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Rare specimens yield DNA to confirm additional members of tribe Stifftieae (Stifftioideae, Asteraceae) on the Guiana Highlands tepuis

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Abstract: Eight of the ten genera of the Guiana Highlands shrubby Mutisieae were sequenced for the ITS and ETS of the nuclear ribosomal DNA. Results from phylogenetic analyses of the sequence data are congruent with earlier reports that support two independent introductions from different lineages of the basal Asteraceae resulting in the diversification of these taxa in the Guiana Highlands. The two clades segregated according to corolla symmetry. The five genera with bilabiate corollas, *Achnopogon*, *Duidaea*, *Eurydochus*, *Gongylolepis* and *Neblinaea,* were supported with members of subfamily Stifftioideae whereas those with actinomorphic corollas, *Chimantaea*, *Stenopadus* and *Stomatochaeta,* constitute a monophyletic group sister to *Wunderlichia* and strongly supported in the Wunderlichioideae. Resolution, but not statistical support, was found for relationships within the Guiana Highlands shrubby Mutisieae.

Introduction

Since the first descriptions of the spectacular table-mountains, or tepuis, of the Guiana Highlands of northern South America by Humboldt (1822) and Schomburgk (1840), multiple expeditions have endeavored to document the diverse flora of the region with a modern account of its diversity recently completed for the Venezuelan Guiana (Berry et al., 1995). Tepuis are predominantly composed of quartz-sandstones (Sauro, 2014) with intrusions of igneous and other rock types and shaped by erosion (Briceño and Schubert, 1990). At least six erosion plains have been identified in the Guiana Highlands with the oldest plains corresponding to the flat summits of tepuis and surrounding slopes dating to the Mesozoic (Briceño and Schubert, 1990). Taking into consideration the age and composition of the basements of these mountains, Huber et al. (2018) groups the tepuis into four categories. Following this classification, the tepuis range in age from the mid Precambrian to the Ordovician with the Roraima group tepuis of the eastern Guiana Highlands being the oldest, followed by the Neblina group of southern Venezuela and the Tunui and Chiribiquete sandstone plateaus of southern Colombia and northern Brazil being the youngest. Vertical walls jutting hundreds to nearly one thousand meters above the landscape variously flank most tepuis (Huber 1995) producing their characteristic shape. However, the plateaus of most tepuis are not completely isolated from the surrounding forests or grasslands (Huber et al., 2018), being connected to them by screes and gently eroded plains that have environmental conditions different than those of the lowland areas and the summits. The collective tepui summits were termed the Pantepui area by Mayr and Phelps (1955). Huber (1987) expanded this concept to include in his biogeographic unit, the Pantepui province, the slopes and summits above 1200 m found within the Guiana Highlands region. Because these sky islands each have a diversity of soils, and a cooler and wetter climate than the surrounding rain forests, a distinctive flora with 34% endemism has evolved in the Pantepui (Riina et al., 2019; Rull et al., 2019).

