METACOMMUNITY SPECIES DELIMITATION AND POPULATION GENETICS IN TROPICAL TANK BROMELIAD PHYTOTELMATA

by

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ABSTRACT OF THE DISSERTATION

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Studying whole communities and ecosystems is seldom performed which makes empirical studies in community and ecosystem ecology difficult to execute. An ecosystem that is often utilized though is the individual ecosystems contained in tank bromeliad phytotelmata that are comprised of mostly larval invertebrates and insects. The inhabitants of these systems are drastically understudied making morphological species identifications very difficult or impossible which inhibits studies pertaining to biodiversity. DNA barcoding and a single-locus maximum likelihood tree-based species delimitation method were used for the first time in this system to estimate patterns of diversity and gene flow in a naturally occurring experimental setup on samples collected along an elevation gradient in the Monteverde region of Costa Rica. This naturally occurring experimental setup contains three different habitat types (cloud forest, wet primary rainforest, and dry primary rainforest). Biodiversity is expected to change along the elevation gradient as temperature, as well as levels of gene flow because of particular dispersal barriers that may exist in the changing landscape. Operational barcode units were successfully delimited, and results suggest the presence of the mid domain effect with further investigation required. Species found along the entirety of the elevation gradient were targeted to assess gene flow among populations. Certain species share

genetic information along the entire gradient and some species showed a level of population divergence indicating a dispersal barrier or perhaps cryptic speciation. Both of which require a more robust dataset to answer those questions.

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To my fiancé, Audrey Biondi, thank you for being at my side unconditionally in times of difficulty and in times of comfort. You are my driving force.

DEDICATION

This work is dedicated in memory of my father, Mark Wilson Kellogg.

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1.1 Objective

The objective of this thesis is to describe the patterns of community diversity in aquatic invertebrate communities from the Monteverde region of Costa Rica and to describe dispersal patterns of, particularly well-distributed species. Community diversity patterns seen here were investigated using DNA barcoding techniques, maximum likelihood tree estimation, and multi-rate Poisson tree processes as a means of species delimitation. Additionally, this work will contribute to a larger effort to study whole bromeliad dwelling communities across latitudinal and longitudinal gradients spanning the tropics of Central and South America using robust phylogenetic frameworks.

1.2 Phytotelmata

Phytotelmata are small bodies of water held by varying parts of plants. The word is derived from the Greek words for plant and pond, *phyton* and *telm* respectively (Maguire Jr, 1971). Phytotelmata are often capable of housing complete, functioning ecosystems and communities. These microcosmic communities are comprised mostly of invertebrates, but there are also some species of dendrobatid frogs that are phytotelm specialists, laying their eggs only within water trapped by these plants. These small, manipulable, and replicable ecosystems are ideal models for ecological studies pertaining to food web structure and ecosystem functions.

Within phytotelmata, there are five main categories, each with their own subcategories. The five types of phytotelmata are as follows: tank bromeliads, pitcher plants, tree holes, bamboo internodes, and axil waters collected by leaves, bracts or petals (Kitching, 2000).

Pitcher plants (Cephalotaceae, Nepenthaceae, and Sarraceniaceae) are unique in that the phytotelm is formed from modified leaf parts or extensions of leaves, adapted specifically to be impermeable and to hold liquids (Fig. 1). These "pitchers" are used as pitfall traps into which prey fall in, drown, and are then are digested by the plant's extracellular enzymes. While these pitchers may seem like hostile environments, it is hospitable for some invertebrates. Depending on the species, these water-filled pitchers can be maintained year-round or for short periods of time. It has been reported that some inhabitants like *Wyeomyia smithii* (Diptera: Culicidae) larvae even overwinter in the frozen environment inside a pitcher for up to four months (Paterson, 1971). These pitchers create a particularly nutrient-rich environment that supports a diverse group of organisms like bacteria, protozoa, rotifers, dragonflies, frogs, mosquito larvae and other Diptera larvae (Gray, 2012; Ratsirarson & Silander Jr, 1996).

Tree holes are a common and widely distributed group of phytotelmata (Frank, 2008; Schmidl, Sulzer, & Kitching, 2008). They are formed by the natural growth patterns of the tree and roots, penetration of bark or heartwood by rot, fallen trees, and very often via buttresses in the roots (Kitching, 2000; Schmidl et al., 2008). Root 'pans' are a widely seen subset of the tree hole phytotelm, characterized by a shallow, broad geometry (Fig. 3). These phytotelmata can occur in almost any species of tree, some more often than others. This also means that these habitats are present on all continents except Antarctica. The holes can be filled and maintained via three different processes: direct rainfall, water that falls from leaves (throughfall), and water that flows down the branches and the trunk (stemflow) (Frank, 2008; Schmidl et al., 2008). Because these are passively filled holes, the majority of nutrients come from leaf detritus and arthropod

cadavers. With nutrients and other compounds coming from water in stemflow, throughfall, leaf detritus and other organic detritus, the water in these holes create another suitable habitat for an array of Diptera larvae, Coleoptera, small crustacea, and protozoa (Albicócco, Carbajo, & Vezzani, 2011; Frank, 2008; Kitching, 2000; Schmidl et al., 2008).

Bamboo shoots are another medium in which these aquatic invertebrate communities can form. Bamboos are grasses that have hollow internodes separated by impermeable nodes in their stems (Frank, 2008; Kitching, 2000). In these hollow internodes, water can accumulate and is impounded by the nodes (Fig. 3). The formation of these phytotelmata can occur via two simple processes. Water can enter from the top of a broken or cut internode, or in some cases, newly emerged beetles will bore their way out of the bamboo after emerging from their endophytically laid eggs inside of the bamboo. The exit hole is typically large enough for water to penetrate and establish a new phytotelm (Kitching, 2000). Bamboos are a diverse group of plants with over 1,200 species being distributed throughout the tropics. They are found in Africa, Madagascar, the Americas, and the Asia-Pacific region (Bystriakova & Kapos, 2006). Given this geographic distribution, their inhabitants are also quite diverse. As with other phytotelmata communities, many Diptera families (Culicidae, Ceratopogonidae, Tipulidae) occupy the habitat, along with some aquatic beetles, and some odonate taxa (Sota & Mogi, 1996). Identification to species level is often difficult and never fully realized in many studies regardless of habitat type.

In no particular order, the last group of phytotelmata that will be discussed here is the axial water group. These communities are formed in water that is impounded by

either the floral bracts, leaf axils, or the flower itself. This section excludes the description of communities formed by tank bromeliads. The axial water group of phytotelmata is the most diverse in part due to the plant kingdom's diversity but also the means by which these phytotelmata are formed. The vast majority of these communities occur in the superorders of Commelinidae, Arecidae, and Liliidae with a predominantly tropical dispersion (Kitching, 2000). A very common form of this habitat comes from the leaf sheath, often seen on Monocotyledons and specifically plants in the *Heliconia* genus (Fig. 4). The leaf-sheath is the extended portion of the leaf-base that is free from the axis (Majumdar, 1956). It is within these leaf-sheaths that the habitat is formed, each of them potentially filling with water and hosting a separate community within each leaf-sheath. These communities are comprised of species from Acarina, Dermaptera, Copepoda, Hemiptera, Coleoptera, and Diptera (Seifert, 1982; Seifert & Seifert, 1976). The base of this food web is supported by shredded plant material and fallen flowers (Seifert & Seifert, 1976). As the plant material degrades and water levels change, so does the community. Sizes of these communities are dependent on the volume of water present which can range from 1.5 cm^3 to 1754.8cm^3 (Kitching, 2000).

1.3 Tank Bromeliads

Among the variety of phytotelmata, the Bromeliaceae is a diverse and well-studied group. From their ecological services offered to their adaptations in different environments, bromeliads offer scientists a tremendous opportunity to study many biological principles as a model organism. They are one of the most diverse and morphologically distinctive clades in flowering plants. Because of their economic

importance and novel adaptations, more is known about their ecophysiology than any other plant family (Benzing & Bennett, 2000).

Of the nearly 3,500 species in this family of monocots, many of them form these phytotelm habitats, and they do so in varying ways (Christenhusz & Byng, 2016). It should be noted that not all species in this large family entrap water. Species like the epiphytic Spanish Moss (*Tillandsia usneoides*) and the largest bromeliad known, Queen of the Andes (*Puya raimondii*), have adapted to their environments in other ways morphologically and do not form phytotelmata.

