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Molecular systematics of the neotropical genus *Psiguria* (Cucurbitaceae): Implications for phylogeny and species identification[1]

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Varying morphological features in many groups of tropical vines confound identification, requiring molecular tools for distinguishing species. Confusion is amplified in *Psiguria*, a small genus found in Central and South America and the Caribbean, because male and female flowers of these monoecious plants are widely separated by time and position on a branch. We present the first phylogeny of *Psiguria* utilizing a combination of eight chloroplast intergenic spacers, the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA repeat, and the intron of the low-copy nuclear gene serine/threonine phosphatase, for a total aligned length of 9456 base pairs. Analyses include multiple accessions of all species in the genus. The data support the monophyly of *Psiguria* and elucidate several species boundaries. Also presented are *Psiguria*-specific DNA barcodes, which include the chloroplast regions: *ndhC-trnV*, *rps16-trnQ*, *rpoB-trnC*, *ndhF-rpl32*, and *psbZ-trnM*. For the first time, systematists, ecologists, and evolutionary biologists will have the tools to confidently identify species of *Psiguria* with DNA barcodes that may be useful in other genera of Cucurbitaceae.

Key words: angiosperms; Caribbean; chloroplast; DNA barcoding; *Gurania*; Guraniinae; *Helmontia*; ITS; low-copy nuclear; phylogenetics.

Vines and lianas are important components of tropical forest ecosystems. They are found in both primary and secondary forests, at all levels of vegetation from forest floor to canopy, and they are most commonly seen without flowers. Adaptations for defense, camouflage, pollinator attraction, or increased exposure to light include leaf mimicry, changes in leaf shape, texture, or size over the life span of an individual, as well as variation in flower shape, size, and color. Such infraspecific transformations make identification based on morphology very challenging, if not impossible. One solution is to use molecular data to define species boundaries. An example is presented here with the genus *Psiguria* Arn. (Cucurbitaceae), whose species identifications are confounded by several morphological variants.

Psiguria is a neotropical genus of monoecious, perennial vines with a geographic distribution extending from southern Mexico to Paraguay and into the Caribbean islands. The genus has been the focus of several ecological and evolutionary studies on the sexual system and the interactions with various insects in the community, but there has never been a phylogeny proposed for the genus. *Psiguria* plays a central role in a suite of plant–animal interactions, including host to several species of *Blepharoneura* Loew fruit flies (Condon, 1984; Condon and Gilbert, 1990; Condon and Norrbom, 1994; Condon and Steck, 1997; Condon et al., 2008). Additionally, most species of *Heliconius* Kluk butterflies rely heavily on nutrients obtained from pollen of *Psiguria* flowers. Unlike other lepidopterans that obtain nitrogenous compounds necessary for egg production as larvae, *Heliconius* butterflies assimilate amino acids and proteins from pollen gathered by adults, principally from *Psiguria* and a few members of the sister genus, *Gurania* Cogn. (Gilbert, 1972, 1975, 1977; Boggs et al., 1981). Although most of the *Heliconius*-collected pollen is destroyed by the butterflies, a sufficient portion is transferred to female flowers (presumably mistaken for a pollen source [Gilbert, 1975]), fulfilling the butterfly's role as *Psiguria*'s primary pollen vector (Condon, 1984; Murawski and Gilbert, 1986). The historical relationship between these two evolving groups of organisms may have influenced the reproductive strategy of the butterflies and the separation of genera in the plant subtribe (Gilbert, 1993).

Psiguria species occur in low densities in neotropical rainforests, commonly growing over shrubs and trees along edges of gaps and streams (Condon, 1984; Murawski and Gilbert, 1986; Condon and Gilbert, 1990). The genus is generally characterized by large, glabrous, palmately 3- to 5-lobed or compound (3- to 5-foliolate), leathery leaves. However, these leaf

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characteristics vary over the life of an individual. For example, first leaves of *P. pedata* (L.) R. A. Howard, found in the Greater Antilles, are simple, shallowly trilobed, and relatively membranous. Subsequent, or even adjacent, leaves may be more deeply lobed or completely separated into two or three leaflets. Leaves on mature *P. pedata* are coriaceous and show further division, typically pedate—three distinct leaflets with side leaflets that are further divided into two subleaflets. *Psiguria* are serially monoecious; i.e., male flowers emerge before the plants switch sex to produce female flowers (Gilbert, 1980; Condon, 1984). Flowers develop in clusters on long, pedunculate, indeterminate racemes or spikes. Both male and female flowers are salverform with tubular calyx and rotate petals; a similarity that, along with the stamen-like structure of the pistil, aids in "training" pollinator butterflies to visit both sexes. Sepals of both male and female flowers are green and short (relative to the sister genus *Gurania*). Petals are typically red with yellow bases, pink, or orange; however, this is another characteristic, along with flower diameter, that can change drastically over the life of an individual. The first male flower of *P. ternata* (M. J. Roem.) C. Jeffrey from Bolivia, for example, is typically light pink or salmon and very large and showy (12–13 cm in diameter). However, subsequent flowers are progressively smaller (down to 0.5–1.0 cm in diameter) with much darker, almost red, petals. Additionally, the calyx tubes of *P. ternata* flowers change from flask-shaped to cylindrical in consecutive flowers in an inflorescence. Furthermore, plants are not always in bloom, and sterile collections can make up approximately 15% of herbarium sheets. For these reasons and the fact that incomplete individuals of *Psiguria* are usually found on a given herbarium sheet, questions have been raised as to how many species there are, where species boundaries exist, and how to identify individual species. The primary goal of this study was to address these questions of species delimitation, a task that is becoming a priority for many systematists (Wiens, 2007).

The last complete taxonomic treatment of *Psiguria* was published by Cogniaux in 1916, using the genus name *Anguria* (Plum.) L. This name is now a nomen rejiciendum because it is a later homonym (published by von Jacquin 1760) of *Anguria* Mill. (1754). In his key to species, Cogniaux (1916) used leaf morphology and anther characteristics such as shape (straight or folded) and appendage texture (glabrous, papillose, blunt, or gradations of these) as major distinctive characters. As discussed above, leaf morphology varies over the life of an individual, and while anther shape may be a good taxonomic character, texture of the anther appendage also varies within a species (Steele, in press). Although Cogniaux (1916) recognized 29 species of *Anguria*, Jeffrey (1978) recognized only eight, and subsequent publications have resulted in 17 names listed in the International Plant Names Index (IPNI, 2008). In a key to *Psiguria* of Panama, Wunderlin (1978) used calyx tube shape and color along with leaf texture (membranous vs. coriaceous) to distinguish species. Again, some of these characters have been observed to vary in individuals throughout their lives. M. Nee (New York Botanical Garden) and C. Jeffrey (Royal Botanic Gardens Kew, now at Russian Academy of Sciences) have annotated most of the specimens located at five of the largest herbaria in the United States (MO, NY, G, F, and US). The majority of those specimens are annotated with five names (*P. pedata*, *P. ternata*, *P. triphylla* (Miq.) C. Jeffrey, *P. umbrosa* (Kunth) C. Jeffrey, or *P. warscewiczii* (Hook f.) Wunderlin), and a few with *P. racemosa* C. Jeffrey. *Psiguria triphylla* and *P. warscewiczii* have been used most often (together making up approximately 72% of annotated specimens). It has not been clear whether this large percentage is due to their having broader geographic distributions, being more common, or being used as catchall names. The remainder of the names has been used for less than 8% of herbarium specimens, and some names have not been used on any specimen other than the type. The question has remained as to whether any of the other 11 names listed in IPNI pertain to species distinct from those six.

Past taxonomic treatments of *Psiguria* listed two species endemic to Caribbean islands: *P. trifoliata* (L.) Alain and *P. pedata* (Wunderlin, 1978; Howard, 1973). If it were confirmed that these are distinct species, many interesting biogeographical questions could be asked. These include where the genus originated, how many times and in which direction it moved between Latin America and the islands, and whether it migrated by dispersal or vicariance. The divergence of the subtribe Guraniinae from sister genus *Wilbrandia* is estimated to be 11 ± 3 million years ago (mya), and the split between *Psiguria* and *Gurania* is estimated to be 6 ± 3 mya (Schaefer et al., 2009). Because of the much greater age of the Caribbean islands (45–46 my) (Inturralde-Vinent and MacPhee, 1999; Graham, 2003), this timing suggests that dispersal played the major role in the distribution pattern of *Psiguria*, and the only question is from where dispersal occurred.

Psiguria is placed in the tribe Coniandreae, subtribe Guraniinae along with only two other genera, *Gurania* and *Helmontia* Cogn. (Jeffrey, 2005). Jeffrey (1978) contended that the integrity and distinctness of the subtribe is supported by pollen and flower morphology. Separation of *Helmontia* is based on palynological evidence (Marticorena, 1963); pollen grains of *Helmontia* are singular, while those of *Psiguria* and *Gurania* occur in tetrads. Additionally, *Helmontia* petals are white, in contrast with the brightly colored orange, red, or yellow petals of its two sister genera. *Psiguria* and *Gurania* are easily distinguished by floral morphology. In contrast to the structure and color of *Psiguria* flowers and leaves, *Gurania* flowers have bright orange, long, divided sepals and inconspicuous yellow petals, and leaves are generally pubescent and not nearly as leathery as those of *Psiguria*. Additionally, *Gurania* are found only on neotropical continents and Trinidad and Tobago (Cheesman, 1940), not in the Greater and Lesser Antilles. A familywide phylogeny of Cucurbitaceae supported the monophyly of subtribe Guraniinae, but relationships at the generic level raised doubts about the monophyly of *Psiguria* (Kocyan et al., 2007). The results of Kocyan et al. (2007) showed *Helmontia* embedded within *Psiguria* and *Gurania*, but taxon sampling (only two *Psiguria*, three *Gurania*, and one *Helmontia*) may have been insufficient to draw strong conclusions (S. Renner, Systematische Botanik, personal communication). The analysis placed subtribe Guraniinae sister to genus *Wilbrandia* Presl., with these, in turn, sister to *Doyerea* Grosourdy (Kocyan et al., 2007).

In recent years, several groups of plant scientists have been working to find a DNA region (or combination of regions) that can serve as a barcode for identifying species across flowering plants (Kress et al., 2005) or land plants (Chase et al., 2005, 2007; Kress and Erickson, 2007). A region of the mitochondrial genome (cytochrome *c* oxidase 1 or *CO1*) has been successfully used in animals for several years (e.g., Blaxter et al., 2004; Hebert et al., 2004; Hajibabaei et al., 2006; Witt et al., 2006), but

TABLE 1. Samples included in the study.

