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Title: Cryptic Diversity in the Red Algal Genus *Actinotrichia* Decaisne (Galaxauraceae, Rhodophyta)

Approved: _____

Tom Schils, Chairman, Thesis Committee

The introduction of non-native algae is of great concern to marine resource managers because of their potential to become invasive. To assess the risk of introducing non-indigenous marine macroalgae and to detect those already present, a thorough understanding of the species richness of island floras and species distribution ranges is required. Traditional (morphological) identification can be expensive and cumbersome, but modern genetic techniques provide fast and cost-effective methods for algal identification and cryptic species recognition.

In this study, I use the red algal genus *Actinotrichia* Decaisne as a proxy to examine algal species diversity in Micronesia and the western Pacific, and demonstrate how genetics-based species delimitation methods can be used to characterize marine floras in the region. For this study, *Actinotrichia* specimens were collected from Okinawa, Guam, Chuuk, Pohnpei, Kosrae, and Hawaii and examined for unique morphological characteristics. Sequences of the mitochondrial marker *cox1* and plastid marker *rbc*L were obtained from these specimens and compared against previously-published sequences and

analyzed using species delimitation methods ABGD, bPTP, and BPP. Finally, the similarity of each island's flora was compared using the unifrac distance metric. The morphological, phylogenetic, species delimitation, and biogeographical analyses reveal a high degree of cryptic diversity in *Actinotrichia.* These results support the description of three new species unique to Micronesia: *A. lenwoi sp. nov., A. carolinia sp. nov.,* and *A. micronesica sp. nov.* These results suggest the marine flora of Micronesia is substantially more diverse than currently recognized and further study of this diversity will aid resource managers in detecting potentially harmful invasive species.

TO THE OFFICE OF GRADUATE STUDIES

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CRYPTIC DIVERSITY IN THE RED ALGAL GENUS *ACTINOTRICHIA* DECAISNE (GALAXAURACEAE, RHODOPHYTA)

BY

ANNA SIMEON

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Table of Contents

List of Tables

List of Figures

Cryptic Diversity in the Red Algal Genus *Actinotrichia* Decaisne (Galaxauraceae, Rhodophyta)

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Introduction

Marine ecosystems are increasingly affected by human activities such as pollution, habitat degradation, fishery exploitation and the accidental introduction of non-indigenous marine species (NIMS) (Pauly et al. 2002; Lotze et al. 2006; Molnar et al. 2008). Over the last several decades, introduced algae have become particularly notorious because of the possibility for them to become invasive and detrimental to coastal habitats (Eldredge and Smith 2001; Pauly et al. 2002). In Hawaii, for example, the introduced algae *Acanthophora spicifera* (M. Vahl) Børgesen and *Gracilaria salicornia* (C. Agardh) E.Y. Dawson have invaded and replaced native species and smothered local reef communities (Smith et al. 2002). Many introduced algae are transported to new areas through many human-related activities (Hewitt et al. 2007), but those associated with boat traffic – such as hull fouling and ballast water contamination – are of greatest concern (Drake & Lodge 2007). Worldwide shipping has increased substantially over the last three decades and is expected to increase even more (Endresen et al. 2007), and geographic regions isolated from one another are now artificially linked by shipping lanes, increasing the opportunities for potentially invasive species to spread.

The first step of evaluating the diversity of any flora, however, requires a solid definition of biodiversity and a protocol for how to delineate and identify species. While some argue that species limits are not important for understanding concepts like speciation and evolution (Winker et al. 2007), a species is an extremely useful taxonomic unit when

comparing floristic similarities between regions, particularly when estimates of species richness, abundance, and distribution are desired (Agapow et al. 2004, Dayrat 2005). Many different species concepts are currently used to define species and each has a separate purpose depending on the methods available. While it is ideal to use multiple species concepts when attempting to categorize taxa (Zuccarello et al. 2002; Alström et al. 2008; Tronholm et al. 2010), multiple approaches are not always practical. As a result, some systematic studies use only one species concept. The biological species concept is perhaps the most widely-accepted method used to define species, and does so based on whether two individuals can reproduce and subsequently produce viable, fertile offspring (Donoghue 1985). However, this quality is not always practical, or even possible, to test. Another one of these, the ecological species concept, considers organisms a member of the same species if they share an ecological niche. Additionally, the phylogenetic species concept is one that has become more popular as genetic studies have become more commonplace. This concept considers genetic sequence data and monophyly within phylogenies (organisms sharing a common ancestor) to determine species boundaries (Mishler 1985; Donoghue 1985).

However, the most popular is the original Linnaean-aged morphological species concept, whereby species are defined by morphological, anatomical, developmental, and physiological characteristics (Alström et al. 2008). But even this more traditional method has its limits. Evolutionary phenomena – such as convergent evolution and conserved morphology – can blur distinctions between morphologically similar species (Keshavmurthy et al. 2013). Individuals of the same species can also look vastly different depending on their environment, a phenomenon known as phenotypic plasticity

(Schlichting 1989; Stewart 2006), and a single species may undergo multiple, morphologically distinct life stages. In marine algae, this is especially true as many taxa have complex life cycles with sometimes radically different morphologies between life stages (Saunders 2005). Many closely-related algal species also appear extremely similar, even after microscopic examination (Saunders 2005; Saunders & Kucera 2010). In such cases, the distinguishing factor is often an elusive trait found only during a certain life stage. These issues make accurate morphological species identifications in algae difficult and, if certain traits are simply not present in a specimen, occasionally impossible.

Perhaps the largest shortcoming of morphological taxonomy is its inability to reveal hidden, or "cryptic," diversity (Bucklin et al. 2010). Cryptic species appear to be the same morphologically, but genetic differences suggest different evolutionary histories and taxonomic statuses. These differences can have significant phenotypic consequences. In snakes, for example, venom can vary enough between cryptic species to require different antidotes (Wuster & Thorpe 1994). Similarly, cryptic algal species can form different secondary compounds, some of which have known medical uses (Dioli 2011). Our understanding of such cryptic species is necessary to fully understand biodiversity and its implications for humans and the natural environment.

One way to explore biodiversity using molecular tools is DNA "barcoding", a technique that has gained popularity in the fields of taxonomy and ecology because of its ability to cheaply and quickly detect new taxa and identify their systematic placement (Saunders 2005). The method involves sequencing a small, specific, and officially agreed-upon portion of an organism's genome with the intent to compare the sequence against those from the same marker in other specimens. To be effective, these barcodes (also

known as markers) must consist of a common gene to be useful in both broad comparisons and those of closely-related species, but must also contain enough genetic divergence between species so that distinctions between them can actually be made (Hebert 2005). This difference between intra-species and inter-species diversity is termed the "barcode gap" and is often used for helping determine the limits on diversity within and between species (Bucklin et al. 2011). The mitochondrial marker cytochrome oxidase subunit I (*cox1* or COI) is considered by many to be a "universal" marker, useful for identifying a wide variety of organisms (Neigel et al. 2007; Lowenstein 2009; Richards et al. 2009), including red (Saunders 2005) and brown algae (Saunders & Hana 2010; McDevit & Saunders 2009). This gene has many advantages over nuclear markers including its lack of introns, limited recombination rate, and haploid character – all of which generally increase amplification success (Bucklin et al. 2011). After successful sequencing, these new barcodes can assist in taxon identification. Once published, barcode sequences are generally deposited in online databases (such as GenBank or BOLD Systems) where they may be publicly accessed (Bucklin et al. 2011). These databases allow for taxon identification by comparing the unknown sequence against others previously uploaded.

This is not to say, however, that barcoding is able to or should completely replace traditional (morphological) taxonomy. Some argue, for instance, that the resolution at which barcoding can correctly identify some species is no better than what is already accomplished through traditional taxonomy (Will & Rubinoff 2004), and molecular species delimitations can vary substantially depending on which markers are used. Furthermore, if the specimens whose sequences are uploaded to online databases are incorrectly identified, (which, for many taxa, is not an unsubstantiated concern), the

resulting list of taxon names with most similar sequences to that of an unidentified organism may also be incorrect. If the newly, and inaccurately, identified specimen is then also added to the database under this incorrect classification, the problem is compounded.

DNA barcoding and phylogenetics are, therefore, not proposed as substitutes to traditional morphological taxonomy, but a valuable supplement that can assist in accurately identifying taxa and evaluating biodiversity.

Objectives and Scope of Work

To explore the diversity of Micronesia's marine flora and assess overlap in floristic composition throughout the region, this study aims to develop a baseline floristic survey by examining potential cryptic species based on morphological and phylogenetic data, using the genus *Actinotrichia* Decaisne as a proxy for the flora of Micronesia. This study evaluates the genetic diversity within *Actinotrichia* specimens from Japan, Guam, Hawaii, Micronesia, and other localities using the mitochondrial *cox1* marker for species delimitation, the chloroplast encoded large subunit RuBisCo (*rbc*L) marker to evaluate phylogenetic placement, and corroborates new species distinctions with morphological characteristics and biogeographical information. Such morphological data, combined with molecular data and information on geographic distributions, allows for a glimpse into the true floristic diversity of the genus in the western Pacific.