The sunflower family Asteraceae has the highest number of endemic species in the

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Pantepui (Riina et al., 2019) and they are an important component of the flora at the higher elevations of this region (Berry et al., 1995, Riina et al., 2019). Although most tepuis are broadly scattered in the Guiana region, preliminary cluster analysis of species comprising their flora shows that there is an affinity in floristic composition among the eastern (Roraima group) and western (Neblina group) tepuis (Riina, et al., 2019). Because of their shrubby habits, leaf features and floral traits, Pruski (1991) considers ten genera of Guiana Highlands Mutisieae to be closely related. He identified this group as the shrubby Guiana Highlands Mutisieae, and includes 58 species, 56 of which are endemic to the Guiana Highlands region and immediately adjacent areas. According to Pruski (1991), four of these genera, *Chimantaea*, *Quelchia*, *Stenopadus* and *Stomatochaeta* are primarily found on the eastern tepuis and mostly have actinomorphic corollas, whereas *Achnopogon*, *Duidaea*, *Eurydochus*, *Glossarion*, *Gongylolepis* and *Neblinaea* are largely found on the western tepuis and have bilabiate corollas. Two species, *Stenopadus andicola* Pruski and *Gongylolepis colombiana* (Cuatrec.) Cuatrec., are endemic to the eastern Andes of Colombia, Ecuador, Peru and Venezuela. Pruski (1991), like Maguire (1956) before him, argued that shared floral symmetry indicates a common evolutionary history in the group and considered the actinomorphic and bilabiate groups to be sister clades. As an exception, the genus *Quelchia*, in spite of having actinomorphic and shallowly bilabiate zygomorphic corollas, was considered by Maguire (1956) to be part of the actinomorphic corolla group. Phylogenetic analyses using morphological characters by Jiménez-Rodríguez et al. (2004) places *Quelchia* in a clade with five of the bilabiate corolla genera. Maguire (1956) believes that these Asteraceae have evolved in the Guiana Highlands from a very ancient introduction and that the closely related genera *Stifftia* and *Moquinia* from the Planalto of Brazil and Amazonia are derived from within the genus *Stenopadus*. Pruski (1991) considers the shrubby Guiana Highlands Mutisieae to be more closely related to *Wunderlichia* and *Stifftia* and distantly related to other genera of the Mutisiinae or Gochnatiinae (sensu Cabrera, 1977) found in other parts of South America.

Molecular phylogenies do not support the monophyly of the shrubby Guiana Highlands Mutisieae (Panero & Funk, 2002; Panero & Funk, 2008; Funk et al., 2014; Panero et al., 2014; Mandel et al., 2019). These studies, incorporating sequence data from multiple markers of the chloroplast and nuclear DNA, show that the traditional circumscription of tribe Mutisieae (sensu Cabrera, 1977) is paraphyletic and the recognition of several novel clades at the subfamily level is required to maintain monophyletic groups (Panero & Funk, 2008). The five genera of the Guiana Highlands Mutisieae sampled in Panero & Funk (2002, 2008) resolved in tribes Stifftieae (Stifftioideae) and Wunderlichieae (Wunderlichioideae). Clades corresponding to these two tribes are not sister in either chloroplast (Panero & Crozier, 2016) or nuclear DNA (Mandel et al., 2019) phylogenies. The genera with actinomorphic corollas, *Chimantaea*, *Stenopadus* and *Stomatochaeta* are sister to *Wunderlichia* and collectively sister to the clade Gochnatioideae-Asteroideae based on analyses of chloroplast DNA (Panero et al., 2014) and sister to Gochnatioideae in phylogenies based on nuclear data (Funk et al., 2014; Mandel et al., 2019). The genera with bilabiate corollas, *Duidaea* and *Gongylolepis* are sister to *Hyaloseris* and *Stifftia* and collectively sister to the clade Wunderlichieae-Asteroideae in the chloroplast DNA (Panero et al., 2014) but strongly supported as sister to Hyalideae and collectively to subfamily Mutisioideae in the nuclear genomic phylogeny of Mandel et al. (2019). Molecular results to date support that there are two lineages of shrubby Guiana Highlands Mutisieae as hypothesized based on floral morphology (Pruski, 1991) but do not support a single introduction of these taxa into the area. Because of the paucity of herbarium and tissue collections, and difficulty in obtaining DNA from specimens

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collected and preserved in alcohol and/or dried under very humid conditions during floristic surveys of the Pantepui and other areas of the Guiana Highlands, only half of the ten genera of shrubby Guiana Highlands Mutisieae have been included in previous molecular studies of the basal lineages of the family (Panero & Funk 2008; Panero et al., 2014). In the intervening years since producing the chloroplast phylogenies, I have continued attempting to sequence the shrubby Guiana Highlands Mutisieae from herbarium specimens with the aim of producing sequence data of the nuclear ribosomal ITS and ETS for these genera. This study reports on those efforts that aim to place those genera of the shrubby Guiana Highlands Mutisieae that have not been included in previous studies in a molecular phylogeny of Asteraceae, and to discover whether these newly sampled genera might have affinities to any clade(s) other than the Stifftieae and the Wunderlichieae.