Genus	Specific-epithet	Numerical designation in tree[c]	Country of collection	Collector and collection no. (herbarium)[d]
Doyerea	*emeto-carthartica*	1*	Antilles	none available – (M)
Doyerea	*emeto-carthartica*	2	Jamaica	Adams 12246 (M)
Gurania	*acuminata*	3*	Peru	Condon P05-77 (MOVC)
Gurania	*costaricensis*	4*	Costa Rica	Steele 1009 (TEX)
Gurania	*eriantha*	5	Peru	Condon P05-40B (MOVC)
Gurania	*insolita*	6	Peru	Steele 1022 (TEX)
Gurania	*lobata*	7*	Bolivia	Steele 1045 (TEX)
Gurania	sp.	8*	Peru	Steele 1046 (TEX)
Gurania	sp.	9*	Peru	Steele 1047 (TEX)
Gurania	sp.	10*	Peru	Steele 1048 (TEX)
Helmontia	*jeffreyi*	11	Venezuela	Steyermark 111716 (F)[a]
Helmontia	*leptantha*	12	Guyana	Clarke 9665 (US)[a]
Psiguria	*pedata*	13*	Cuba	Shafer 10523 (NY)
Psiguria	*pedata*	14*	Puerto Rico	Steele 1030 (TEX)
Psiguria	*pedata*	15*	Dominican Republic	Steele 1035 (TEX)
Psiguria	*pedata*	16*	Dominican Republic	Steele 1036 (TEX)
Psiguria	*pedata*	17*	Dominican Republic	Alain 14123 (NY)
Psiguria	*pedata*	18*	Dominican Republic	Alain 17756 (NY)
Psiguria	*pedata*	19*	Puerto Rico	Alain 32529 (NY)
Psiguria	*pedata*	20	Costa Rica	Haber 4860 (MO)
Psiguria	*racemosa*	21*	Colombia	Barriga 13400[b] (NY)
Psiguria	*racemosa*	22*	Venezuela	Steele 1018 (TEX)
Psiguria	*racemosa*	23	Venezuela	Steyermark 120279 (MO)
Psiguria	*racemosa*	24	Colombia	Romero 9685 (MO)
Psiguria	*racemosa*	25	Venezuela	Steyermark 88837 (NY)
Psiguria	sp.	26	Brazil	Noblick 3356 (MO)
Psiguria	*triphylla*	27	Mexico	Dillon 1780 (TEX)
Psiguria	*triphylla*	28*	Mexico	Steele 1004 (TEX)
Psiguria	*triphylla*	29*	Mexico	Steele 1038 (TEX)
Psiguria	*triphylla*	30	Mexico	Steele 1058 (TEX)
Psiguria	*triphylla*	31*	Mexico	Abbott 23877 (FLAS)
Psiguria	*triphylla*	32*	Mexico	Torres 3281 (MO)
Psiguria	*ternata*	33*	Peru	Steele 1039 (TEX)
Psiguria	*ternata*	34*	Bolivia	Steele 1040 (TEX)
Psiguria	*ternata*	35*	Bolivia	Steele 1043 (TEX)
Psiguria	*ternata*	36*	Brazil	Steele 1066 (TEX)
Psiguria	*ternata*	37*	Bolivia	Rivero 244 (LPB)
Psiguria	*ternata*	38*	Bolivia	Nee 33826 (LPB)
Psiguria	*ternata*	39	Bolivia	Nee 38959 (TEX)
Psiguria	*ternata*	40*	Bolivia	Nee 40367 (LPB)
Psiguria	*ternata*	41	Bolivia	Nee 47832 (NY)
Psiguria	*triphylla*	42	Guatemala	Lundell 16872 (TEX)
Psiguria	*triphylla*	43*	Costa Rica	Steele 1007 (TEX)
Psiguria	*triphylla*	44*	Costa Rica	Steele 1008 (TEX)
Psiguria	*triphylla*	45*	Peru	Steele 1052 (TEX)
Psiguria	*triphylla*	46*	Peru	Steele 1054 (TEX)
Psiguria	*triphylla*	47*	Trinidad	Steele 1056 (TEX)
Psiguria	*triphylla*	48*	Costa Rica	Steele 1059 (TEX)
Psiguria	*triphylla*	49*	Costa Rica	Steele 1060 (TEX)
Psiguria	*triphylla*	50*	Costa Rica	Steele 1062 (TEX)
Psiguria	*triphylla*	51*	Costa Rica	Steele 1063 (TEX)
Psiguria	*triphylla*	52	Costa Rica	Steele 1068 (TEX)
Psiguria	*triphylla*	53	Brazil	Sterling 6217 (NY)
Psiguria	*triphylla*	54*	Costa Rica	Croat 22130 (MO)
Psiguria	*triphylla*	55*	Ecuador	Cerón 7619 (MO)
Psiguria	*triphylla*	56*	Ecuador	Gilbert (TEX)
Psiguria	*triphylla*	57	Guatemala	Jones 3361 (NY)
Psiguria	*triphylla*	58	Guyana	Boom 7305 (NY)
Psiguria	*triphylla*	59	Belize	Gentle 5469 (TEX)
Psiguria	*triphylla*	60*	Ecuador	Condon JS05-97 (MOVC)
Psiguria	*triphylla*	61	Mexico	Martinez 12486 (GH)
Psiguria	*triphylla*	62*	Panama	Croat 12218 (MO)
Psiguria	*triphylla*	63*	Panama	Croat 16518 (MO)
Psiguria	*umbrosa*	64	Trinidad	Steele 1002 (TEX)
Psiguria	*umbrosa*	65	Trinidad	Steele 1017 (TEX)
Psiguria	*umbrosa*	66*	Trinidad	Steele 1065 (TEX)
Psiguria	*umbrosa*	67*	Brazil	Plowman 8512 (MO)
Psiguria	*umbrosa*	68*	St. Lucia	Howard 19874 (GH)
Psiguria	*umbrosa*	69	Venezuela	Berry 3789 (MO)
Psiguria	*warscewiczii*	70*	Guatemala	Contreras 8809 (TEX)
Psiguria	*warscewiczii*	71	Panama	Hammel 3218 (MO)
Psiguria	*warscewiczii*	72*	Costa Rica	Steele 1006 (TEX)
Psiguria	*warscewiczii*	73*	Belize	Steele 1055 (TEX)
Psiguria	*warscewiczii*	74	Belize	Steele 1061 (TEX)
Psiguria	*warscewiczii*	75*	Costa Rica	Steele 1064 (TEX)
Psiguria	*warscewiczii*	76*	Colombia	Callejas 2271 (NY)
Psiguria	*warscewiczii*	77*	Costa Rica	Hammel 20374 (MO)
Psiguria	*warscewiczii*	78*	Guatemala	Contreras 6339 (TEX)
Psiguria	*warscewiczii*	79*	Honduras	Poole 1167 (TEX)
Psiguria	*warscewiczii*	80*	Honduras	Molina 32001 (MO)
Psiguria	*warscewiczii*	81*	Mexico	Martínez 26070 (MO)
Psiguria	*warscewiczii*	82*	Panama	Croat 8499 (MO)
Wilbrandia	*ebracteata*	83	Paraguay	Wooston 980 (NY)
Wilbrandia	*longisepala*	84*	Brazil	Mikich 38568 (NY)

[a]Leaf samples obtained by T. S. Quedensley, Plant Biology Graduate Program, University of Texas at Austin

[b]Note on label indicates that the collection information may be incorrect.

[c]Samples marked with an asterisk (*) are those in which sequences for all three markers were obtained and are included in "reduced" data sets.

[d]Specimens are housed in herbaria indicated in parentheses. F = Field Museum of Natural History, FLAS = Florida Museum of Natural History, GH = Harvard University, LPB = Herbario Nacional de Bolivia, M = Botanische Staatssammlung München, MOVC = Cornell College, NY = New York Botanical Garden, TEX = University of Texas at Austin, US = Smithsonian Institution.

not in all groups. In a study where this marker was tested in Diptera, Meier et al. (2006) successfully determined less than 70% of all species. The task of finding a suitable barcoding region for plants has provided a great challenge for several reasons: (1) the mitochondrial genome in plants generally has extremely low levels of variability, (2) the effectiveness of regions in the nuclear genome is questionable due to complex evolutionary patterns, and (3) the chloroplast genome has low levels of variability (but greater than the mitochondrial genome) and is usually uniparentally inherited (Chase et al., 2005, 2007; Kress et al., 2005; Kress and Erickson, 2007; Edwards et al., 2008). Nevertheless, the nuclear ribosomal internal transcribed spacer (ITS) and several chloroplast genes and intergenic spacers (IGS) have been suggested as potential DNA barcodes: ITS + *trnH-psbA* (Kress et al., 2005), ITS + *rbcL* (Chase et al., 2005), *rpoC1*+ *rpoB* + *matK* or *rpoC1*+ *matK* + *psbA-trnH* (Chase et al., 2007), and a portion of *rbcL* + *trnH-psbA* (Kress and Erickson, 2007). It has also been noted that even these regions are lacking sufficient levels of variation in many genera; therefore, additional regions are required for distinguishing

species in groups with extremely low levels of detectable molecular variation or in those that have undergone recent, rapid speciation (Chase et al., 2007).

In this study, we investigated the molecular evolutionary history of *Psiguria* using three independent markers: (1) a concatenated chloroplast marker made up of eight intergenic spacers, (2) both internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA repeat, and (3) the intron of nuclear serine/threonine phosphatase gene. Our goals were to test the monophyly of *Psiguria*, to determine the number of species in the genus, and to detect sister relationships. Additionally, we set out to identify regions of the chloroplast genome that could be used as a barcode to distinguish species quickly and efficiently.

MATERIALS AND METHODS

Taxon sampling—Because a phylogeny of *Psiguria* has never been published and the number of species in the genus has been debated, several individuals from each species were studied. Regardless of names listed on herbarium sheets, sampling spanned the geographical and morphological breadth of the genus and included field collections made by P. R. Steele and L. E. Gilbert, collections from plants in the greenhouses of L. E. Gilbert at The University of Texas at Austin, and leaf material from herbarium collections (Table 1). In addition to 70 samples of *Psiguria*, 10 of the sister genera *Gurania* (eight) and *Helmontia* (two) were included. Outgroups were chosen based on the Cucurbitaceae family phylogeny (Kocyan et al., 2007) and included two samples of *Doyerea emeto-cathartica* and two species of *Wilbrandia*.

Fresh leaf material was dried over silica. Total DNA was extracted using either the CTAB protocol of Doyle and Doyle (1987) or the DNeasy Plant DNA Extraction Kit (Qiagen, Germantown, Maryland, USA). CTAB products were purified by ultracentrifugation in cesium chloride and ethidium bromide gradients (Sambrook et al., 1989).

Primers, PCR amplification, and DNA sequencing—Listed in Appendix S1 (see Supplemental Data with the online version of this article) are the eight chloroplast DNA intergenic spacer regions, two internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA repeat, and the intron of the nuclear serine/threonine phosphatase gene (*s/t phos*) used as phylogenetic markers in this study, along with primer sequences and PCR annealing temperatures. With the exception of those for *trnS-trnG* taken from Shaw et al. (2005), all chloroplast primers were designed based on the genome sequence of *Cucumis sativus* L. (DQ119058; Kim et al., 2006). The ITS primers were as described by Kim and Jansen (1994). Original primers for amplifying *s/t phos* were obtained from Padolina (2006; primer combination #96). After this region was successfully amplified in several samples, primers specific to this study group were designed to eliminate end regions that had little or no variation and to increase amplification success. All nested primers were designed based on sequences of *Psiguria* samples for which initial primers amplified the region. Also shown in SAppendix S1 (see online Supplemental Data) are internal primers used to sequence some regions to obtain 2× sequence coverage. Finally, the nuclear *s/t phos* intron in *Psiguria umbrosa* contains a large (>500 bp) insert relative to all other samples; therefore, internal primers for sequencing were designed specifically for those samples (Pumb96I-F and Pumb96I-R in Appendix S1).

Chloroplast marker amplifications were performed using PCR in 25-μL volumes containing 14.6 μL of double-distilled (dd) H_2O, 7.5 μL of FailSafe buffer–PreMix E (EPICENTRE Biotechnologies, Madison, Wisconsin, USA), 0.25 μL of a 20 μM solution of each forward and reverse primer, 0.4 μL of *Taq* polymerase (produced in the laboratory of R. K. Jansen following the protocol of Pluthero (1993) and diluted to 1 unit), and 2 μL of unquantified DNA template. The PCR reaction conditions were as follows: one round of amplification consisting of denaturation at 96°C for 3 min; annealing at the temperature for each region shown in online SAppendix S1 for 45 s; and extension at 72°C for 1 min; followed by 35 cycles of 94°C for 35 s, annealing temperature for 45 s, and 72°C for 1 min; with a final extension step of 72°C for 12 min. Amplifications were visualized on 1% agarose gels with ethidium bromide and a size standard to estimate fragment sizes and DNA concentration. The PCR amplicons were cleaned using Exo-SAP by adding a 3 μL solution of 2.25 μL of ddH_2O, 0.25 μL of Exonuclease I (New England Biolabs, Ipswich, Massachusetts, USA), and 0.50 μL of Shrimp Alkaline Phosphotase (Promega, Madison, Wisconsin, USA) to each product, and processing on a thermocycler at 37°C for 30 min, followed by 80°C for 15 min. Sequencing was conducted at the ICMB Core Facilities at The University of Texas at Austin, using ABI (Applied Biosystems, Foster City, California, USA) Big Dye chemistry.