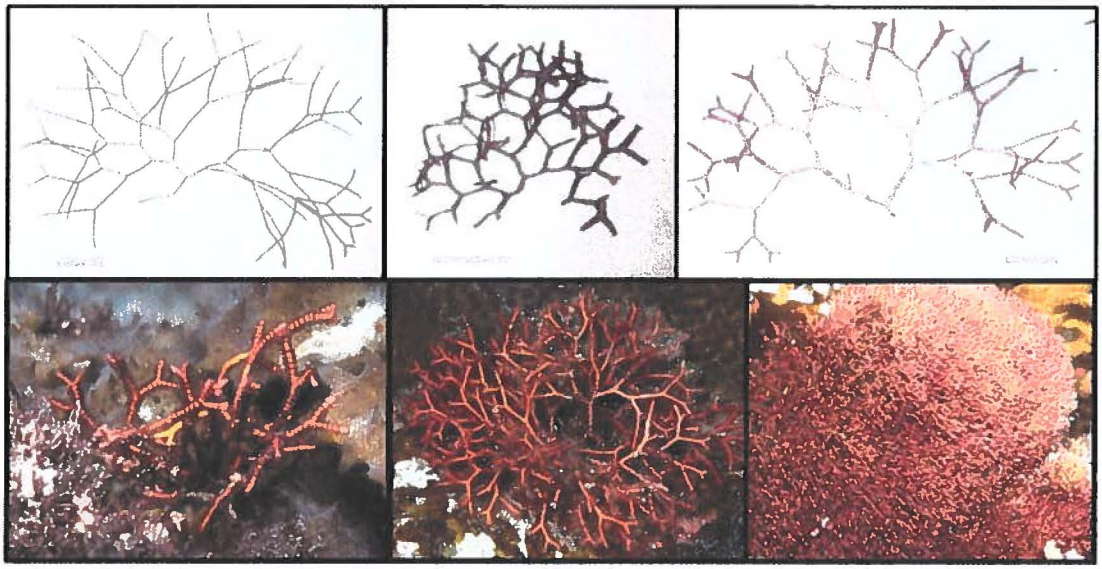
Actinotrichia is a member of the family Galaxauraceae, which currently contains four genera: *Actinotrichia* Decaisne, *Dichotomaria* Lamarck, *Galaxaura* Lamouroux, and *Tricleocarpa* Huisman et Borowitzka (Huisman et al. 2004; Wang et al. 2005; Huisman 2006), all of which are widely distributed throughout the world's tropical waters (Papenfuss et al. 1982; Liu & Wang 2009). This family has been the focus of considerable

research in the past decade including a systematic revision completed in 2005 by Wang and colleagues, as well as an order-level phylogenetic appraisal in 2004 (Huisman et al. 2004).

Unlike some red algae, members of the Galaxauraceae are relatively large in size, easily recognized in the field, and generally abundant on tropical reef ecosystems in a variety of habitats, making them ideal for collection and study. Specimens of the genus *Actinotrichia* are easily recognized by their bright orange to red thallus, which is thin, calcified, and dichotomously branching with small assimilatory filaments forming whorls on the branches (Plate 1). The genus is distinct from others in the Galaxauraceae because of its isomorphic life history, assimilatory filaments found in whorls on the thallus surface, and only two sterile branches issuing from the hypogynous cell. (Liu and Wang 2009).

The genus *Actinotrichia* was originally established by Decaisne (1842) based on the species *Galaxaura rigida* Lamouroux (now *A. fragilis*) and currently contains four species worldwide: *A. calcea* Pham-Hoàng Hô, *A. fragilis* (Forsskål) Børgesen , *A. robusta* Itono, and *A. taiwanica* Liu & Wang (Guiry & Guiry 2012). *A. fragilis* was originally described from Yemen and has the broadest distribution range of all *Actinotrichia* species, while the remaining species are reported to be more restricted in their distributions. This observation is probably compromised because few studies have looked at *Actinotrichia* distributions and some of the species have only recently been described. Of these species, only *A. fragilis* is currently reported for Micronesia (Lobban & Tsuda 2003). Specimens of the type species *A. fragilis* from Taiwan were recently investigated by Liu and Wang (2009), who noted two more species in specimens previously identified as *A. fragilis*, i.e. *A. robusta* and *A. taiwanica*. These species distinctions were independently assessed and

Figure 1: Photos displaying morphological diversity in *Actinotrichia* specimens. Scale bar on herbarium specimens equals 1 cm.



confirmed by Wiriyadamrikul et al. (2013) for *Actinotrichia* individuals from other locations in the Indian and western Pacific Oceans. The same study also concluded that *A. robusta* is more widely distributed than previously known.

While *A. fragilis, A. robusta,* and *A. taiwanica* have thorough descriptions, *A. calcea* arguably does not. Its original, and only, description from Vietnam provides insufficient detail about how the species differs from others in the genus and no known material from the type specimen is available for further morphological or genetic analysis. Furthermore, no type material of *A. calcea* has been directly compared against the other *Actinotrichia* species to date (and none was obtained for this study), so its taxonomic standing within the genus remains in question.

Although the species of *A. fragilis, A. robusta, and A. taiwanica* are well-described, distinguishing and identifying specimens using only their morphological features is difficult. Some features are obviously unique to a certain species; for instance, the thallus

width and arrangement of assimilatory filaments of *A. taiwanica* are reported to be notably different than those of the other species. However, most published measurements of the diagnostic characteristics of *Actinotrichia* species overlap substantially between species, especially between *A. fragilis* and *A. robusta*. For example, the range of all metrics and anecdotal observations that supposedly distinguish the species *A. fragilis* and *A. robusta* overlap substantially and – except in the most extreme of cases – do not provide enough morphological difference to identify a specimen.

Given that morphology alone is not enough to positively identify many *Actinotrichia* specimens, this study approaches the issue of species identification and delineation in several ways. First, I examined phylogenetic relationships between previously-published *Actinotrichia* sequences and our own using the *rbc*L marker singularly, and also concatenated with the *cox1* marker. Next, using primarily the *cox1* marker, I employed three species delimitation methods: automatic barcode gap analysis (ABGD, Puillandre et al. 2011) to estimate which clades may constitute individual species, followed by Bayesian Poisson tree process (bPTP, Zhang et al. 2013), and Bayesian Phylogenetics and Phylogeography (BPP, Yang & Rannala 2013) to verify these choices. Morphological characteristics of newly recognized phylogenetic species are then compared to their groups in a search for diagnostic features.

Methods and Study Design

The main challenge of this project is to determine which criteria and barcoding gaps are most helpful in identifying new species of *Actinotrichia*. This topic has been debated extensively for years (Agapow et al. 2004) and still does not have a universal solution. Effective barcode gaps vary across taxa (Knox et al. 2012), and in some cases, no true

"gap" exists (Meyer & Paulay 2005). However, supplementing genetic data with morphological, phylogenetic, and geographical data has shown to be a successful way to define species in several studies focusing on algae and other organisms (Zuccarello et al. 2002, Alström et al. 2008, Tronholm et al. 2010). This study describes the species diversity of *Actinotrichia* throughout tropical north Pacific islands, using these three types of data.

Actinotrichia samples were collected from islands in the tropical north Pacific. Sampling efforts occurred in Okinawa in June 2010, June 2011, and May 2012 at twelve different sites on the main island. Samples from Guam were collected at various times throughout 2011, 2012, and 2013. The diversity gradient from the western to the central Pacific is especially interesting because relatively little work has focused on cryptic diversity in algae along this dispersal-limited longitudinal gradient. To investigate this aspect of diversity, this study included *Actinotrichia* samples collected in July and August 2012 from the islands of Chuuk, Kosrae, and Pohnpei in the Federated States of Micronesia, and Oahu (Hawaiian Islands). Additionally, we traveled to the Majuro and Arno atolls in the Marshall Islands, but surprisingly found no *Actinotrichia* specimens despite records stating it is found there. Additional specimens from the University of Guam's Herbarium collection were included along with samples contributed by an international group of collaborators. Most specimens were collected in waters down to 10 m depth, although some sites were explored to 30 m.

The question regarding how many specimens should be sampled per site has been addressed in a number of studies. In a barcoding marker test, Kress & Erikson (2009) deemed it necessary to only sample one specimen per species, while others make the case that sampling even five to ten specimens cannot uncover most genetic diversity (Zhang et

al. 2010). Because this study aimed to uncover cryptic species that maybe uncommon, it was necessary to examine and sequence as many specimens as possible from each sampled location. Based on this goal, the time required for sample processing, and the sometimes-low amplification and sequencing success rates, this study employed an opportunistic sampling approach where we collected as many samples from each site as was possible – up to as many as 20 samples per site. In areas where *Actinotrichia* was abundant, we selected specimens that were distant from each other and–when possible–appeared to be morphologically distinct.

Each specimen was photographed either *in situ* or after collection before processing and placed in an individual plastic bag with ample seawater to prevent spoiling and contamination. An epiphyte-free portion of each specimen was cleaned, rinsed in fresh water, wrapped in sterile tissue paper, and dried in silica gel for later DNA sequencing. Most of each remaining thallus was subsequently stored in a solution of 5% formaldehyde to seawater (v/v) as a voucher specimen. Exceptional or unique specimens were also pressed and dried on herbarium paper and deposited in the University of Guam Herbarium (GUAM).

Molecular Methods

While the mitochondrial *cox1* is the official primary barcode for red algae, the chloroplast marker *rbc*L has been used extensively in past phylogenetic studies of red algae (Freshwater et al. 1994) and has also successfully identified and delimited species in the Galaxauraceae and other red algae taxa (Freshwater et al. 1994; Gurgel et al. 2003; Wang et al. 2005; Liu & Wang 2009; Wiriyadamrikul et al. 2013). Furthermore, not all *Actinotrichia* taxa have published sequences from both markers – for instance, *A. taiwanica*

is only described using *rbcL* sequences. By primarily using *cox1* for species delimitation, and then *rbc*L for species identity verification of sequences for which no *cox1* sequences exist, this study compares phylogenies of all currently-sequenced species with other previously published sequences.

cox1 Extraction and Sequencing

DNA sequencing of red algae has been a focus of considerable research in the past decade and machine-based DNA extraction and sequencing techniques are faster and more economical than manual extraction for large quantities of samples. For this reason, most specimens' DNA was extracted through a machine-based technique at the lab of Dr. Gary Saunders at the University of New Brunswick. For extraction, approximately 5 mm^2 of each dried sample was loaded into one of the 1.1 mL mini tubes of the 96-well plate of the PROgene Mini Tube System (UltiDent Scientific, St. Laurent, QC), covered, and shipped in a sealed bag containing silica gel to prevent any moisture from degrading the tissue. Samples were ground using the Tissuelyser II (Qiagen Inc., Valencia, CA) and DNA extraction was completed using the Qiaxtractor DNA purification robot (Quigen Inc., Valencia, CA) with the protocol described in Saunders & McDevit (2012). All extracted DNA that was not used for sequencing at the University of New Brunswick was lyophilized and returned to the University of Guam for future study.