Bromeliads are native to the Neotropics with a few species being found in the southern continental United States. Within the Neotropics, their distribution in forest systems also varies widely with some species being terrestrial and some species being epiphytic, reaching heights into the canopy. This allows them to be vertically stratified within the forest, occupying many niches. In regard to vertical stratification, Pittendrigh (1948) described three ecophysiological types based on shade tolerance and related shoot architecture: exposure type, sun type, and shade-tolerant type (Fig. 5). 'Exposure' bromeliads occupy the upper reaches of the canopy with the highest amount of sunlight exposure out of the three groups (Benzing & Bennett, 2000; Pittendrigh, 1948). The 'sun' type bromeliads are found at intermediate heights within the forest and have broad and shallow shoots (Benzing & Bennett, 2000; Pittendrigh, 1948). The final 'shade' group occupies the lower reaches of tree trunks and forest floor (Benzing & Bennett, 2000; Pittendrigh, 1948). While these trends were described based on a study in Trinidad, similar patterns have been described in other parts of the tropics (Benzing & Bennett, 2000).

Faunal diversity within these phytotelmata has been difficult to measure. These systems are ideal to study ecological processes at the community level because they are contained by borders, they are small, abundant, and manipulable. However, understanding the true faunal diversity in these systems is quite difficult in part because the invertebrate inhabitants are primarily larval stages of insects. Few diagnostic characters exist for many insect larvae, and larval descriptions and keys are uncommonly available; the identification of these larvae to species often requires the larvae to be matched to its adult form. This requires expert taxonomic identification based on many morphological structures and due to the relatively low number of individuals with this taxonomic expertise, identification based on morphology alone is a challenge. Because these communities are comprised of individuals hatching from eggs that were laid at different times, although each community may have the same species present, species may be at different life stages, making it difficult to consistently identify these organisms. Currently, there is still a shortage of tropically focused literature for many of these groups although it is being generated (Brown, 2009a, 2009b; Garrison, Ellenrieder, & Louton, 2006, 2010; Miller & Bergsten, 2016). And even as more literature and guides are generated focusing only on the adult forms, there is still a lack of knowledge, linking larval stages to adult stages.

1.3.1 Community Dynamics

The aquatic habitats within bromeliads are dominated by cross-ecosystem organisms. These organisms, such as insects, are those whose life-stages take place in multiple ecosystems; some insects develop as larvae in aquatic habitats, but their adult

life stages are spent in terrestrial ecosystems (Romero & Srivastava, 2010). Other inhabitants of these aquatic ecosystems are not cross-ecosystem species. These species, like leeches and small crustaceans, spend their ontogeny in a single ecosystem. All ecosystems have both cross-ecosystem residents and single-ecosystem residents. This, in turn, means that these ecosystems have inhabitants that are capable of dispersal from their birth site, and others that are not.

Dispersal can have varying effects on community and metacommunity structure (Chase, 2007; Evans, Martiny, & Allison, 2017). These effects can be influenced by spatial and temporal scale as well. Dispersal of a species can increase local species diversity causing a rescue effect for species that have gone locally extinct (Cadotte & Fukami, 2005; Verreydt et al., 2012). Dispersal can also increase local diversity through source-sink effects by supplying a sustainable amount of immigrants to the population (Cadotte & Fukami, 2005; Holyoak, Leibold, & Holt, 2005). Dispersal can also lead to a homogenization of local community structure which can, in fact, decrease regional diversity (Cadotte & Fukami, 2005). Considering that much of the species pool at the local and regional level is made up of insects with moderate to high dispersal mechanisms (mosquitoes, crane flies, damselflies), the role of dispersal and the level at which these species are capable of moving from their birth community to new communities is very important. Not only does dispersal affect diversity, but it also inflicts changes at different trophic levels (Verreydt et al., 2012).

This system presents a unique opportunity to create a molecular dataset to be used in DNA barcoding and other phylogenetic and population genetic analyses. To test the applicability of these new methods, we used samples collected from a naturally occurring

experimental set up. Samples were collected along an elevation gradient that contains different habitat types (cloud forest and non-cloud forest), and we would expect differences in species diversity along this gradient. It would also be expected that species distributed along the entire gradient would show signs of population-level divergence because of these different habitat types that are present. Here, we use DNA analyses to evaluate genetic structure along these gradients and provide the first molecular-based species delimitation effort in this region for this system.

2 Materials and Methods

2.1 Study Site and Sample Collection

Samples from bromeliad communities were collected from Costa Rica in the Monteverde Cloud Forest Reserve. Bromeliads were collected opportunistically from varying heights above the ground and from the ground. Collection took place at 9 individual sites within the Monteverde region of Costa Rica and were named as the following: Aleman, El Valle, Brilliante, Pocosol, Eladios, San Gerardo, San Luis, Research Trail, and Dos Ases. The characteristics of each site can be found in Table 1. All sites were sampled in 2015 and 2016 except for Dos Ases which was not sampled in 2016. In 2015 a total of 142 individual plants (communities) were surveyed. In 2016 a total of 48 individual plants were sampled. Every bromeliad was characterized in terms of their size (water-holding capacity, plant diameter), taxonomic identity, and habitat (location, exposure, and height above ground). Contents of the bromeliad were separated (detritus from inhabitants) when they were rinsed. All individuals were identified to morphospecies via morphology. Specimens were stored in 95% ethanol to preserve flesh

and prevent tissue decay and dehydration. Of the non-terrestrial or aquatic organisms on which this study focuses 30,399 individuals were collected in total.

The nine collection sites within Costa Rica were assigned an elevation category based on the minimum and maximum elevation in which bromeliads were collected. For this study, there are three elevation categories (low, mid, and high). Low elevations range from 750 meters above sea level to 1,000 meters. Mid elevations range from 1,001 meters to 1,300 meters. High elevation communities range from 1,301 meters to 1,610 meters above sea level. Low elevation sites are as follows: Aleman, Pocosol, and Eladios. Mid elevation sites are as follows: San Gerardo, San Luis, and Dos Ases. High elevation sites are as follows: Brilliante, Research Trail, and El Valle.

2.2 DNA Extraction

DNA extractions were performed on whole-body tissue of most larval samples, with the exception of Odonata and Coleoptera samples in which extractions were performed on leg tissue. All extractions were performed using a Qiagen DNeasy Blood and Tissue Kit. Samples were incubated overnight in 180µl of ATL Buffer and 20µl of proteinase K at a temperature of 56°C. All steps of the manufacturer protocol were followed but with an added 15-minute, room temperature, incubation step in the elution buffer before the final centrifugation instead of the outlined 1-minute.

2.3 PCR Amplification

Amplification of the mitochondrial gene cytochrome c oxidase subunit I (*coi*) was performed in 25µl reactions with each reaction consisting of 12.5µl of Taq 2x master mix

solution, 1µl of each primer (forward and reverse, both diluted to 1x concentration), 5µl of DNA template, and 5.5µl of Milli-Q water. Amplification was carried out in an Eppendorf Mastercycler pro S with forward and reverse primers as listen in Table 2, C1-J-1751 and C1-N-2191 respectively. The following 8-step protocol was used: 94ºC for 120 seconds followed by 20 cycles of 94ºC for 15 seconds, 48ºC for 15 seconds, 72ºC for 30 seconds, then an additional 20 cycles at 94ºC for 15 seconds, 50ºC for 45 seconds, 72ºC for 30 seconds, with a final step at 72ºC for 300 seconds. PCR products were visualized with a 1% agarose gel run at a constant 110V for 40 minutes using 3µl GelRed loading buffer. Successfully amplified samples were both purified and sequenced by Macrogen (Brooklyn, NY, USA) for forward and reverse primer sequences. A total of 391 samples were successfully sequenced (# of sequences at each gradient and slope).

2.4 Sequence Analysis

Each forward and reverse sequence was assembled and edited to reconstruct contig consensus sequences using Sequencher software version 5.0.1. Consensus contig sequences were initially automatically aligned using ClustalX 2.1 followed by manual alignment in Mesquite version 3.51 (Larkin et al., 2007; Maddison & Maddison, 2018).

Candidate species delimitation was performed using a tree-based approach that utilized maximum likelihood and Poisson processes which was performed using the server-based version of mPTP, https://mptp.h-its.org/#/tree (Kapli et al., 2017). A final alignment of all samples from Costa Rica totaling 312 sequences was used for both methods. To identify sequences that were not fit for this analysis (i.e. contaminated sequences, those of insufficient length) we created a reference tree using IQTree web

servers (http://iqtree.cibiv.univie.ac.at/) and sequences mined from GenBank and BOLD. This tree was an ultrafast bootstrap maximum likelihood tree with an automatically detected substitution model. Alignments for both trees were trimmed to 429 base pairs.

For mPTP, the maximum likelihood tree was obtained using the same 312 sequence alignment in raxmlGUI 2.0 (Silvestro & Michalak, 2012). The GTRGAMMAI nucleotide substitution model was selected based on Akaike information criterion results from ModelTest-NG v0.1.3. The raxmlHPC binary was also implemented along with *Machilis rubrofusca* being selected as the outgroup.