Amplifications of both ITS and the *s/t phos* intron were performed using PCR in 25-μL volumes containing ingredients as described above. Reaction conditions were as follows: one round of amplification consisting of denaturation at 94°C for 1 min 30 s; annealing at 53°C for 30 s; and extension at 72°C for 1.5 min; followed by 29 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min 30 s; with a final extension step of 72°C for 30 min.

Cloning was conducted using the TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA). Colonies were amplified using PCR in 25-μL volumes containing 16.1 μL of ddH_2O, 8.0 μL of FailSafe buffer PreMix E, 0.2 μL of a 20 μM solution of each pUC-18 plasmid primer (sequences listed in online SAppendix S1), 0.5 μL of *Taq* polymerase, and 1 colony. Reaction conditions were as follows: one hot-start cycle at 95°C for 3 min 30 s; followed by 35 cycles consisting of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 50 s; with a final extension step of 72°C for 3 min. The PCR products that did not clone successfully were directly sequenced. All sequences were submitted to GenBank, and accession numbers are listed in Appendix 1.

Phylogenetic analyses—Forward, reverse, and internal sequences of all PCR products were assembled and edited with the program Geneious Pro 4.0.4 (Biomatters Ltd., 2005–2009), and then aligned with the program CLUSTAL_X (Thompson et al., 1997), followed by manual adjustments that minimized the number of gaps. The alignment for each data set was uploaded to the TreeBase database (http://treebase.org/treebase/index.html, see Table 3 for alignment numbers). Because chloroplasts are maternally inherited in Cucurbitaceae (Corriveau and Coleman, 1988), the eight IGS were combined into one concatenated data set for analyses. Unalignable regions were cut from sequences prior to phylogenetic analyses (number of base pairs cut: *ndhF-rpl32*, 25; *ndhC-trnV*, 0; *rps16-trnQ*, 56; *trnS-trnG*, 1; *psbZ-trnM*, 13; *psbM-trnD*, 0; *rpoB-trnC*, 10; *psbE-petL*, 36; ITS1, 42; and ITS2, 35.

Nuclear ITS and *s/t phos* data sets were analyzed independently. Nuclear regions were initially analyzed with all clones sequenced. In most cases, clones of multiple samples of the same species mixed within a clade. Because these clones came from different individuals of the same species, this was expected. To compare, contrast, and combine trees from these three data sets, we chose one representative clone at random to be analyzed in an abridged data set. Selection of random clones was conducted three times with different clones to determine if certain sets of clones would give different results. To evaluate total evidence, we conducted phylogenetic analyses on a combined data set that included the concatenated chloroplast marker and the two abridged nuclear data sets. Finally, to directly compare trees resulting from each marker, we conducted additional analyses with reduced data sets that included only those samples successfully amplified and sequenced in all three data sets.

Maximum parsimony (MP) analyses were performed with the program PAUP* 4.0b10 (Swofford, 2002). For each data set, heuristic searches were conducted using 100 random addition replicates with tree-bisection-reconnection (TBR) branch swapping with either a 10-min limit enforced on each replicate or the maximum number of trees set to 10 000. Additionally, searches used characters equally weighted, gaps treated as missing, and the MulTrees option. Insertions and deletions were coded in the program SeqState 1.40 (Müller, 2005) using Simmons and Ochoterena (2000) simple coding. Support for internal nodes was assessed using bootstrap analysis (Felsenstein, 1985) of 100 replicates with one random addition per replicate, and a 10-min limit enforced on each replicate.

Models of evolution were selected based on the Akaike information criterion (AIC) implemented in the program MrModeltest 2.2 (Nylander et al., 2004). Maximum likelihood (ML) analyses were performed using the program Garli 0.951 (Zwickl, 2006) with the model of evolution as selected above. Each data set was analyzed in five separate runs as suggested by Zwickl (2006). The ML analyses used the automated stopping criterion, terminating a search when the likelihood score remained constant for 20 000 consecutive generations. Likelihood scores of the optimal tree generated by Garli were calculated using PAUP*, which better optimizes branch lengths (Zwickl, 2006). ML bootstrap analyses were performed in Garli (Zwickl, 2006) with 100 replicates using an automated stopping criterion set at 5000 generations. Shimodaira-Hasegawa (SH) tests (Shimodaira and Hasegawa, 1999) were conducted in PAUP* to test alternative topologies and to compare trees from different data sets. Incongruence length difference (ILD) tests (Farris et al., 1995) were used to compare reduced data sets.

Identifying DNA barcodes—To discover *Psiguria*-specific DNA barcodes, we visually scanned the chloroplast sequences for base pairs that were unique to each species. Ideally, this set would constitute a single marker with base pairs unique to all species in the genus or a minimum number of regions to delineate them.

RESULTS

Phylogenetic analysis—The model of evolution chosen for our various data sets was either the general time reversible model with proportion of invariant sites and gamma shape parameter (GTR + I + G; for the concatenated chloroplast, *s/t phos* with 385 clones, and combined data set) or general time reversible model with only gamma shape parameter (GTR + G; for the ITS with 246 clones, abridged ITS, and abridged *s/t phos* data sets). Sequences were not successfully obtained from all samples for all markers (marked with "—" in Appendix 1), and a few regions had undetermined internal gaps. A list of all regions that were coded as missing is shown in Appendix S2 (see Supplemental Data with the online version of this article).

Chloroplast data—Aligned sequence characteristics for the eight IGS are shown in Table 2. Sequence characteristics and tree statistics for the concatenated chloroplast data set are given in Table 3. The tree resulting from the ML analysis is shown in Fig. 1. Our data strongly support the monophyly of *Psiguria* (shown with a thick branch in Fig. 1). *Helmontia* is nested within *Gurania*, but it has a very long branch. Some clades within *Psiguria* are well supported (*P. pedata* and *P. umbrosa*), *P. ternata* has moderate support, and *P. warscewiczii* is weakly supported. Most *P. triphylla* samples form a strongly supported clade; however, *P. racemosa* and *P. triphylla* are found in several places in the tree (specifically, *P. triphylla* [54], *P. triphylla* [58], *P. racemosa* [23], and *P. racemosa* [24], shaded and marked with an asterisk). The identity of one *Psiguria* sample, designated as *P*. sp. (26), is uncertain. This sample was taken from an herbarium specimen annotated as *P. ternata* that was collected in Brazil. It does not group with the *P. ternata* clade, and it does not have identifiable morphological characteristics that suggest it belongs to any of the other named clades in the tree. There is <50% support for relationships among clades throughout the backbone. One exception is a weakly supported sister relationship between *P. warscewiczii* and *P. umbrosa*. In two of the five ML analyses, the *P. ternata* and *P. pedata* clades switched positions, but this difference is not well supported because the branch separating them received less than 50% bootstrap support in both MP and ML analyses, and it collapses in strict consensus trees.

Serine/threonine phosphatase intron data—This nuclear marker was successfully amplified in 60 of the 84 samples. Cloning was successful with all but 12 amplicons. Shown in Table 3 are sequence characteristics and tree statistics for the data set containing 385 clones and the abridged *s/t phos* data set. Both numbers and percentages of parsimony informative characters were much greater for the data set including all clones than the abridged set, due to variation between clones of a single sample. Alignment of sequences revealed a large (> 500 bp) insert in two of three *P. umbrosa* samples (from Trinidad-66 and St. Lucia-68) but not in a third sample (from Brazil-67).

The ML tree for the full data set of *s/t phos* clones is shown in online SAppendices S3–S5, and the ML tree for the abridged data set is shown in Fig. 2. *Wilbrandia* grouped with *Gurania* with strong bootstrap support, but with a very long branch. This marker does not support the monophyly of *Psiguria*. Four clades of *Psiguria* have strong bootstrap support: *P. pedata*, *P. warscewiczii*, *P. umbrosa*, and *P. ternata*. *Psiguria warscewiczii* and *P. umbrosa* are strongly supported as sister species. As with the chloroplast tree, *P. racemosa* and *P. triphylla* samples are paraphyletic. However, with this data set, *P. triphylla* (54) is associated with other samples of *P. triphylla*. As mentioned above, random clone selection was conducted three times for the *s/t phos* abridged data set. Each of the three abridged data sets was analyzed individually, and ML trees reconstructed (data not shown). The trees gave the same results as described above.

ITS data—Both ITS1 and ITS2 were successfully amplified in 70 of 84 samples. Cloning was successful with all but seven amplicons. The number of successful clones that was sequenced varied from one to five per sample. Putative pseudogenes were identified as those sequences having more than one base pair different in the 5.8S region from all other sequences in the same genus and were not included in analyses. In several cases, if the direct sequence of the amplified region was clean, with no ambiguous sites, it was included in the analysis.

Shown in Table 3 are sequence characteristics and tree statistics for the data set containing 246 clones and the abridged ITS data set. As with the *s/t phos* data, numbers and percentages of parsimony informative characters were much greater for the ITS data set including all clones than the abridged set due to the variation between clones of a single sample.

The ML tree for the full data set of ITS clones is shown in online Appendices S6 and S7, and the ML tree for the abridged data set is shown in Fig. 3. The abridged ML tree is identical to one of 6803 MP trees, except in the MP trees, the *Wilbrandia* clade is sister to the *Doyerea* clade, and the *P. warscewiczii* clade is sister to the *Gurania* clade but with only 57% bootstrap support. In the ML tree, *Wilbrandia* is sister to *P. warscewiczii*, and both are sister to *Gurania*, but neither has bootstrap

TABLE 2. Sequence characteristics of the eight chloroplast intergenic spacers.

Intergenic spacer		*ndhF-rpl32*	*ndhC-trnV*	*rps16-trnQ*	*trnS-trnG*	*psbZ-trnM*	*psbM-trnD*	*rpoB-trnC*	*psbE-petL*
Aligned length, w/ indels		725	743	1253	724	851	988	1148	1233
Number of coded indels		9	15	29	19	19	11	6	12
Guraniinae only, w/o indels	No. PI characters	27	27	38	17	8	14	16	29
	% PI characters	3.77	3.71	3.10	2.41	0.96	1.43	1.40	2.38
Guraniinae only, w/ indels	No. PI characters	29	35	47	22	15	19	18	34
	% PI characters	4.05	4.81	3.84	3.12	1.80	1.94	1.58	2.78
All samples, w/indels	No. PI characters	49	46	69	30	29	20	24	51
	% PI characters	6.84	6.32	5.64	4.26	3.49	2.05	2.10	4.18

Note: PI = parsimony informative, w/ = with, w/o = without

TABLE 3. Sequence characteristics and tree statistics for all six data sets.

Statistic		Concatenated chloroplast	*s/t phos* clones	*s/t phos*: abridged	ITS clones	ITS: abridged	All 3 data sets combined
Aligned length, with indels		7700	1183	1164	813	796	9660
Number of coded indels		155	43	24	42	25	204
Guraniinae [a] only, w/o indels	No. PI characters	176	257	87	157	84	356
	% PI characters	2.33%	22.54%	7.63%	20.36%	10.89%	3.76%
Guraniinae [a] only, w/ indels	No. PI characters	219	275	94	187	92	411
	% PI characters	2.84%	23.25%	8.08%	23.00%	11.56%	4.25%
All samples, w/indels	No. PI characters	408	278	99	227	117	626
	% PI characters	5.30%	23.50%	8.51%	27.92%	14.70%	6.48%
Number of samples included		84	385	60	246	70	84
Number of MP trees [b]		8900	400	10000	6400	6803	164124
Tree length		830	798	272	606	289	1465
CI [c]		0.7104	0.6491	0.7554	0.5411	0.6029	0.6434
RI [c]		0.9087	0.9640	0.9394	0.9143	0.8628	0.8830
−ln L		14466.81	7270.04	3331.88	4827.42	2706.05	21602.42
Figure		1	S3, S4, S5	2	S6, S7	3	4
TreeBase alignment no.		M4677	M4681	M4678	M4682	M4679	M4680

Note: An "indel" may be an individual base pair (bp) or a set of contiguous bps.

[a] Guraniinae included only samples of *Psiguria*, *Gurania*, and *Helmontia*.

[b] Maximum number of trees was set to 10000 on all data sets except the combined set, upon which a 10-min time limit was enforced on each replicate.