For the *cox1* marker, polymerase chain reaction (PCR) DNA amplification was performed using a portion of the extracted DNA using the markers described in Saunders & McDevit (2012) using Ex Taq DNA polymerase. Sequencing was completed using the AB I Big Dye (Foster City, CA) kit following the manufacturer's protocol. Trace files were

uploaded to the Barcode of Life Data Systems webpage (BOLD; www.boldsystems.org), where they were accessed and downloaded for this study.

*rbc*L Extraction and Sequencing

Many of the resulting *cox1* sequences were identical for specimens sampled from nearby locations. As such, one specimen of each *cox1* haplotype was chosen for *rbc*L sequencing, with preference given to gametophytes and geographically distinct specimens. DNA was extracted from those chosen specimens with the DNeasy Plant Mini Kit (Qiagen). The DNA was amplified using the end primers F57 (5'-GTAATTCCATATGCTAAAATGG–3') and rbcLrevNEW (5'-ACATTTGCTGTTGG AGTYTC-3') and two new internal primers specifically designed for *Galaxauraceae* specimens based on previously-published sequences and the TLF and TLR primer series described in Saunders and Moore (2013). The new forward and reverse primers, respectively, are TLFa (5'-TCYCARCCWTTTATGCGYTG-3') and TLRb (5'-AAYTCAGCTCTYTCATAC AT-3').

The PCR reaction mix contained 0.5 µL of each respective forward and reverse primer, 10.5 µL of nuclease-free water, and 12.5 µL of AmpliTAQ® Gold 360 Master Mix (Thermo Fisher Scientific, Inc.) for each sample. The PCR amplification profile was modified from that described in Saunders & Moore (2013): 95 °C for 2 minutes; 40 cycles of 93 °C for 1 minute, varying annealing temperatures for 30 seconds (47 °C for primers F57 and TLRb, 51 °C for primers rbcLrevNEW and TLFa), 72 °C extension for 55 seconds; followed by a final extension at 72 °C for two minutes.

To determine if the specimens' DNA were successfully amplified, 3 µL of each resulting PCR product was mixed with 2 µL of loading dye and run on a 1.5% agarose

electrophoresis gel along with a 1000 base pair DNA ladder for fragment length comparison. Successfully amplified templates were purified using either the Qiagen QIAquick PCR Purification Kit's standard procedure or ethanol precipitation.

Purified PCR products were prepared for sequencing using a sequence reaction using the Thermo Sequenase Cycle Sequencing Kit (Affymetrix, Santa Clara, California) and fluorescently labeled primers synthesized with 5' IRDye® modifications: forward primers (TLFa, F57) with IRDye® 700 and reverse primers (TLRb, rbcLrevNEW) with IRDye® 800. The sequence reaction profile is as follows: 92 °C for 2 minutes followed by 35 cycles of 92 °C for 30 seconds, 52 °C annealing for 1 minute, 70 °C extension for 1 minute. Afterwards, 3 µL of stop solution was added to each product, denatured at 95 °C for two minutes, and immediately stored at -20 °C.

Sequencing was performed on a LI-COR 4300 DNA Analysis System using a 40 cm gel made with a premixed 4.5% acrylamide gel matrix and a 48-well sharks tooth comb. The gel underwent a 30-minute pre-run to calibrate the machine before 0.4 µL of each product was loaded by hand into the wells. The sequencing process ran for 9 hours.

Sequence Editing

Raw image files obtained from the LI-COR system were automatically sequenced using the LI-COR e-Seq software (version 3.1). The resulting forward and reverse sequence trace files were aligned, assembled, checked for congruency, and edited using LI-COR AlignIR software (version 2.1). The final consensus sequences were exported to Geneious (version 5.5) for all subsequent editing and organization. New consensus sequences - as well as those obtained from the University of New Brunswick - were aligned with and

checked against verified reference sequences for gaps and insertions. Before further analysis, all sequences were screened through the BOLD website; this ensured that all sequences considered for analysis were actually from *Actinotrichia* specimens and not from epiphytes or some other mislabeled specimen. Poor-quality sequences with multiple incongruences were not considered in the analysis.

Phylogenetic Analyses

Alignment Construction

Five sequence alignments were created for this study; all sequences were aligned and trimmed in Geneious and include *Galaxaura* and *Dichotomaria* sequences as outgroups. Alignments were created with all sequences available for each of the *cox1* and *rbc*L markers, and then also alignments with identical sequences removed. Gametophytic specimens and those from unique geographical areas were prioritized when selecting sequences for the *rbc*L alignment.

Finally, one alignment was created using concatenated sequences of both genes. Only specimens for which both *cox1* and *rbc*L sequences were available were used in this alignment. Some duplicates were encountered in this alignment and were removed.

Model Selection

All single-gene alignments were evaluated for the best evolutionary model based on likelihood (-lnL) scores in jModelTest (version 2.1.2) using the Akaiki Information Criterion (AIC). The program does not currently support partitioned analysis for multi-gene alignments. For the *cox1* and *rbc*L alignments, the general time-reversal model including invariable sites and the gamma distribution parameter (GTR+I+G) was chosen. Although the likelihood value for this model was not the best for the *rbc*L alignment, its likelihood

scores were very close to the first choice and was chosen for ease of analysis; many online "black box" phylogenetic programs include GTR in their analysis options, but the same is not the case for other models. Using the GTR+I+G model, jModelTest calculated the model parameters for each marker as shown in Table 1.

Table 1: GTR+I+G model parameters as calculated using the Akakiki Information Criterion (AIC) in jModelTest. Values fA, fC, fG, and fT represent the proportions of each nucleotide base; "p-inv" is the proportion of invariable sites found in each alignment.

Marker	Likelihood	fA	fC	fG	fT	p-inv	shape
cox1	2913.86	0.3271	0.1070	0.1388	0.4271	0.6110	0.9940
***rbc*L**	3337.15	0.3209	0.1527	0.2192	0.3071	0.6900	0.9200

Tree Construction

For each of the five alignments, both a maximum likelihood (ML) tree and a Bayesian tree were constructed. Bayesian phylogenetic trees and support values were constructed for all alignments in MrBayes (version 3.2.6 via CIPRES; www.phylo.org) using the parameters in Table 1. Each alignment's set of trees was constructed through 5,000,000 generations (sampling every 1,000), two independent runs, six chains (five hot, one cold), and with a 25% burn-in. Both the *cox1* and *rbc*L Bayesian analyses finished with the average standard deviation of split frequencies < 0.01 – a strong indication of convergence.

Maximum likelihood trees and bootstrap values were computed using RAxML version 8.2 on the CIPRES server. Each analysis completed 1,000 bootstrap replications.

Species Delimitation

The process of delimiting species using phylogenetic data is a much-debated subject, with most authors agreeing that an integrative approach using multiple delimitation methods is most likely to accurately determine species. This study used three methods to

delimit new *Actinotrichia* species. First, I used the genetic distance-based model "Automatic Barcode Gap Discovery" (ABGD) to determine an estimated threshold for the barcode gap between species. This rough estimate was used to help guide the subsequent delimitation methods. Second, the Bayesian Poisson Tree Process model (bPTP) was used to identify potential distinct species. Finally, the Bayesian Phylogenetics and Phylogeography (BPP; Yang & Rannala, 2010) method was used to test if these hypothesized species distinctions are statistically supported. Once these potential phylogenetic species were identified, their morphological measurements and biogeographical data were examined to support final species distinctions.

Automatic Barcode Gap Detection (ABGD)

For this analysis, only the alignments containing unique sequences for each of the *cox1* and *rbc*L data sets were used; outgroups were removed from each alignment. Each analysis was run on the ABGD online server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) using the Jukes-Cantor (JC69) model (no GTR model is available for this program's analysis) with the set parameters noted in Table 2.

Table 2: Parameters used for the ABGD analysis

Marker	Pmin	Pmax	Steps	X	Nbins
cox1	0.005	0.1	21	1	20
*rbc*L	0.005	0.1	100	.8	20

Bayesian Poisson tree process (bPTP)

Each ML tree was tested for species through the online Bayesian Poisson tree process (bPTP; http://species.h-its.org) using 250,000 generations, a thinning parameter of 100, a burn-in of 0.25, and with the "remove outgroups" option selected.

Bayesian Phylogenetics and Phylogeography (BPP)

This analysis was conducted using the program BPP (Rannala and Yang, 2003; Yang and Rannala, 2010) and the *cox1* haplotype alignment. This method accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. A gamma prior G (2, 1000), with mean 2/2000 = 0.001, was used for the population size parameters. The age of the root in the species tree was assigned the gamma prior G (2, 1000), while the other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala, 2010: equation 2). The analysis for the alignment was run twice to confirm consistency between runs.

This analysis relies heavily on a user-specified "guide tree" on which the user assigns sequences to a certain species, creates a simplified input "tree" using those species, and then tests the validity of those selections. Due to the congruency and consistency of the *rbc*L and concatenated trees' topologies, that was the topology used for the BPP guide tree.

Morphological Analysis

Standard morphological measurements reported in previous *Actinotrichia* publications were measured in this study for analysis and comparison. Specimen height and the distances between branch dichotomies were measured for each voucher specimen using a standard metric ruler. Overall height was measured from the base of the holdfast to the furthest point perpendicular to the attachment substrate. If the holdfast was broken off and not retrieved during collection, overall height was not measured and the dichotomy distance was measured starting after the first branching dichotomy. Thallus width, distances

between assimilatory filament whorls, and branching angle were measured using microscopy. Because branching angles can appear to change depending on how a specimen is stored and handled, only distal branching angles were considered for this analysis since itis less likely they were bent out of their original shape. Finally, reproductive structures and the internal cortical features were identified and examined under a microscope after a portion of the thallus was sectioned by hand with a razor blade, stained with 1% aqueous aniline blue, and decalcified in 1% HCl solution. All measurements and observations were compared against available literature.

Basic summary statistics were calculated for all morphological data. Additionally, a linear discriminant analysis (LDA) was completed in R using the "lda" function in the "MASS" package. For the LDA, the lengths between branching dichotomies were averaged for each specimen instead of analyzed individually. The analysis was run first for all specimens collected by this study, and then only those that ended up in the "*A. robusta*" clade.

