been drastically transformed since 1997. *Coccoloba uvifera* (L.) L. and *Podopterus mexicanus* Bonpl. were selected as outgroups according to Burke & al. (2010). All Mexican specimens were deposited in the herbarium UADY. For further details see Table 1. Foliar tissue from new leaves was collected from three or four individuals at each population and maintained in silica gel to transport it to the Biodiversity and Ecophysiology Laboratory of the Universidad Autónoma de Yucatán. Then, in the lab, we proceeded to extract and isolated DNA from each sample.

DNA isolation, amplification, sequencing — DNA was purified from 50 mg of foliar tissue of three or four individuals of each Mexican population and from each herbarium specimen from Belizean populations (Table 1) using the DNeasy Plant mini Kit (QIAGEN). Genomic DNA was amplified for *ITS1, 5.8S* and *ITS2*, using ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers from White & al. (1990); and *lfy* (exon2 and intron 2) using lfy2i1 (CCTGCCGACATANTGGCGCATCTTGGGCTT) and lfy23 (TGCAAGGGGTAAGAAGAACGGCCTTGA) primers from Sánchez & Kron (2011). All amplifications were conducted in a 10 µl reaction using the standardized protocol of Sánchez & Kron (2009). Amplified fragments were visualized in 0.8 % agarose gels. Bands with the expected size for the amplicons were purified with the Pure Link ® Quick Gel PCR Purification Combo Kit for Sanger sequencing in Macrogen Inc., South Korea. Homology of the sequences was tested using the sequence from *Ancona & Ortiz 186* in BLAST using the module megaBLAST search (Morgulis & al. 2008) from NCBI and confirmed with the retrieval of sequences of the same region from *Gymnopodium floribundum* and other *Polygonaceae* species mostly of the genera *Ruprechtia* C. A. Mey. and *Triplaris* Loefl.

Sequences for *ITS1, 5.8S* and *ITS2* did not include ambiguous nucleotide calls, suggesting that each individual is homozygous for this locus. All *lfy* (exon2 and intron 2) sequences did include ambiguous nucleotide calls for several sites, suggesting that each individual is heterozygous for this locus. Alleles per individual were inferred using DNAsp v.6 (Rozas & al. 2017).

Phylogenetic analyses — The nucleotide data matrix for *ITS1, 5.8S* and *ITS2* contained 32 sequences, as did that for *lfy* (exon2 and intron 2). For each region, the alignment was obtained using ClustalW algorithm (Thompson & al. 1994), as implemented in MEGA v 6.0 (Tamura & al. 2013), and then visually checked.

To delimit this new species, we employed the phylogenetic species concept, which defines a species as: "the smallest aggregation of (sexual) populations or (asexual) lineages diagnosable by a unique combination of character states" (Wheeler & Platnick 2000). The distance approach was used to infer if *Gymnopodium floribundum* and *G. toledense* aggregate as unique or separate entities (monophyletic groups) in the neighbour-joining trees obtained for each one of the regions. The neighbour-joining trees was computed by the maximum composite likelihood method (Tamura & al. 2004), and clustering of the branches were tested using 500 bootstrap replications (Felseinstein 1985), as implemented in MEGA v 6.0 (Tamura & al. 2013). For the final analyses, all positions containing gaps and missing data were excluded.

Distribution map — The distribution map of *Gymnopodium floribundum* and *G. toledense* was made using the SimpleMappr program (Shorthouse 2010) and geographic coordinates of herbarium specimens databased in Tropicos (http://www.tropicos.org).