[c] Uninformative characters were excluded.

support above 50%. Two clades have moderate to strong bootstrap support: *P. pedata* and *P. ternata*. As with the other data sets, *P. racemosa* and *P. triphylla* samples are paraphyletic. There is moderate bootstrap support for a sister relationship between *P. umbrosa* and some samples of *P. warscewiczii*. *Psiguria warscewiczii* split into two clades—one sister to *Wilbrandia* (with <50% bootstrap support) and the other sister to *P. umbrosa*. The two clades of *P. warscewiczii* are split along geographical lines. The group sister to *Wilbrandia* is from southern Central America/northern South America (Costa Rica, Panama, and Columbia), and the samples sister to *P. umbrosa* are from northern Central America (Mexico, Guatemala, Belize, and Honduras). Random clone selection was conducted three times for the abridged ITS data set. Each of the three abridged data sets was analyzed individually, and ML trees were reconstructed (data not shown). Trees gave the same results as described above.

Combined data—Shown in Table 3 are sequence characteristics and tree statistics for the combined data set, which includes all genomic regions that amplified and were sequenced in all samples. The tree resulting from the ML analysis is shown in Fig. 4. The combined topology is not radically different from the chloroplast tree except that branches are generally longer with more support for individual clades. This tree provides strong support for the monophyly of *Psiguria*. *Helmontia* is nested within *Gurania*, again with very long branches leading to *Helmontia*. *Psiguria pedata*, *P. ternata*, and *P. umbrosa* are well supported. *Psiguria triphylla* has moderate support (except sample *P. triphylla* [58]), and *P. warscewiczii* has low to moderate support. Four of the five samples of *P. racemosa* grouped together (excluding *P. racemosa* [24]), but with <50% bootstrap support. As before, the one sister relationship with bootstrap support above 50% is *P. warscewiczii* and *P. umbrosa*. The unidentified *Psiguria* sample (26) again does not fall into any of the well-supported clades.

Although bootstrap support for the monophyly of *Psiguria* is slightly lower in the combined tree than in the chloroplast tree, all other clades were enhanced by the addition of nuclear data, with one exception; the support for the clade of *P. pedata*, which was 100/100 (MP/ML) in both data sets. The bootstrap support for *P. triphylla* and *P. racemosa* clades decreased, but one sample of each (*P. triphylla* [54] and *P. racemosa* [23]) that was not grouping with the species clade of the same name in the chloroplast tree, moved into the putatively correct clade in the combined tree. Additionally, support for a sister relationship between *Gurania* and *Helmontia* increased.

Marker comparison with reduced data sets—Sequences for all three markers were obtained for 57 samples (marked with an asterisk in Table 1). These included one of each outgroup (*Doyerea* and *Wilbrandia*), six *Gurania*, 49 *Psiguria* including some from each species, and no *Helmontia*. The trees resulting from ML analyses are shown in online SAppendices S8 (chloroplast), S9 (*s/t phos*), and S10 (ITS). These data sets do not result in topologies different from the full data sets.

DNA barcoding—It was not possible to identify a single chloroplast region that had nucleotide changes unique to each species. The smallest number of noncoding chloroplast regions required to distinguish all species of *Psiguria* was four. Table 4 lists the markers and specific nucleotide positions that distinguish the species. If the sequence has a nucleotide other than the one listed in the table for that position, it is not the listed species. Only one region (*ndhC-trnV*) is necessary to identify *P. pedata*, and it has three unique nucleotides. One region (*rps16-trnQ*) identifies *P. ternata* with one unique nucleotide, and one region (*rpoB-trnC*) identifies *P. racemosa* with two unique nucleotides. Three chloroplast regions (*rps16-trnQ* [3 bp], *ndhC-trnV* [2 bp], and *ndhF-rpl32* [1 bp]) are listed for *P. triphylla*. Any one or all of these regions can be used to identify *P. triphylla*. At least two chloroplast regions are required to identify *P. umbrosa* and *P. warscewiczii* (*ndhF-rpl32* plus *rpoB-trnC* or *psbZ-trnM*). Two nucleotide bases in *ndhF-rpl32* are unique to both *P. umbrosa* and *P. warscewiczii*. Then, to distinguish *P. umbrosa* from *P. warscewiczii*, either *rpoB-trnC* or *psbZ-trnM* must be as listed in Table 4.

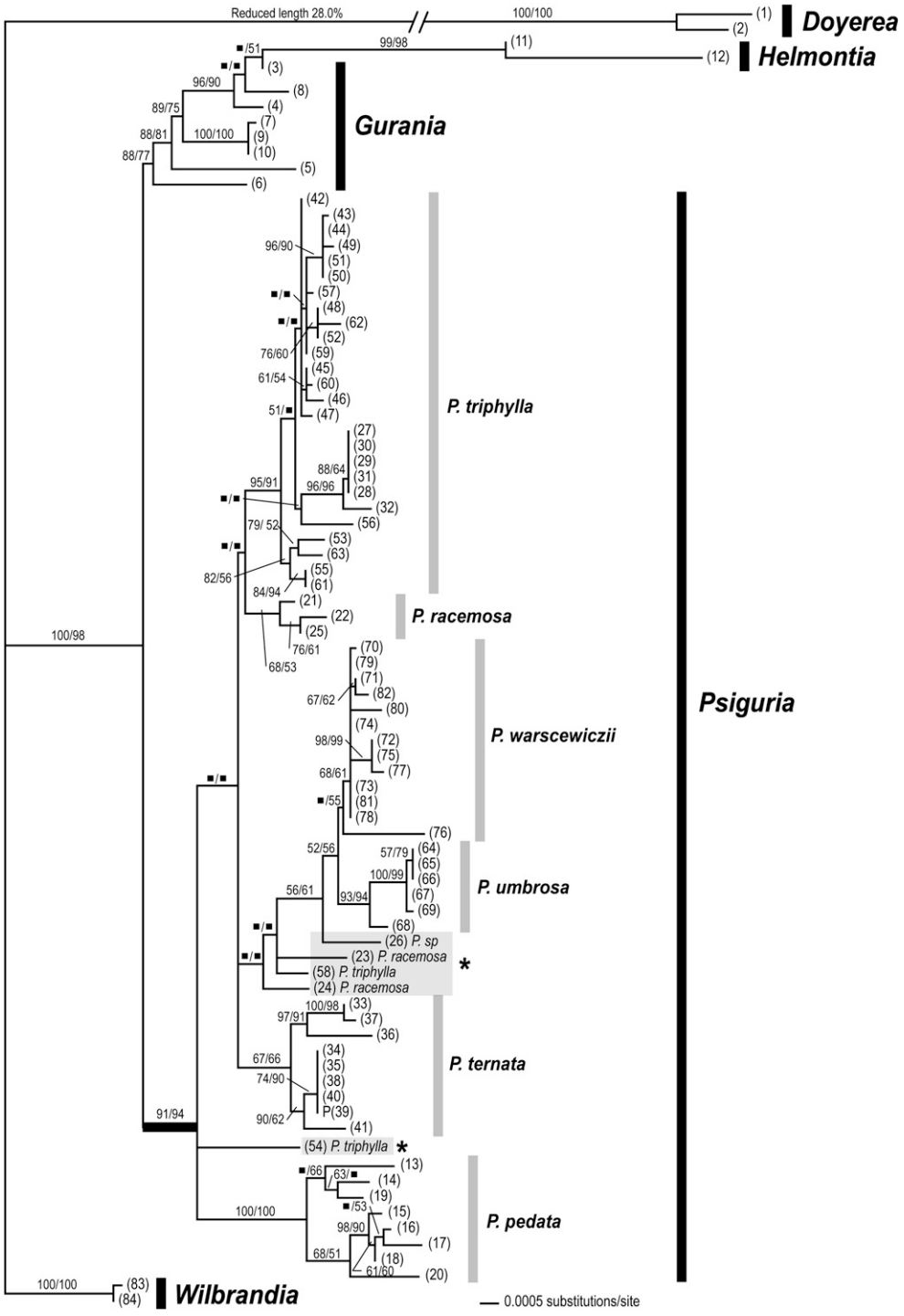

Fig. 1. Maximum likelihood (ML) tree (−ln L = 14 466.81) inferred from the concatenated chloroplast data set, identical to one of the 8900 maximum parsimony (MP) trees (length = 830; CI = 0.71; RI = 0.91). The data set includes 84 samples, with *Doyerea* and *Wilbrandia* used to root the tree. Numbers above the lines indicate MP/ML bootstrap values; a black square indicates a bootstrap value <50%. Shaded samples with asterisks fall outside the clades of the same name. The parenthetical number indicates the sample designation in Table 1.

DISCUSSION

Marker selection—*Chloroplast*—Prior to embarking on the phylogenetic study of *Psiguria*, it was first necessary to identify variable regions that were sufficiently informative. For a useful number of informative characters to be obtained at this low taxonomic level, multiple noncoding regions had to be sequenced and combined to represent one chloroplast marker. In a pilot study including three *Psiguria* and one *Gurania* sample, 26 regions of the chloroplast genome were investigated based on a

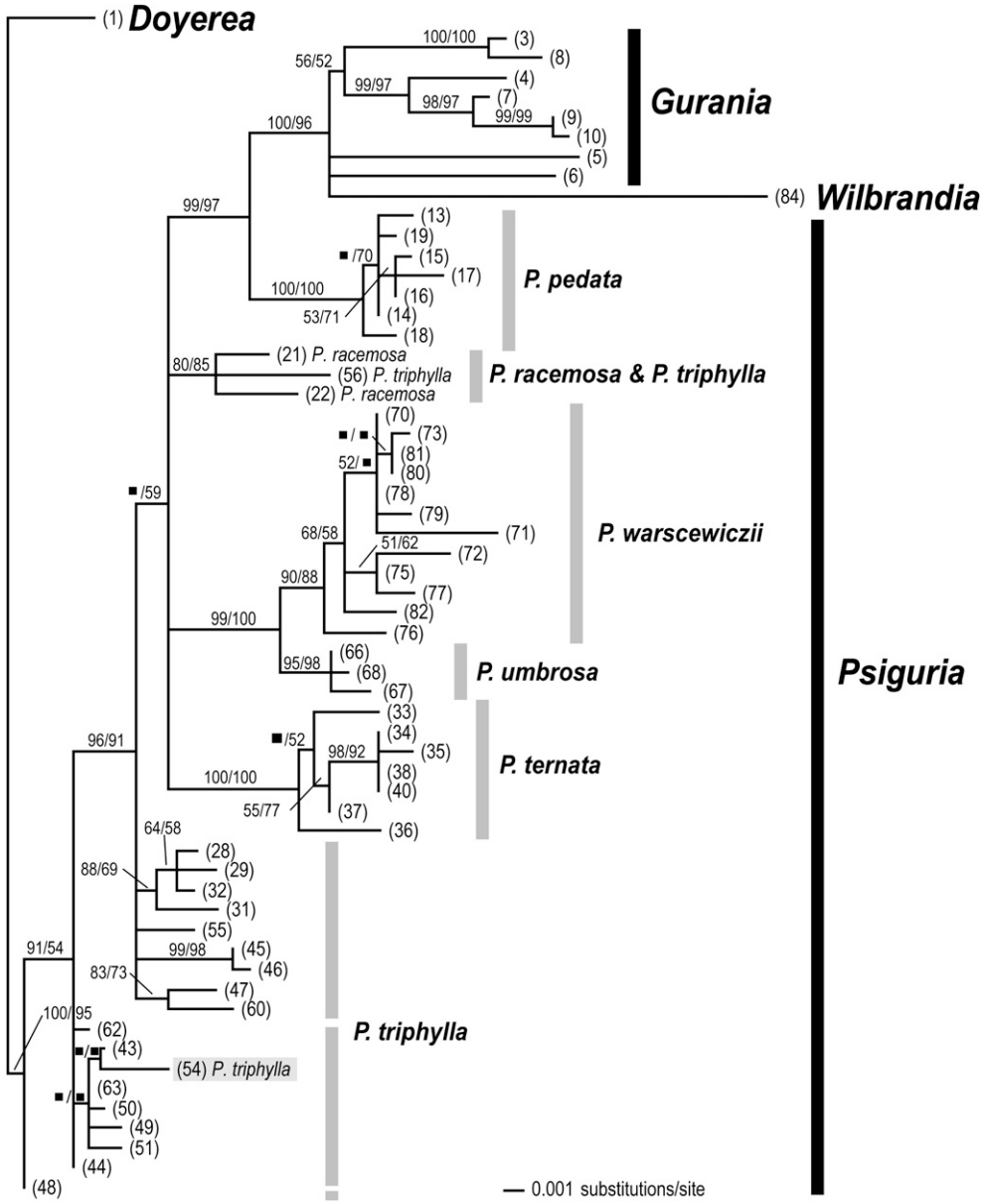

Fig. 2. Maximum likelihood (ML) tree (−ln L = 3331.88) inferred from an abridged set of the clones (one per sample) of the serine/threonine phosphatase gene intron. This tree is identical to one of the 10 000 maximum parsimony (MP) trees (length = 272; CI = 0.76; RI = 0.94). The data set includes 60 samples, with *Doyerea* and *Wilbrandia* used to root the tree. Numbers above the lines indicate MP/ML bootstrap values; a black square indicates a bootstrap value <50%. The shaded samples fell outside the clades of the same name in the chloroplast data set. The parenthetical number indicates the sample designation in Table 1.

