

PROJECT SUMMARY

I. INTELLECTUAL MERIT OF THE PROPOSED ACTIVITY

Psiguria (Cucurbitaceae) is a Neotropical genus of vines that grows in a variety of habitats, and displays an interesting distribution between Central and South America with two endemic species in the geologically complex Caribbean region. The ecological importance of *Psiguria* is highlighted by the mutualistic association with its pollinators, *Heliconius* butterflies. Since a phylogeny of the butterflies has been proposed, the missing piece of information needed to test this important system is the phylogeny of their pollen hosts. A systematic study of *Psiguria* will provide a basis for studying its biogeographical history and a phylogenetic framework for ecologists and evolutionary biologists to address a multitude of important questions about plant-insect interactions in this model system.

Three major objectives are proposed in this project. The first is to reconstruct the phylogenetic history of *Psiguria* utilizing chloroplast, nuclear, and morphological data sets. The goals are to determine if *Psiguria* is monophyletic and to estimate phylogenetic relationships within the subtribe *Guraniinae*. DNA has been isolated from 14 taxa including outgroups, and multiple regions from chloroplast and nuclear genomes have been amplified with PCR. A chloroplast data set has been assembled from eight noncoding regions totaling 7,185 base pairs (bp), and several phylogenetically informative, low-copy nuclear markers have been identified. Preliminary phylogenetic analyses suggest that *Psiguria* is monophyletic with moderate to strong bootstrap support. The second objective is to write a comprehensive monograph of *Psiguria*. Despite the multitude of recent and ongoing ecological studies surrounding the genus, the last comprehensive taxonomic treatment was published in 1916 by Cogniaux under the *nomen rejiciendum* genus name *Anguria*. The monograph will include a revised classification based on the phylogenetic reconstruction. The third objective is to study the biogeographic history of the genus comparing four different reconstruction methods including parsimony, area cladograms, DIVA, and a maximum likelihood algorithm. The goals are to determine how the genus moved between the continents and the islands (vicariance or dispersal), the number of times it migrated, and in which direction. With its overall small size, exemplar geographic distribution, and extensive ecological interest, *Psiguria* is an ideal genus for this type of examination.

II. BROADER IMPACTS RESULTING FROM THE PROPOSED ACTIVITY

The results of this study will provide important scientific contributions beyond the systematics of *Psiguria*. As the impact of humanity on the Earth escalates, scientists are predicting that, without sincere conservation efforts, a third of the world's terrestrial species could become extinct this century. Nearly half of the world's species are concentrated in the 6% of the land area covered by tropical rainforests, and these species face the most serious risk of extinction. Effective conservation programs must include systematic and biogeographic considerations in the development of conservation strategies. A monograph of the Neotropical genus *Psiguria* will contribute to conservation efforts of rainforest ecosystems. Likewise, the biogeographic history of *Psiguria* will contribute to ongoing studies addressing Caribbean biogeography, which are severely lacking in phylogenies focused on plant groups.

The phylogenetic framework for *Psiguria* will provide a basis for ecologists and evolutionary biologists to ask questions about the multitude of associated plants, insects, and other animals in the biological network. These include three interactions with insect species in agricultural pest families – tephritid fruit flies, chrysomelid flea beetles, and pyralid moths. Knowledge gained from studying these interactions with *Psiguria* will add to our understanding of the related negative insect interactions with agriculturally important members of Cucurbitaceae such as *Cucumis sativus*, *Citrullus lanatus*, and *Cucurbita pepo*.

This study will also serve as a training opportunity for two undergraduates and a graduate student. A UT-Austin undergraduate student and one hosted at UT-Austin from Ithaca College will aid in data acquisition from collected specimens, and will benefit by working in a large research laboratory. In addition, the visiting student will benefit from meeting and working with scientists outside of his/her undergraduate institution. In return, these students will provide valuable assistance to the project. Through her GK-12 Fellowship, the graduate student co-PI will share the knowledge gained from her field and lab research experiences with her associated middle-school science students. Additionally, the graduate student will be supported and trained in a wide variety of fields and tasks including flowering plant systematics, molecular evolution, field work, and involvement in preparing papers and presentations for local, state, national, and international scientific meetings. The process of conducting and completing this project will give the graduate student the education, skills, and tools she needs to conduct additional important and meaningful research in her future career in biology.

PROJECT DESCRIPTION

RESPONSE TO REVIEWER COMMENTS – Reviewers had four primary criticisms that are highlighted here and discussed in more detail on the pages listed in parentheses. Other minor suggestions are addressed in the text. **1) Hypotheses could have been framed better.** Hypotheses to be tested are now explicitly stated (pp. 1, 2, & 7), **2) There was little variation in the low-copy nuclear gene preliminary data.** Three additional nuclear regions have been identified (p. 6), all with greater percentages of parsimony-informative characters. **3) Preliminary results from morphological phylogenetic work would have strengthened the proposal.** A discussion about this has been added (p. 7). **4) Timeline seems unrealistic.** In the last year, considerable work has been completed on the project including an extensive investigation to identify additional low-copy nuclear markers, plant collecting trips to two countries, and the addition of four *Psiguria* collections to phylogenetic analyses. The goal to investigate the evolution of morphological characters was removed, and the timeline was adjusted (p. 8).

I. INTRODUCTION

Psiguria (Cucurbitaceae) is a genus of 17 species of Neotropical vines. It has important and interesting ecological relationships with other members of the ecosystem and an exemplar pattern of distribution. This study will focus on the systematics and biogeography of *Psiguria* culminating in a comprehensive monograph. Since there has never been a phylogeny estimated for *Psiguria*, the results will provide the framework necessary for biologists to address an array of ecological and evolutionary questions and contribute to our growing knowledge of plant evolutionary histories in the Caribbean.

Psiguria has flowers and fruits that are important to insects, birds, and mammals of rainforest ecosystems (1,2,3,4,5). Moreover, it has a mutualistic association with its pollinators, *Heliconius* butterflies, making it a model system for investigating coevolutionary relationships. The historical relationship between these two evolving groups of organisms has influenced both the reproductive strategy of the butterflies and, potentially, the separation of *Psiguria* as a genus distinctive from others in the subtribe *Guraniinae* (L. Gilbert, pers. comm.). Since the phylogeny of *Heliconius* butterflies has been proposed (6,7), a phylogenetic framework of *Psiguria* is the missing information needed to fully investigate the evolutionary significance of this system. Other interactions involving *Psiguria* include interactions with insect species in agricultural pest families – tephritid fruit flies, chrysomelid flea beetles, and pyralid moths. A phylogeny of *Psiguria* will provide a necessary framework for asking questions about these relationships and many other evolutionary biology questions about rainforest ecosystems.