Biogeographical Analysis

This study examines the geographical patterns of the data through two methods. First, I employed the weighted UniFrac distance metric (Lozupone & Knight 2005) on the new haplotypes collected from this study using the "phyloseq" program in the "vegan" package in R. This package compares phylogenetic and geographic data along with preliminary species distinctions to identify which localities resemble each other in species and phylogenetic composition. In this study, I used the program to help identify which island's assemblages of *Actinotrichia* specimens were most similar.

Results

Phylogenetic Analyses

The *cox*1 and *rbc*L sequences use in this analysis were 601 base pairs (bp) long and contain representatives from the previously-described species *A. fragilis,* the "*A. robusta*" specimen identified as "*A. robusta*" by Liu & Wang (2009), and an unidentified specimen from Thailand simply named *Actinotrichia sp.* (Wiriyadamrikul et al. 2013). To date, no published *A. taiwanica cox1* sequences exist and no material of *A. calcea* is available for sequencing or examination. In this study, 148 *cox*1 sequences were generated from Okinawa (24), Guam (24), Chuuk (15), Pohnpei (43), Kosrae (26), and Hawaii (16). In these new sequences, 34 unique haplotypes were discovered. (Table 3)

The final *rbc*L sequences were 1,308 bp long representing *A. fragilis, A. robusta, A. taiwanica,* and the same unconfirmed specimen from Taiwan denoted as *Actinotrichia sp.* Twenty-three new *rbc*L sequences were generated from Okinawa (3), Guam (5), Chuuk (4), Pohnpei (7), Kosrae (1), and Hawaii (3), containing 14 unique haplotypes. (Table 3)

Overall, the topologies indicate four moderately to highly-supported main clades containing: (1) the single *A. taiwanica* specimen, (2) the specimens that cluster with the previously-reported *A. robusta* in Liu & Wang (2009) and Wiriyadamrikul et al. (2013), (3) the specimen named *A. sp.* by Wiriyadamrikul et al. (2013; accession number CNU21550), and (4) all other sequences including those that have been traditionally

Table 3: Final sequence alignment lengths and composition

Marker	Purpose	Length	Description	*n* Sequences
***rbc*L**	Phylogenetic placement	1,308 bp	All Available Sequences	43
			New (This Study)	23
			All Unique Haplotypes	30
cox1	Species delimitation	601 bp	All Available Sequences	172
			New (This Study)	148
			All Unique Haplotypes	51
Concatenated	Phylogenetic placement	1,909 bp	All Available, Unique Sequences	70
			Only Complete Sequences	33

identified as *A. fragilis*. All new specimens acquired from our sampling on Okinawa and Hawaii clustered in the *A. fragilis* clade as did 21 of the 24 new specimens from Guam. Of the 83 obtained from Micronesia, only 11 clustered in the *A. fragilis* clade (Figure 2).

The tree topologies using the concatenated and the *rbcL*-only alignments were consistent among the ML and Bayesian tree-building techniques with only a few exceptions in the *A. fragilis* species complex, but nothing notable given this study is focusing on only the clade containing the supposed "*A. robusta*" specimen published by Liu & Wang (2009). Additionally, within the clade containing the traditionally-named *A. fragilis* specimens there was some shuffling of sequences between tree building methods, but nothing radical enough to affect the outcome of the study. The tree topologies using only the *cox*1 sequences, however, were considerably different between Bayesian and ML methods and even between different analysis programs; given the higher evolution rate of cox1 versus *rbc*L, this was to be expected. While sequences from the same geographic regions did generally group together, the exact locations of these clades on the tree varied

between runs and had low support values. Because the concatenated and *rbc*L-only sequence topologies were consistent with each other and with those found in previous studies, and have higher support values, they were regarded as the most correct and were used as the reference phylogram for the rest of the study.

Save for the "*A. robusta*" specimens named in Liu & Wang (2009) and Wiriyadamrikul et al. (2013), all other sequences in this clade were new from Micronesia and each smaller clade within it is highly supported in both the rbcL (ML & Bayesian methods) and concatenated alignments with only one exception (Sp. B has low support in the Bayesian analysis. Nearly the opposite was true of the "A. fragilis" clade: the topology in this clade is not consistent across tree-building methods and while many of this study's new sequences cluster together, the branch support values are low, indicating relationships with previously-recorded sequences are not clear. Additionally, while general morphometric measurements are published for each currently-described species of Actinotrichia, specimens that do not belong to the University of Guam Herbarium were not morphologically examined and compared with the newly collected specimens of this study. Without further sampling and study, these issues make new species identifications within the "A. fragilis" clade difficult and potentially unreliable – especially since it may contain a species complex (Wiriyadamrikul et al. 2013). For these reasons, the species delimitations from this study focus only on the clade containing most of the Micronesian specimens (including Sp. A, Sp. B, Sp. C, and the "A. robusta" specimens from Liu & Wang (2009) and Wiriyadamrikul et al. (2013))

Figure 2: Tree depicting phylogeny of *Actinotrihia* sequences. The three node support values are (1) *cox1* and *rbc*L concatenated maximum likelihood bootstrap values, (2) *rbc*L maximum likelihood bootstrap values, and (3) *rbc*L Bayesian analysis posteriors. The tree topologies for each of these analyses varied only within the *A. fragilis* species complex. Species determined by species delimitation programs are shown in different colors. Specimen names reflect taxonomic recommendations prior to this study and field observations. Abbreviations are Chuuk (CH), Guam (GU), Hawaii (HI), Indonesia (IN), Korea (KR), Kosrae (KO), Okinawa (OK), Philippines (PH), Pohnpei (PO), and Thailand (TH).

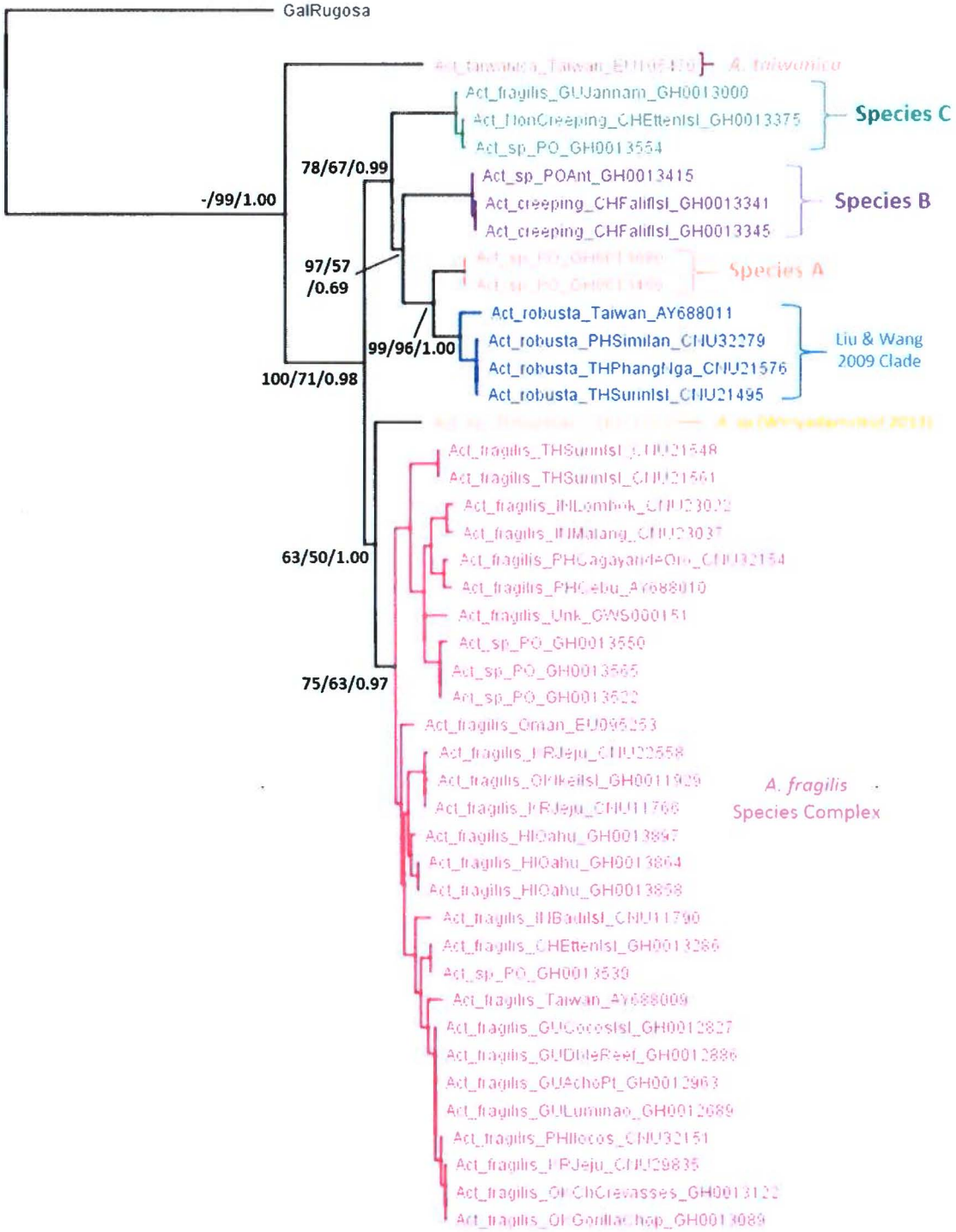
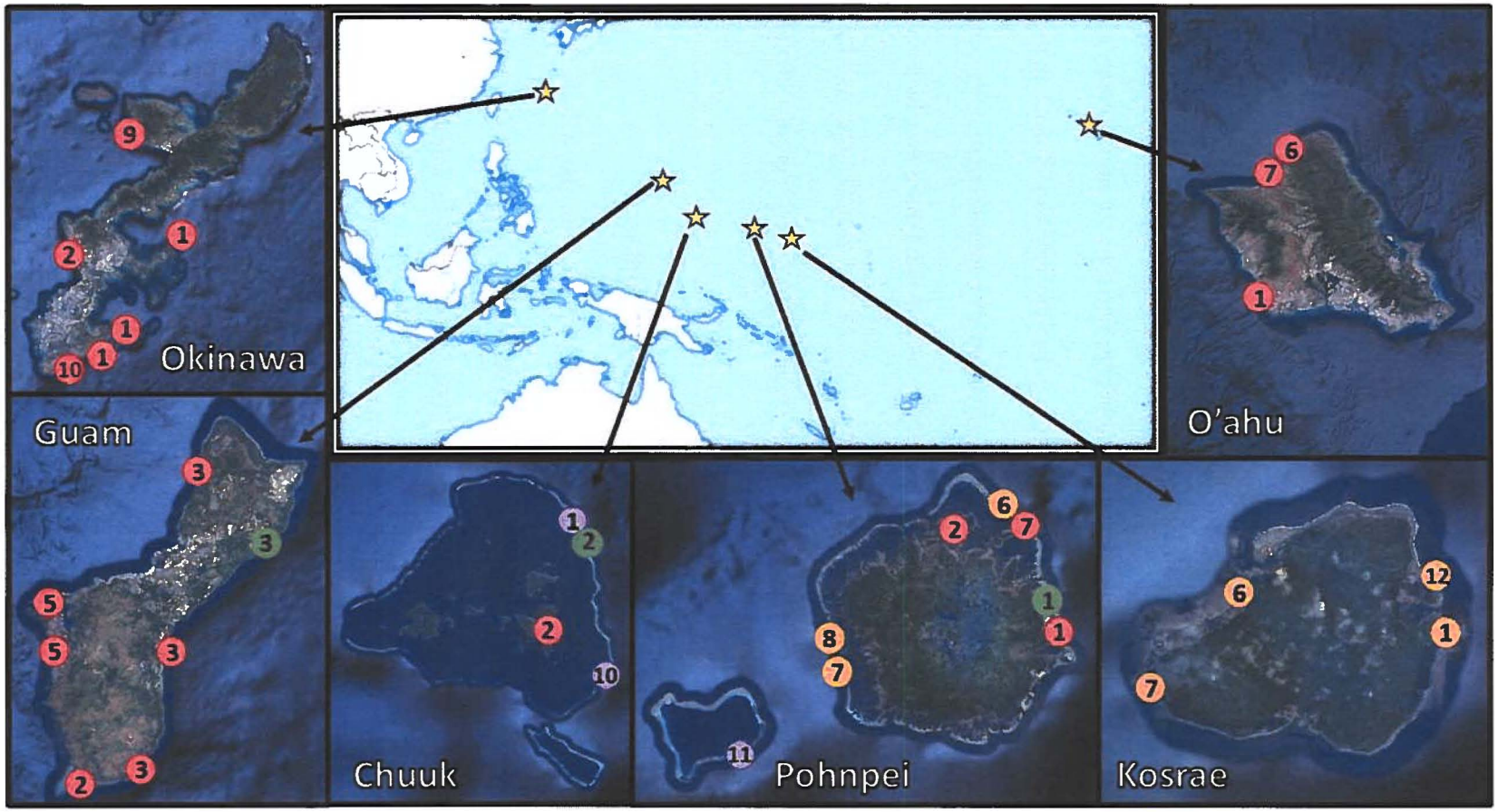