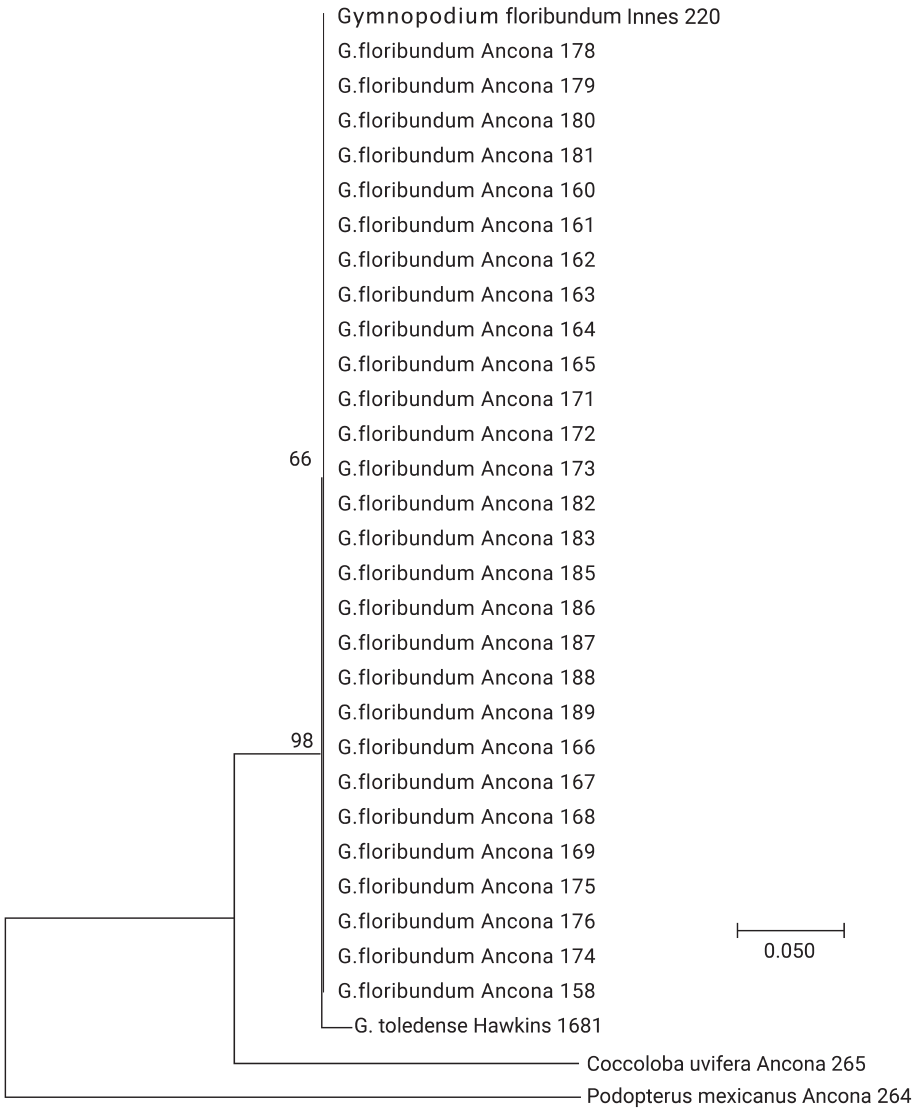

Fig. 1. Neighbour-joining optimal tree using *ITS1, 5.8S* and *ITS2* sequences; percentage of 500 replicates that subtended nodes are shown next to branches; collector and number refer to specimen voucher column in Table 1.

Results and Discussion

The diagnostic morphological characters between *Gymnopodium floribundum* and *G. toledense* are shown in Table 2. *Gymnopodium toledense* differs from *G. floribundum* by, e.g., its sparse or dense glandular trichomes on the leaves, inflorescences and flowers, and its longer inflorescences and perianth segments. All the *G. floribundum* specimens examined lack the basal gland on the trichomes, and their racemes and perianth segments are shorter than those of *G. toledense* (see also Ortiz-Diaz 1994).

The total length of the *ITS1*, *5.8S* and *ITS2* sequence was 745 sites including gaps and for the *lfy* sequence 562 sites including gaps. The sequence from *Ancona & Ortiz 186* obtained from the BLAST search for *ITS1*, *5.8S* and *ITS2* has a 98 % identity to the sequence of the same region from *Gymnopodium floribundum* (GB GQ206251). The *lfy* (exon2 and intron 2) sequence from *Ancona & Ortiz 186* has a 92 % identity to sequences of the same region from *G. floribundum* (GB HQ693138.1), and included sequences of the same region from *Coccoloba swartzii* Meisn. (GB EF442787.1) and *Gilmania luteola* (Coville) Coville (GB EF438069.1).