In mPTP, we used the multi-rate poisson tree processes model with a cropped outgroup of *Machilis rubrofusca*. Results were output as a maximum likelihood tree and a .txt file with the best score for multi-coalescent rate, the null-model score, and the number of delimited species and the corresponding specimens. Delimited species in this study will be referred to by one of two terms; species and operational barcode unit (OBU). The operational barcode unit is a term adapted from Rahman et al. 2019 wherein the species level DNA sequence clusters can be interpreted as candidates for DNA barcodes.

To assess whether or not populations from different elevations are mixing and dispersing, minimum spanning haplotype networks were constructed using PopART 1.7 (Leigh & Bryant, 2015). An analysis of molecular variance was carried out in GenAlEx 6.503 (Peakall & Smouse, 2006).

3 Results

3.1 Species Delimitation and Community Diversity

Results of the maximum likelihood tree reconstructed by RaxML and the species delimited tree produced by mPTP have revealed previously-unknown levels of diversity within these metacommunities. Found among only 312 sequences, a total of 50 potential species have been defined across 3 orders of Insecta: one species of *Mecistogaster* (Zygoptera, Pseudostigmatidae), 12 species of mosquitoes, 4 species of beetles from two families (Dytiscidae and Scirtidae), and 33 other species of Diptera from 7 families (Tipulidae, Chironomidae, Ceratopogonidae, Syrphidae, Tabonidae, Corethrellidae, and Psychodidae) (Table 2 and Fig. 6). It should be noted that the amplification of the *coi* region for many individuals failed and that this work is only based on 312 sequences from a total of 30,399 individuals collected from 190 bromeliads.

This analysis also displayed many instances of morphological identification shortcomings as many of the morphology-based identifications may be incorrect. For many specimens though, prior morphological identification was only completed to the family taxonomic level. However, maximum likelihood analysis was able to place these essentially unidentified specimens within an OBU, some of which had morphospecies identification.

In the case of OBU 3, this clade shows many individuals only identified to family Culicidae placed among three different morphospecies: *Anopheles* aleman, *Anopheles* stubby, and *Anopheles* collar (Fig. 6). This clade also shows no evidence of divergence between the four morphospecies identified, indicating a lack of support for the defining characters used to classify these individuals. This variation in defining characters that were chosen may be explained by variation in morphology through the ontogeny of these

larvae. Some features may be present or absent depending on the time elapsed since oviposition. This information is not presently available in wild phytotelmata.

A reference tree composed of mined sequences from GenBank and BOLD was used in an attempt to classify these morphospecies to the level of genus (Fig. 7). This method proved difficult because genera from mined sequences did not always form clades. For example, the genus *Aedes* was found in two different clades of the tree (Fig. 7a). *Aedes* was placed among *Ochlerotatus*, *Haemogogus*, and among other *Aedes* sp. This may be a result of an error in the original identification of the NCBI sequence or discrepancy in nomenclature. *Ochlerotatus* was recognized as a subgenus until 2000 when it was elevated to genus (Reinert, 2000). Another example of this problem can be seen in the case of *Larsia* sp. Sequence MPCB05509|Larsia_sp.|COI5P|HM379557 is placed next to a group of Tanypodinae individuals. It would be reasonable to assume that these unidentified Tanypodinae species may, in fact, belong to the genus *Larsia*. There is, however, another sequence, CHMNO21515|Larsia_atrocincta|COI5P, that is not in the same clade as MPCB05509|Larsia_sp.|COI5P|HM379557 (Figure 7b). This lack of support at the level of genus makes it difficult to hypothesize any possible identification.

Results of the mPTP analysis allow species richness estimates along the elevational gradient to be performed. These results show that most species are found in locations within the mid-elevation range, with 34 OBU's indicating the presence of a mid-domain effect. High elevations contained 28 different OBU's. Low elevation sites contained 30 distinct OBU's. 10 OBU's (15, 17, 24, 26, 30, 38, 40, 42, 43, and 50) were found in each elevation range (low, mid, and high). 7 OBU's (16, 19, 21, 29, 35, 36, and 48) were found only in low elevations. 4 OBU's (11, 12, 18, and 31) were only found at

mid-elevations. Finally, a total of 7 OBU's (5, 9, 14, 27, 32, 39, and 41) can be categorized as high elevation specialists.

3.2 Population Genetics Analysis

Haplotype networks show mixed patterns of dispersal in different OBU's, with some networks supporting panmictic populations and with other populations isolated by elevation. In the case of OBU 17 (a mosquito), high-elevation individuals show no evidence of dispersal or gene flow to lower elevations, while low and mid-elevation individuals display evidence of gene flow and dispersal (Fig. 8). OBU 24, (another mosquito), displays patterns of dispersal along the entire elevation gradient. However, the number of substitutions among haplotypes is substantial. The reason for the distinctness among haplotypes in this species may be caused by the presence of cryptic species. Alternatively, it may, in fact, be due to true population divergence. The haplotype network for OBU 30, a tipulid, again indicates a large amount of dispersal along the entire elevation gradient with the primary haplotype being shared among elevations (Fig. 10. OBU 1, *Mecistogaster* sp., disperses along the elevation gradient but this OBU may also contain up to 3 distinct species. The maximum likelihood tree also supports this hypothesis (Figure #) with high branch support. In any instance of possible cryptic speciation, more evidence would be needed to classify or describe said species.

4 Discussion

Studying the diversity of entire communities is an often challenging task yet is often required for conservation efforts and building upon ecological principles. Here we

present the first reference tree for an entire metacommunity-level analysis of the tank bromeliad phytotelmata. Never before have bromeliad communities been sequenced with such vigor. Major studies using strictly morphological identification have been performed before but with no molecular basis for species identification (Richardson, 1999). Our current study presents the first major reference tree for tropical invertebrate phytotelm communities and adds to population-level analysis of dispersal capabilities of multiple species from these systems. Our results prove the necessity and utility of single-locus molecular barcodes even as metabarcoding and multilocus datasets dominate the systematics and ecological fields. These analyses suggest high levels of taxonomic diversity are present in these communities as well as extensive dispersal capabilities among the more cosmopolitan species identified in this study. In some cases, like with OBU 30, there is no evidence of population-level divergence suggesting a completely connected population. In other instances, like with OBU 17, there is preliminary evidence for population-level divergence indicating two separate populations at higher and lower elevations. However, because little is known of the life history of many of these tank bromeliad inhabitants, and because it is quite likely that many of these species are being barcoded for the first time, discerning between population divergence and species divergence requires further insight. DNA barcoding, haplotype network analysis and molecular based species delimitation has allowed us to identify species which may hold cryptic diversity and also groups that were not as diverse as expected species. Creating a more robust genetic dataset for these groups would allow us to uncover the true diversity and uncover more population level patterns. This would provide valuable insights into how these populations are interacting or not interacting.

This study is a particularly good example of how morphology-based identification and weighting of characters can lead to misidentification. There are many instances in which morphospecies are placed at different locations on the tree, and thus, different operational barcode units. This indicates a lack of explanatory power for the characters chosen for morphological identification. However, in many instances where individuals were only identified to the taxonomic level of family, these individuals were able to be placed among species or barcode units.

The results of this study can be used in two ways for future projects requiring species identification in these phytotelm systems. Future work can be done to identify easily recognizable intraspecific morphological characters for these barcode units identified here. This would create a new set of characters by which specimens could be identified. This would allow identification to carry on at no extra cost to researchers which is desirable for many reasons. However, morphological identification can often be a time-consuming process that is not capable of being performed in large batches. Each individual would still need to be thoroughly examined. If sufficient funds and equipment are readily available, molecular barcode datasets could be generated and compiled to create one inclusive barcode database that would potentially span large geographic ranges throughout the tropics. As this work is in collaboration with the Bromeliad Working Group (BWG), which has carried out extensive sampling across the tropics, the insights developed in this study have the potential to be applied to global analyses of the system.