Figure 3: Map depicting the number of sequences obtained per sampling location and which clades they cluster in.



and will from here on be referred to as the Micronesian clade, leaving taxonomic clarifications within the *A. fragilis* species complex to be resolved at a later date.

Species Delimitation

Automatic Barcode Gap Detection (ABGD) Results

The ABGD analysis of *cox1* alignment suggested a barcode gap of around 6.5% with 9 distinct groups identified using that gap. The *rbc*L alignment analysis confirmed the sole *A. taiwanica* species is a separate group than any other specimens. In the *rbc*L analysis, all specimens that group in the *A. fragilis* species complex were considered one species. Different interpretations of the *cox*1 alignment indicated there may be anywhere from 9 to 19 distinct groups within the phylogeny. The extra 10 groups suggested by the larger estimate were found entirely within the *A. fragilis* complex; the groups identified among the Micronesian clades did not differ.

Bayesian Poisson Tree Process (bPTP) Results

Results from the bPTP were similar to those from the ABGD analyses, but suggested more groups as potential species. The number of species recommended by this algorithm for both the *cox1* maximum likelihood and Bayesian trees were 21 (the *rbc*L analyses supported the *A. taiwanica* specimen as its own species). Like with the ABGD analyses, most of the extra groups were found in the *A. fragilis* complex, although some in the Micronesian clade were split further, all with strong support values. As with ABGD, the bPTP analyses suggest the specimen simply named *Actinotrichia* sp. (Wiriyadamrikul et al. 2013; accession number CNU21550) should also be considered a unique species.

Bayesian Phylogenetics and Phylogeography (BPP) Results

The results from the ABGD and bPTP analyses suggest anywhere from nine to 21 species within the sequences used for this study. Most of those "extra" groups were within the *A. fragilis* species complex; since this study focuses only on the Micronesian clade, the discordance in that species complex is not investigated further. However, a few splits occurred in this Micronesian clade in the bPTP analysis. In deciding which to identify as preliminary species in BPP, I ultimately decided to choose conservatively as is prudent when delimiting species (Carstens et al. 2013). Given all this, six groups were tested for species validity using the *cox1* alignment in BPP, preliminarily identified as Sp. A, Sp. B, Sp. C, and the full *A. fragilis* clade; the fifth and sixth groups are the previously published "*A. robusta*" specimens and *A. sp.* (CNU21550) sequences. *Actinotrichia taiwanica* was not tested because no *cox1* sequence is available for it.

All BPP runs (both the *rbc*L and concatenated analyses) supported these distinctions with posteriors greater than 0.95 (with the exception of Sp. A which had a support value of 0.89

Morphological Results

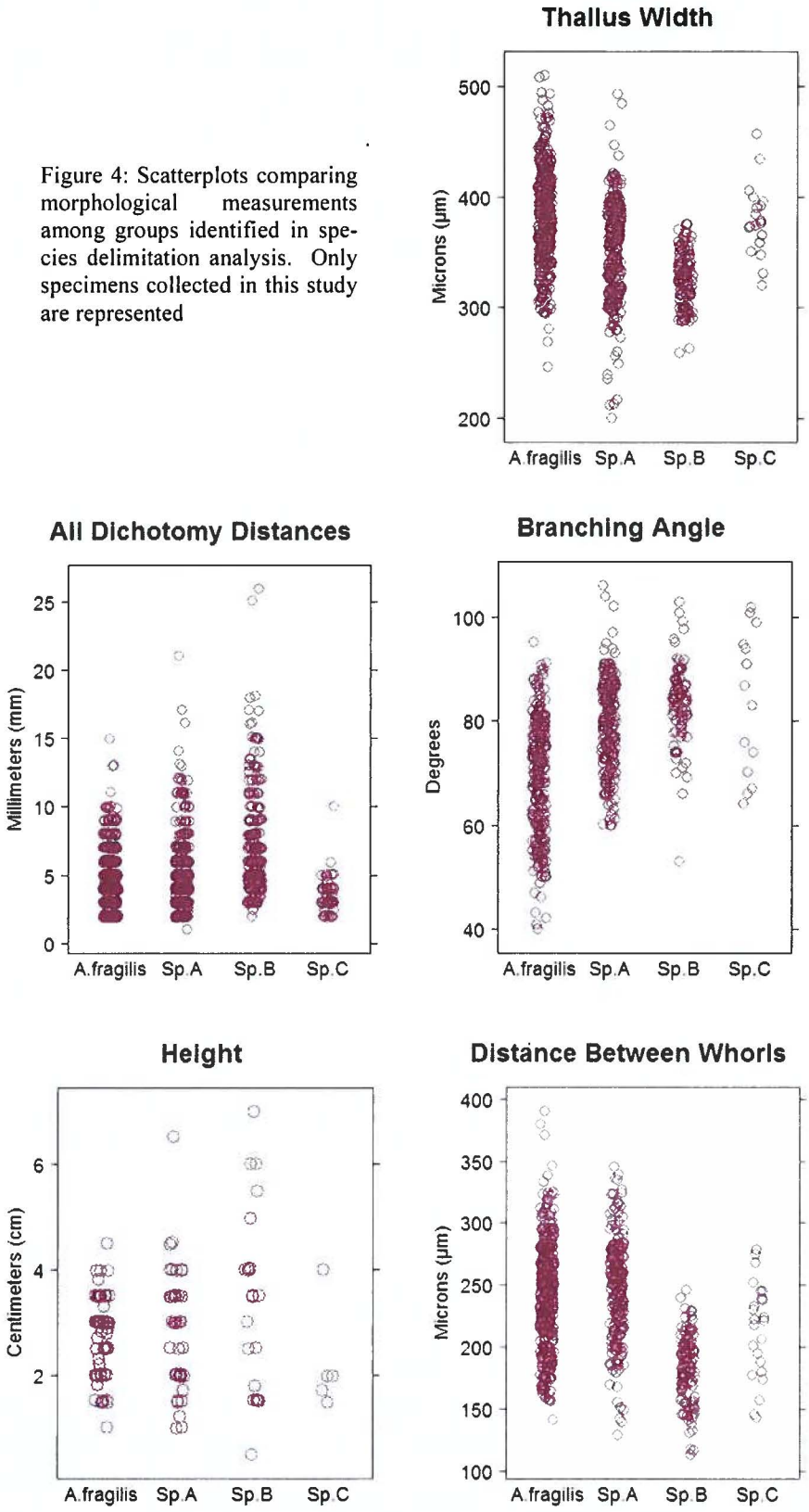
All specimens collected for this study were examined for morphological characters. Of the 148 specimens collected for this study, 71 were tetrasporophytes, 11 were male gametophytes, 3 were female gametophytes, and the remainder were not visibly fertile. Overall growth morphologies ranged from small, compact "lattice-like" forms to long and spindly.

Using the same 6 groups suggested in the species delimitation analyses, the morphological data were analyzed by group to determine if any of those groups had unique measurements. Four of those groups contain specimens collected in this study.

The results of the descriptive statistics are shown in Figure 4. In general, the ranges of each morphological trait overlapped considerably across all four groups with no clear differences between them. However, a few interesting observations are worth noting. First, the distance between the branching dichotomies in Sp. B appear to be generally larger than the other groups (except in the 6th dichotomy, the sixth most distant dichotomy from the holdfast). Also, the specimens that cluster with the group considered to be *A. fragilis* have a much wider range of branching angles compared to the other groups.