How did flora and fauna of the West Indies cross the Caribbean Sea? What was the pattern of movement between islands? What is the evolutionary history of organisms in the area? How did successful adaptations evolve? None of these questions can be adequately addressed without phylogenies of the organisms. The Caribbean islands have strikingly high levels of biotic diversity and endemism (8), and the basis for their uniquely rich flora most certainly involves the complex geologic and environmental history of the region. This complex history provides evolutionary biologists the daunting but exciting challenge of reconstructing the historical biogeography of the region (9), but studies on the classification, evolution, and geographic history of Caribbean biota are seriously deficient in those focusing on plants (9,10,11,12). *Psiguria* grows throughout Latin America and the Caribbean, including two species endemic to the Greater Antilles. Biogeographic history of *Psiguria* will contribute a much-needed plant study to ongoing debates about how organisms migrated between disjunct landmasses.

A family-wide phylogeny of Cucurbitaceae supported the monophyly of the subtribe *Guraniinae*, but relationships at the generic level raised doubts about the monophyly of *Psiguria* (13). In the proposed study, the monophyly of *Psiguria* will be tested using multiple data sets, greatly expanded taxon sampling, and several outgroups. Preliminary results from molecular data sets support the monophyly of *Psiguria* with moderate to strong bootstrap support. Results of phylogenetic and biogeographic analyses along with an intense morphological investigation of individuals from field collections, greenhouses, and herbarium specimens will contribute to a monograph of the genus.

II. RESEARCH GOALS – The goals include comprehensive phylogenetic and taxonomic analyses of *Psiguria* allowing an investigation into the processes that have shaped the biogeography and evolutionary history of the genus. The three major objectives of this study and specific hypotheses to be tested are:

1. reconstruct the phylogenetic history of *Psiguria* using multiple independent data sets to test the monophyly of the genus, estimate species relationships, and develop a phylogenetic classification
 - $H_0 = \textit{Psiguria}$ is monophyletic, $H_0 = \textit{Gurania}$ is sister to *Psiguria*
2. examine biogeographic history of *Psiguria* to infer the geographic origin of the genus and determine distribution patterns between the continents and island chains of the Caribbean

- $H_0 = Psiguria$ originated on the continent, $H_0 = Psiguria$ migrated to the Caribbean islands via dispersal, $H_0 = Psiguria$ colonized the Caribbean islands once with subsequent radiation
3. prepare a comprehensive monograph of *Psiguria* (hypotheses not applicable).

III. PROPOSED RESEARCH

A. Background Information

1. Study System. *Psiguria* is a Neotropical genus of vines including approximately 17 species (14). It is confined to the New World tropics with a geographical distribution extending from southern Mexico to South America with four species in the Caribbean islands including at least two endemics. The genus has been the focus of several ecological and evolutionary studies on the sexual systems and the interactions with various insects in the community, but the systematics, biogeography, and taxonomy of *Psiguria* have not been addressed extensively since Cogniaux's treatment in 1916.

Psiguria plays a role in a suite of plant-animal interactions. Most species of *Heliconius* butterflies could not reproduce without the nutrients obtained from pollen of *Psiguria* flowers (1). Unlike other lepidopterans that obtain necessary egg-laying, nitrogenous compounds from larval feeding, *Heliconius* butterflies as adults assimilate amino acids and proteins obtained from *Psiguria* and a few *Gurania* (1). Although most of the pollen produced by *Psiguria* is actually consumed by the butterflies, a sufficient portion is transferred to female flowers (which are mistakenly seen as pollen sources) fulfilling the butterfly's role as the pollen vector (1,2). The historical relationship between these two evolving groups of organisms has influenced both the reproductive strategy of the butterflies and, potentially, the separation of *Psiguria* as a genus distinct from others in the subtribe *Guraniinae* (L. Gilbert, pers. comm.).

Some members of *Psiguria* have commanded additional attention from evolutionary biologists, ecologists, and agricultural scientists. Condon and Gilbert (15) elucidated the details of sex expression in *Psiguria*, and Condon (16) further studied the various visitors to *Psiguria* including the seed-dispersing, fruit-eating bats. Murawski (4) studied the floral resource variation and pollen flow in *P. warszewiczii*. Condon and Steck (17) more recently discovered an exciting system in which some species of *Psiguria* and sister genus *Gurania* are hosts to several species of *Blephroaneura* (Diptera: Tephritidae) flies, each of which feeds on a separate part of the same plant. The phylogenetic work being conducted by Susan Swensen at Ithaca College (see attached letter) is part of a larger project researching coevolution between *Blephroaneura* flies and plants of the subtribe *Guraniinae*. *Psiguria* is also host to other pests such as the leaf-feeding flea beetle *Diabrotica* (Chrysomelidae), coreid bugs (e.g. *Paryphes*), and pickleworm moth larvae *Diaphnia* (Pylalidae) (5). These insects have close relatives that are pests on agriculturally important members of Cucurbitaceae. Studying relationships between these parasites and *Psiguria* will help agricultural scientists understand the interactions between cucumber plants and their insect pests. Several of these authors emphasize the need for a complete taxonomic revision of *Psiguria* and a phylogenetic history to aid in answering some of their questions and inspiring new ones (18,19).

Psiguria contains perennial vines occurring in low densities in Neotropical rainforests, commonly growing over shrubs and trees in the understory (3). The genus is generally characterized by large, glabrous, palmately 3- to 5-lobed or compound, leathery leaves. Plants are serially monoecious; male flowers emerge before the plants switch sex to produce female flowers (20,21). Flowers develop in clusters on long, pedunculate, indeterminate racemes. Both male and female flowers are salverform with fused sepals and petals – a similarity that, along with the stamen-like structure of the pistil, aids in “training” the pollinator butterflies to visit both sexes (L. Gilbert, pers. comm.). Sepals of both male and female flowers are green and short (relative to the sister genus *Gurania*). Petals are red with yellow bases, pink, or orange. Male flowers have two stamens with extremely short filaments fused to the interior of the hypanthium. Roubik and Moreno (22) found pollen of *Psiguria bignoniacea* and *P. warszewiczii* to be arranged symmetrically in groups of four with tetrads measuring around 120 μm . Individual grains are periporate (5-porate), apolar-asymmetric with a psilate surface and an exine layer 1.5 μm thick. One species of *Psiguria*, along with the monotypic related genus *Helmontia*, is reported to produce pollen as single grains (1,18,23). Female flowers are epigynous with a 2-carpellate ovary containing numerous ovules maturing into seeds inside a small, fleshy pepo that often has longitudinal green and white stripes. Although flowers of both sexes last only a single day (3), plants flower throughout the year (4,15,16,19,24,25). A single male inflorescence can flower for six months to three years, and plants can fluctuate between female and vegetative conditions a number of times over several years (2,19). Photographs of representative species can be found at www.sbs.utexas.edu/roxisteele.

2. Taxonomic History. The last complete taxonomic treatment of *Psiguria* was published by Cogniaux in 1916, using the genus name *Anguria*. Although Cogniaux (26) recognized 29 species of

Anguria, Charles Jeffrey (23) recognized only eight in his treatment. More recent work has resulted in 17 validly published *Psiguria* species (14); a list of these can be found at www.sbs.utexas.edu/roxisteele. Several authors assert that additional species still need to be moved from *Anguria* to *Psiguria* (18,27).