Materials and Methods

Taxon and Character Sampling. The data matrix contained 52 taxa. The 46 Asteraceae species sampled represent 10 of the 13 subfamilies of the family. Eight of the 10 Guiana Highlands genera belonging to subfamilies Wunderlichioideae and Stifftioideae were sampled. Outgroups included five species of Calyceraceae and a species of *Scaevola* of the Goodeniaceae. I sequenced the ITS and ETS of the nuclear ribosomal DNA (PCR primers listed below). Outgroup taxa sequences were obtained from Genbank (Appendix). The data matrix is available from the author.

Total DNA was obtained from herbarium specimens using the CTAB extraction method (Doyle & Doyle, 1987). DNA was further cleaned using a phenol-chloroform-isoamyl alcohol (25-24-1) solution followed by two 70% ETOH washes. DNA was also isolated from herbarium specimens using the DNeasy Plant Kit (QIAGEN, Hilden, Germany).

Polymerase chain reactions were performed with 2 units of Taq polymerase, 0.2 M Tris-HCl, 8 mM $(NH_4)_2SO_4$, 0.2 mM dNTPs, 5 mM $MgCl_2$, 20 μM of primers, target DNA and water to a volume of 50μl. PCR amplification protocols for the ITS and ETS included the following steps: 95 °C for 4 min, 50 °C for 45 s, 72 °C for 1 min followed by 36 cycles of 95 °C for 1 min, 50 °C for 45 s, 72 °C for 1 min with a 2 s extension per cycle and a final extension of 72 °C for 8 min (Rivera et al., 2016).

Amplification and sequencing of the ITS was performed using primers ITS-4 (TCCTCCGCTTATTGATATGC) and ITS-5 (GGAAGTAAAAGTCGTAACAAGG) of White et al., (1990) and occasionally primer ITS 7.5 (GAGTCATCAGCTCGCGTTGACTA, Plovanich and Panero, 2004) in lieu of ITS 5 in amplification and sequencing reactions. For the ETS region we used primers Ast-1 (CGTAAAGGTGTGTGAGTGGTTT, modified from Markos & Baldwin 2001), primer 18S-Alt (TGAGCCATTCGCAGTTTCACAGTC, a modified version of primer 18-S of Baldwin & Markos, 1998), and ETS-Goch (GATGTCTGCTTGCGCAGCAACG, Panero 2019) for amplification and sequencing. PCRs were cleaned with the QIAquick PCR purification kit (QIAGEN) following manufacturers specifications. Cleaned PCR products were sequenced at the University of Texas Sequencing facility using an ABI 3730 xl sequencer.

Sequences were edited using Sequencher v 4.9 (Gene Codes Corporation, Ann Arbor, USA) and assembled into primer-based matrices. Matrices were exported as Nexus interleaved files and concatenated in Paup* (4.0a, build 163) (Swofford, 2002) then imported into Mesquite (Maddison & Maddison, 2015) to produce nbrf files. Alignment of the nbrf files was performed at the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/mafft) using the program MAFFT (Katoh & Standley, 2013). The aligned matrices were imported into Mesquite, edited and subsequently exported as simplified Nexus interleaved files and concatenated in Paup*. The concatenated matrix was exported as a simpli-

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fied Nexus sequential file to use in Bayesian analyses.