Another interesting observation, although not quantified, was found in specimens from several clades. In many "advanced" tetrasporophyte specimens (i.e. those with many tetraspores), the assimilatory filament whorls were indistinguishable in some portions of the thallus because many filaments bearing tetraspores grew out of the thallus in patches, rather than whorls. These short, dense tetraspore patches in some places appeared to break through the cortex and grow directly out of the medulla, rather than the cortical cells (as is the case with the standard assimilatory filaments). Advanced gametophytes do not exhibit this same level of cortex-breakdown. This observation has not been noted in any previous study and brings into question the validity of irregular whorls as a defining characteristic of *A. robusta* and *A. taiwanica.* Because this observation occurred in multiple specimens in several clades (including those in the *A. fragilis* complex, Sp. A, and , it may simply be

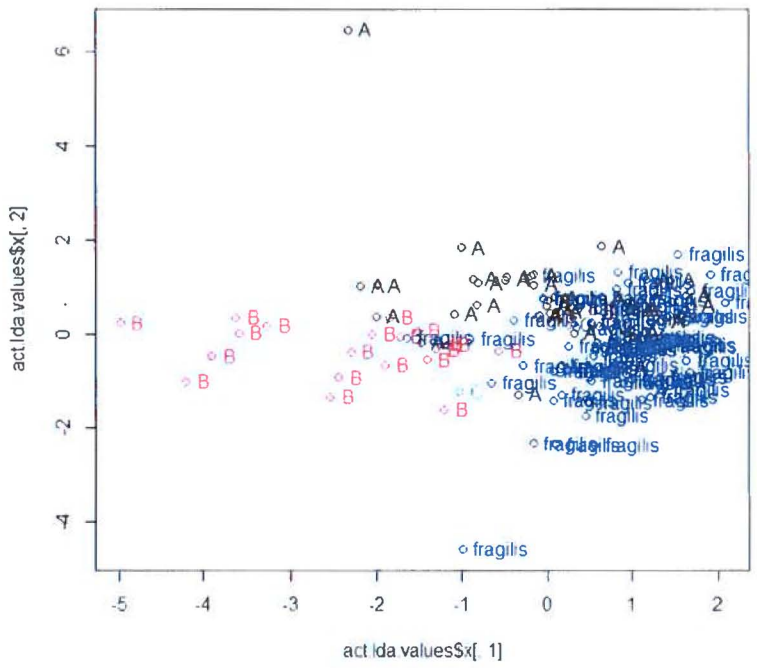
Figure 4: Scatterplots comparing morphological measurements among groups identified in species delimitation analysis. Only specimens collected in this study are represented



a result of advanced tetraspore production in *Actinotrichia*. Further studies into tetrasporophyte development are required to clarify this.

The results of the first linear discriminant analysis (Figure 5) performed on all samples seem to support the morphological results mentioned above. Overall, there is considerable overlap between the species considered, except for Sp. B that was more separated from the other three species. The "*A. fragilis*" group displays the broadest variability in character of all species considered. Additionally, the analysis suggests that the length between branching dichotomies is the most important factor when distinguishing between

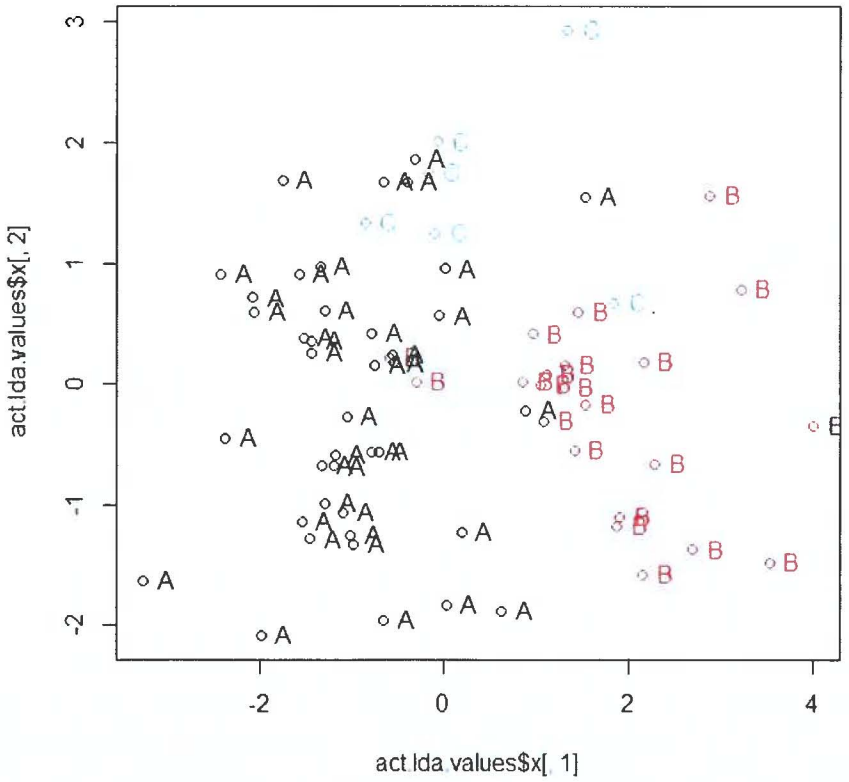
Figure 5: LDA analysis of morphological characters comparing measurements from Species A, Species B, Species C, and the *A. fragilis* species complex.



groups with a linear discriminant coefficient of 0.740, with height being the next most important (-0.55). The trace proportion indicates that these combined measurements account for 74.0% of the species variance.

Because this study does not address the potential cryptic diversity in the "*A. fragilis*" clade and the wide range of morphological values shown for this group from this study could be from multiple cryptic species, the next LDA was completed excluding this group (Figure 6). With this analysis, the differences between the other three potential are more defined with the data explaining 84.3% of the variance. As with the previous LDA, the length between branch dichotomies is the most influential factor with a linear discriminant coefficient of -0.379, with height coming in second (0.227).

Figure 6: Linear discriminant analysis of the three groups in the Micronesian clade sampled in this study.



Biogeographical Results

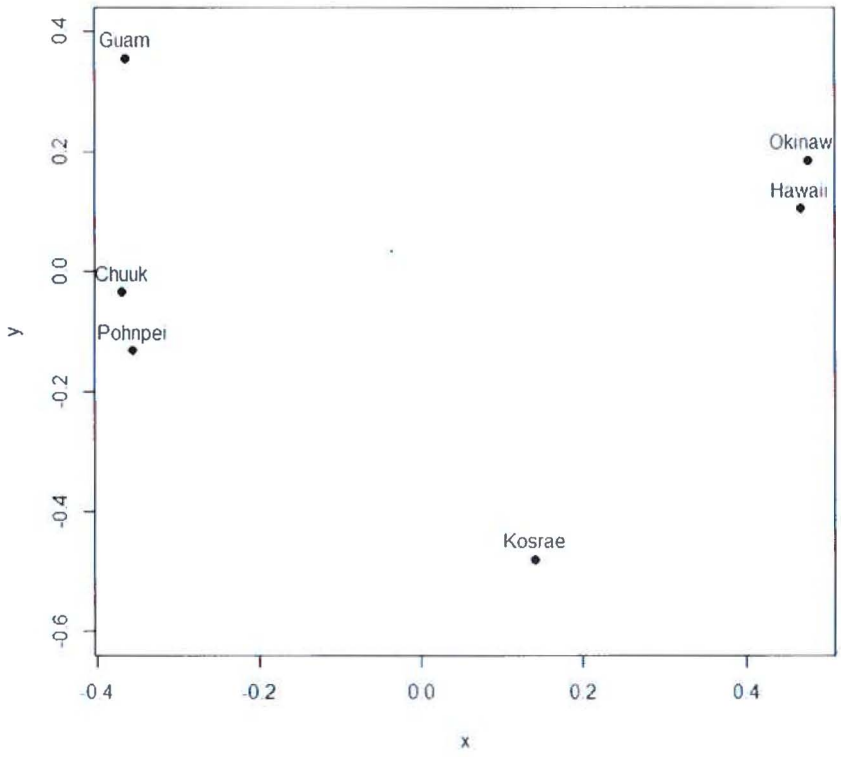
The unifrac MDS analysis confirmed the similarity between several island's *Actinotrichia* flora (Figure 7). Okinawa and Hawaii, which have the highest proportion of "*A. fragilis*" specimens, grouped closely together. Chuuk and Pohnpei, the only locations where Sp. B is found, also cluster closely together. Not surprisingly, Kosrae is the most dissimilar of all the other islands, having only one species (Sp. B) shared with Pohnpei.

Discussion

Existing *Actinotrichia* Species Descriptions

For nearly 75 years after its initial introduction as *Fucus fragilis* by Forskål in 1775, the genus we now call *Actinotrichia* was described simply by the dichotomous branching

Figure 7: Unifrac MDS plot of genetic similarity between *Actinotrichia* specimens collected in this study.



of a calcified thallus with small whorls of colored bristly hairs. This general description was gradually expanded upon by various authors (Table 4) to include measurements defining maximum height (Decaisne 1842), branch width (Okamura 1916), and even rudimentary depictions of reproductive structures (Weber-Van Bosse 1928, Svedelius 1952). As more species (*A. calcea, A. robusta,* and *A. taiwanica*) were described within the genus, more morphological characteristics were considered as diagnostic features. However, a thorough review of literature describing *Actinotrichia* species morphology reveals that many of these previously reported species descriptions are incomplete and conflicting – calling into question the validity of the four currently-accepted species.

This problem is well-illustrated by the description of *A. calcea.* By the time Phạm Hoàng Hộ described this new species from Vietnam in 1978, the measurements attributed to *A. fragilis* had expanded considerably: its reported height and branch width ranges were then 1 – 10 cm and 300 – 1000 μm, respectively. The published description of *A. calcea,* however, was so rudimentary that it is unclear what distinguished it enough to warrant being named a new species: the only morphological values listed fall entirely within the range of *A. fragilis* (Table 4). Additionally, the herbarium code of the voucher specimen in the publication is incorrect and there are no genetic sequences available from the only once recorded specimen. Given these issues, it is impossible to identify any current specimen as *A. calcea* without a fresh sample from the original locality in Vietnam; it may in fact not be a valid species at all, but future work should sort that out. Given the uncertain description of *A. calcea* and the taxon's type locality outside of my study area, I have chosen to proceed with the study as if *A. calcea* is not represented in our collection and give the species no further consideration.