Psiguria is placed in the tribe *Coniandreae*, subtribe *Guraniinae* along with only two other genera, *Gurania* and *Helmontia* (28). Jeffrey (23) contended that the integrity and distinctness of the subtribe was upheld by pollen and flower morphology. The separation of *Helmontia* is based on palynological evidence (29), whereas *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 with yellow petals, and leaves are generally pubescent and not nearly as leathery as those of *Psiguria*. Additionally, *Gurania* is found only on Neotropical continents, not on Caribbean islands.

A family-wide phylogeny of Cucurbitaceae supported the monophyly of subtribe *Guraniinae*, but relationships at the generic level raised doubts about the monophyly of *Psiguria* (13). Their results show *Helmontia* embedded within *Psiguria* and *Gurania*, but they believe their taxon sampling may be insufficient to draw any strong conclusions about the results (S. Renner pers. comm.). The analysis placed the subtribe *Guraniinae* sister to the genus *Wilbrandia*, and these are, in turn, sister to *Doyerea* (13). Swensen's lab at Ithaca College is currently working to reconstruct the phylogeny of the subtribe *Guraniinae*, including some representative species of *Psiguria* (see attached letter).

3. Distribution and Biogeography. With an estimated 12,000 species of vascular plants, plant diversity in the islands of the Caribbean is so extraordinary that the region is considered a distinctive phytogeographic unit within the Neotropics (30). These islands have relatively high levels of endemism for many species of vascular plants; perhaps as many as one-third (31,32). These phenomena combined with a remarkably complex geologic history inspire great interest in the biogeography of the region (11).

More than two decades ago it was recognized that studies on the classification, evolution, and geographic history of the Caribbean biota were seriously deficient in those focusing on plant groups, particularly phylogenetic studies (10). Even today, most projects conducted on the biogeography of the Caribbean region focus on animal groups (9,11,12). Yet, information we gain from studies of plant evolution in the Caribbean can aid in addressing many other questions about the history of the flora in the region as well as contributing to an indirect understanding of the geological evolution of the islands. Because fossil data from the Caribbean are sparse (12), bringing together phylogenies of various groups found there can help elucidate the general biogeographic patterns that underlie the distributions of plant taxa as well as the biological processes involved in their development (9).

Hypotheses about the formation and geologic history of the Caribbean islands are constantly being debated in the literature. Because of this, questions regarding even basic knowledge such as which areas were above sea level during the history of the islands remain unsolved (33). Recently, a plate tectonic model has been advanced in which a volcanic island arc that extended from Mexico to Ecuador gradually moved through the developing passageway between North and South America and collided with the Bahamas Platform ca. 45 million years ago (mya) (34). This model agrees with the inference of Inturralde-Vinent and MacPhee (36) that the Greater Antilles are no more than about 46 million years old. Approximately 34 mya, there was a land bridge connecting the Greater Antilles and northwestern South America called GAARlandia (the Greater Antilles + the Aves Ridge) (11,12). This period was followed by high sea levels, and approximately 15 mya, tectonic activity further subdivided the Greater Antillean land blocks (12). Because of this ongoing debate about the geologic history, questions about the processes leading to the observed distributions of flora and fauna are equally debated.

Two competing models have been cited to explain the processes responsible for the spatial and temporal patterns of diversity found in the Caribbean islands – vicariance and dispersal (11,34,35). In the vicariance model, proto-Antillean biota in North and South America were fragmented by plate tectonic movements forming the current island diversity, and in the dispersal model, organisms arrived by over-water migration (11). Over the past 30 years, the favored hypothesis has shifted several times, but recently, a combination of both has been used to explain diversity patterns (37). Although many researchers are open to either scenario, two prominent, recent reviews argued that dispersal was the more likely explanation (11,34). Graham (34) argued that since the most recent models of geological history in the Antilles reveal that they were most likely submerged until around 55 mya, dispersal played a larger role. Likewise, Hedges (11) inferred that nearly all plant lineages originated more recently than the vicariance model would predict, and in an earlier study of amphibians and reptiles (33), he concluded that the likely source for lineages was South America. This is supported by the nearly unidirectional current flow into the Caribbean Sea from rivers in South America, which likely brought flotsam to the region (11).

In contrast, tropical storms and hurricanes may have also played a role in dispersal, with an unknown direction of transfer (11). Estimations of the direction of dispersal can be inferred from a phylogeny of the group of organisms, whether dispersal, vicariance, or a mix is assumed (12). Finally, Hedges (11) raises the important point that multiple origins are possible for some observed distribution patterns.

In the few biogeographic studies conducted for plant taxa in the Caribbean, a mixture of results emerged. A tabular overview of thirteen studies can be found at www.sbs.utexas.edu/roxisteele. In seven studies, researchers concluded that dispersal was likely the process by which the distribution arose, and in none of the studies was vicariance alone given as the justification for the observed distribution pattern. In five cases they suggested a mixture of both dispersal and vicariance, and the final one simply revealed the number of distribution events necessary to explain the tree topology. The studies involved a variety of families and genera with members in the Caribbean, and they included an assortment of geographical distributions, number of species, data types, and analyses. The combined analysis of these results with many more systematic and biogeographic studies conducted on plants in the Caribbean, along with physical and climatic histories of the region, will aid in producing a model of ecosystem evolution (34). Phylogenetic estimates of additional organisms such as *Psiguria* with appropriate distributions and life history parameters can provide data complementary to geological evidence for testing hypotheses on Antillean historical biogeography (9,38).

Psiguria grows throughout the Neotropics. Taxonomic treatments list two species endemic to the Caribbean islands – *P. trifoliata* and *P. pedata* – while two species occur on both the continents and the islands – *P. trilobata* and *P. ottoniana* (27,39). Individuals have been found from 100 m to 1400 m above sea level and in both dry and wet forests. Because species of *Psiguria* are native to three different, major geographical areas, and at least two of the species are endemic to Caribbean islands, many interesting biogeographical questions arise. 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 tribe *Guraniinae* from sister genus *Wilbrandia* is estimated at 19 mya (S. Renner pers. comm.) implying that the divergence between *Psiguria* and *Gurania* is even more recent. This timing suggests that dispersal played the major role in the distribution pattern of *Psiguria* seen today.

B. Experimental Strategy

1. Taxon Sampling. An intense field survey of *Psiguria* will be conducted to collect as many individuals with unique morphological, geographical, and molecular variation as possible. Because of the discrepancies in the delineation of species, it is vital that multiple populations of each are collected to clarify species-level relationships. Multiple samples of *Gurania* (one from each subgenus) and the species from the monotypic genus *Helmontia* will be collected for comparison and used as outgroups in phylogenetic analyses. Additionally, taxa outside the subtribe *Guraniinae*, such as the sister genus *Wilbrandia* (13), will be included to test the monophyly of *Psiguria*. In the summer of 2005, a plant-collecting trip was conducted in Costa Rica, where four species were collected – *Psiguria bignoniacea*, *P. warscewiczii*, *P. triphylla*, and *Gurania costaricensis*. In January 2007, three collections were made in Puerto Rico and the Dominican Republic. In addition to the taxa available in Larry Gilbert's greenhouses at UT-Austin, samples of some species will be obtained through our collaboration with Susan Swensen (see attached letter). It remains that new collections still need to be made in Peru and Bolivia. Contacts with local botanists in these two countries have already been made, permits have been secured from Dirección General de Biodiversidad (DGB) in Bolivia, and those for Peru are well underway (see attached e-mails). Many herbarium specimens will be studied for the monograph and used in the phylogenetic analyses. Loans of herbarium specimens of all *Psiguria* and *Anguria* including type specimens have been received from US, F, MO, NY, GH. Additional loans will be requested from other major herbaria in the US, Europe, the Caribbean, and Central and South America.