Phylogenetic analysis. Bayesian phylogenetic analyses were performed for the ITS and ETS concatenated dataset. Models of molecular evolution were evaluated under the Akaike Information Criteria (AIC) using the program jModeltest 2 (Guindon & Gascuel, 2003; Darriba et al., 2012). Number of informative characters was calculated by PAUP* (Swofford, 2002). The concatenated dataset was treated as a single partition with the model of evolution chosen using jModeltest 2 specified as TVM + G + I. Two independent runs of four Markov chains, each starting with a random tree for 10 million generations was implemented, sampling trees at every 5,000th generation. Stationarity of the chains was ascertained using ESS (Effective Sample Size) values above 200 as viewed in Tracer v1.7.1 (Rambaut et al., 2018) and Mr. Bayes 3.2.6 (Ronquist et al., 2012). The first 10% of the trees were discarded as burn-in samples. The 50% majority rule consensus tree was calculated by MrBayes 3.2.6 (Ronquist et al., 2012). Analyses were performed in the Cyber infrastructure for Phylogenetic Research (CIPRES) cluster (Miller et al., 2015, https://www.phylo.org). A maximum clade credibility (MCC) tree was constructed in TreeAnnotator v1.10 (Rambaut et al., 2018) depicting the maximum sum of posterior clade probabilities and visualized and edited in FigTree v.1.4.3 (Rambaut et al., 2018, http://tree.bio.ed.ac.uk/software/figtree/).

Results

New sequences obtained of the ITS for taxa of the shrubby Guiana Highlands Mutisieae included one species each of *Achnopogon*, *Eurydochus*, *Neblinaea*, and *Stomatochaeta* and the of the ETS included one species of the genus *Duidaea*. Five of the eight Guiana Highlands genera sampled, namely *Achnopogon*, *Duidaea*, *Eurydochus*, *Gongylolepis*, and *Neblinaea,* comprised a clade sister to *Hyaloseris* and these sister to *Stifftia* of the Stifftioideae. The other three, *Chimantaea*, *Stenopadus* and *Stomatochaeta,* were sister to *Wunderlichia* of the Wunderlichieae (Wunderlichioideae) (Fig. 1).

Aligned ITS and ETS were 1179 base pair (bp) long (748 bp for ITS, and 431 bp for ETS), 398 characters were constant, 121 were variable but not parsimony-informative, and 660 were parsimony-informative. All parameters of the MCMC chain were above 200.

The maximum clade credibility tree produced by Bayesian analysis is shown in Fig. 1. The Hyalideae (Wunderlichioideae) were sister to Stifftioideae (Posterior Probability (PP) 0.99). Wunderlichieae were sister to Gochnatioideae and the latter was not monophyletic with *Cyclolepis* (Gochnatioideae) sister to Hecastocleidoideae, although this relationship has no statistical support. The monophyly of Mutisioideae was strongly supported (PP 1.00), as well as that of its tribes Onoserideae and Nassauvieae, whereas that of Mutisieae was not. The monophyly of the Cichorioideae and placement of *Sonchus* sister to other Cichorioideae (*Arctotis*, *Sinclairia*) was not significantly supported (PP 0.71) and there was not support for the monophyly of Carduoideae. *Calycera* was sister to all other Calyceraceae and that family was strongly supported as monophyletic. The two species of *Gongylolepis* sampled were sister.

Significant statistical support for relationships within each group was lacking, but the maximum clade credibility tree placed *Chimantaea* sister to *Stenopadus* and *Stomatochaeta*, congruent with well-supported phylogenetic results from chloroplast DNA (Panero & Funk 2008, Panero et al., 2014). *Achnopogon* was more closely related to *Gongylolepis* than other bilabiate genera. The two species of *Gongylolepis* sampled were sister but had at least 20 base pair differences between them.