The neighbour-joining trees built with molecular data show *Gymnopodium toledense* separated from *G. floribundum* (Fig. 1 & 2). The sum of the branch lengths was 0.60421319 for the *ITS1, 5.8S* and *ITS2* and 4.68968630 for the *lfy* (exon2 and intron 2) trees. The bootstrap support values for the nodes grouping *G. toledense* and *G. floribundum* were 98 % for *ITS1, 5.8S* and *ITS2* and 97% for *lfy* (exon2 and intron 2). The bootstrap support values for the nodes connecting the *G. floribundum* terminal branches were 66 % for *ITS1, 5.8S* and *ITS2* and 38% for *lfy* (exon2 and intron 2). The molecular data of *ITS1, 5.8S* and *ITS2* and *lfy* (exon2 and intron 2) markers provided numerous substitutions to delimit both species. The neighbour-joining trees showed *G. toledense* as the sister species of *G. floribundum* (Fig. 1 & 2). The *ITS1, 5.8S* and *ITS2* and *lfy* (exon2 and intron 2) markers have been successfully used to infer relationships between species of *Ruprechtia* and *Triplaris* (Sánchez & Kron 2011) and of *Fallopia* Adans., *Muehlenbeckia* Meisn. and *Reynoutria* Houtt. (Schuster & al. 2011).

The morphological and molecular characters allowed us to clearly define the boundaries between *Gymnopodium floribundum* and *G. toledense*. Such evidence supports the proposal of *G. toledense* as a new species. Moreover, both phylogenetic trees built with the *ITS1, 5.8S* and *ITS2* and *lfy* (exon2 and intron 2) markers suggest that *G. floribundum* is a polymorphic taxon. Further studies including morphometry and chloroplast markers

could provide new evidence to confirm this hypothesis.

Taxonomic treatment

Gymnopodium toledense Ancona & Ortiz-Díaz, **sp. nov**. – Fig. 3.

Holotype: Belize, Toledo, Las Sierritas, 20 km W of Big Creek Settlement, ridge and W slopes of Cerrito in Las Sierritas hills, 16°31'45"N, 88°36'05"W, 160–213 m, ridge-top vegetation of mixed hardwood species growing on thin soils over exposed limestone, vegetation severely damaged by recurrent fires, 6 Dec 1997, *T. Hawkins 1681* (MO [barcode MO-321695 accession no. 04950838]; isotypes: BM [barcode BM000565699], MEXU [catalogue no. 898235]).

Morphological diagnosis — *Gymnopodium toledense* differs from *G. floribundum* by bearing trichomes with basal glands on the petioles, leaf blades, inflorescence rachis, pedicels and ovary, prominent veins on the abaxial surface of the leaf blade, and the basal segment of the pedicel hidden by the ochreole (see also Table 2).