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Table 1:

Site	Site Code	Elevation Category
Aleman	A	Low
El Valle	B	High
Brilliante	C	High
Pocosol	D	Low
Eladios	E	Low
San Gerardo	F	Mid
San Luis	G	Mid
Research Trail	H	High
Dos Ases	I	Mid

Table 2:

COI Primers	Forward/Reverse	Sequence 5’ to 3’	Tm ºC
C1-J-1751 (alias Ron)	Forward	GGAGCTCCTGACATAGCATTCCC	62.8
C1-N-2191 (alias Nancy)	Reverse	CCCGGTAAAATTAAAATATAAACTTC	56.7

Table 3:

Sequence ID	Operational Barcode Unit	Collection Site	Elevation Category
15A5COI_Zygoptera	1	Aleman	Low
15D6COI_M.modesto	1	Pocosol	Low
15E13COI_M_modesta	1	Eladios	Low
16E23COI_Zygoptera_8	1	Eladios	Low
16E15COI_Zygoptera	1	Eladios	Low
16A16_COI_Zygoptera	1	Aleman	Low
16D19_COI_Zygoptera_#23	1	Pocosol	Low
16A6COI_Zygoptera	1	Aleman	Low
16E6COI_Zygoptera	1	Eladios	Low
16E7COI_Zygoptera	1	Eladios	Low
16F22COI_Zygoptera_#12	1	San Gerardo	Mid
16A2COI_Zygoptera	1	Aleman	Low
16E9COI_Mecistogaster_modesta	1	Eladios	Low
16F12COI_Zygoptera	1	San Gerardo	Mid
16F4COI_Zygoptera_#14	1	San Gerardo	Mid
16F8COI_Zygoptera_#13	1	San Gerardo	Mid

16D5COI_Zygoptera_vv_small	1	Pocosol	Low
16E17COI_Zygoptera_#4	1	Eladios	Low
16_E19_COI_Zygoptera_#2_large	1	Eladios	Low
16A10COI_Zygoptera	1	Aleman	Low
15A5.2COI_Zygoptera	1	Aleman	Low
15D6.2COI_M_modesto	1	Pocosol	Low
15E13.2COI_M_modesta	1	Eladios	Low
15F27.2COI_Mecistogaster_modesta	1	San Gerardo	Mid
15F27COI_Mecistogaster_modesta	1	San Gerardo	Mid
15C15COI_Anopheles_stubby	2	Brilliante	High
16G19COI_Anopheles_V	2	San Luis	Mid
15H13COI_Anopheles_collar	2	Research Trail	High
15C14.2COI_Anopheles_v	2	Brilliante	High
15F10COI_Anopheles	2	San Gerardo	Mid
15_G15_COI_Anopheles_v	2	San Luis	Mid
15C14COI_Anopheles_v	2	Brilliante	High
15E12COI_Anopheles_collar	3	Eladios	Low
15E18COI_Anopheles_stubby	3	Eladios	Low
16F3COI_Anopheles	3	San Gerardo	Mid

16A5COI_Anopheles	3	Aleman	Low
16D7COI_Anopheles	3	Pocosol	Low
16E8COI_Anopheles	3	Eladios	Low
15E12.2COI_Anoph_collar	3	Eladios	Low
15E18.2COI_Anoheles_stubby	3	Eladios	Low
15E25.2COI_Anopheles	3	Eladios	Low
15E25COI_Anopheles	3	Eladios	Low
15F22.2COI_Anoph_collar	3	San Gerardo	Mid
15F22COI_Anoph_collar	3	San Gerardo	Mid
15F24.2COI_Anoph_aleman	3	San Gerardo	Mid
15F26.2COI_Anopheles_stubby	3	San Gerardo	Mid
15G12COI_Anopheles_aleman	3	San Luis	Mid
15G19COI_Anophees_collar	3	San Luis	Mid
16A5.2COI_Anopheles	3	Aleman	Low
16E8.2COI_Anopheles	3	Eladios	Low
16F3.2COI_Anopheles	3	San Gerardo	Mid
16G9.2COI_Anopheles_aleman	3	San Luis	Mid
15G14COI_Anopheles_stubby	3	San Luis	Mid
15F26COI_Anopheles_stubby	3	San Gerardo	Mid

15F24COI_Anoph_aleman	3	San Gerardo	Mid
15G19.2COI_Anopheles_collar	3	San Luis	Mid
15G12.2COI_Anopheles_aleman	3	San Luis	Mid
15E1COI_Tanypodinae	4	Eladios	Low
16D18COI_Tanypodinae	4	Pocosol	Low
16E5COI_Tanypodinae	4	Eladios	Low
16A9COI_Tanypodinae	4	Aleman	Low
16F19COI_Tanypodinae	4	San Gerardo	Mid
15D5.2COI_Psychodid_zebra	4	Pocosol	Low
15D8.2COI_Corithrellidae	4	Pocosol	Low
15E1.2COI_Tanypodinae	4	Eladios	Low
15F9.2COI_Tanypodinae	4	San Gerardo	Mid
15F9COI_Tanypodinae	4	San Gerardo	Mid
16D18.2COI_Tanypodinae	4	Pocosol	Low
16E5.2COI_Tanypodinae	4	Eladios	Low
16F19.2COI_Tanypodinae	4	San Gerardo	Mid
15C6COI_Tanypodinae	5	Brilliante	High
16G15COI_Tanypodinae	6	San Luis	Mid
16H6COI_Tanypodinae_pink	6	Research Trail	High

15G7COI_Tanypodinae	6	San Luis	Mid
16G15.2COI_Tanypodinae	6	San Luis	Mid
15H9COI_Tanypodinae	6	Research Trail	High
15H9.2COI_Tanypodinae	6	Research Trail	High
15G7.2COI_Tanypodinae	6	San Luis	Mid
16G7COI_Polypdilum	7	San Luis	Mid
15H10.2COI_Polypedilum_short_tail	7	Research Trail	High
15G6.2COI_Polypedilum_short_tail	7	San Luis	Mid
16D14COI_Polypedilum	8	Pocosol	Low
15F8COI_Polypedilum_short_tail	8	San Gerardo	Mid
15F14COI_Polypedilum_long_tail	8	San Gerardo	Mid
15E10COI_Polypedilum_short	8	Eladios	Low
15A1COI_Polypedilum_short_tail	8	Aleman	Low
15C3COI_Polypedilum_short_tail	9	Brilliante	High
15C3.2COI_Polypedilum_short_tail	9	Brilliante	High
15B3COI_Polypedilum	10	El Valle	High
15C2COI_Polypedilum	10	Brilliante	High
16F11COI_Polypedilum	10	San Gerardo	Mid
15C2.2COI_Polypedilum	10	Brilliante	High

15F14.2COI_Polypedilum_long_tail	10	San Gerardo	Mid
15G2.2COI_Polypedilum	10	San Luis	Mid
15G2COI_Polypedilum	10	San Luis	Mid
16F11.2COI_Polypedilum	10	San Gerardo	Mid
16F14.2COI_Orthoclad	11	San Gerardo	Mid
15G5COI_Orthoclad_short_head	11	San Luis	Mid
16G20COI_Orthoclad	12	San Luis	Mid
15F13.2COI_Orthoclad_short_head	13	San Gerardo	Mid
15F13COI_Orthoclad_short_head	13	San Gerardo	Mid
15H3COI_Orthoclad_short_head	13	Research Trail	High
16G20.2COI_Orthoclad	13	San Luis	Mid
15H3.2COI_Orthoclad_short_head	13	Research Trail	High
15C7COI_Orthoclad_short_head	14	Brilliante	High
16H7COI_Orthoclad_short_head	14	Research Trail	High
16B11COI_Orthoclad	14	El Valle	High
16C13COI_Orthoclad	14	Brilliante	High
16_C13.2_COI_Orthoclad	14	Brilliante	High
16F14COI_Orthoclad	15	San Gerardo	Mid
15B5COI_Orthoclad_short_head	15	El Valle	High

16A3COI_Orthoclad	15	Aleman	Low
16D11COI_Orthoclad_short_head	16	Pocosol	Low
15A2COI_Culicidae_el_valle	17	Aleman	Low
15B1COI_Culicidae	17	El Valle	High
16H5COI_Culicidae_el_valle	17	Research Trail	High
16A13COI_Culicidae_el_valle	17	Aleman	Low
15A2.2COI_Culicidae_el_valle	17	Aleman	Low
15B1.2COI_Culicidae	17	El Valle	High
15F5.2COI_Culicidae	17	San Gerardo	Mid
15F5COI_Culicidae	17	San Gerardo	Mid
15F23.2COI_Mosquito_stripey	17	San Gerardo	Mid
15G18COI_Culicidae_el_valle	17	San Luis	Mid
16C16COI_Culicidae_el_valle	17	Brilliante	High
16A13.2COI_Culicidae_el_valle	17	Aleman	Low
16_G3.2_COI_Culicidae_diab_amer	17	San Luis	Mid
16H5.2COI_Culicidae_el_valle	17	Research Trail	High
15G18.2COI_Culicidae_el_valle	17	San Luis	Mid
16G5COI_Culicidae_lfs	18	San Luis	Mid
16F17COI_Culicidae_DiabAmer	18	San Gerardo	Mid