2. Data Acquisition

a) DNA processing. DNA will be extracted from plant specimens, variable genetic markers will be amplified, and regions from both the chloroplast and nuclear genomes will be sequenced and aligned. Total DNA will be isolated from all taxa in *Psiguria* and the outgroup species using fresh, silica-dried, or herbarium material. DNA will be extracted using either the DNeasy Plant DNA Extraction Kit (Qiagen Inc.) or standard CTAB protocols (40,41,42), with some modifications. Standard PCR amplification techniques will be used, and the products cleaned with Exo-Sap. Sequencing reactions will be conducted, the products will be cleaned, and DNA sequence data will be obtained from the DNA Analysis Facility at UT-Austin. DNA sequences will be edited and aligned using Sequencher (Gene Codes Corporation), Clustal

X (43), and MacClade 4.0 (45). To date, DNA has been extracted from 11 *Psiguria*, two *Gurania*, and *Ibervillea lindheimeri*, including two samples from 16 and 37-year-old herbarium sheets.

b) Marker Selection. It is first necessary to identify variable regions that are sufficiently informative for the hypotheses being tested. In order to obtain a useful number of informative characters at low taxonomic levels, multiple noncoding regions must be sequenced and may be grouped together to represent one chloroplast marker since they are maternally inherited as a unit (45), and there is no known recombination. Nineteen different regions of the chloroplast genome were investigated based on a consensus of results in the literature (46,47,48,49). After testing these regions for phylogenetic utility, the top eight were selected and are shown in Table 1. One reviewer commented that perhaps some regions could be excluded if they did not contribute to the reconstruction. As seen in the table, some regions provide greater percentages of parsimony informative characters than others, so we conducted analyses with several combinations until there was little increase in bootstrap support. As a result, these eight markers will be used, and the three least variable markers reported previously (*trnT-psbD*, *trnLE1-trnF*, and *trnD-trnE*) were eliminated. The chloroplast data set contains a sufficient number of informative characters to resolve the species relationships within *Psiguria* (Fig. 1), and the chloroplast tree supports the monophyly of *Psiguria*.

In addition to a large combined chloroplast marker, the species-level relationships will be investigated using at least two nuclear markers. One hundred forty-four primer combinations for amplifying low-copy

nuclear (LCN) regions were screened for phylogenetic usefulness in *Psiguria*. Primer pairs were designed by comparing the whole nuclear genome of *Oryza* with that of *Arabidopsis* to identify conserved, LCN regions in distantly related angiosperms (50). In *Psiguria*, 11 regions amplified successfully, and clones were sequenced in at least three *Psiguria* species plus outgroups. Three regions were found to be potentially phylogenetically informative – *atp*

intergenic spacer	# char.	# p.i. char.	% p.i.
<i>ndhF-rpL32</i>	734	19	2.59
<i>ndhC-trnV</i>	669	14	2.09
<i>3rps16-trnQ</i>	1218	16	1.31
<i>trnS-trnG</i>	710	10	1.41
<i>psbZ-trnM</i>	722	10	1.39
<i>psbM-trnD</i>	934	8	0.86
<i>rpoB-trnC</i>	1096	7	0.64
<i>psbE-petL</i>	1102	9	0.82
Total	7185	93	1.29

Table 1: Eight noncoding regions making up the chloroplast marker for phylogenetic analysis of *Psiguria*. char. = character and p.i. = parsimony-informative

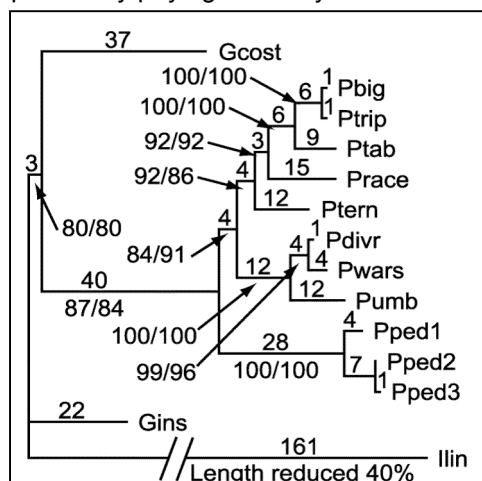


Fig. 1 Maximum Likelihood tree (-ln L = 11,716.73), which is congruent with the single most parsimonious tree (length=381, CI=0.79, RI=0.86) reconstructed from the chloroplast data set. Numbers above the lines indicate the number of changes, and numbers below the lines indicate ML/MP bootstrap values.

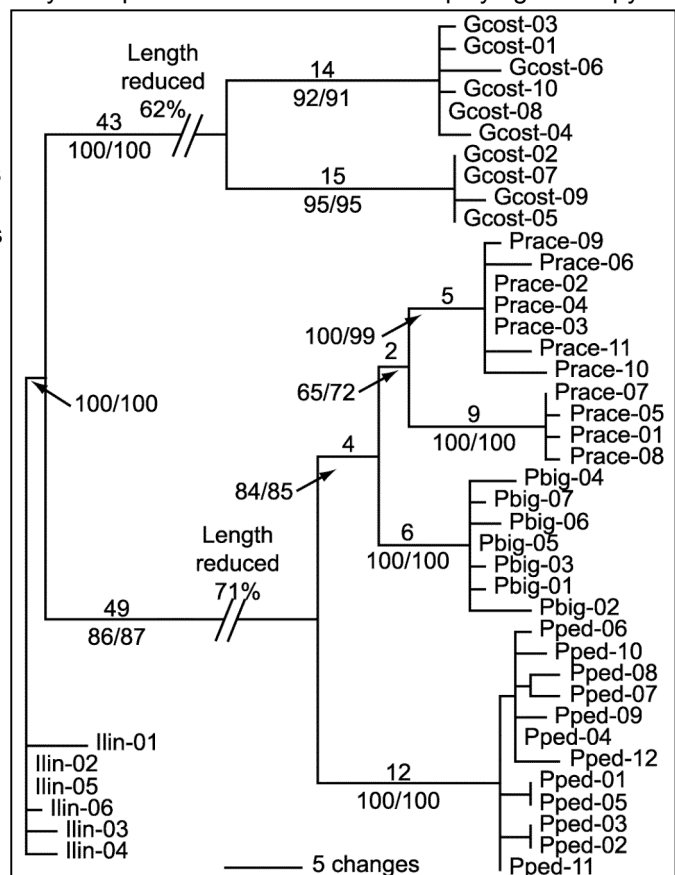


Fig. 2 Maximum Likelihood tree (-ln L = -3028.24), which is congruent with 1 of 2 most parsimonious trees (length=168, CI=0.95, RI=0.99) reconstructed from the s/t phos data set. Numbers above the lines indicate the number of changes, and numbers below the lines indicate ML/MP bootstrap values.

synthase, *actin*, and *serine/threonine phosphoesterase (s/t phos)*. The preliminary phylogenetic reconstruction from *s/t phos* sequences in three *Psiguria* species plus *Gurania costaricensis* and *Ibervillea lindheimeri* is shown in Figure 2. For complete details on this screening study, see the manuscript available at www.sbs.utexas.edu/roxisteele (51). Clones of each taxon group together, *Psiguria* is monophyletic, and all clades have moderate to strong bootstrap support. Tree statistics for analyses generated from two other LCN regions plus ITS are shown in Table 2.