Discussion

With eight of the 10 genera of Guiana Highlands shrubby Mutisieae sampled, the ITS+ETS phylogeny reinforces earlier findings in Panero & Funk (2008) and Mandel et al. (2019) that support two independent

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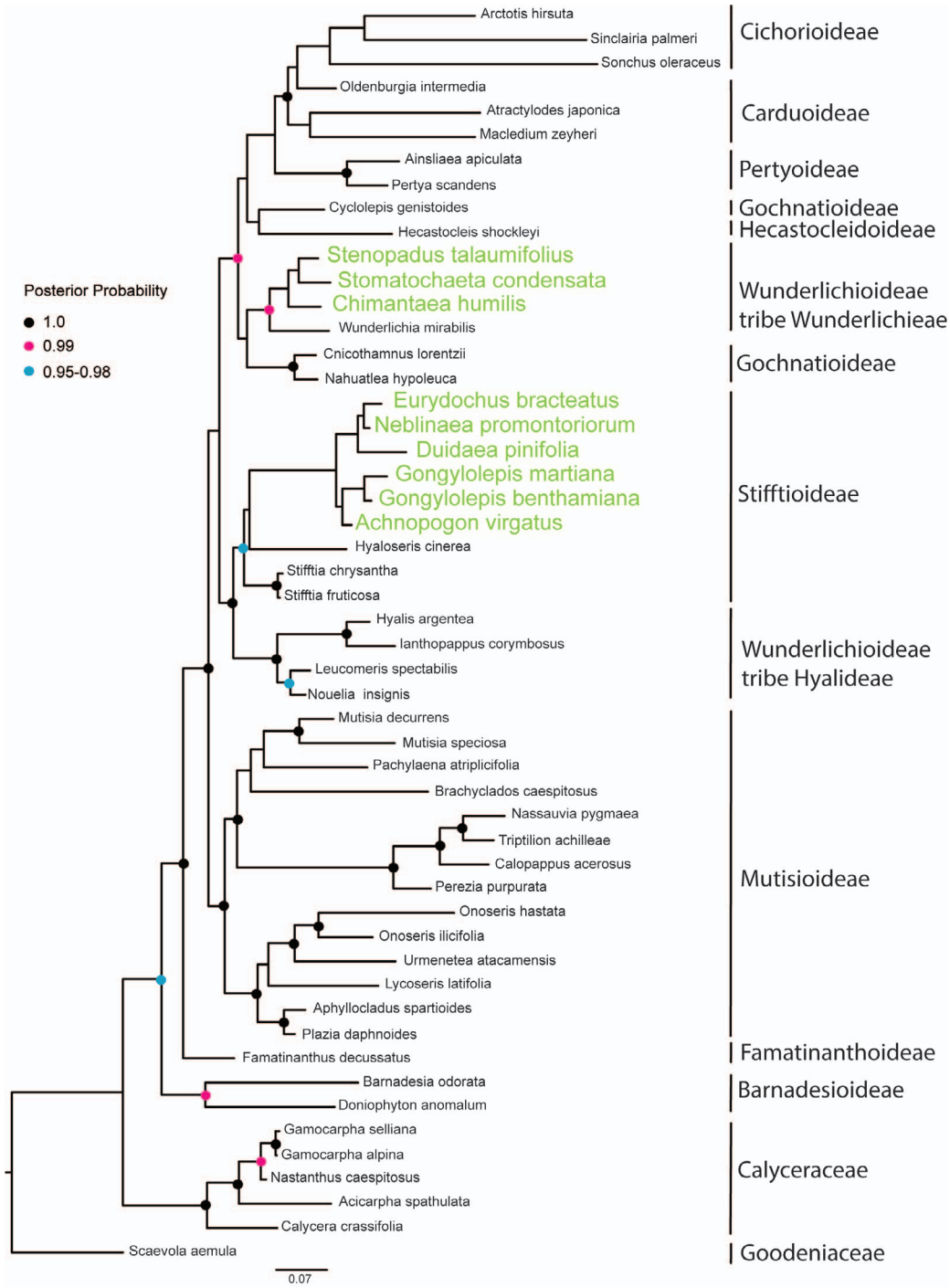


FIG. 1. Relationships of Guiana Highlands Stifftieae and Wunderlichieae (Asteraceae). Genera collectively known as the Guiana Highlands shrubby 'Mutisieae' are shown in green on a maximum clade credibility tree produced by Bayesian analysis of the nr ITS+ETS. DNA samples of *Glossarion* and *Quelchia* from herbarium specimens were too degraded to include. Subfamily taxonomy follows Panero & Crozier 2016.