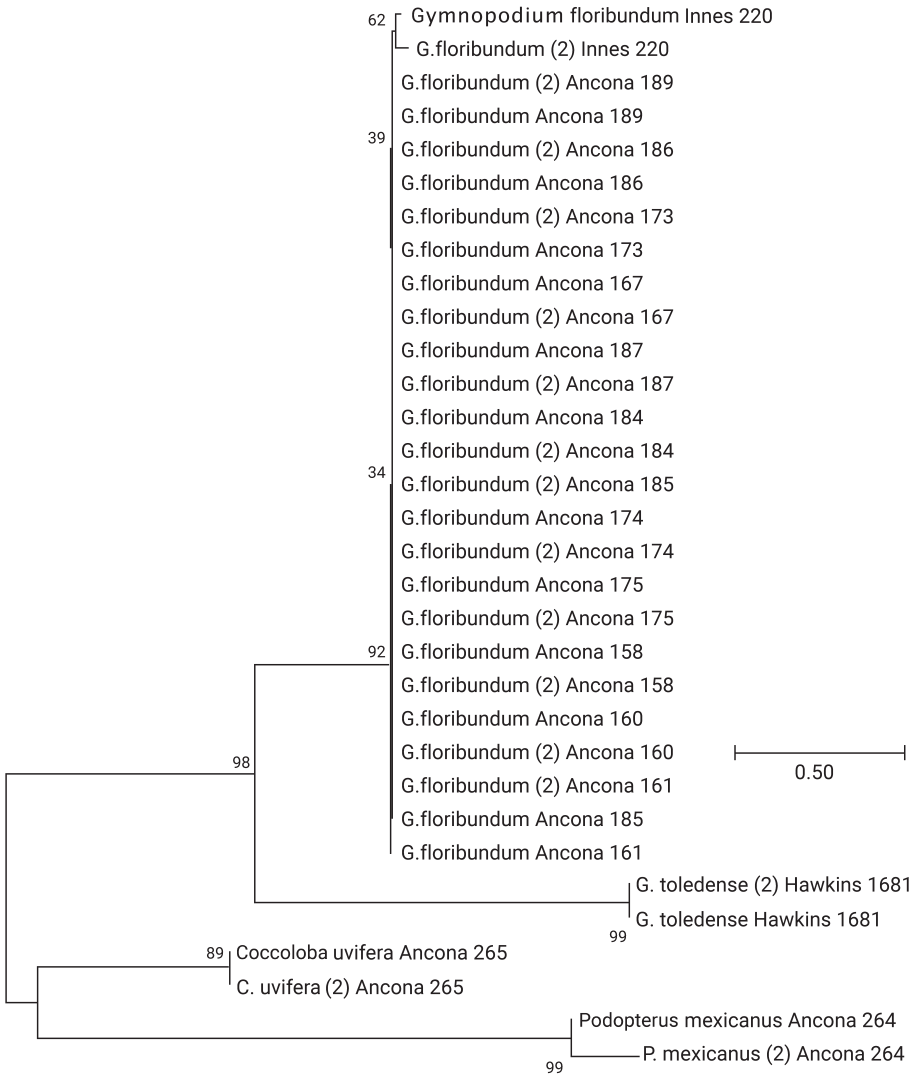

Fig. 2. Neighbour-joining optimal tree using *lfy* (exon2 and intron 2) sequences; percentage of 500 replicates that subtended nodes are shown next to branches; collector and number refer to specimen voucher column in Table 1; "(2)" indicates second allele.

Morphological description — *Shrubs* often scrambling, 2–4 m tall; *bark* grey to dark brown, fissured; *young branches* divaricate, flexuous, grey to pale brown; *internodes* 2–3 cm long; *ochrea* deciduous, annular, c. 1 mm long, sparsely pubescent, trichomes with basal glands. *Leaves* alternate, arising from ochrea, fasciculate (2 or 3 together) on small vegetative shoots (brachyblasts), simple; *petiole* 1.5–2 × c. 1 mm, canaliculate, densely pubescent, trichomes dark brown, 0.3–0.5(–7) mm long with basal glands; *leaf blade* obovate to obpyriform, 5–7 × 3–4 cm, chartaceous, base obtuse, margin entire, apex obtuse to slightly emarginate; veins prominent abaxially; abaxial surface of leaf blade densely pubescent, trichomes 0.3–0.5 mm long with basal glands, adaxial surface glabrous except puberulent on midvein. *Inflorescence* terminal; *racemes* single or paired, on brachyblasts, 12–20 cm long; *rachis* 15–18 cm long, densely pilose, trichomes 0.3–0.5(–0.7) mm long with light yellow basal glands; *flowers* in fascicles of 2–4(–6); *ochreoles* lanceolate, 1–2 mm long, membranous, sparsely to densely pubescent, trichomes with basal glands; *pedicels* articulated below middle, basal segment 0.5–1 mm long, hidden by ochreole, distal segment 5–6.5(–8) mm long, densely pubescent, trichomes 0.3–0.5(–8) mm long with basal glands. *Flowers* hermaphrodite; *perianth segments* 6, 3 outer and 3 inner; *outer segments* greenish yellowish, ovate-orbicular to cordate, 8.5–9(–9.5) × 6–6.5(–7) mm, papery, sparsely to densely pubescent when young, glabrous to pubescent when mature, persistent and accrescent in fruit, reticulate; *inner segments* subulate-lanceolate, smaller than outer ones, (5–)5.5–6 × 1.5–1.8(–2) mm, papery, glabrous when young to scarcely pubescent when mature, apex long acuminate; *stamens* 6, filiform, outer 3 inserted into a basal disk, inner 3 arising opposite ovary grooves; *filaments* c. 2 mm long; *anthers* versatile, suborbicular, 0.5–0.7 mm long, bilocular, dehiscence longitudinal. *Ovary* superior, sessile, trigonous, compressed, to 1