If the data from these markers do not resolve the phylogeny with strong bootstrap support, additional regions will be investigated. The advantages of low-copy nuclear sequences over chloroplast sequences include a higher rate of evolution, the potential to accumulate datasets from multiple unlinked loci, and biparental inheritance (52). Small et al. (52) listed a number of gene families that have been widely investigated for their phylogenetic utility in plants including: *Adh*, G3PDH, GBSSI, PHY, PGI, and MADS-box genes including *pistillata*, *apetala 1*, *apetala 3*, and *leafy*. If necessary, these regions will be examined for variation and reasonable length (550-1300 bp) in *Psiguria*.

LCN region	Tree Length	# char.	# p.i. char.	% p.i. char.	# taxa	# clones
<i>s/t phos</i>	168	1384	104	7.5	5	46
<i>actin</i>	108	1132	61	5.4	6	48
<i>atp synthase</i>	75	622	30	4.8	6	42
ITS	408	778	140	18	11	109

Table 2: Phylogenetic tree statistics for ITS and three LCN regions found to be informative for *Psiguria*. char. = character and p.i. = parsimony-informative

One reviewer commented that the discussion on determining if a region was phylogenetically useful and the differences between orthology and paralogy were confusing. The following is written for clarification. In order to determine if a nuclear region is phylogenetically informative, first amplification is attempted in a few test species. If successful, the region is cloned in four or five taxa including an outgroup. Once clones have been sequenced, phylogenetic analyses are conducted, and a region is considered phylogenetically useful if the tree topology meets two criteria. First, it has to be single-copy, or it must be possible to identify an orthologous copy. If the clones of each taxon form a monophyletic group without any clones outside of that group, the region is considered to be single-copy. If clones are not all in monophyletic groups by taxon, but an isolated, monophyletic clade of the tree includes multiple clones of all the species, and all of those clones are in monophyletic groups by taxon, then this is an orthologous copy of the region that can be distinguished from other paralogous copies. Furthermore, if the orthologous copies are sufficiently divergent, specific primers could be designed. Second, there has to be sufficient variation to provide moderate to strong bootstrap support for individual clades, and contain more changes between taxa than between the individual clones of a species.

c) Monograph and Data for Morphological Phylogeny. A preliminary list of twenty-two morphological characters was assembled, each with two to six character states. A table of these characters can be found at www.sbs.utexas.edu/roxisteele. In addition to this matrix of discrete characters, the list will be expanded further by measuring and databasing quantitative characters surveyed from multiple field collections and herbarium specimens. These characters will be used in both the taxonomic descriptions and the morphological phylogenetic analysis. Once categorized, a random sample of at least 15 specimens per species will be measured for each character.

Additionally, a survey of pollen will be conducted including a comparison with *Helmontia*. Pollen characteristics will be studied via scanning electron microscopy using a Zeiss Supra 40 VP SEM at the ICMB Microscopy and Imaging Facility, UT-Austin. A comparison of pollen morphology from all three genera will contribute to understanding generic boundaries. Pollen of both *Psiguria ternata* and *P. umbrosa* were harvested and viewed under a microscope. Pollen in both of these taxa was found to be tetrahedral tetrads, radiosymmetrical, isopolar, periporate, psilate, and 116-128 μm . No differences were observed in this preliminary investigation.

A chromosome study has not been conducted on *Psiguria* or any of its closest relatives. Studies in Cucurbitaceae have revealed haploid chromosome counts of 11, 12, or 13 in most genera, but a haploid count of seven was found in the agriculturally important *Cucumis sativus* (53), and chromosome counts suggesting polyploidization have been reported in the family (54). Chromosome counts for as many *Psiguria* species as possible will be conducted. These will be pollen mother cell meiotic counts from bud material or mitotic counts from root tips of seed grown in the greenhouse. Buds and root tips will be fixed in 4:3:1 chloroform: absolute ethyl alcohol: glacial acetic acid solution. Young anthers will be removed, stained in aceto-carmine, and squashed. Root tips will be placed in a saturated aqueous paradichlorobenzene solution for two hours, then fixed in 3:1 absolute ethyl alcohol: glacial acetic acid

solution for 30 minutes, hydrolyzed in a 1N hydrochloric acid solution at 55°C for 20 minutes, stained in leucobasic fuchsin and squashed (55). Counts of chromosomes in prepared squashes will be conducted, and photographs of slides taken. One reviewer asked how the chromosome study would be used in the phylogenetic analysis. The primary purposes for conducting the chromosome study are for completeness of the monograph, and to detect polyploidization. We do not plan to use the counts in phylogenetic analyses; however, if polyploidy is detected, it can provide valuable information about hybridization.

3. Phylogenetic Analyses. Two hypotheses will be tested using both molecular and morphological data sets: $H_0 = Psiguria$ is monophyletic and $H_0 = Gurania$ is sister to *Psiguria*.

a) Molecular. Phylogenetic analyses will be conducted with several different computer algorithms including Maximum Parsimony using PAUP* (56), Maximum Likelihood (ML) using GARLI (57), and Bayesian analysis using Mr. Bayes (58). Prior to running ML and Bayesian analyses, Modeltest (59) will be used to select the most appropriate model of DNA substitution. Because chloroplasts are inherited as a unit, analyses using the eight chloroplast regions will be conducted in a concatenated data set, but the analyses on the nuclear, morphological, and concatenated chloroplast data sets will be conducted separately. The resulting tree topologies will be inspected for incongruence. If incongruence is detected, three different methods will be used to evaluate whether the incongruence is statistically significant – the incongruence length difference (ILD) test (60), the Templeton test, and the KH test (61). If incongruence is significant, and trees are not combinable, hypotheses will be tested on each dataset separately. If incongruence is not statistically significant, the trees will be combined to represent the species tree. Support for individual clades in each ML and MP tree will be assessed using bootstrapping (62). For trees constructed using Bayesian analysis, support will be measured by calculating posterior probabilities (58).