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lineages of Asteraceae giving rise to these taxa. All taxa with actinomorphic corollas were monophyletic and those with bilabiate corollas formed another clade (Fig. 1) as predicted by floral morphology (Maguire, 1956; Pruski, 1991) and earlier molecular phylogenetic analyses of chloroplast DNA (Panero et al., 2014). Genera with actinomorphic corollas were strongly supported as sister to the Brazilian shield genus *Wunderlichia* (Wunderlichieae) with *Chimantaea* sister to *Stenopadus* and *Stomatochaeta*. This topology is identical to well-supported results obtained using chloroplast DNA (Panero & Funk 2008, Panero et al., 2014). The bilabiate corolla group, sister to the Andean genus *Hyaloseris* and those to the Brazilian genus *Stifftia,* is congruent with the chloroplast phylogeny (Panero et al., 2014), although without statistical support. This differs from the nuclear genomic phylogeny (Mandel et al., 2019) where *Gongylolepis* is strongly supported as sister to *Stifftia* and the two to *Hyaloseris*.

These two lineages represent successive introductions of Asteraceae into the Guiana Highlands probably during the Oligocene an 11 My epoch characterized by climate cooling and great changes in biodiversity and plant communities, as well as the expansion of mammal diversity. *Gongylolepis* is estimated to have diverged from *Stifftia* approximately 34 My in the early Oligocene, and *Stenopadus* from *Wunderlichia* almost nine My later in the late Oligocene (Mandel et al., 2019). The two groups have similar number of extant species and genera in the Guiana Highlands with the actinomorphic group having 33 species in four genera and the bilabiate corolla group 23 species in six genera including one species each in the northern and central eastern Andes. The largest genus of Guiana Highlands Stifftieae, *Gongylolepis*, and the largest of Guiana Highlands Wunderlichieae, *Stenopadus*, have most of their species widely distributed in the Guiana Highlands and these are sympatric with narrowly endemic, smaller genera belonging to the same clades (Fig. 2). Based on morphology and species distributions Maguire (1956) proposes that *Gongylolepis* and *Stenopadus* each have given rise to smaller genera via founding events in multiple areas of the Guiana Highlands. Testing if these two large diverse genera are polyphyletic was beyond the scope of the present study due to limited species sampling. *Gongylolepis* is very weakly supported as sister to the Dominican genus *Salcedoa* based on the presence of style branch ribs in phylogenetic analyses of morphological characters (Jiménez-Rodríguez et al., 2004), but *Salcedoa* was not included here.

Future phylogenetic studies to determine species relationships and generic boundaries within the Guiana Highlands Stifftieae and Wunderlichieae will require sampling much more broadly and will need to rely upon new field collections preserved with molecular studies in mind. Studies of species occupying the Pantepui province will benefit from the undisturbed environmental conditions of this tropical region. Although human-induced fires and mining have transformed several areas of the Guiana Highlands, the Pantepui has remained essentially free of destructive human activities and most of its flora and fauna remains unspoiled (Rull et al., 2019). This makes the Pantepui an excellent outdoor laboratory in which to conduct evolutionary studies. Of particular interest would be to document the parallel evolution of the Stifftieae and Wunderlichieae on the Pantepui to assess rates of molecular evolution, the importance of environmental heterogeneity in the process of upslope speciation, the rate of gene flow between populations, and differences in floral morphology and pollination syndromes between narrow endemics and widespread species.