15E17COI_Mosquito_diablo	19	Eladios	Low
16_A4_COI_Culicidae_diab_amer	19	Aleman	Low
16G6COI_Toxorhynchites	20	San Luis	Mid
16D17COI_Toxorhynchites	20	Pocosol	Low
16D4COI_Culicidae	21	Pocosol	Low
16E13COI_Culicidae_x-mas	21	Eladios	Low
16D4.2COI_Culicidae	21	Pocosol	Low
16D13.2COI_Culicidae_adult	21	Pocosol	Low
16_E13.2_COI_Culicidae_x_mas	21	Eladios	Low
15C17COI_Culicidae_-_chubby	22	Brilliante	High
16F7COI_Culicidaechubby	22	San Gerardo	Mid
15H17COI_Culicidae_chubby	22	Research Trail	High
15F15.2COI_Culicidae	22	San Gerardo	Mid
15F15COI_Culicudae_double_check	22	San Gerardo	Mid
16F7.2COI_Culicidae_chubby	22	San Gerardo	Mid
15H17.2COI_Culicidae_chubby	22	Research Trail	High
16G18COI_Culicidae_polka_dot	23	San Luis	Mid
15G16COI_Culicidae_polka-dot	23	San Luis	Mid
16F2.2COI_Culicidae_polka_dot	23	San Gerardo	Mid

15F17COI_Culicidae_polka_dot	23	San Gerardo	Mid
15E20COI_Culicidae_polka_dot	23	Eladios	Low
16A15COI_Culicidae_polka_dot	23	Aleman	Low
15C16COI_Culicidae_-_yfs	24	Brilliante	High
16C4COI_Culicidae_yfs	24	Brilliante	High
16G4COI_Culicidae_yfs	24	San Luis	Mid
16A14COI_Culicidae_yfs	24	Aleman	Low
16E10COI_Polypedilum_short	24	Eladios	Low
16F9COI_Culicidae_lfs	24	San Gerardo	Mid
15C16.2COI_Culicidae_yfs	24	Brilliante	High
15F3.2COI_yfs	24	San Gerardo	Mid
15F3COI_Yfs	24	San Gerardo	Mid
15G17COI_Culicidae_yfs	24	San Luis	Mid
16D9.2COI_Culicidae_yfs	24	Pocosol	Low
16_E10.2_COI_Culicidae_yfs	24	Eladios	Low
16F9.2COI_Culicidae_lfs	24	San Gerardo	Mid
16G4.2COI_Culicidae_yfs	24	San Luis	Mid
15H16.2COI_Culicidae_yfs	24	Research Trail	High
15C13COI_Culicidae_collar	25	Brilliante	High

16C11COI_Culicidae_collar	25	Brilliante	High
16H8COI_Culicidae_collar	25	Research Trail	High
16G11COI_Culicidaecollar	25	San Luis	Mid
15H14COI_Culicidae_collar	25	Research Trail	High
15C13.2COI_Culicidae_collar	25	Brilliante	High
15G11COI_Culicidae_collar	25	San Luis	Mid
16C11.2COI_Culicidae_collar	25	Brilliante	High
16G11.2COI_Culicidae_collar	25	San Luis	Mid
15H14.2COI_Culicidae	25	Research Trail	High
15D9COI_Culex_big_eye	26	Pocosol	Low
15E6COI_Culex_big_eye	26	Eladios	Low
16D3COI_Culex_big_eye	26	Pocosol	Low
16H3COI_Culex_big_eye	26	Research Trail	High
16F5COI_Culex_big_eye	26	San Gerardo	Mid
15E6.2COI_Culex_big_eye	26	Eladios	Low
15F16.2COI_Culex_big_eye	26	San Gerardo	Mid
15F16COI_Culex_big_eye	26	San Gerardo	Mid
16D3.2COI_Culex_big_eye	26	Pocosol	Low
16F5.2COI_Culex_big_eye	26	San Gerardo	Mid

16G16.2COI_Culex_big_eye	26	San Luis	Mid
15G10COI_Culex_big_eye	26	San Luis	Mid
15H19.2COI_Culex_big_eye	26	Research Trail	High
15G10.2COI_Culex_big_eye	26	San Luis	Mid
16C6.2COI_Tipulid_antennae	27	Brilliante	High
15E2COI_Tipulid	28	Eladios	Low
15E7COI_Tipulid_adult	28	Eladios	Low
16E11COI_Psychodid_short_double_spike	28	Eladios	Low
16G16COI_Culex_big_eye	28	San Luis	Mid
16D10COI_Tipulidae_xs	28	Pocosol	Low
15F21.2COI_Tipulidae	28	San Gerardo	Mid
16E11.2COI_Tipulidae_xs	28	Eladios	Low
16A11COI_Tipulid_ys	29	Aleman	Low
16H13COI_Tipulid_small	30	Research Trail	High
16F21COI_Tipulid	30	San Gerardo	Mid
16C3COI_Tipulid	30	Brilliante	High
15H7COI_Tipulid	30	Research Trail	High
15G3.2COI_Tipulidae	30	San Luis	Mid
16B1.2COI_Tipulid_xs	30	El Valle	High

16C3.2COI_Tipulid	30	Brilliante	High
16C10.2COI_Orthoclad_black_head	30	Brilliante	High
16H13.2COI_Tipulid_small	30	Research Trail	High
15F21COI_Tipulidae	30	San Gerardo	Mid
15D1COI_Tipulidae	30	Pocosol	Low
15C4COI_Tipulid	30	Brilliante	High
15I5COI_Tipulidae	30	Dos Ases	Mid
15H7.2COI_Tipulid	30	Research Trail	High
16G14COI_Tipulid	31	San Luis	Mid
15G3COI_Tipulidae	31	San Luis	Mid
16G14.2COI_Tipulid	31	San Luis	Mid
16B12COI_Corethrellidae	32	El Valle	High
16B12.2COI_Corethrellidae	32	El Valle	High
15D8COI_Corithrellidae	33	Pocosol	Low
16F18COI_Corethrellidae	33	San Gerardo	Mid
16E3COI_Cortithrellidae	33	Eladios	Low
15E23COI_Corethrellidae	33	Eladios	Low
15F29.2COI_Corithrellidae	33	San Gerardo	Mid
15F29COI_Corithrellidae	33	San Gerardo	Mid

16E21.2COI_Corethrellidae	33	Eladios	Low
15I3COI_Corethrellidae	33	Dos Ases	Mid
15E8COI_Psychodid_siphon_tail	34	Eladios	Low
15E8.2COI_Psychotid_siphon_tail	34	Eladios	Low
15F11.2COI_Psychodid_siphon_tail	34	San Gerardo	Mid
15F11COI_Psychodid_siphon_tail	34	San Gerardo	Mid
15F20.2COI_Psychodid_zebra_stripe	34	San Gerardo	Mid
15E24COI_Black_siphon_psychodid	35	Eladios	Low
16A1COI_Psychodid__zebra_stripe	36	Aleman	Low
16H11COI_Psychodid_zebra	37	Research Trail	High
16G12COI_Psychodid_zebra	37	San Luis	Mid
16B6COI_Psychodid_zebra_stripe	37	El Valle	High
15C11.2COI_Psychodid_zebra_stripe	37	Brilliante	High
15G1.2COI_Psychodid_zebra_striped	37	San Luis	Mid
16C14.2COI_Orthoclad	37	Brilliante	High
16H11.2COI_Psychodid_zebra	37	Research Trail	High
15H2COI_Psychodid_zebra_stripe	37	Research Trail	High
15G1COI_Psychodid_zebra_striped	37	San Luis	Mid
15I12COI_Psychodid_zebra_striped	37	Dos Ases""	Mid

15H2.2COI_Psychodid_zebra_striped	37	Research Trail	High
16F15COI__Mycetophiliade_banana	38	San Gerardo	Mid
16C2COI_Mycetophilidae	38	Brilliante	High
16D16COI_Worm_with_head_noID	38	Pocosol	Low
15F12.2COI_Chironomid_black_head	38	San Gerardo	Mid
15F12COI_Chironomid_black_head	38	San Gerardo	Mid
15C1COI_Orthoclad_black_head	39	Brilliante	High
16C10COI_Diptera_angel_wing	40	Brilliante	High
15H11COI_Orthoclad_black_head	40	Research Trail	High
15C1.2COI_Orthoclad_black_head	40	Brilliante	High
15F25COI_Orthoclad_black_head	40	San Gerardo	Mid
16E1.2COI_Syrphid_rat_tail_maggot	40	Eladios	Low
15I7COI_Polypedilum_short_tail	40	Dos Ases""	Mid
15H11.2COI_Orthoclad_black_head	40	Research Trail	High
16H10COI_Forcipomyiinae	41	Research Trail	High
16C5COI_Forcipomyiinae	42	Brilliante	High
16F10COI_Forcipomyiinae	42	San Gerardo	Mid
16D6COI_Forcipomyiinae	42	Pocosol	Low
16C5.2COI_Forcipomyiinae	42	Brilliante	High