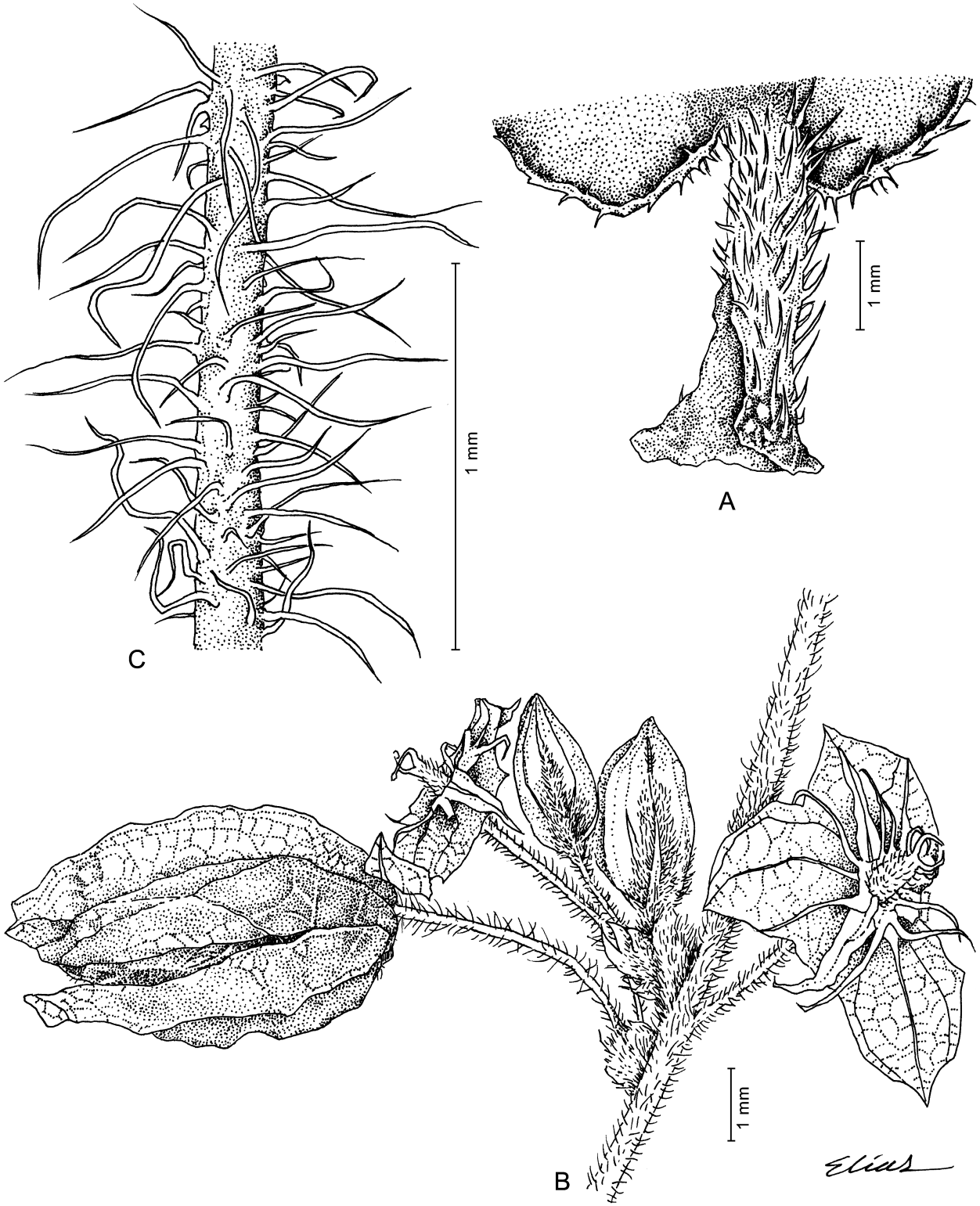

Fig. 3. *Gymnopodium toledense* – A: petiole and basal portion of leaf blade; B: young and mature flowers, showing rachis, outer and inner perianth segments and pubescent ovary; C: detail of pedicel, showing trichomes with basal glands. – Drawn by Jesús Elías García López based on the holotype.

× c. 0.5 mm, unicarpellate, unilocular, densely pubescent at vertices, trichomes with basal glands; *styles* 3, filiform, 1.5–1.7 mm long; *stigmas* 3, capitate. *Fruit* an achene, light brown, lustrous, trigonous, 5(–5.5) × 2(–2.5) mm, smooth, included in perianth segments; *seed* 1.