consensus of results in the literature (Panero and Crozier, 2003; Shaw et al., 2005; Daniell et al., 2006; Timme et al., 2007) and a study of the published chloroplast genome for *Cucumis sativus* (Kim et al., 2006). After testing these regions for amplification success, suitable length, and phylogenetic utility, we selected the most informative eight (Table 2) by conducting phylogenetic analyses with several combinations until there was little increase in bootstrap support. Regions that were eliminated are *rpl16* intron, *trnT-psbD*, *trnT-trnL*, *ycf3-trnS*, *trnC-ycf6*, *trnG2G-trnG*, *rpl20-5′rps12*, *rps16* intron, *trnD-trnE*, *trnG* intron, *trnT-psbD*, *trnE-trnT*, *atpF-atpH*, *psbA-trnH, psbJ-petA*, *ndhA* intron, *rpl32-trnL*, and *trnL-trnF*, which includes the *trnL* intron and the IGS, *trnL-trnF*. Each of these regions had <1% parsimony informative characters within Guraniinae.

Shaw et al. (2005, 2007) investigated potential variability in 34 noncoding chloroplast regions in three groups of angiosperms: asterids, rosids, and monocots. Of the nine regions that showed the greatest variation in their study, four represented the most variable regions for *Psiguria*: *rps16-trnQ*, *ndhC-trnV*, *ndhF-rpl32*, and *psbE-petL*. Three others (*rpl32-trnL*, *trnT-psbD*, and *psbJ-petA*) showed <1% variation in Guraniinae, one (*atpI-atpH*) was not annotated in the *Cucumis sativus* genome, and the other (*rps16-trnK*) did not amplify successfully. Therefore, as concluded by Shaw et al. (2007), there is no chloroplast

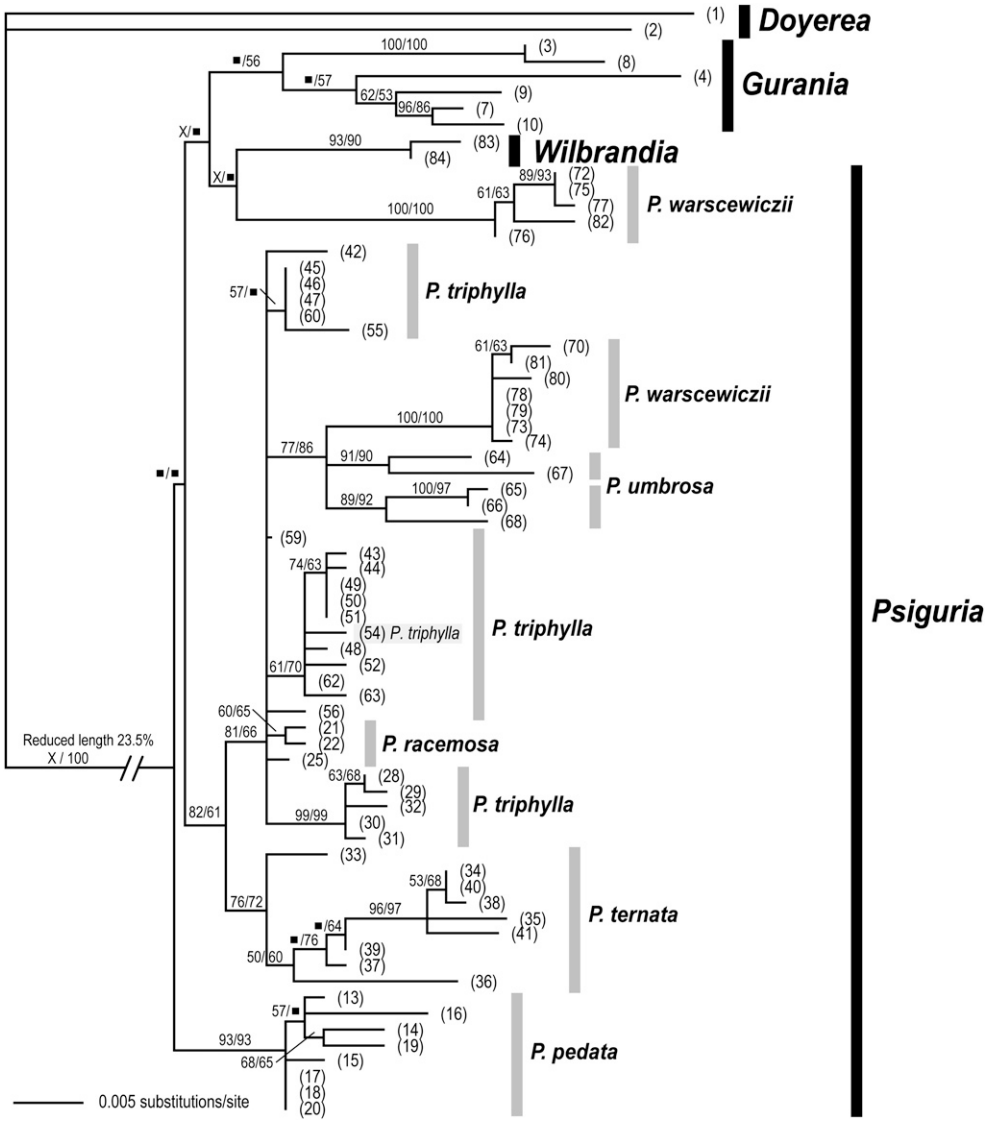

Fig. 3. Maximum likelihood (ML) tree (−ln L = 2706.05) inferred from an abridged set of the clones (one per sample) of the nuclear ribosomal internal transcribed spacers (ITS1 and ITS2). This tree is identical to one of the 6803 maximum parsimony (MP) trees (length = 289; CI = 0.60; RI = 0.86), except in the MP trees, the *Wilbrandia* clade is sister to the *Doyerea* clade, and the *P. warscewiczii* clade is sister to the *Gurania* clade. The data set includes 70 samples, with the *Doyerea* and the *Wilbrandia* samples used to root the tree. Numbers above the lines indicate MP/ML bootstrap values; a black square indicates a bootstrap value <50%. The shaded samples fell outside the clades of the same name in the chloroplast data set. The parenthetical number indicates the sample designation in Table 1. "X" indicates that this branch does not occur in this result.

region (or combination of regions) that will have phylogenetic utility in all lineages, so several must be screened in a reduced set of samples before committing to a studywide sequencing effort.

Low-copy nuclear—One hundred forty-one primer combinations for amplifying low-copy nuclear (LCN) regions were screened for phylogenetic usefulness in *Psiguria* (Steele et al., 2008). These primer pairs were designed by comparing the whole nuclear genome of *Oryza sativa* L. to *Arabidopsis thaliana* (L.) Heynh. to identify conserved regions (Padolina, 2006). In *Psiguria*, 11 regions amplified successfully, and clones were originally sequenced in at least three *Psiguria* species plus outgroups. Steele et al. (2008) discovered three regions that were potentially phylogenetically informative: genes for ATP synthase, actin, and serine/threonine phosphatase (*s/t phos*). Actin was not tested further for phylogenetic utility because there were at least two copies of the gene. After adding many samples to the ATP synthase data set, it was discovered that there was insufficient variation between species, and little bootstrap support for any clades.

Only one copy of serine/threonine phosphatase (*s/t phos*) was detected in a preliminary study by Steele et al. (2008). It showed the greatest potential for phylogenetic utility after adding many samples to the data set. The *s/t phos* gene codes for an enzyme that dephosphorylates serine and threonine residues in proteins (Wera and Hemmings, 1995). Phosphorylation of structural and regulatory proteins is a major intracellular con-

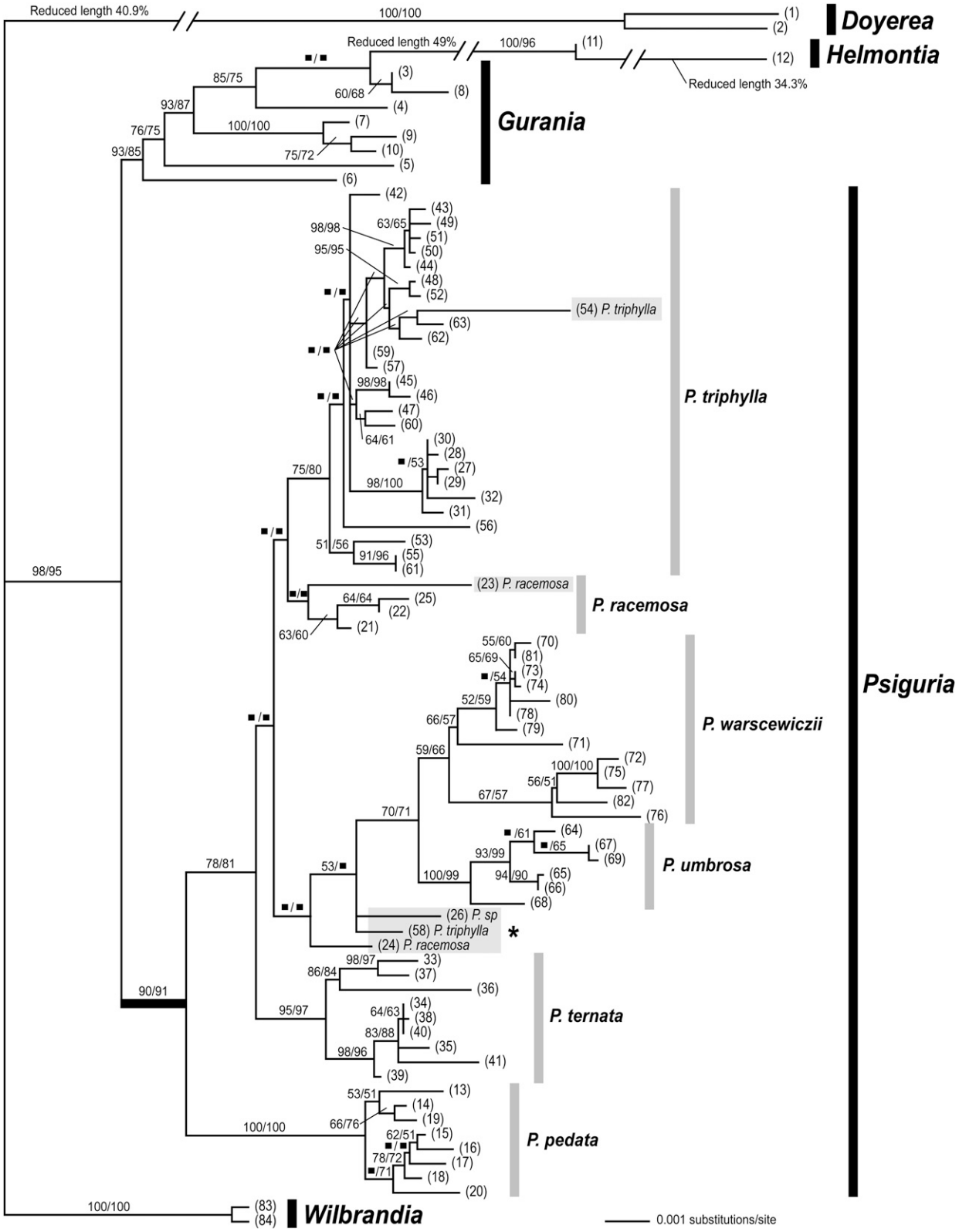

Fig. 4. Maximum likelihood (ML) tree (−ln L = 21 602.42) inferred from the combined data set (chloroplast + *s/t phos* + ITS), identical to one of the 164 124 maximum parsimony (MP) trees (length = 1465; CI = 0.64; RI = 0.88). The data set includes 84 samples, with *Doyerea* and *Wilbrandia* used to root the tree. Numbers above the lines indicate MP/ML bootstrap values; a black square indicates a bootstrap value <50%. Shaded samples with asterisks fall outside the clades of the same name. Shaded-only samples fall outside the clades of the same name in the chloroplast data set. The parenthetical number indicates the sample designation in Table 1.

trol mechanism in eukaryotes. The phosphorylation state of a protein is a dynamic process controlled by both protein kinases and protein phosphatases (Wera and Hemmings, 1995). We designed a *Psiguria*-specific set of primers such that most of the amplified region contained an intron within *s/t phos*.