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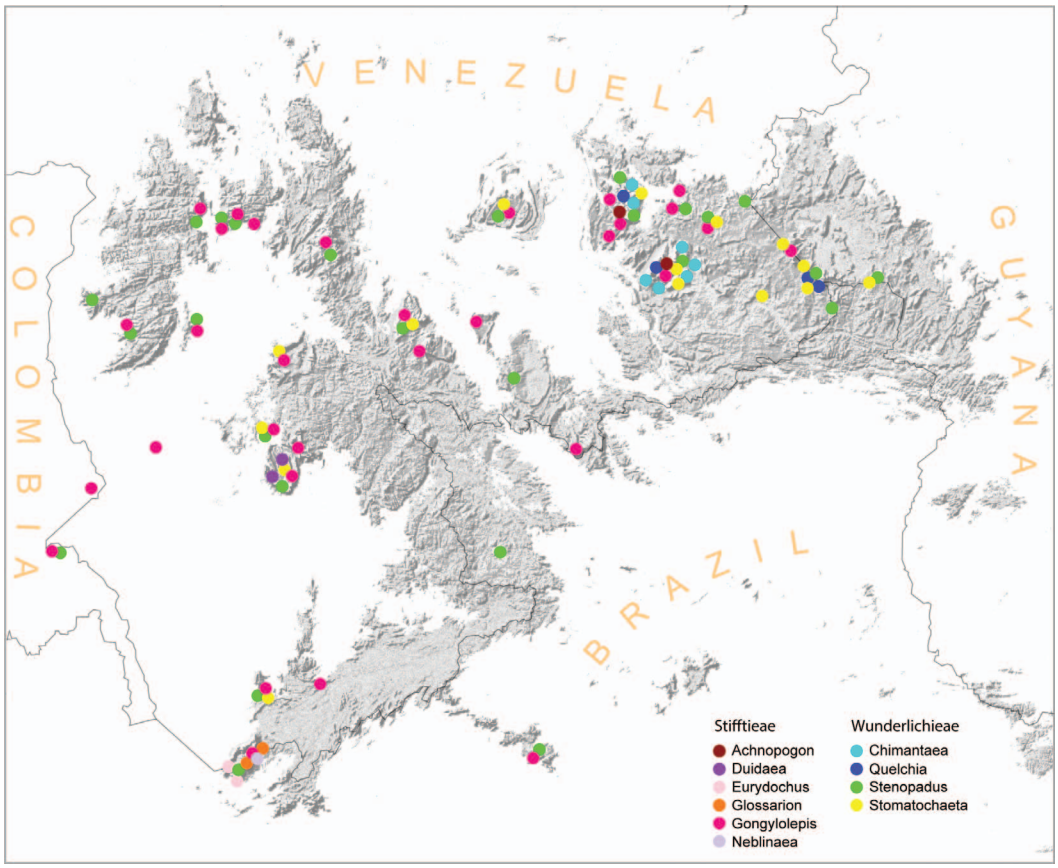


FIG. 2. Stifftieae and Wunderlicheae (Asteraceae) occurring in the Guiana Highlands. The widespread distributions of *Gongylolepis* (pink dots), *Stenopadus* (green dots) and *Stomatochaeta* (yellow dots) broadly overlap across the Guiana Highlands. *Duidaea* and its sister clade comprising *Neblinaea*, *Eurydochus* and probably *Glossarion* are restricted to the western tepuis of Duida, Marahuaca and Neblina, whereas *Achnopogon* is restricted to Chimantá and Auyan–tepui in the east. *Chimantaea* and *Quelchia* are also restricted to eastern tepuis. The ranges of *Gongylolepis* and *Stenopadus* extend beyond the map area to the Andes, and *Gongylolepis* to northern Brazil. Elevations above 500m are shown in shaded relief. Black lines mark national boundaries. Information was sourced from Pruski (1991, 1997), label data from New York Botanical Garden specimens, and base map from https://www2.jpl.nasa.gov/srtm/guiana.htm, downloaded Apr 29, 2020.