By including several species from related genera *Gurania* and *Helmontia*, species from closely related genera sister to *Guraniinae* such as *Wilbrandia*, and the distant outgroup *Ibervillea lindheimeri*, the monophyly of *Psiguria* will be tested. Both the chloroplast and LCN datasets (Fig. 1-2) provide resolution of the taxa, and suggest that *Psiguria* is monophyletic with moderate to strong bootstrap support. AU (63) or SOWH (64) tests will be used to test the stated hypotheses. Various tree topologies in which *Gurania* and *Helmontia* are forced into the ingroup will be tested to determine if they can be rejected in lieu of a monophyletic *Psiguria*.

b) Morphological. Morphological data are currently used less frequently than molecular data for phylogenetic analyses because of the limited number of unambiguous characters available and obstacles to accurate homology assessment and character coding (65). Despite these limitations, morphological analyses can add support to phylogenetic reconstructions from molecular data sets. To address the fundamental problems of morphological character analysis (definition, delimitation, and ordering of character states), Weins (66) suggests coding morphological characters as continuous quantitative states. Once coded, the characters may be analyzed using parsimony, distance, and likelihood methods for quantitative traits. Mentioned previously, a preliminary list of possible characters to include in morphological analyses is available at www.sbs.utexas.edu/roxisteele.

Reviewers commented that they would like to see some preliminary results from morphological phylogenetic work. Although we look forward to finally teasing out the complexity of characters in the genus, this is not yet possible. Eleven of 22 characters shown in the table pertain to leaf characteristics, but an initial study of *Psiguria* plants has revealed a very complex life history in this genus in which most of these characters, in addition to flower color, change over the life of the individual in many species. Obviously a deep understanding of these characteristics and the changes they undergo is vital to writing a monograph. Our understanding of the morphological variation is improving, but it will take some time and experience with plants in the field, greenhouses, and herbarium to clarify the morphological complexity in the genus. Once this is achieved, it will be possible to subject these data to phylogenetic analyses.

4. Biogeographic Analysis. Three hypotheses will be tested: 1) $H_0 = Psiguria$ originated on the continent, 2) $H_0 = Psiguria$ colonized the Caribbean islands once, and 3) $H_0 = Psiguria$ migrated to the Caribbean islands via dispersal. These hypotheses will be investigated by comparing and contrasting the results from four methods: 1) parsimony reconstruction of the ancestral pattern of distribution, 2) comparison of area cladograms between unrelated groups of organisms (such as 67 & 68), 3) Dispersal Vicariance Analysis (DIVA) (69), and 4) a likelihood method for inferring geographic range (70). This fourth approach takes into account information on the timing of lineage splits and putative dispersal routes. Although the likely means of distribution was dispersal based on the estimated timing of the genus origin and the equivalent results of other plant groups studied, vicariance cannot be ruled out *a priori*.

Using several outgroup species in the analysis will lead to a determination of the direction of migration within the genus. Those taxa that are more basal have earlier divergence times, and those closer to the tips of the tree are more recent. This fact will establish the direction of movement. If the early diverging species are the island endemics, then the genus is estimated to have originated in the islands, and movement occurred

from the islands to the continent. If the island endemics are the more recent taxa, the reverse is inferred. The number of times that *Psiguria* moved between the disjunct land masses will be inferred from the topology of the tree. Three hypothetical topologies for the relationships of the two endemic Caribbean species are shown in Figures 3-5, although other topologies are also possible including ones that take into account species that co-occur on the islands and the continent. If the topology looks like Figure 3, one would conclude that only one migration event had occurred. If the topology in Figure 4 is generated, the most likely conclusion is that two migration events to the islands occurred rather than the less parsimonious situation of one movement to the islands, and three back to the continent. The topology in Figure 5 would make it difficult to draw any strong conclusions since it seems feasible that there could have been one move to the Caribbean and one back to the continent or two independent movements to the islands. Alternative hypotheses will be tested using AU (63) and SOWH (64) tests. For species that occur on both the continents and the Caribbean islands, multiple individuals from the different geographic regions will be included to aid in elucidating the relationships and the migration events.

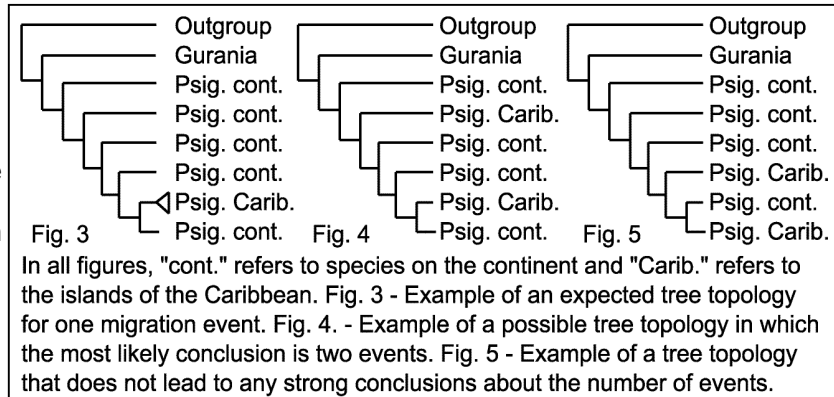
IV. SIGNIFICANCE

Scientists generally acknowledge that the world's tropical rainforests are decreasing at an alarming rate (71). Conservation efforts are underway to database and understand the flora and fauna of the few remaining tropical forests. Funk et al. (72) noted that recently the interest in effective conservation efforts has offered unique opportunities for interactions between biosystematics and conservation biology as well as other disciplines. Information from systematic biology and phylogenetics can be used in conservation studies to answer questions about population genetics, population biology, speciation of rare and endangered taxa, community-level studies of patterns of endangerment, and the use of spatial patterns to identify critical areas for biodiversity (72,73). A monograph of the Neotropical genus *Psiguria* will contribute to conservation efforts of rainforest ecosystems.

With its overall small size and two endemic species in the Greater Antilles, *Psiguria* is an ideal genus for studying the phylogenetic and biogeographic history of plants with members in both Latin America and the Caribbean. Adding this information to the growing collection of phylogenetic reconstructions of organisms in the Caribbean, and combining it with geologic and climatic histories, will aid in producing a model of ecosystem evolution for the region (34). Brought together with the estimated phylogeny of its butterfly pollinators, a systematic study of *Psiguria* will provide the missing information needed to address ecological questions about this model system for investigating coevolutionary relationships.

Given the availability of specimens and lab facilities, this project allows for the opportunity to train and utilize the skills of an enthusiastic undergraduate from a collaborating teaching university, and one from UT-Austin. And, finally, the process of conducting and completing this project will give the graduate student the education, skills, and tools she needs to conduct additional important and meaningful research in her future career in biology.

V. TIMELINE – Before Activation of Funds: Group the borrowed herbarium specimens into morphological categories to confirm taxon sampling and build understanding of morphological characters; conduct pollen studies; continue sequencing nuclear and chloroplast markers. **Year 1, First Six Months:** Collect taxa in Bolivia and Peru; continue sequencing nuclear and chloroplast markers; conduct chromosome studies. **Year 1, Second Six Months:** Complete sequencing nuclear and chloroplast markers; complete phylogenetic analyses using molecular data. **Year 2, First Six Months:** Complete biogeographic and morphological analyses; begin writing thesis including monograph. **Year 2, Second Six Months:** Complete writing; Defend thesis.