The sequence encoding serine/threonine phosphatase is a member of a large gene family in *Arabidopsis*. It was not possible to identify which member of the family was amplified/sequenced in this study because sequences obtained did not match any of those in the GenBank *Arabidopsis* database. Perhaps the lack of match similarity is because the sequences amplified here consist mainly of noncoding DNA. The >500 bp insert in two samples (66 and 68) occurred in the center of the sequence and, therefore, would not interfere with putative splicing sites of the exons.

Marker utility—It is surprising that, despite the large number of genomic regions (eight chloroplast, ITS, and one LCN gene intron) used in this study, bootstrap support for several clades is only weak or moderate. Systematic studies of other neotropical genera often include fewer molecular markers, yet result in higher bootstrap support for individual species. For example, Saslis-Lagoudakis et al. (2008) used ITS plus the plastid *trnL* intron, *trnL-F* intergenic spacer, and partial exon of *matK* to investigate the systematics of *Platymiscium* Vogel (Fabaceae), a genus of rainforest trees. In most cases, these regions provide enough phylogenetic information to delineate species and, in some cases, varieties of species with high bootstrap support. Lack of support for species of *Psiguria* may be due to the estimated young age of the group (6 ± 3 mya; Schaefer et al., 2009) or it may indicate that the genus has a low rate of molecular evolution.

Taxonomic implications—*Molecular phylogeny and alternative hypothesis testing*—Phylogeny reconstructions from the concatenated chloroplast (Fig. 1) and combined (Fig. 4) data sets show strong bootstrap support for the monophyly of *Psiguria*. Monophyly is not supported by the *s/t phos* (Fig. 2) or ITS (Fig. 3) trees. Although the tree resulting from the combined data set (Fig. 4) has the longest branches and most well-supported clades, the large amount of chloroplast data (7700 bp) may be overwhelming the signal contributed by the two nuclear data sets (*s/t phos*-1164 bp and ITS-796 bp). To directly compare results from the three markers, reduced data sets were created that included only those samples in which all three markers were successfully amplified and sequenced (online SAppendices S8–S10). Both SH and ILD tests were conducted to pairwise compare all three data sets, and in all comparisons, trees were significantly different ($P < 0.05$).

Both the monophyly of *Psiguria* and its distinction as a genus separate from *Gurania* are supported by several morphological characters discussed above (and more thoroughly in Steele, in press). The lack of support by the nuclear data for the monophyly of *Psiguria* may be caused by one or more phenomena. These include incomplete lineage sorting or mistaken orthology. Although as many as 12 *s/t phos* clones were included for each sample, those regions sequenced in *P. pedata* may be paralogous to those from other species. This paralogy is more likely because this gene is a member of a large gene family and could also explain the split in *P. warscewiczii* samples in ITS trees and the grouping of one clade with *Gurania*. Furthermore, although no hybrids have been identified in *Psiguria* based on morphological characters, it is possible that the nuclear genes are indicating their presence. Additional nuclear markers are required to test this hypothesis. Low bootstrap support, predominantly in the backbone of the trees, may be due to the lack of informative characters. Nonetheless, the nuclear data sets support several of the same species clades supported by the chloroplast data.

Two collections, *P. triphylla* (58) and *P. racemosa* (24), do not group with other collections of those species, but branches separating them all have <50% bootstrap support and collapse in strict consensus trees. We conducted SH tests forcing *P. triphylla* (58) with the *P. triphylla* clade, and another forcing *P. racemosa* (24) with the *P. racemosa* clade. In both of these tests, alternative trees were rejected ($P = 0.001$ and $P = 0.042$, respectively). There are several possible explanations for the positions of these two collections. They may represent distinct species, they could be hybrids, or there may not be enough variation for correct placements. In contrast, two collections that did not group with other samples of the same species, *P. triphylla* (54) and *P. racemosa* (23), in the chloroplast tree, are grouping in those clades in the combined tree.

Trees inferred from *s/t phos* and ITS clones have, for the most part, the same topologies as the abridged trees (Figs. 2 and 3). One discrepancy is with sample *P. triphylla* (56) from Ecuador. In the *s/t phos* tree, all 10 *P. triphylla* (56) clones group together but within a clade of *P. racemosa* clones with moderate support (online Appendix S4). However, in the ITS tree, one clone of *P. triphylla* (56) (C04; online Appendix S6) falls into a moderately supported clade of *P. ternata* clones, while the other four group into a weakly supported clade of *P. triphylla* clones. Although hybridization has never been reported in wild *Psiguria*, artificial hybrids have been made in the greenhouse (L. E. Gilbert, unpublished data). It is possible that this is an example of a natural hybrid, but the parent species cannot be determined. Also in the nuclear trees, *Wilbrandia* is sister or near to *Gurania*. Utilizing the combined data, the alternative topology placing *Wilbrandia* sister to *Gurania* was rejected ($P = 0.035$). Some topological difference between the *s/t phos* and ITS trees may be due to long-branch attraction.

The *P. warscewiczii*, *P. ternata*, and *P. pedata* clades each contain two weakly to moderately supported groups (Fig. 4). The ITS tree separates *P. warscewiczii* along geographical lines, but this weak result is not supported in the combined tree. There is no morphological evidence that suggests that any of these clades should be split into two. Finally, the unidentified sample, *Psiguria* sp. (26), is isolated in the tree. This sample was collected in Brazil and annotated as *P. ternata*, but this placement was rejected in an SH test ($P = 0.005$). It is possible that this collection represents a seventh species of *Psiguria*, but characteristics must be identified to recognize it as distinct. Furthermore, additional samples from this geographic region should be included in future phylogenetic analyses.

In the chloroplast tree, *Helmontia* groups with *Gurania* on a very long branch. A previous phylogeny of Cucurbitaceae showed *Helmontia* embedded within *Psiguria* and *Gurania* (Kocyan et al., 2007). Because we included an expanded sampling of *Psiguria* and a greater number of markers, we used the combined data set to test the alternative topology that *Helmontia* is sister to *Psiguria*, and this alternative was rejected ($P = 0.020$). It will still be necessary to expand sampling of *Gurania*—estimated to contain approximately 75 species (A. Neill, Botanical Research Institute of Texas, personal communication)—to determine if this placement of *Helmontia* is a good one or is due to long-branch attraction.

TABLE 4. DNA barcodes with the unique nucleotides that identify species of *Psiguria*. The representative sequences and associated GenBank accession numbers necessary for comparison are given along with the particular base number in that sequence. In some cases, a combination of sequences is required for identification; see "Discussion" for more details.

Species uniquely identified	Representative sequence [a]	Chloroplast marker	GenBank accession	Base pair number	Unique nucleotide	Primers [b]	
						Forward	Reverse
P. pedata	*P. pedata* (14)	ndhC-trnV	GQ489273	50	C	ndhC-trnV forward	ndhC-trnV nested-R
				315	C		
				434	C		
P. triphylla	*P. triphylla* (28)	ndhF-rpl32	GQ489356	180	T	ndhF-rpl32 forward	ndhF-rpl32 nested-R
P. triphylla	*P. triphylla* (28)	ndhC-trnV	GQ489280	54	T	ndhC-trnV forward	ndhC-trnV nested-R [c]
				65	T		
P. triphylla	*P. triphylla* (28)	rps16-trnQ	GQ489768	30	C	rps16-trnQ forward	rps16-trnQ nested-R [d]
				638	T		
				713	T		
P. racemosa	*P. racemosa* (22)	rpoB-trnC	GQ489682	855	C	rpoB-trnC internal-F	rpoB-trnC reverse
				942	C		
P. ternata	*P. ternata* (34)	rps16-trnQ	GQ489773	434	C	rps16-trnQ forward	rps16-trnQ nested-R [d]
P. umbrosa	*P. umbrosa* (64)	psbZ-trnM	GQ489641	196	C	psbZ-trnM forward	psbZ-trnM nested-R [e]
P. umbrosa	*P. umbrosa* (64)	rpoB-trnC	GQ489724	721	G	rpoB-trnC internal-F	rpoB-trnC reverse
P. umbrosa / *P. warscwiczii*	*P. umbrosa* (64)	ndhF-rpl32	GQ489394	422	T	ndhF-rpl32 forward	ndhF-rpl32 nested-R
				504	T		

[a] The parenthetical number indicates its designation in Table 1.

[b] Unless noted otherwise, the indicated primer from SAppendix S1 (see Supplemental Data with the online version of this article) may be used.

[c] As an alternative, the following proposed reverse primer may be used to amplify a 400-bp-shorter region: GCA TTG GGT TAT GGT GGA G.

[d] As an alternative, the following proposed reverse primer may be used to amplify a 300-bp-shorter region: AAT AGG TAG GAA CAA TCG.

[e] As an alternative, the following proposed reverse primer may be used to amplify a 150-bp-shorter region: CCA TTC AAC TAT ATC CGC.

The number of species in *Psiguria* has been estimated to be between eight (Jeffrey, 1978) and 29 (Cogniaux, 1916), and IPNI lists 17. Taking into consideration both the molecular phylogeny and results of an extensive morphological study (Steele, in press), we recognize six species: *P. pedata*, *P. ternata*, *P. umbrosa*, *P. warscewiczii*, *P. triphylla*, and *P. racemosa*. Although one could argue that the molecular results may suggest more than six species, morphological studies (Steele, in press) do not support splitting of any species. Nonetheless, future collections of *Psiguria* and additional sequencing of molecular markers may contribute to the discovery of additional species.

On the basis of our results, we propose the simplified phylogeny of *Psiguria* shown in Fig. 5. The monophyly of Guraniinae and *Psiguria* have strong bootstrap support. *Psiguria pedata* is sister to the remainder of *Psiguria* with moderate bootstrap support. *Psiguria warscewiczii* and *P. umbrosa* are sister species with moderate bootstrap support, and there are six well-defined species. It turns out that Jeffrey's (1978) circumscription of *Psiguria* species matches fairly closely with the results obtained here.

Implications for ecological and biogeographical studies—The phylogenetic framework shown in Fig. 5 will assist ecologists and evolutionary biologists with questions surrounding this rainforest vine. For example, Condon et al. (2008) are investigating the pattern of host use and diversity in *Blepharoneura* fruit flies, whose larvae feed within flowers or fruits of some *Psiguria* and *Gurania*. Additionally, *Psiguria* has a mutualistic association with its pollinators, *Heliconius* butterflies, making it a model system for investigating coevolution. An evolutionary history of the butterflies has been proposed (Beltrán et al., 2007), and now a missing piece of information needed to understand this important system (the genealogy of their coevolved pollen hosts) is available. Ecologists and biologists can now address important questions about the evolutionary history of this interaction. As an extension of this work, studies of a multitude of plants, insects, and other animals in the biological network can be conducted in a phylogenetic context. These may include insect species in agricultural pest families such as tephritid fruit flies, chrysomelid flea beetles, coreid bugs, and pyralid moths. Knowledge gained from studying such interactions involving *Psiguria* may add to our general understanding of parallel interaction networks involving agriculturally important members of Cucurbitaceae such as *Cucumis sativus*, *Citrullus lanatus* (Thunb.) Matsum. & Nakai, and *Cucurbita pepo* L.