	Height	Branch Width	Branching Angle	Dichotomy Length	Cortex Thickness	Distance Between Whorls	Assimilatory filaments	Paraphyses Intermixed with Gonimoblast Filaments	DNA	Sampled Localities	Source
Actinotrichia fragilis	1 – 3 cm	-	-	--	-	-	whorled	-	-	Mocha, Yemen	Decaisne 1842
	5 – 8 cm	800 – 1000 µm	-	-	-	-	whorled, deciduous leaving annular marks	-	-	Japan	Okamura 1916
	-	300 – 450 µm	-	-	-	-	whorled	-	-	Hawaii	Svedelius 1952
	< 10 cm	< 1000 µm	-	-	-	-	whorled; deciduous	-	-	Philippines	Cordero 1975
	4 – 6 cm	< 2000 µm*	-	-	-	-	whorled	-	-	Hawaii	Abbott 1999
	1.5 – 5 cm	-	-	-	-	-	whorled	No	-	Taiwan	Wang & Chiang 2001
	< 7 cm	250 – 600 µm	-	2 – 10 mm	-	150 - 200 µm	-	-	-	Australia	Huisman 2006
	< 10 cm	400 – 650 µm	40 - 60°	3 – 7 mm	50 – 85 µm	-	whorled	No	Yes	Taiwan	Liu & Wang 2009
	< 8 cm	400 – 600 µm	40 - 60°	3 – 6 mm	-	-	whorled	No	Yes	Philippines, Japan, Korea, Indonesia, Thailand	Wiriyadamrikul et al. 2013
Actinotrichia calcea	2 - 3 cm	400 µm	-	-	-	-	whorled	-	-	Vietnam	Pham-Hoàng Hô 1978
Actinotrichia robusta	< 7.5 cm	400 – 600 µm	"narrower than *A. fragilis*"	2 – 9 mm	75 – 90 µm	-	whorled or not present; deciduous; no annular marks	-	-	Japan, Marquesas Islands	Itono 1979
	< 8 cm	400 - 600 µm	20 – 40°	3 – 6 mm	65 - 100 µm	-	mostly whorled	Yes	Yes	Taiwan	Liu & Wang 2009
	< 8 cm	400 - 600 µm	30 – 70°	3 – 6 mm	-	-	"indistinct" whorled	Yes	Yes	Thailand	Wiriyadamrikul et al. 2013*
Actinotrichia taiwanica	< 7.5 cm	600 – 1000 µm	20 – 40°	4 – 7 mm	90 - 150 µm	-	not whorled; irregular	Yes	Yes	Taiwan	Liu & Wang 2009

Table 4: Published morphological descriptions of *Actinotrichia* species. (*The value of 2 mm for the thallus width described in Hawaii by Abbott (1999) is likely an error and is here considered as such; no reported *Actinotrichia* specimens have a thallus diameter more than half this value, including the specimens collected in Hawaii for this study.)

The next species description of *A. robusta* was published by Itono (1979); at the time, he was not aware of the *A. calcea* description). The distinction between *A. fragilis* and *A. robusta,* according to Itono, is four-fold. First, *A. fragilis* is more calcified and therefore more fragile than *A. robusta*. Second, *A. robusta* has "narrow" branching angles when compared to the "wide" branching angles of *A. fragilis.* Itono unfortunately offers no numerical data to support these two character states, making modern comparisons difficult by just using these qualitative descriptions. The third distinction mentioned in the paper is that the cortical cell lumens are circular, wheras in *A. fragilis* they are angular except for the cells the assimilatory filaments grow out of.. Finally, the fourth distinction – and the one most quoted by subsequent studies – is *A. robusta*'s assimilatory filaments are not always produced in regular, concentric whorls throughout the thallus. Although previous studies had described *A. fragilis* as having "deciduous" filaments that drop off in the older parts of the thallus (Okamura 1916; Cordero 1975), this was the first attempt to use them as a diagnostic feature. Indeed, Itono (1979) addresses this by saying that *A. fragilis* thallus axes have "Clear annular marks on the axes after the loss of the assimilatory filaments", whereas *A. robusta* specimens lack them. These are arguably the first species-specific diagnostic traits published about *Actinotrichia.*

The last species described to date was *A. taiwanica* (Liu & Wang 2009), which was the first *Actinotrichia* species described using both molecular and morphological systematics. Morphologically, *A. taiwanica* is noticeably distinct from the other species in that its branch diameter and cortex thickness are wider, and that the assimilatory filaments are almost non-existent on the thallus. This last descriptor makes it appear to be *Galaxaura* (another genus of the family Galaxauraceae), but Liu & Wang indicate that the female

reproductive structures in *A. taiwanica* are indeed consistent with those described in other *Actinotrichia* species (Wang & Chiang 2001). Because of its nearly filament-less morphology and consistently thick thallus, *A. taiwanica* is the only species in the genus that can be identified using gross morphology alone.

Individually, the descriptions of each species seem to be valid, but complications arise when cross-comparing them. Table 4 describes the published characteristics of each species and demonstrates the significant overlap in trait characteristics: height, thallus width, branching angle, and dichotomy lengths are – for the most part – indistinguishable between all four species. Further complicating the matter, the species description of the *Actinotrichia fragilis* type specimen collected from Yemen in 1776 provides no morphometric measurements of features, and none of the authors in subsequent publications report having examined the type specimen. Essentially, every observation attributed to supposed "*Actinotrichia fragilis*" specimens in subsequent morphological studies did so based only on the genus' gross morphology and as Itono (1979) described, this alone is not enough to identify them to species level. With the currently available morphological information, no "*A. fragilis*" identifications can be trusted other than the type.

Itono (1979) distinguished *A. robusta* based on the four observations mentioned above: that (1) filaments are deciduous on the older parts of the thallus and leave no annular marks (2) the thallus is less calcified and less brittle, (3) the branching angle is "less" than *A. fragilis,* and (4) the epidermal cells have round lumens rather than angled ones. The second and third distinctions are subjective and were only compared against *"A. fragilis"* specimens whose identity can't be currently verified, therefore rendering them useless.

However, although Okamura identified his *"A. fragilis"* specimens as also having deciduous filaments, the difference (as noted by Itono) is that Okamura's specimens had annular marks where the filaments had once been, whereas the newly-described *A. robusta* did not. This distinction, combined with the round lumen observation, are the two consistent observations that distinguish *A. robusta.*

The next publication to tackle taxonomy was written by Liu & Wang (2009), where they described *A. taiwanica.* This was the first study to include molecular data in *Actinotrichia* taxonomy. As mentioned, the morphology of *A. taiwanica* is different from any other *Actinotrichia*. Even *A. fragilis* specimens *sensu lato* with similarly wide thalli do not share the smooth branches of *A. taiwanica* with few or no filaments that never occur in whorls. Because of the species' unique morphology, the sequence for *A. taiwanica* in this publication is reliable.Liu & Wang (2009) also published three sequences for *A. fragilis* specimens from Oman, Taiwan, and the Philippines. While the original type specimen was not referenced in this study, the specimen from Oman is the closest specimen to the type locality available and because of the relative proximity likely represents the same or a closely related taxon to the type specimen.

However, although Liu & Wang (2009) also report one *A. robusta* sequence from Taiwan, they do not show how this specimen fits Itono's description of the genus. They describe a branching angle of less than 40 degrees and irregularly whorled deciduous filaments (no mention of annular marks or lumen shapes), but neither of these features alone is enough to positively identify the specimen as *A. robusta* Itono. While their supposed *"A. **robusta**"* specimen from Taiwan is indeed phylogenetically different from the Oman *A. fragilis* specimen, there is not enough morphological evidence to tie its

morphology to the description of Itono (1979) in any definitive way. As such, its sequence and morphological observations should currently not be considered from or attributed to *A. robusta* (Itono).

The most recent publication regarding *Actinotrichia* taxonomy was completed by Wiriyadamrikul et al. (2013). These authors also attribute some of their specimens to *A. robusta* (Itono) through similarity to sequences from Liu & Wang (2009). Because Liu & Wang's cannot be definitively associated with *A. robusta* Itono. Additionally, the pictures of the supposed "*A. robusta*" (and *A. fragilis*) specimens identified in Wiriyadamrikul et al. (2013) contradict their own descriptions of whorl regularity and branching angles – two characteristics upon which they base the identifications. They also introduce an unknown *Actinotrichia sp.* as a potential *A. calcea,* but with no good description of the original type, it also cannot be identified as such.

In summary, only two published *Actinotrichia* species are linked to their original type specimens and have been sequenced: *A. taiwanica* and *A. fragilis*. Although clearly phylogenetically different from those two species, none of the supposed *"A. robusta"* specimens collected by Liu & Wang (2009) and Wiriyadamrikul et al. (2013) are shown to be the same as those described by Itono (1979), and therefore should be reevaluated.

Morphology of *Actinotrichia* Specimens from Micronesia

When comparing previously published morphological data to those collected in this study, several observations stand out. First, many of the thallus width measurements in this study (and especially for the three new groups from Micronesia) are substantially less than previously described measurements (Table 4). The sole exception of this is in Huisman

(2006) stating a thallus width as narrow as 250 μm in Australian specimens, but no published Australian *Actinotrichia* sequences are available to confirm whether these specimens are actually *A. fragilis*. Additionally, the branching angles of the Micronesia taxa are generally much larger than described elsewhere – up to 100 degrees in some specimens – whereas the maximum previously reported value is only 70 degrees. Finally, the distance between branching dichotomies is much longer in some Micronesian taxa: up to 25 mm, compared to a maximum of 10 mm in previous studies. While some of these data do overlap with previously published morphological data (Table 4), the significant differences in thallus width, branching angles, and distance between dichotomies suggest the morphologies of these new taxa are distinct from those previously reported.