Distribution and ecology — *Gymnopodium toledense* is so far known as an endemic species of the seasonal forests of southern Belize (Fig. 4), in the biogeographic region of Eastern Central America. It could possibly be found also in Guatemala and Honduras.

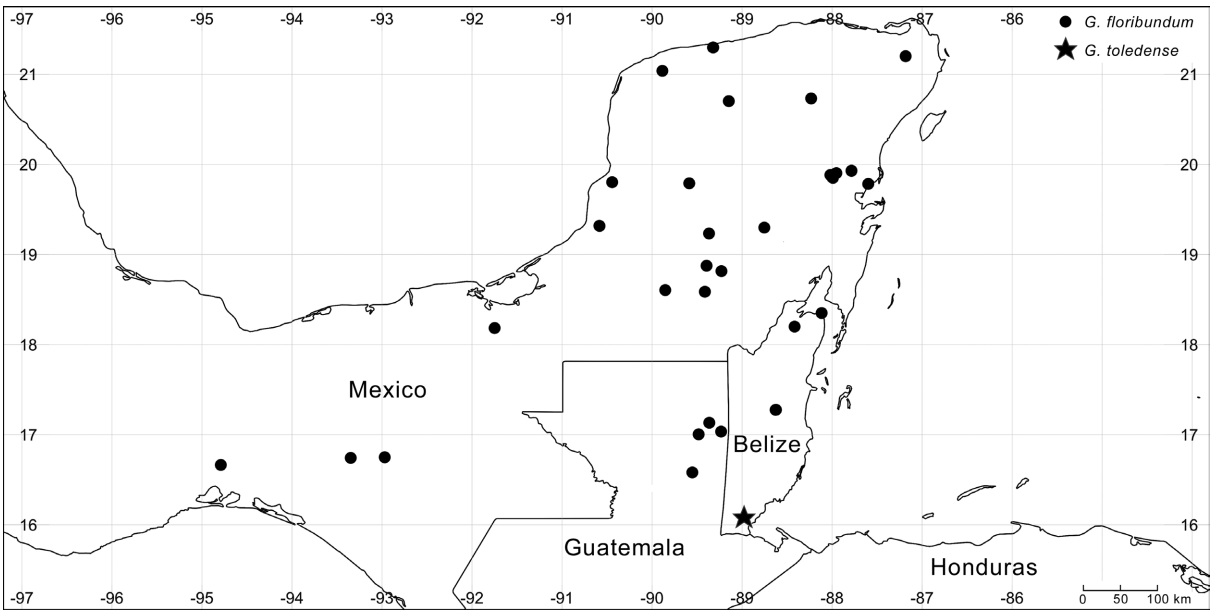

Fig. 4. Distribution map of *Gymnopodium floribundum* (●) and *G. toledense* (★).

Conservation status — *Gymnopodium toledense* is currently known only of the population last recorded in December 1997 at Las Sierritas in the Toledo district of southern Belize. Our efforts to find the population during our field work in 2016 were not successful owing to the landscape having undergone a drastic transformation since 1997, and indeed the field notes of the type gathering included "vegetation severely damaged by recurrent fires". Therefore, the new species meets the following IUCN (2012) criteria for the category Critically Endangered (CR): B1 = Extent of Occurrence estimated to be less than 100 km^2; a = severely fragmented or known to exist at only a single location; and b(iii) = continuing decline, observed, inferred or projected, in area, extent and/or quality of habitat; i.e. CR B1ab(iii). Further exploration is needed through the seasonal forests of southern Belize and adjacent areas of Guatemala and Honduras to assess the frequency and status of any extant subpopulations.

Etymology — The specific epithet refers to the area where this species was collected, Toledo district, Belize.