15E11.2COI_Pshchodid_short_double_spike	43	Eladios	Low
15F28.2COI_Ortho	43	San Gerardo	Mid
15F28COI_Ortho	43	San Gerardo	Mid
16D6.2COI_Forcipomyiinae	43	Pocosol	Low
15E11COI_Psychodid_short_double_spike	43	Eladios	Low
15D4COI_Psychodid_spikey	43	Pocosol	Low
16B9COI_Forcypomyiinae_pronged_tail	43	El Valle	High
15F6.2COI_Tabonid	44	San Gerardo	Mid
15F6COITabonid	44	San Gerardo	Mid
15D7COI_Tabanid	44	Pocosol	Low
16E18COI_Scirtidae	45	Eladios	Low
15F4COI_Scirtidae	45	San Gerardo	Mid
16_E18.2_COI_Scirtidae	45	Eladios	Low
16D15COI_Scirtid	46	Pocosol	Low
16A7COI_Scirtid_immature	46	Aleman	Low
16D15.2COI_Scirtidae	46	Pocosol	Low
15C9COI_Scirtid	46	Brilliante	High
15D10COI_Scirtidae	47	Pocosol	Low
16B5.2COI_Scirtes_immature	47	El Valle	High

15D11COI_Dytiscid_adult	48	Pocosol	Low
15D3COI_Dytiscid	48	Pocosol	Low
15E15COI_Dytiscid_larvae	48	Eladios	Low
16D2COI_Dytiscidae_larvae	48	Pocosol	Low
16D12COI_Dytiscid_adult	48	Pocosol	Low
15D3.2COI_Dytiscid	48	Pocosol	Low
15E15.2COI_Dytiscid_larvae	48	Eladios	Low
16D2.2COI_Dytiscidae_larvae	48	Pocosol	Low
16D12.2COI_Dytiscid_adult	48	Pocosol	Low
15E9COI_Dytiscid_adult	48	Eladios	Low
15C10COI_Diptera_angel_wing	49	Brilliante	High
15H6COI_Diptera_angel_wing	49	Research Trail	High
15I9COI_Diptera_angel_wing	49	Dos Ases""	Mid
15H6.2COI_Diptera_angel_wing	49	Research Trail	High
15E3COI_Syrphid_RT_maggot	50	Eladios	Low
16E1COI_Syrphid_RT_maggot	50	Eladios	Low
16G17COI_Syrphid_RT_maggot	50	San Luis	Mid
15_E3.2_COI_Syrphid_RT_maggot	50	Eladios	Low
15E16.2COI_Syrphid_hard_tail	50	Eladios	Low

15G9COI_Syrphid_rat_tail	50	San Luis	Mid
16G17.2COI_Syrphid_rat_tail_maggot	50	San Luis	Mid
15F2COI_Syrphid_hard_tail	50	San Gerardo	Mid
15E16COI_Syrphid_hard_tail	50	Eladios	Low
15_I6_COI_Syrphid_-_hard_tail	50	Dos Ases	Mid
15H8.2COI_Syrphid_RT_maggot	50	Research Trail	High
15_G9.2_COI_Syrphid_RT_maggot	50	San Luis	Mid

Figure 1: Depiction of pitcher plant community from *Nepenthes bicalcarata* (Kitching, 2000).

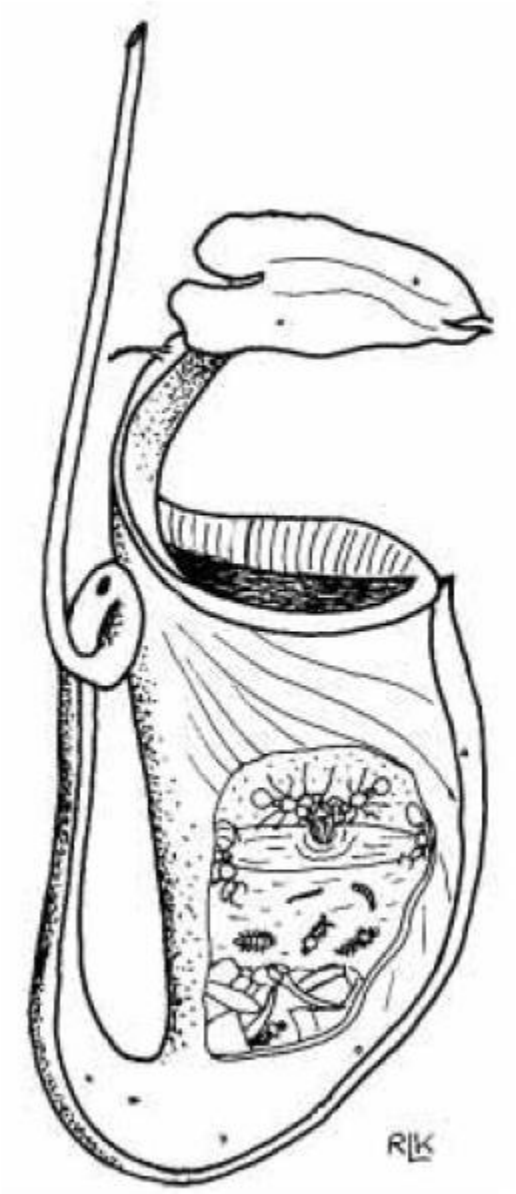

Figure 2: Depiction of water filled tree hole community based on one found in Lamington National Park, south-east Queensland (Kitching, 2000).

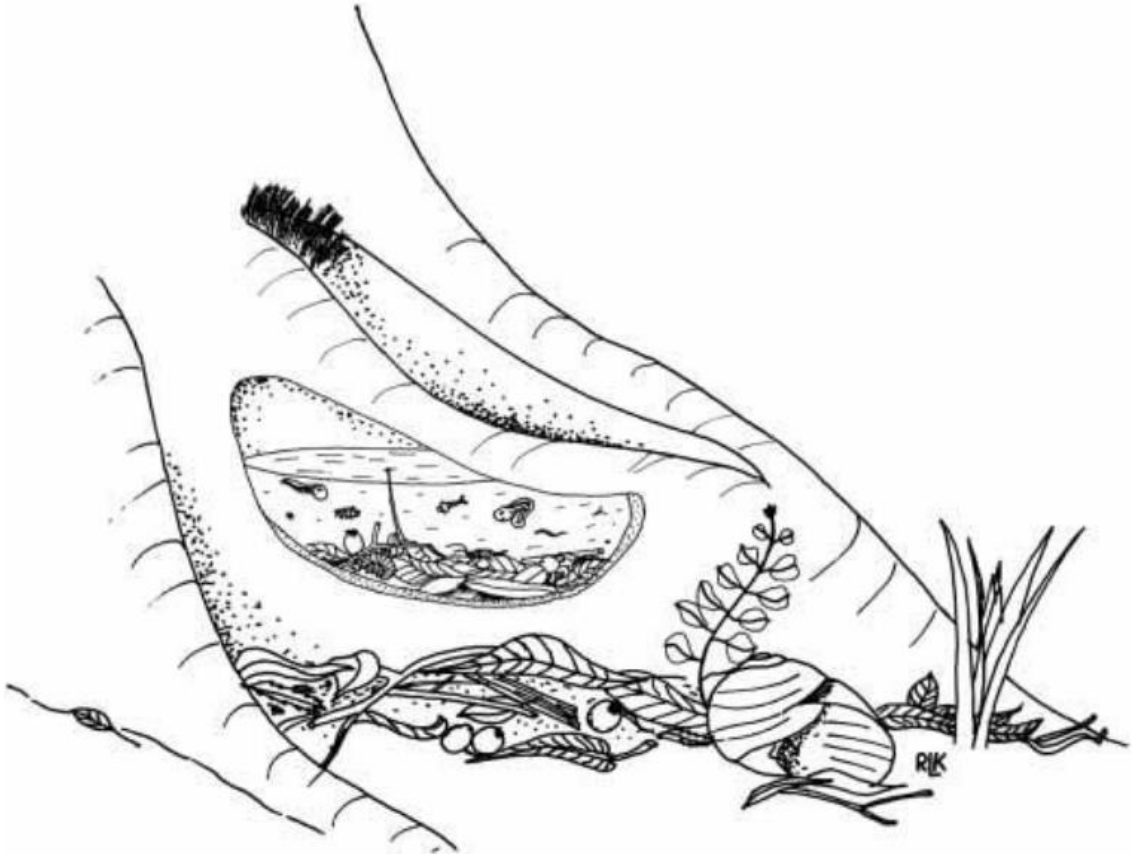

Figure 3: Depiction of a bamboo internode community content based on one from New Guinea (Kitching, 2000).