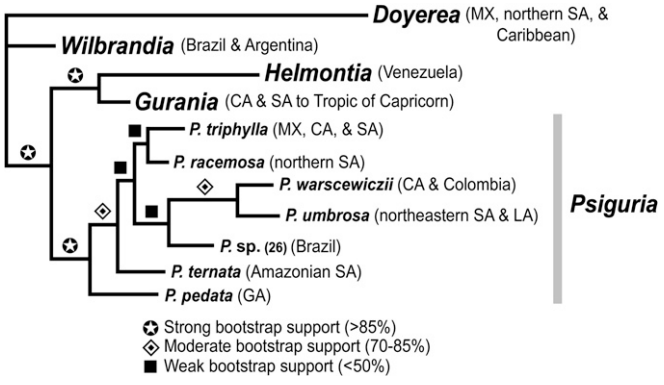

Fig. 5. Phylogeny of *Psiguria*—a simplified version of the tree inferred from a combination of eight chloroplast intergenic spacers, ITS, and the nuclear *serine/threonine phosphatase* gene intron—showing geographic distributions. Symbols represent bootstrap values as indicated. MX = Mexico, CA = Central America, SA = South America, LA = Lesser Antilles, and GA = Greater Antilles.

Species of *Psiguria* are found throughout the New World tropics with a geographic distribution extending from southern Mexico to Paraguay and in the Caribbean islands. The distribution of each species is shown in Fig. 5. Although it was previously believed that there were two species endemic to the Caribbean islands, our data suggest that there may be only one or none. There is only one taxon in the Greater Antilles, *P. pedata* (*P. trifoliata* is synonymous), and at least one sample of this species has been collected in Costa Rica (sample no. 20). However, it is possible that this specimen was collected from a recent introduction. Two other species, *P. triphylla* and *P. umbrosa*, are predominantly continental, but they extend into the Lesser Antilles.

Unlike its closest relatives, *Gurania* and *Helmontia*, which are mostly confined to the continent, *Psiguria* is found both on the continent and throughout the West Indies. *Wilbrandia*, the sister genus to the subtribe Guraniinae, is found only in Brazil and northern Argentina (Cogniaux, 1916), while the next closest genus, *Doyerea*, has approximately the same range (range data provided by Missouri Botanical Garden, accessed through GBIF [2009] Data Portal) as *Psiguria*, although shifted slightly northward. With the geographic distribution of *Psiguria*'s closest relatives predominantly covering South America and Central America (SA/CA), the most likely scenario is that *Psiguria* originated in SA/CA. Subsequently, the genus dispersed to the Greater Antilles (GA), leading to *P. pedata*. Then, some members of the genus migrated into the Lesser Antilles (LA) (*P. umbrosa*), and others migrated northward to Mexico (MX).

Morphological characters—The six species of *Psiguria* supported by the molecular phylogeny are also distinguished by a few morphological characters, predominantly in male flowers (Steele, in press). *Psiguria pedata* is characterized by anthers folded backward, whereas all other species have straight anthers. *Psiguria triphylla* has >0.75 male flowers per millimeter and pedicels absent. *Psiguria ternata* and *P. racemosa* both have pink flowers, but *P. ternata* has trifoliolate leaves, and *P. racemosa* has simple, trilobed leaves. *Psiguria umbrosa* and *P. warscewiczii* both have calyces and peduncles that are light green with darker green speckles and orange petals, but *P. umbrosa* has longer pedicels (>2.0 mm) and thin, linear sepals, while *P. warscewiczii* has short pedicels (<2.0 mm) and thick, triangular sepals. For taxonomic keys and complete descriptions of all species, see Steele (in press). The reliance upon male flowers to distinguish species of *Psiguria* morphologically argues for the need to use DNA barcodes to help identify species, especially since flowers are not always available in the field or on herbarium specimens.

***Psiguria*-specific DNA barcodes**—While it has been suggested that there should be a region (or regions) of DNA that can be used as a barcode to identify species across land plants (Chase et al., 2005, 2007; Kress and Erickson, 2007), these authors also noted that some groups may require additional DNA regions (for example, in *Aspalathus* [Fabaceae] [Edwards et al., 2008] and in *Solanum* sect. *Petota* [Solanaceae] [Spooner, 2009]). We suggest that *Psiguria* is one of those groups because

regions that have been proposed so far are not variable enough to distinguish its species. ITS copies have nearly as much variation between some clones as between some species (see online SAppendices S6 and S7). There is so little variation in noncoding chloroplast regions between species of *Psiguria*, and even between genera within Guraniinae (Table 2), that one could not expect enough variation between coding regions of the chloroplast genome such as *rbcL* (Chase et al., 2005), *rpoC1*, *rpoB*, or *matK* (Chase et al., 2007) to be useful as DNA barcodes. The IGS *psbA-trnH* has been suggested in conjunction with other regions (Kress et al., 2005; Chase et al., 2007; Kress and Erickson, 2007). As opposed to the 300–400 bp length described in other groups, this noncoding region in *Cucumis sativus*, the species most closely related to *Psiguria* whose whole chloroplast genome has been sequenced (Kim et al., 2006), is only 150 bp long. It would be very surprising if this region were useful for distinguishing species of *Psiguria*.

Spooner (2009) found similar difficulties in *Solanum* sect. *Petota* (Solanaceae). ITS had too much intraspecific variation, and the plastid markers lacked sufficient variation. Edwards et al. (2008) concluded that at least three molecular regions would be necessary for species discrimination in *Aspalathus*. As with *Psiguria*, species recognition in *Aspalathus* is dependent upon floral characters; therefore, in most cases, identification of sterile specimens is not possible from morphological characters (Edwards et al., 2008). In their assessment of potential DNA barcodes for *Aspalathus*, species identification depended upon a "threshold of sequence divergence" (p. 1318). In *Psiguria*, because of confounding intraspecific variation, it was necessary to be more explicit. We looked through sequences of the chloroplast markers used in the phylogenetic study and identified a nucleotide position (or positions) with a base unique to each species. At least four regions are necessary to distinguish species of *Psiguria* (Table 4), none of which is a region that has been suggested for potential plant barcodes. This result indicates that multiple regions will be necessary to identify groups with little detectable molecular variation, but it also underlines the notion that different regions may be necessary for each plant group.

DNA barcoding has attracted much controversy in recent years (Ebach and Holdrege, 2005; Hebert and Gregory, 2005; Marshall, 2005; Meyer and Paulay, 2005; Will et al., 2005). Many argue that the use of DNA barcodes to identify species will overshadow the need for morphological keys or characters and will take funding away from classical taxonomic studies (Ebach and Holdrege, 2005). The DNA barcodes presented here will lend support to systematists, ecologists, and evolutionary biologists trying to identify species of *Psiguria* that are not in an appropriate life history stage for morphological identification (i.e., without male flowers) and will help to identify many sterile herbarium specimens or those that have been damaged. DNA barcodes can be used in conjunction with morphological characters, when they are available. This combined use of molecular and morphological data is one of the major advantages espoused by proponents of DNA barcodes (Gregory, 2005; Hebert and Gregory, 2005; Schindel and Miller, 2005). Another argument is that there may be limited confidence in a particular DNA barcode (or set of barcodes) unless a comprehensive sample of specimens is tested for conformity (Meyer and Paulay, 2005). For each *Psiguria* species delimited in this study, we sampled multiple individuals with quantities relative to geographical range and morphological variation (8: *P. pedata*, 5: *P. racemosa*, 9: *P. ternata*, 28: *P. triphylla*, 6: *P. umbrosa*, and 13: *P. warscewiczii*). We feel confident that these regions will successfully identify species of *Psiguria*.

Conclusions—A combined molecular data set including regions from two genomes and totaling nearly 10 000 bp strongly supports the monophyly of *Psiguria*. These data also support the recognition of six species within *Psiguria*. Sister relationships are resolved, although some are not well supported. Further information about sister relationships and additional splitting of species will require the identification of additional molecular and morphological characters. Four chloroplast DNA barcodes can be used to distinguish the six species of *Psiguria*, and they may be useful in other groups of Cucurbitaceae. For the first time, systematists, ecologists, and evolutionary biologists have the tools to identify species of *Psiguria*, even of sterile specimens, and pursue questions surrounding these vines of rainforest ecosystems.

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APPENDIX 1. GenBank accession numbers for all samples and all regions that amplified successfully.