Key to the species of *Gymnopodium*

1. Leaves, inflorescence rachises and pedicels glabrous or covered with sparse to dense simple trichomes without basal glands; veins not prominent on abaxial surface of leaf blade; pedicel basal segment not hidden by ochreole; outer perianth segments 6.5–8 × 5–6.5(–7) mm; inner perianth segments (4–)4.5(–5) × 1–1.5 mm; fruit 4–4.5(–5) × 1.5(–2) mm . . . . . ***G. floribundum***
– Leaves, inflorescence rachises and pedicels covered with sparse to dense simple trichomes with basal glands; veins prominent on abaxial surface of leaf blade; pedicel basal segment hidden by ochreole; outer perianth segments 8.5–9(–9.5) × 6–6.5(–7) mm; inner perianth segments (5–)5.5–6 × 1.5–1.8 mm; fruit 5(–5.5) × 2(–2.5) mm . . . . . ***G. toledense***

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Table 1. Taxa, localities, specimen vouchers, herbaria and GenBank accession numbers for the nuclear markers *ITS1*, *5.8S* and *ITS2* and *lfy* (exon2 and intron 2).

Taxon	Country	State	Locality	Latitude, longitude	Specimen voucher	Herb.	ITS	LFY
Gymnopodium floribundum	Mexico	Yucatán	Dzemul	21°17'53.9"N, 89°19'25.5"W	*Ancona & Ortiz 158*	UADY	MK098142	MK097205, MK097206
G. floribundum	Mexico	Yucatán	Dzemul	21°17'53.9"N, 89°19'25.5"W	*Ancona & Ortiz 160*	UADY	MK098143	MK097207, MK097208
G. floribundum	Mexico	Yucatán	Dzemul	21°17'53.9"N, 89°19'25.5"W	*Ancona & Ortiz 161*	UADY	MK098144	MK097209, MK097210
G. floribundum	Mexico	Yucatán	Huhi	20°42'11.8"N, 89°08'54.8"W	*Ancona & Ortiz 162*	UADY	MK098145	
G. floribundum	Mexico	Yucatán	Huhi	20°42'11.8"N, 89°08'54.8"W	*Ancona & Ortiz 163*	UADY	MK098146	
G. floribundum	Mexico	Yucatán	Huhi	20°42'11.8"N, 89°08'54.8"W	*Ancona & Ortiz 164*	UADY	MK098147	
G. floribundum	Mexico	Yucatán	Huhi	20°42'11.8"N, 89°08'54.8"W	*Ancona & Ortiz 165*	UADY	MK098148	
G. floribundum	Mexico	Quintana Roo	Vigía Chico	19°55'54.9"N, 87°47'03.6"W	*Ancona & Camara 166*	UADY	MK098149	
G. floribundum	Mexico	Quintana Roo	Vigía Chico	19°55'54.9"N, 87°47'03.6"W	*Ancona & Camara 167*	UADY	MK098150	MK097211, MK097212
G. floribundum	Mexico	Quintana Roo	Vigía Chico	19°55'54.9"N, 87°47'03.6"W	*Ancona & Camara 168*	UADY	MK098151	
G. floribundum	Mexico	Quintana Roo	Vigía Chico	19°55'54.9"N, 87°47'03.6"W	*Ancona & Camara 169*	UADY	MK098152	
G. floribundum	Mexico	Campeche	Calakmul	18°52'31.6"N, 89°23'42.6"W	*Ancona & Ortiz 171*	UADY	MK098153	
G. floribundum	Mexico	Campeche	Calakmul	18°52'31.6"N, 89°23'42.6"W	*Ancona & Ortiz 172*	UADY	MK098154	
G. floribundum	Mexico	Campeche	Calakmul	18°52'31.6"N, 89°23'42.6"W	*Ancona & Ortiz 173*	UADY	MK098155	MK097213, MK097214
G. floribundum	Mexico	Campeche	Xtampac	19°47'26.2"N, 89°35'09.2"W	*Ancona & Camara 174*	UADY	MK098156	MK097215, MK097216
G. floribundum	Mexico	Campeche	Xtampac	19°47'26.2"N, 89°35'09.2"W	*Ancona & Camara 175*	UADY	MK098157	MK097217, MK097218
G. floribundum	Mexico	Campeche	Xtampac	19°47'26.2"N, 89°35'09.2"W	*Ancona & Camara 176*	UADY	MK098158	
G. floribundum	Mexico	Chiapas	El Chorreadero	16°45'04.6"N, 92°58'12.6"W	*Ancona & Ortiz 178*	UADY	MK098159	
G. floribundum	Mexico	Chiapas	El Chorreadero	16°45'04.6"N, 92°58'12.6"W	*Ancona & Ortiz 179*	UADY	MK098160	
G. floribundum	Mexico	Chiapas	El Chorreadero	16°45'04.6"N, 92°58'12.6"W	*Ancona & Ortiz 180*	UADY	MK098161	
G. floribundum	Mexico	Chiapas	El Chorreadero	16°45'04.6"N, 92°58'12.6"W	*Ancona & Ortiz 181*	UADY	MK098162	
G. floribundum	Mexico	Chiapas	Ocozocuautla	16°44'33.9"N, 93°20'49.3"W	*Ancona & Hernández 182*	UADY	MK098163	
G. floribundum	Mexico	Chiapas	Ocozocuautla	16°44'33.9"N, 93°20'49.3"W	*Ancona & Hernández 183*	UADY	MK098164	
G. floribundum	Mexico	Chiapas	Ocozocuautla	16°44'33.9"N, 93°20'49.3"W	*Ancona & Hernández 184*	UADY		MK097219, MK097220
G. floribundum	Mexico	Chiapas	Ocozocuautla	16°44'33.9"N, 93°20'49.3"W	*Ancona & Hernández 185*	UADY	MK098165	MK097221, MK097222
G. floribundum	Mexico	Oaxaca	Las Anonas	16°39'50.1"N, 94°47'27.5"W	*Ancona & Ortiz 186*	UADY	MK098166	MK097223, MK097224
G. floribundum	Mexico	Oaxaca	Las Anonas	16°39'50.1"N, 94°47'27.5"W	*Ancona & Ortiz 187*	UADY	MK098167	MK097225, MK097226
G. floribundum	Mexico	Oaxaca	Las Anonas	16°39'50.1"N, 94°47'27.5"W	*Ancona & Ortiz 188*	UADY	MK098168	
G. floribundum	Mexico	Oaxaca	Las Anonas	16°39'50.1"N, 94°47'27.5"W	*Ancona & Ortiz 189*	UADY	MK098169	MK097227, MK097228
G. floribundum	Belize	Cayo	Belmopan	17°16'35.3"N, 88°37'20.9"W	*R. R. Innes 220*	MO	MK098170	MK097229, MK097230
Gymnopodium toledense	Belize	Toledo	Toledo	16°05'25.8"N, 88°58'43.2"W	*T. Hawkins 1681*	MO	MK098171	MK097231, MK097232
Podopterus mexicanus	Mexico	Yucatán	Dzibilchaltun	21°05'29.9"N, 89°35'44.2"W	*Ancona & Ortiz 264*	UADY	MK098172	MK097233, MK097234
Coccoloba uvifera	Mexico	Yucatán	Merida	20°52'00.2"N, 89°37'22.7"W	*Ancona & Ortiz 265*	UADY	MK098173	MK097235, MK097236