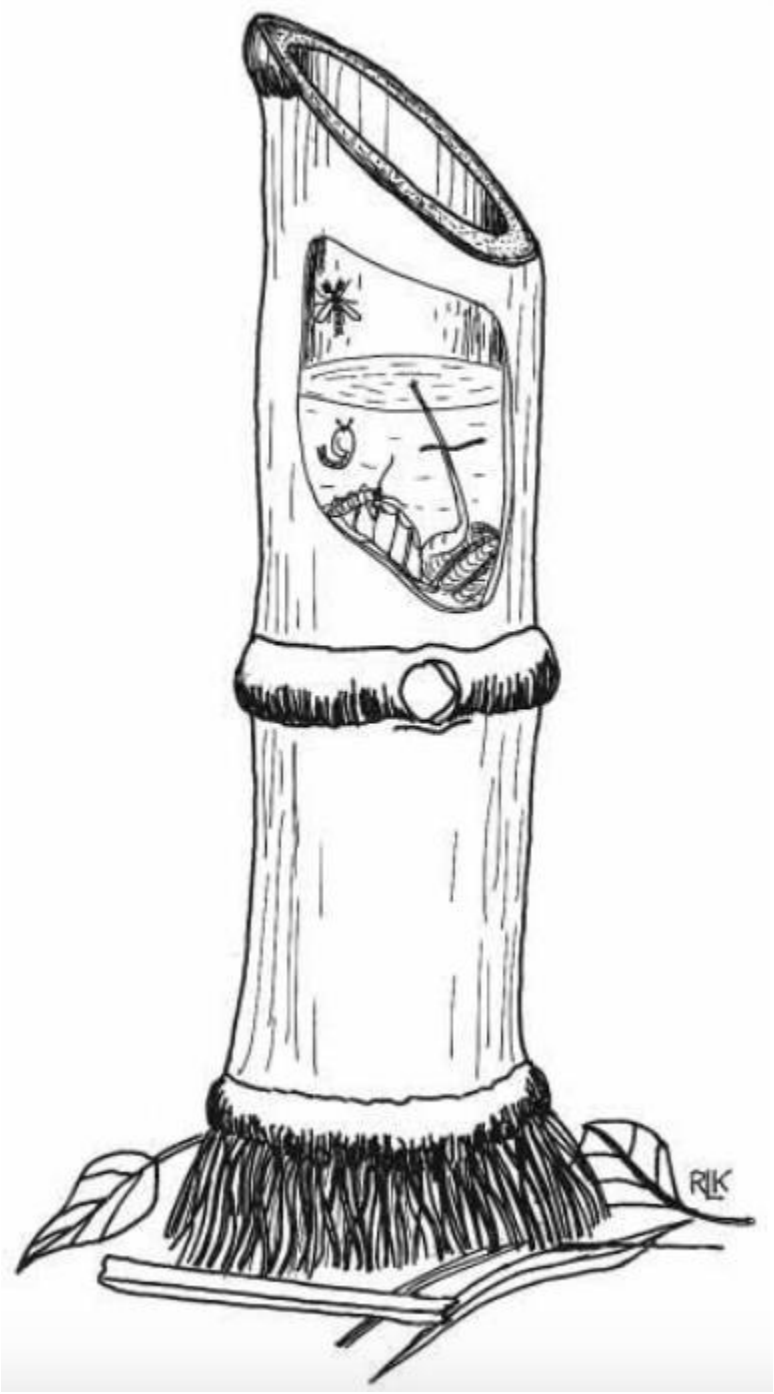

Figure 4: Cut-away schematic of an actual *Heliconia caribaea* community found in Venezuela (Kitching, 2000).

Figure 5: Diagram illustrating the vertical stratification of the different tank bromeliad types (Benzing, 2000).

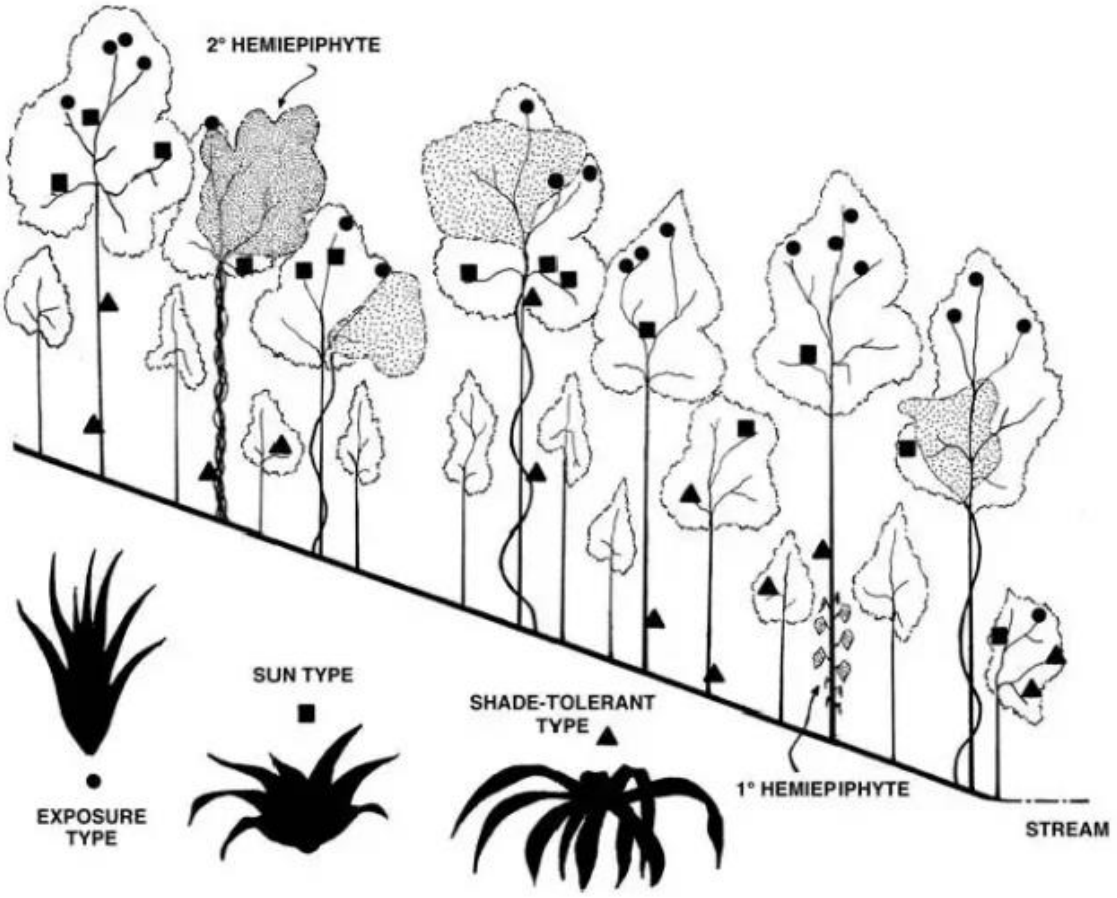

Figure 6: mPTP phylogram of bromeliad dwelling insects based on standard fragment of the mitochondrial *coi* gene. The scale bar indicates the expected number of substitutions per site. Clusters of red tips, or singular green tips indicate delimited barcode units or species that were resolved from mPTP analysis.

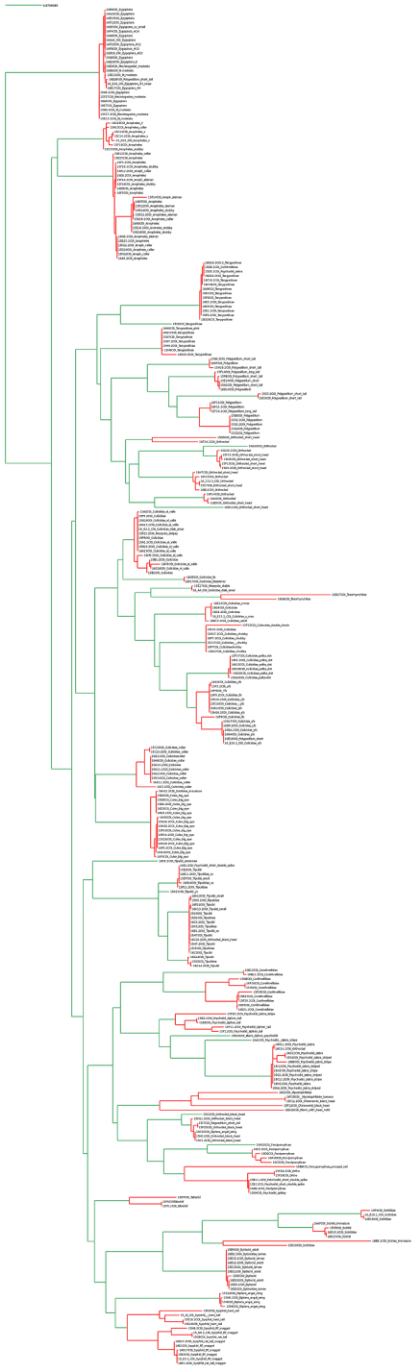

Figure 7: Rapid bootstrap reference tree of all samples from GenBank, BOLD, and all sequences generated from this study. IQTree analysis based on standard fragment of the mitochondrial *coi* gene. (a) Blue clade from the reference tree. (b) Red clade from reference tree.