REFERENCES CITED

1. Gilbert, L. E. 1972. Pollen Feeding and Reproductive Biology of *Heliconius* Butterflies. *Proc. Natl. Acad. Sci. USA*. Vol. 69. No. 6. pp.1403-1407.
2. Gilbert, L. E. 1975. Ecological Consequences of a Coevolved Mutualism Between Butterflies and Plants. In L. E. Gilbert and P. H. Raven eds. *Coevolution of Animals and Plants*. UT Press, Austin. pp.210-240.
3. Murawski, D. A. and L. E. Gilbert. 1986. Pollen flow in *Psiguria warscewiczii* a comparison of *Heliconius* butterflies and hummingbirds. *Oecologia* 68:161-167.
4. Murawski, D. A. 1987. Floral resource variation, pollinator response, and potential pollen flow in *Psiguria warscewiczii*. *Ecology* 68(5) pp.1273-1282.
5. Gilbert, L. E. 1992. An Evolutionary Food Web and Its Relationship to Neotropical Biodiversity. *Animal-Plant Interactions in Tropical Environments* (ed. by Barthlott, W., Naumann, C.M., Schmidt-Loake, K., & K.-L. Schuchmann). Results of the annual Meeting of the German Society for Tropical Ecology, held at Bonn, February 13-16, 1992.
6. Brower, A. V. Z. 1994. Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylog. Evol.* 3:159-174.
7. Brower, A. V. Z. and M. G. Egan. 1997. Cladistic Analysis of *Heliconius* Butterflies and Relatives (Nymphalidae: Heliconiini): A Revised Phylogenetic Position for *Eueides* Based on Sequences from mtDNA and a Nuclear Gene. *Proc. R. Soc. Lond.: Biol. Sci.* Vol. 264(1384):969-977.
8. Myers, N., R. A. Mittermeier, C. G. Mittermeier, G. A. B. da Fonseca, and J. Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403:853-858.
9. Fritsch, P. W. and T. D. McDowell. 2003. Biogeography and Phylogeny of Caribbean Plants – Introduction. *Syst. Bot.* 28(2): pp.376-377.
10. Humphries, C. J. 1982. Vicariance biogeography in Mesoamerica. *Ann. Missouri Bot. Gard.* 69: pp.444-463.
11. Hedges, S. B. 2001. Biogeography of the West Indies: An Overview. In Woods, C.A. and F. E. Sergile eds. *Biogeography of the West Indies: Patterns and Perspectives*. CRC Press, Boca Raton. pp.15-33.
12. Santiago-Valentin, E. and R. G. Olmstead. 2004. Historical biogeography of Caribbean plants: introduction to current knowledge and possibilities from a phylogenetic perspective. *Taxon.* 53(2) pp.299-319.
13. Kocyan, A., L. Zhang, H. Schaefer, and S. Renner. 2007. A multi-locus chloroplast phylogeny for the Cucurbitaceae and its implications for character evolution and classification. *Mol. Phylog. Evol.* 44 (2):553-577.
14. International Plant Names Index (IPNI). 2004. Published on the Internet <http://www.ipni.org> (accessed April 2006). Collaboration between The Royal Botanic Gardens, Kew, The Harvard University Herbaria, and the Australian National Herbarium.
15. Condon, M. A. and L. E. Gilbert. 1988. Sex Expression of *Gurania* and *Psiguria* (Cucurbitaceae): Neotropical vines that change sex. *Amer. J. Bot.* 75(6). pp.875-884.
16. Condon, M. A. 1992. Coevolution revisited: variation in patterns of visitation to flowers of *Gurania* and *Psiguria* (Cucurbitaceae). Department of Biology, University of North Carolina, Chapel Hill 27599.
17. Condon, M. A. and G. J. Steck. 1997. Evolution of host use in fruit flies on the genus *Blephroaneura* (Diptera: Tephritidae): cryptic species on sexually dimorphic host plants. *Biol. J. Linn. Soc.* 60:443-466.

18. Condon, M. A. and L. E. Gilbert. 1990. Reproductive Biology and natural history of the Neotropical vines *Gurania* and *Psiguria*. In Biology and Utilization of the Cucurbitaceae. Cornell Univ. Press, Ithaca. pp.150-166.
19. Gilbert, L. E. 1983. *Anguria* and *Gurania* (Rain-forest cucumber). In D. H. Janzen ed. Costa Rican Natural History. The University of Chicago Press. pp.190-191.
20. Gilbert, L. E. 1980. Coevolution of animals and plants: a 1979 postscript. In L. E. Gilbert and P. H. Raven, eds. Coevolution of animals and plants. Revised edition. University of Texas Press, Austin, TX, USA. pp.247-263.
21. Condon, M. A. 1984. Reproductive biology, demography, and natural history of Neotropical vines *Gurania* and *Psiguria* (*Guraniinae*): a study of the adaptive significance of size-related sex change. Dissertation. University of Texas, Austin, TX, USA.
22. Roubik, D. W. and J. E. Moreno P. 1991. Pollen and Spores of Barro Colorado Island. Monographs in Systematic Botany from the Missouri Botanical Garden. Volume 36. pp. 8-9, 83-84, and 205-206.
23. Jeffrey, C. 1978. Further notes on Cucurbitaceae: IV Some New-World taxa. *Kew Bulletin* Vol. 33(2) pp.347-380.
24. Croat, T. B. 1978. Flora of Barro Colorado Island. Stanford University Press, Stanford, California, USA. pp.835-837.
25. Stevens, W. D. 2001. Flora de Nicaragua. Missouri Botanical Garden Press, St. Louis, MO. pp.689, 711-712.
26. Cogniaux, A. 1916. Cucurbitaceae – Fevilleae et Melothrieae. Das Pflanzenreich Regni vegetabilis conspectus. IV. 275. I.
27. Wunderlin, R. P. 1978. Flora of Panama Part IX: Family 182 Cucurbitaceae. *Ann. Missouri Bot. Gard.* 65:285-288, 344-349, & 365.
28. Jeffrey, C. 2005. A new system of Cucurbitaceae. *Bot. Zhurn.* 90: 332-335.
29. Marticorena, C. 1963. Material para una monografia de la morfologia del pollen de Cucurbitaceae. *Grana Palynol.* 4(1):78-91.
30. Gentry, A. H. 1982. Neotropical floristic diversity: phytogeographical connections between Central and South America, Pleistocene climatic fluctuations, or an accident of the Andean orogeny? *Ann. Missouri Bot. Gard.* 69: 557-593.
31. Adams, C. D. 1972. Flowering Plants of Jamaica. The University of the West Indies, Mona, Jamaica.
32. Gentry, A. H. 1992. Tropical forest biodiversity: distributional patterns and their conservational significance. *Oikos* 63:19-28.
33. Hedges, S. B. 1996. Historical biogeography of West Indian vertebrates. *Ann. Rev. Ecol. Syst.* 27: 163-196.
34. Graham, A. 2003. Historical phytogeography of the Greater Antilles. *Brittonia.* 55(4):357-383.
35. Zink, R. M., R. C. Blackwell-Rago, and F. Ronquist. 2000. The shifting roles of dispersal and vicariance in biogeography. *Proc. R. Soc. L.* 267:497-503.
36. Inturralde-Vinent, M. A. and R. D. E. MacPhee. 1999. Paleogeography of the Caribbean region: implications for Cenozoic biogeography. *Bull. Amer. Mus. Nat. Hist.* 238:1-95.
37. Ronquist, F. 1997. Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. *Syst. Biol.* 46:195-203.
38. Fritsch, P. W. 2003. Multiple Geographic Origins of Antillean *Styrax*. *Syst. Bot.* 28(2):421-430.
39. Howard, R. A. 1973. The Enumeration and Selectarum of Nicolaus von Jacquin. *Journ. Arn. Arb.* 54:437-438 & 440-442.