Table 2. Morphological comparison between *Gymnopodium toledense* and *G. floribundum*.

Character	***Gymnopodium toledense***	***Gymnopodium floribundum***
Ochrea indumentum	densely pubescent, trichomes with basal glands	glabrous to densely pubescent, trichomes without basal glands
Petiole indumentum	densely pubescent, trichomes with basal glands	glabrous to densely pubescent, trichomes without basal glands
Leaf blade texture	chartaceous	papery to coriaceous
Leaf blade indumentum	abaxially densely pubescent, trichomes with basal glands, adaxially glabrous except puberulent on midvein	abaxially densely pubescent, trichomes without basal glands, adaxially glabrous
Leaf blade apex	obtuse to slightly emarginate	acute to rounded
Leaf blade veins on abaxial surface	prominent	not prominent
Raceme length [cm]	12–20	(3–)5–10(–12)
Pedicel basal segment length [mm]	0.5–1, hidden by ochreole	1.5–2, exserted from ochreole
Outer perianth segments length × width [mm]	8.5–9(–9.5) × 6–6.5(–7)	6.5–8 × 5–6.5(–7)
Inner perianth segments length × width [mm]	(5–)5.5–6 × 1.5–1.8(–2)	(4–)4.5(–5) × 1–1.5
Ovary indumentum	densely pubescent at vertices, trichomes with basal glands	glabrous
Fruit length × width [mm]	5(–5.5) × 2(–2.5)	4–4.5(–5) × 1.5(–2)

Willdenowia
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