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Appendix. List of specimens of Asteraceae and outgroup taxa sequenced in this study. Genbank accession numbers are as follows: ETS; ITS. NS = no sequence available. *Achnopogon virgatus* Maguire, Steyerm. & Wurdack, NS, MN582001. *Acicarpha spathulata* R. Br. MN582018, AY102728. *Ainsliaea apiculata* Sch. Bip. ex. Zoll., MN582019, AB288430. *Aphyllocladus spartioides* Wedd., MN582020, MN582002. *Arctotis hirsuta* (Harv.) P. Beauv., MN582021, EU846366. *Atractylodes japonica* Koidz., MN582022, AY548203. *Barnadesia odorata* Griseb., MN582023, EU841144. *Brachyclados caespitosus* (Phil.) Speg., MN582024, MN582004. *Calopappus acerosus* Meyen, MN582025, FJ979685. *Calycera crassifolia* Hicken, MN528026, JN874692. *Chimantaea humilis* Maguire, Steyerm. & Wurdack, MN528027, NS. *Cnicothamnus lorentzii* Griseb., MN457761, MN457799. *Cyclolepis genistoides* D. Don, MN457791, MN457827. *Doniophyton anomalum* (D. Don) Kurtz, MN528028, EU841164. *Duidaea pinifolia* S. F. Blake, MN582029, NS. *Eurydochus bracteatus* Maguire & Wurdack, NS, MN582005. *Famatinanthus decussatus* Ariza & S. E. Freire, MN528030, MN582006. *Gamocarpha alpina* (Poepp. Ex Less.) H. V. Hansen, NS, JN874693. *Gamocarpha selliana*, NS, MN582003. *Gongylolepis benthamiana* R. H. Schomb., NS, KF989515. *Gongylolepis martiana* (Baker) Steyerm & Cuatrec., NS, KF989515. *Hecastocleis shockleyi* A. Gray, MN528031, AY190282. *Hyalis argentea* D. Don ex Hook., MN457797, MN457833. *Hyaloseris cinerea* (Griseb.) Griseb., MN528032, MN582007. *Ianthopappus corymbosus* (Less.) Roque & D. J. N. Hind, MN457796, MN457832. *Leucomeris spectabilis* D. Don, MN457794, MN457830. *Lycoseris latifolia* (D. Don) Bentham, MN528033, MN582008. *Macledium zeyheri* (Sond.) S. Ortiz, MN528034, MN582009. *Mutisia decurrens* Cav. NS, EU841169. *Mutisia speciosa* Aiton ex Hook., MN528035, MN582010. *Nahuatlea hypoleuca* (DC.) V. A. Funk, MN457782, MN457812. *Nassauvia pygmaea* (Cass.) Hook. f., NS, EU239267. *Nastanthus caespitosus* (Phil.) Reiche, MN528036, MN582011. *Neblinaea promontoriorum* Maguire & Wurdack, NS, MN582012. *Nouelia insignis* Franch., MN457795, MN457831. *Oldenburgia intermedia* Bond, MN528037, AY826303. *Onoseris hastata* Wedd., MN528038, MN582013. *Onoseris ilicifolia* (Cabrera) Panero, MN528039, MN582014. *Pachylaena atriplicifolia* D. Don ex H. A., MN528040, EF530250. *Perezia purpurata* Wedd., NS, FJ979643. *Pertya scandens* Sch. Bip., MN528041, AB288467. *Plazia daphnoides* Wedd., MN457793, MN457829. *Scaevola aemula* R. Br., NS, AY102728. *Sinclairia palmeri* (A. Gray) B. L. Turner, NS, JN837190. *Sonchus oleraceus* L., MN528042, AY458001. *Stenopadus talaumifolius* S. F. Blake, NS, KF989518. *Stifftia chrysantha* Mikan, MN457798, MN457834. *Stifftia fruticosa* (Vell.) D. J. N. Hind & Semir, MN528043, MN582015. *Stomatochaeta condensata* (Baker) Maguire & Wurdack, NS, MF785313. *Triptilion achilleae* DC., MN528044, MN582016. *Urmenetea atacamensis* Phil., MN528045, MN582017. *Wunderlichia mirabilis* Riedel, MN457792, MN457828.

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