Sample	*ndhC-trnV*	*ndhF-rpl32*	*psbE-petL*	*psbM-trnD*	*psbZ-trnM*	*rpoB-trnC*	*rps16-trnQ*	*trnS-trnG*	ITS	*s/t phos*
Doyerea emeto-carthartica (1)	GQ489256	GQ489337	GQ489410	GQ489492	GQ489576	GQ489660	GQ489743	GQ489821	GQ489902–905	GQ490148–154
Doyerea emeto-carthartica (2)	GQ489257	GQ489338	GQ489411	GQ489493	GQ489577	GQ489661	GQ489744	GQ489822	GQ489906–909	—
Gurania acuminata (3)	GQ489260	GQ489341	GQ489414	GQ489496	GQ489580	GQ489664	GQ489747	GQ489825	—	GQ490164
Gurania costaricensis (4)	GQ489259	GQ489340	GQ489413	GQ489495	GQ489579	GQ489663	GQ489746	GQ489824	GQ489915–918	—
Gurania eriantha (5)	GQ489258	GQ489339	GQ489412	GQ489494	GQ489578	GQ489662	GQ489745	GQ489823	GQ489910–914	GQ490155–163
Gurania insolita (6)	GQ489261	GQ489342	GQ489415	GQ489497	GQ489581	GQ489665	GQ489748	GQ489826	—	GQ490165–174
Gurania lobata (7)	GQ489262	GQ489343	GQ489416	GQ489498	GQ489582	GQ489666	GQ489749	GQ489827	GQ489919–921	GQ490175–185
Gurania sp. (8)	GQ489263	GQ489344	GQ489417	GQ489499	GQ489583	GQ489667	GQ489750	GQ489828	GQ489922–925	GQ490186–192
Gurania sp. (9)	GQ489264	GQ489345	GQ489418	GQ489500	GQ489584	GQ489668	GQ489751	GQ489829	GQ489926–930	GQ490193–200
Gurania sp. (10)	GQ489265	GQ489346	GQ489419	GQ489501	GQ489585	GQ489669	GQ489752	GQ489830	GQ489931–934	GQ490201–208
Helmontia jeffreyi (11)	GQ489266	—	—	GQ489502	GQ489586	GQ489670	GQ489753	—	—	—
Helmontia leptantha (12)	GQ489267	—	—	GQ489503	GQ489587	GQ489671	GQ489754	—	—	—
Psiguria pedata (13)	GQ489272	GQ489348	GQ489424	GQ489508	GQ489592	GQ489676	GQ489759	GQ489835	GQ489946–951	GQ490214–220
Psiguria pedata (14)	GQ489273	GQ489349	GQ489425	GQ489509	GQ489593	GQ489677	GQ489760	GQ489836	GQ489952–956	GQ490221–230
Psiguria pedata (15)	GQ489274	GQ489350	GQ489426	GQ489510	GQ489594	GQ489678	GQ489761	GQ489837	GQ489957–959	—
Psiguria pedata (16)	GQ489275	GQ489351	GQ489427	GQ489511	GQ489595	GQ489679	GQ489762	GQ489838	GQ489960–964	GQ490231–241
Psiguria pedata (17)	GQ489276	GQ489352	GQ489428	GQ489512	GQ489596	GQ489680	GQ489763	GQ489839	GQ489960–964	GQ490242–251
Psiguria pedata (18)	—	GQ489353	GQ489429	GQ489513	GQ489597	GQ489681	GQ489764	GQ489840	GQ489969–972	GQ490252–263
Psiguria pedata (19)	GQ489292	GQ489369	GQ489447	GQ489531	GQ489615	GQ489698	GQ489781	GQ489858	GQ490023–026	GQ490326–327
Psiguria pedata (20)	GQ489305	GQ489382	GQ489460	GQ489544	GQ489628	GQ489711	GQ489792	GQ489871	GQ490067	—
Psiguria racemosa (21)	GQ489271	—	GQ489423	GQ489507	GQ489591	GQ489675	GQ489758	GQ489834	GQ489941–945	GQ490210–213
Psiguria racemosa (22)	GQ489277	GQ489354	GQ489430	GQ489514	GQ489598	GQ489682	GQ489765	GQ489841	GQ489973–976	—
Psiguria racemosa (23)	GQ489278	—	GQ489431	GQ489515	GQ489599	—	GQ489766	GQ489842	—	—
Psiguria racemosa (24)	GQ489279	GQ489355	GQ489432	GQ489516	GQ489600	GQ489683	GQ489767	GQ489843	—	—
Psiguria racemosa (25)	GQ489317	GQ489393	GQ489472	GQ489556	GQ489640	GQ489723	GQ489801	GQ489882	GQ490093	—
Psiguria sp. (26)	—	—	GQ489446	GQ489530	GQ489614	GQ489697	GQ489780	GQ489857	—	—
Psiguria triphylla (27)	GQ489270	—	GQ489422	GQ489506	GQ489590	GQ489674	GQ489757	GQ489833	—	—
Psiguria triphylla (28)	GQ489280	GQ489356	GQ489433	GQ489517	GQ489601	GQ489684	GQ489768	GQ489844	GQ489977–980	GQ490264–268
Psiguria triphylla (29)	GQ489281	GQ489357	GQ489434	GQ489518	GQ489602	GQ489685	GQ489769	GQ489845	GQ489981–983	GQ490269–275
Psiguria triphylla (30)	GQ489282	GQ489358	GQ489435	GQ489519	GQ489603	GQ489686	GQ489770	GQ489846	GQ489984–987	—
Psiguria triphylla (31)	GQ489283	GQ489359	GQ489436	GQ489520	GQ489604	GQ489687	GQ489771	GQ489847	GQ489988–991	GQ490276–285
Psiguria triphylla (32)	GQ489313	GQ489389	GQ489468	GQ489552	GQ489636	GQ489719	GQ489797	GQ489878	GQ490079–082	GQ490415–422
Psiguria ternata (33)	GQ489284	GQ489360	GQ489437	GQ489521	GQ489605	GQ489688	GQ489772	GQ489848	GQ489992–996	GQ490286–291
Psiguria ternata (34)	—	GQ489361	GQ489438	GQ489522	GQ489606	GQ489689	GQ489773	GQ489849	GQ489997–000	GQ490292–300
Psiguria ternata (35)	GQ489285	GQ489362	GQ489439	GQ489523	GQ489607	GQ489690	GQ489774	GQ489850	GQ490001–004	GQ490301–310
Psiguria ternata (36)	GQ489286	GQ489363	GQ489440	GQ489524	GQ489608	GQ489691	GQ489775	GQ489851	GQ490005–009	GQ490311–322
Psiguria ternata (37)	GQ489287	GQ489364	GQ489441	GQ489525	GQ489609	GQ489692	GQ489776	GQ489852	GQ490010012	GQ490323
Psiguria ternata (38)	GQ489288	GQ489365	GQ489442	GQ489526	GQ489610	GQ489693	GQ489777	GQ489853	GQ490013–014	GQ490324
Psiguria ternata (39)	GQ489289	GQ489366	GQ489443	GQ489527	GQ489611	GQ489694	GQ489778	GQ489854	GQ490015–016	—
Psiguria ternata (40)	GQ489290	GQ489367	GQ489444	GQ489528	GQ489612	GQ489695	GQ489779	GQ489855	GQ490017–018	GQ490325
Psiguria ternata (41)	GQ489291	GQ489368	GQ489445	GQ489529	GQ489613	GQ489696	—	GQ489856	GQ490019–022	—
Psiguria triphylla (42)	GQ489268	—	GQ489420	GQ489504	GQ489588	GQ489672	GQ489755	GQ489831	GQ489935–937	—
Psiguria triphylla (43)	GQ489293	GQ489370	GQ489448	GQ489532	GQ489616	GQ489699	GQ489782	GQ489859	GQ490027–030	—
Psiguria triphylla (44)	GQ489294	GQ489371	GQ489449	GQ489533	GQ489617	GQ489700	GQ489783	GQ489860	GQ490031–033	GQ490328–336
Psiguria triphylla (45)	GQ489295	GQ489372	GQ489450	GQ489534	GQ489618	GQ489701	GQ489784	GQ489861	GQ490034–038	GQ490337–346
Psiguria triphylla (46)	GQ489296	GQ489373	GQ489451	GQ489535	GQ489619	GQ489702	GQ489785	GQ489862	GQ490039–042	GQ490347–355
Psiguria triphylla (47)	GQ489297	GQ489374	GQ489452	GQ489536	GQ489620	GQ489703	GQ489786	GQ489863	GQ490043–047	GQ490356–365
Psiguria triphylla (48)	GQ489298	GQ489375	GQ489453	GQ489537	GQ489621	GQ489704	GQ489787	GQ489864	GQ490048–050	GQ490366–368
Psiguria triphylla (49)	GQ489299	GQ489376	GQ489454	GQ489538	GQ489622	GQ489705	GQ489788	GQ489865	GQ490051–053	GQ490369–372
Psiguria triphylla (50)	GQ489300	GQ489377	GQ489455	GQ489539	GQ489623	GQ489706	GQ489789	GQ489866	GQ490054–056	GQ490373–383
Psiguria triphylla (51)	GQ489301	GQ489378	GQ489456	GQ489540	GQ489624	GQ489707	GQ489790	GQ489867	GQ490057–060	GQ490384–393
Psiguria triphylla (52)	GQ489302	GQ489379	GQ489457	GQ489541	GQ489625	GQ489708	GQ489791	GQ489868	GQ490061–062	—
Psiguria triphylla (53)	GQ489303	GQ489380	GQ489458	GQ489542	GQ489626	GQ489709	—	GQ489869	—	—
Psiguria triphylla (54)	GQ489304	GQ489381	GQ489459	GQ489543	GQ489627	GQ489710	—	GQ489870	GQ490063–066	GQ490394–395

APPENDIX 1. Continued.

Sample	*ndhC-trnV*	*ndhF-rpl32*	*psbE-petL*	*psbM-trnD*	*psbZ-trnM*	*rpoB-trnC*	*rps16-trnQ*	*trnS-trnG*	ITS	*s/t phos*
Psiguria triphylla (55)	GQ489306	GQ489383	GQ489461	GQ489545	GQ489629	GQ489712	—	GQ489872	GQ490068	GQ490396
Psiguria triphylla (56)	GQ489307	GQ489384	GQ489462	GQ489546	GQ489630	GQ489713	GQ489793	GQ489873	GQ490069–073	GQ490397–406
Psiguria triphylla (57)	GQ489308	GQ489385	GQ489463	GQ489547	GQ489631	GQ489714	GQ489794	GQ489874	—	—
Psiguria triphylla (58)	GQ489309	GQ489386	GQ489464	GQ489548	GQ489632	GQ489715	—	—	—	—
Psiguria triphylla (59)	GQ489310	—	GQ489465	GQ489549	GQ489633	GQ489716	GQ489795	GQ489875	GQ490074	—
Psiguria triphylla (60)	GQ489311	GQ489387	GQ489466	GQ489550	GQ489634	GQ489717	GQ489796	GQ489876	GQ490075–078	GQ490407-414
Psiguria triphylla (61)	GQ489312	GQ489388	GQ489467	GQ489551	GQ489635	GQ489718	—	GQ489877	—	—
Psiguria triphylla (62)	GQ489314	GQ489390	GQ489469	GQ489553	GQ489637	GQ489720	GQ489798	GQ489879	GQ490083–087	GQ490423–425
Psiguria triphylla (63)	GQ489315	GQ489391	GQ489470	GQ489554	GQ489638	GQ489721	GQ489799	GQ489880	GQ490088–092	GQ490426–427
Psiguria umbrosa (64)	GQ489318	GQ489394	GQ489473	GQ489557	GQ489641	GQ489724	GQ489802	GQ489883	GQ490094–097	—
Psiguria umbrosa (65)	GQ489319	GQ489395	GQ489474	GQ489558	GQ489642	GQ489725	GQ489803	GQ489884	GQ490098–101	—
Psiguria umbrosa (66)	GQ489320	GQ489396	GQ489475	GQ489559	GQ489643	GQ489726	GQ489804	GQ489885	GQ490102–106	GQ490429–439
Psiguria umbrosa (67)	GQ489321	GQ489397	GQ489476	GQ489560	GQ489644	GQ489727	GQ489805	GQ489886	GQ490107–111	GQ490440
Psiguria umbrosa (68)	GQ489322	GQ489398	GQ489477	GQ489561	GQ489645	GQ489728	GQ489806	GQ489887	GQ490112–115	GQ490441–446
Psiguria umbrosa (69)	GQ489323	GQ489399	GQ489478	GQ489562	GQ489646	GQ489729	GQ489807	GQ489888	—	—
Psiguria warscewiczii (70)	GQ489269	GQ489347	GQ489421	GQ489505	GQ489589	GQ489673	GQ489756	GQ489832	GQ489938–940	GQ490209
Psiguria warscewiczii (71)	GQ489316	GQ489392	GQ489471	GQ489555	GQ489639	GQ489722	GQ489800	GQ489881	—	GQ490428
Psiguria warscewiczii (72)	GQ489324	GQ489400	GQ489479	GQ489563	GQ489647	GQ489730	GQ489808	GQ489889	GQ490116–119	GQ490447–448
Psiguria warscewiczii (73)	GQ489325	GQ489401	GQ489480	GQ489564	GQ489648	GQ489731	GQ489809	GQ489890	GQ490120–122	GQ490449–459
Psiguria warscewiczii (74)	GQ489326	GQ489402	GQ489481	GQ489565	GQ489649	GQ489732	GQ489810	GQ489891	GQ490123–126	—
Psiguria warscewiczii (75)	GQ489327	GQ489403	GQ489482	GQ489566	GQ489650	GQ489733	GQ489811	GQ489892	GQ490127–131	GQ490460–471
Psiguria warscewiczii (76)	GQ489328	GQ489404	GQ489483	GQ489567	GQ489651	GQ489734	GQ489812	GQ489893	GQ490132	GQ490472
Psiguria warscewiczii (77)	GQ489329	GQ489405	GQ489484	GQ489568	GQ489652	GQ489735	GQ489813	GQ489894	GQ490133–137	GQ490473
Psiguria warscewiczii (78)	GQ489330	—	GQ489485	GQ489569	GQ489653	GQ489736	GQ489814	GQ489895	GQ490138	GQ490474
Psiguria warscewiczii (79)	GQ489331	GQ489406	GQ489486	GQ489570	GQ489654	GQ489737	GQ489815	GQ489896	GQ490139	GQ490475
Psiguria warscewiczii (80)	GQ489332	—	GQ489487	GQ489571	GQ489655	GQ489738	GQ489816	GQ489897	GQ490140–142	GQ490476
Psiguria warscewiczii (81)	GQ489333	GQ489407	GQ489488	GQ489572	GQ489656	GQ489739	GQ489817	GQ489898	GQ490143	GQ490477–480
Psiguria warscewiczii (82)	GQ489334	GQ489408	GQ489489	GQ489573	GQ489657	GQ489740	GQ489818	GQ489899	GQ490144–145	GQ490481–491
Wilbrandia ebracteata (83)	GQ489335	—	GQ489490	GQ489574	GQ489658	GQ489741	GQ489819	GQ489900	GQ490146	—
Wilbrandia longisepala (84)	GQ489336	GQ489409	GQ489491	GQ489575	GQ489659	GQ489742	GQ489820	GQ489901	GQ490147	GQ490492

Note: "—" indicates the region was not sequenced for this sample.