40. Doyle, J. J. and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bull.* 19:11-15.
41. Loockerman D. and R. K. Jansen. 1996. The use of herbarium material for molecular systematic studies. In T. F. Stuessy and S. Sohmer eds. Sampling the Green World. Columbia University Press, New York, NY, USA.
42. Scott, K. D. and J. Playford. 1996. DNA extraction technique for PCR in rain forest plant species. *Biotechniques* 20(6):974.
43. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 24:4876-4882.
44. Maddison, W. P. and D. R. Maddison. 2005. MacClade. Sinauer Associates, Inc., Sunderland, MA.
45. Corriveau, J. L. and A. W. Coleman. 1988. Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *Amer. J. Bot.* 75(10):1443-1458.
46. Panero J. L. and B. S. Crozier. 2003. Primers for PCR amplification of Asteraceae chloroplast DNA. *Lundellia* 6:1-9.
47. Shaw J., E. B. Lickey, J. T. Beck, S. B. Farmer, W. Liu, J. Miller, K. C. Siripun, C. T. Winder, E. E. Schilling, R. L. Small. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *Amer. J. Bot.* 92(1):142-166.
48. Daniell H., S-B. Lee, J. Grevich, C. Saski, T. Quesada-Vargas, C. Guda, J. Tomkins, R.K. Jansen. 2006. Complete chloroplast genome sequences of *Solanum bulbocastanum*, *Solanum lycopersicum* and comparative analyses with other Solanaceae genomes. *Theor. Appl. Genet.* 112:1503-1518.
49. Timme, R. E., J. V. Kuehl, J. L. Boore, and R. K. Jansen. 2007. Comparative analysis of the *Lactuca* and *Helianthus* (Asteraceae) plastid genomes: identification of divergent regions and categorization of shared repeats. *Amer. J. Bot.* 94 (3):302-312.
50. Padolina, J.M. 2006. Phylogenetic reconstruction of *Phalaenopsis* using nuclear and chloroplast DNA sequence data and Using *Phalaenopsis* as a natural system for assessing methods to reconstruct hybrid evolution in phylogenetic analyses. Ph.D. dissertation, The University of Texas at Austin.
51. Steele, P.R., Guisinger-Bellian, M., Linder, C.R., and Jansen, R.K. Phylogenetic utility of 144 low-copy nuclear regions in taxa at different taxonomic levels in two distantly related families of rosids. *Taxon*, submitted.
52. Small, R. L., R. C. Cronn, and J. F. Wendel. 2004. Use of nuclear genes for phylogeny reconstruction in plants. *Austral. Syst. Bot.* 17:145-170.
53. Index to Plant Chromosome Numbers. 1956-present. Missouri Botanical Garden, St. Louis, MO. Published by the International Organization of Plant Biosystematists by its Committee for Plant Chromosome Numbers.
54. Renner, S.S., Schaefer, H., and Kocyan, A. 2007. Phylogenetics of *Cucumis* (Cucurbitaceae): Cucumber (*C. sativus*) belongs in an Asian/Australian clade far from melon (*C. melo*). *BMC Evol. Biol.* 7:58.
55. Nevlng, L. I., Jr. 1962. Chromosome counts of two Thymelaceae. *Rhodora* 64:277-281.
56. Swofford, D. L. 2001. PAUP*:Phylogenetic analysis using parsimony (*and other methods). Academic Press, Sunderland, MA, USA.
57. Zwickl, D. J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas at Austin. www.bio.utexas.edu/faculty/antisense/garli/Garli.html

58. Ronquist, F. and J. P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572-1574.
59. Posada, D. and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*. 14(9):817-818.
60. Farris, J. S., M. Källersjö, A. G. Kluge, and C. Bult. 1995. Constructing a significance test for incongruence. *Syst. Bot.* 44:570-572.
61. Kishino, H. and M. Hasegawa. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* 29:170-179.
62. Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. 39:783-791.
63. Shimodaira, H. 2002. An approximately unbiased test of phylogenetic tree selection. *Syst. Biol.* 51(3):492-508.
64. Swofford, D., G. Olsen, P. Waddell, and D. Hillis. 1996. Phylogenetic inference. In D. Hillis, C. Moritz, and B. K. Mable, eds. Molecular Systematics. Sinauer, Sunderland, Mass. pp.407-514.
65. Scotland, R. W., R. G. Olmstead, and J. R. Bennett. 2003. Phylogeny Reconstruction: The Role of Morphology. *Syst. Biol.* 52(4):539-548.
66. Weins, J. J. 2001. Character Analysis in Morphological Phylogenetics: Problems and Solutions. *Syst. Biol.* 50(5):689-699.
67. Patterson, C. Methods of paleobiogeography. 1981. In Nelson, G. and D. E. eds. Vicariance Biogeography: a Critique. New York: Columbia University Press. pp.446-489.
68. Brundin, L. Z. 1988. Phylogenetic biogeography. In Myers, A. A. and P. S. Giller eds. Analytical Biogeography. London: Chapman & Hall. pp.343-369.
69. Ronquist, F. 1996. DIVA version 1.1. Computer program and manual available by anonymous FTP from Uppsala University (<ftp.uu.se> or <ftp.systbot.uu.se>).
70. Ree, R. H., B. R. Moore, C. O. Webb, and M. J. Donoghue. 2005. A likelihood framework for inferring the evolution of geographic range on phylogenetic trees. *Evolution*. 59(11):2299-2311.
71. Ferraro, P. J. and R. D. Simpson. 2001. Cost-effective Conservation: A Review of What Works to Preserve Biodiversity. *Resources* (143):17-20.
72. Funk, V. A., A. K. Sakai, and K. Richardson. 2002. Biodiversity: The Interface Between Systematics and Conservation. *Syst. Biol.* 51(2):235-237.
73. Desmet, P. G., R. M. Cowling, A. G. Ellis, and R. L. Pressey. 2002. Integrating Biosystematic Data into Conservation Planning: Perspectives from Southern Africa's Succulent Karoo. *Syst. Biol.* 51(2):317-330.