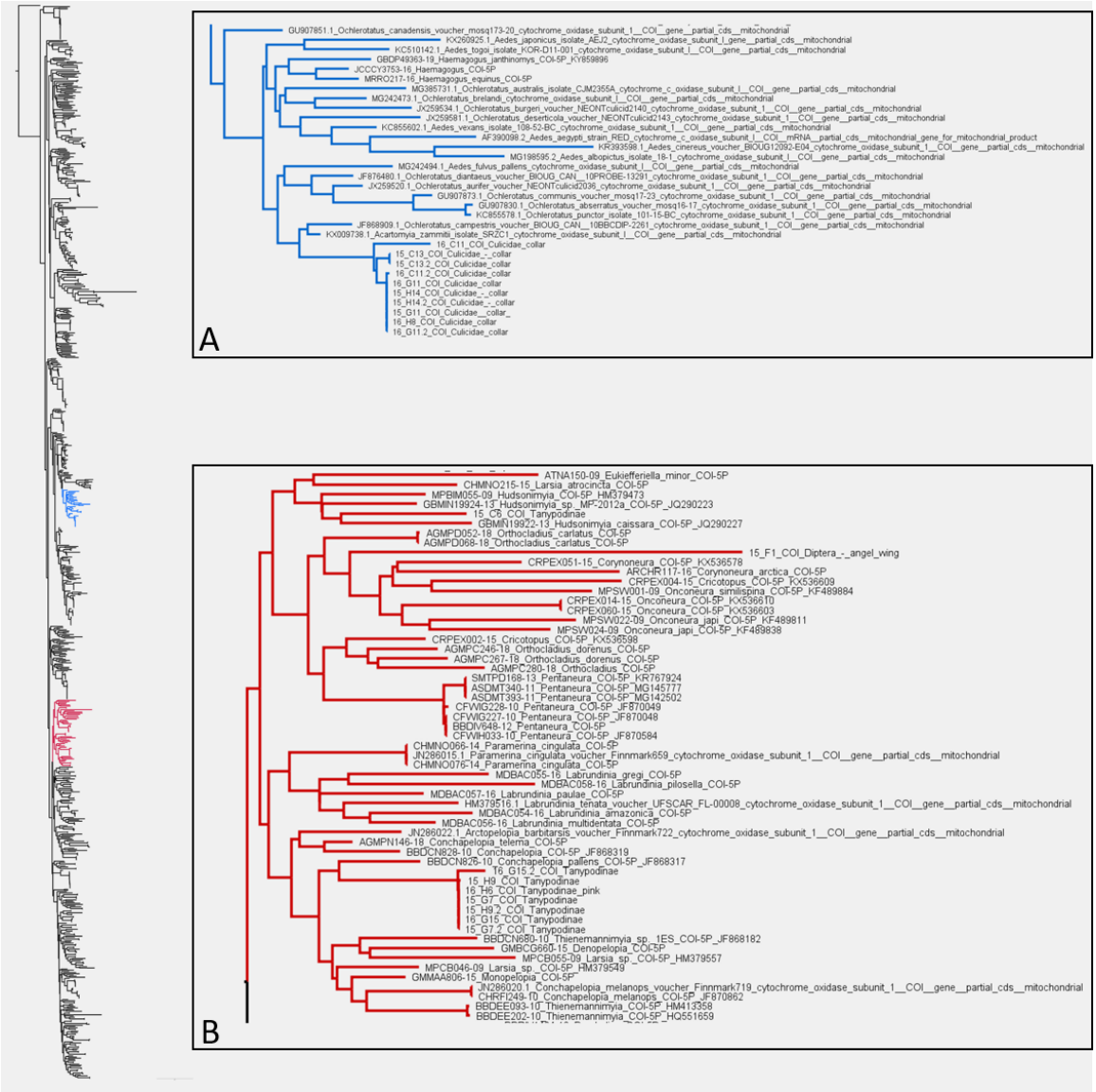

Figure 8: PopART Minimum Spanning Haplotype Network for OBU 17.

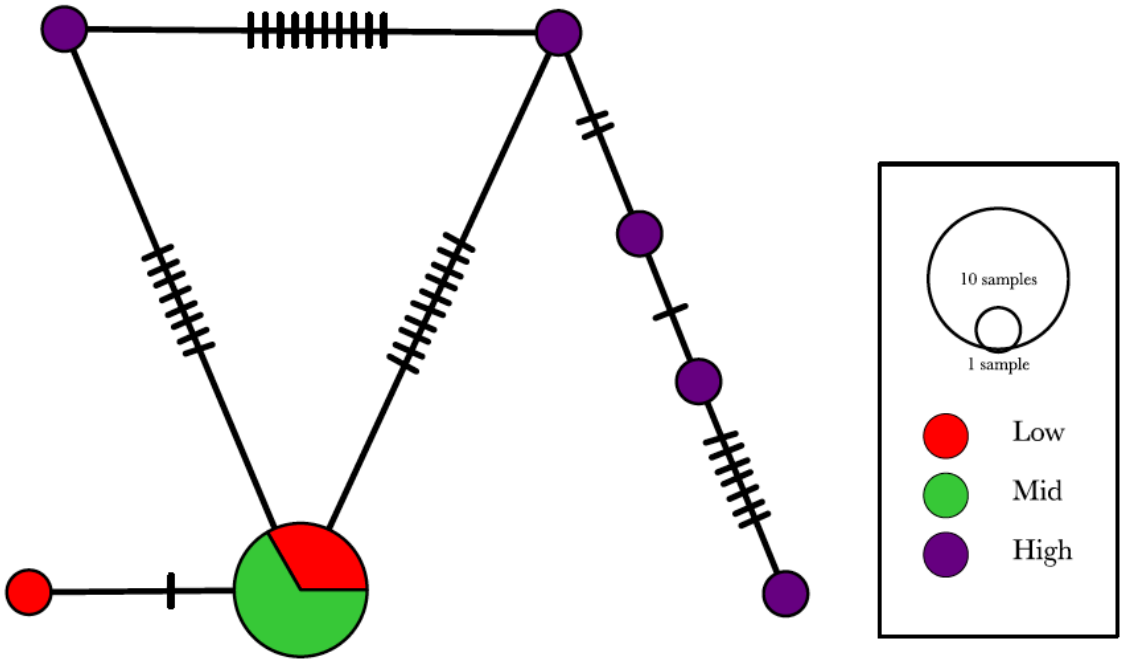

Figure 9: PopART Minimum Spanning Haplotype Network for OBU 24.

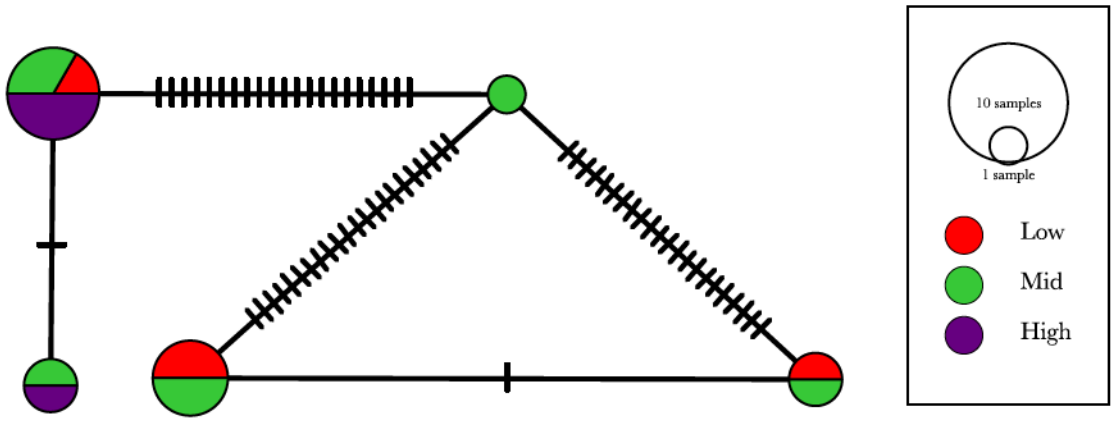

Figure 10: PopART Minimum Spanning Haplotype Network for OBU 30.