The LDA analysis confirms differences between these groups as well. When including the *A. fragilis* species complex in the analysis, the distinctions between all groups are muddled, but removing this group clarifies that there are indeed differences between Sp. A, Sp. B, and Sp. C. (Figure 6). This further suggests that the *A. fragilis* complex may be made up of many different species because the range of morphological values from this study are so wide. And although Sp. A, Sp. B, and Sp. C do not appear to have clear-cut differences between them, the LDA analysis suggests otherwise. The relatively distinct boundaries between each species indicate those three are different from each other morphologically.

While the LDA analyses do suggest that the aggregate morphology is different between groups, the practicality of this result for identification is limited: the morphological overlap between all groups make strict morphological identification difficult except in only

the most extreme examples. This is illustrated by specimens in Sp. B, where overall morphology of the specimens range from very small, decumbent plants with short interdichotomies to large specimens with over 2 cm between some dichotomies (Figure 4). This wide range of morphologies even within the same species further suggests that identifying *Actinotrichia* specimens by morphology alone is a difficult, if not impossible task. Thus, sequence data and is necessary for correct identification.

Phylogenetic Support for *Actinotrichia* Species

The most recently published *Actinotrichia* phylogeny in Wiriyadamrikul et al. (2013, Figure 8) shows that each of the three species for which verified sequences exist (*A. fragilis*, the *"A. robusta"* specimen described by Liu & Wang (2009), and *A. taiwanica)* are clearly delineated by three well-supported monophyletic clades (Figure 8). This study confirmed those relationships with several additions; in both the *rbc*L and concatenated trees, all specimens grouped in seven main monophyletic clades, including the single *A. taiwanica* and *A. sp.* (Wiriyadamrikul et al. 2013) specimens that are the only member of their respective clades. Species B (from Chuuk and Pohnpei) had lower support than the others in the *rbcL* and Bayesian analyses (Figure 2), but the concatenated ML analysis returned a high support value, aligning with the results of the species delimitation methods. The lone *A. sp.* sequence has the lowest ML support of any of the clades, but does consistently end up in the same position in the *rbc*L and concatenated trees.

Figure 8: The most recent phylogenetic tree available using *rbc*L sequences from Wiriyadamrikul et al. (2013). Support values are (1) maximum likelihood bootstrap values and (2) Bayesian posteriors.

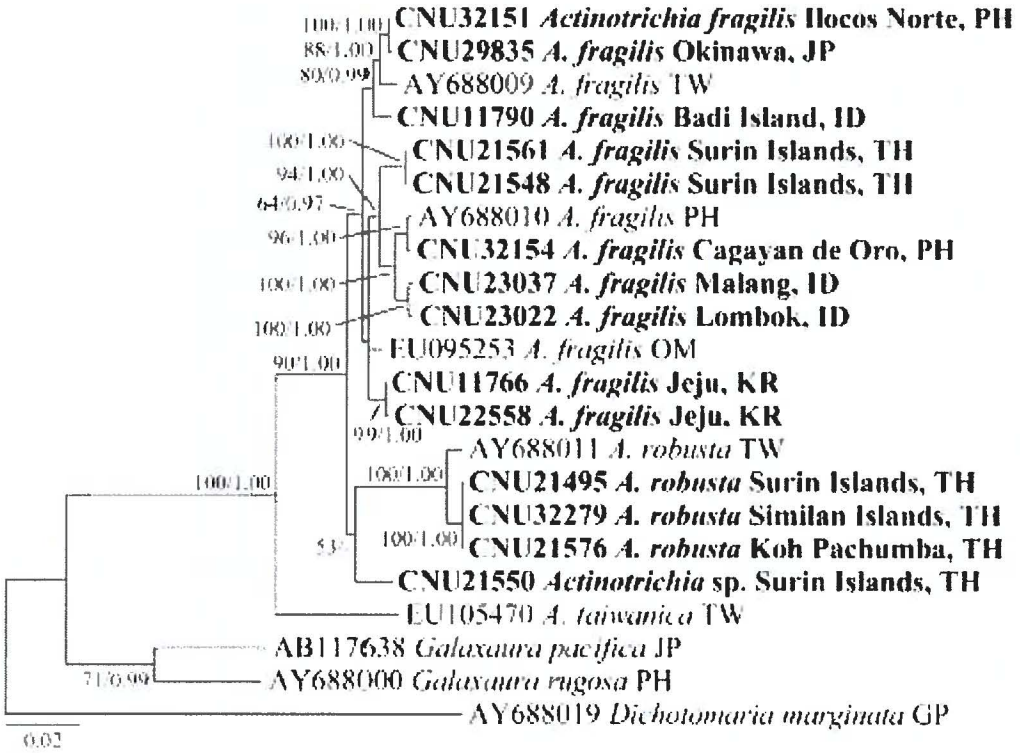
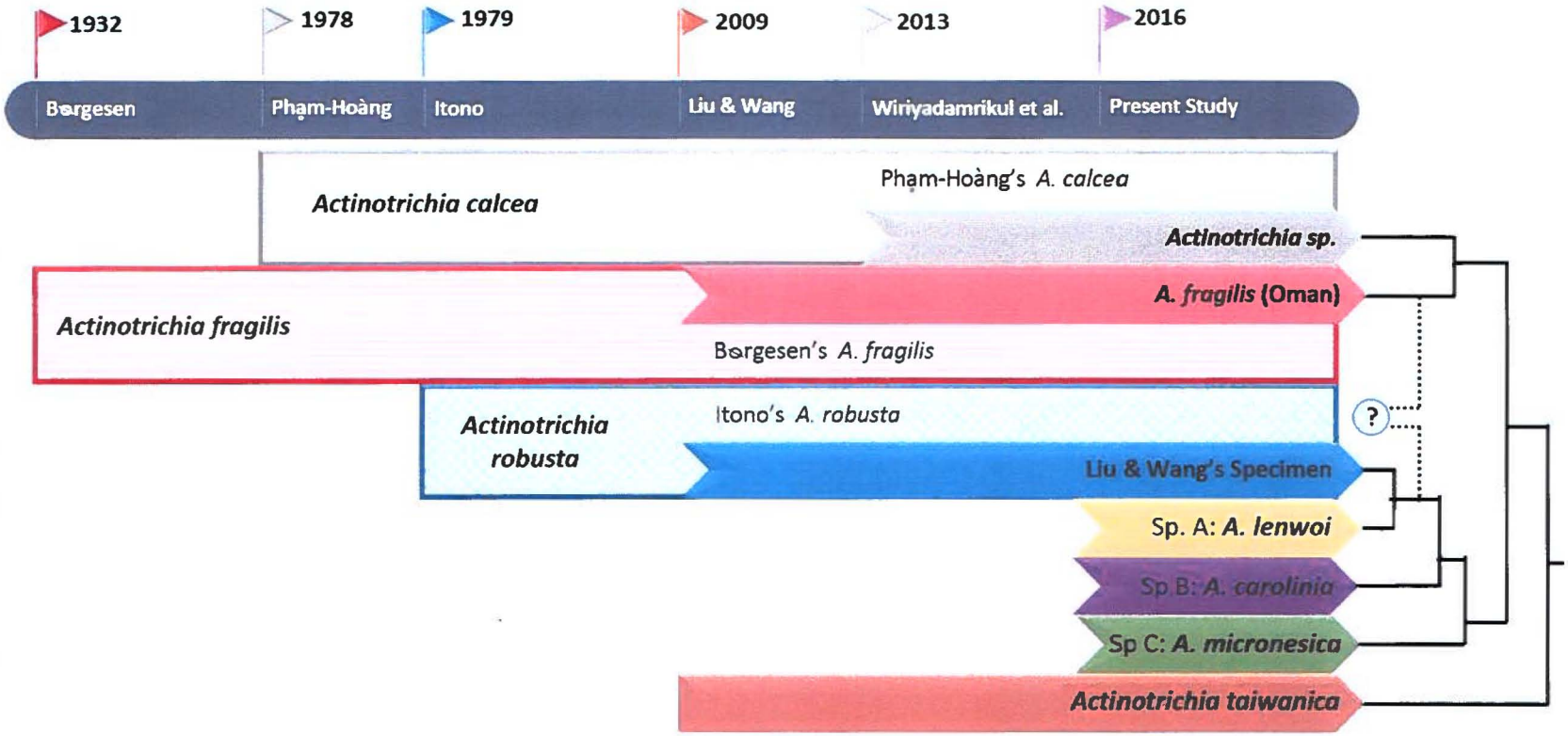


Figure 9: Timeline depicting *Actinotrichia* species described and their phylogenetic relationships. Solid color represents species and timeframes for which molecular data are available; shaded represents species for which only morphological descriptions exist. The dashed lines indicate the phylogenetic placement of *A. robusta* Itono is unknown.



Taxonomic Recommendations

Based on the data presented and examined in this study, I propose the following taxonomic and study recommendations for *Actinotrichia* species (represented in Figure 9)

Actinotrichia fragilis

With such a large geographical area inhabited by this species complex, it is unknown how many potential species have yet to be distinguished and described. While the specimen from Oman is the most likely candidate for being the "true" *A. fragilis,* further studies will need to sequence and examine large numbers of *Actinotrichia* from many diverse locations to unearth this currently unknown diversity. In the meantime, this species complex will likely remain a "catch-all" for specimens that do not align with the morphologies or sequences of others in the genus.

Actinotrichia calcea

Given the extremely vague description of this species and that there is no type specimen or genetic material to examine, *A. calcea* should be reevaluated when a specimen from the same locality as the type in Con Dao, Vietnam is obtained. Given the seemingly high degree of cryptic speciation in the genus, it is possible this species is distinct (although not for the morphological reasons originally described) and requires investigation using genetic techniques.

Actinotrichia robusta Itono

This species described by Itono (1979) is markedly different than *A. fragilis.* However, the specimens on which he based those descriptions are from both the Ryukyu Islands and Marquesas Islands. The geographic distance between these locations combined with

our current understanding of genetic diversity would suggest that it is unlikely these two specimens are of the same species, but no genetic data is available to examine (attempts to extract DNA from Itono's Marquesas specimen stored in the University of Guam Herbarium were unsuccessful). With no genetic information linking the original *A. robusta* (Itono) specimens to current sequences, its phylogenetic placement within the genus is unclear. Further sampling and genetic investigation is required.

"Actinotrichia robusta" Liu & Wang

The specimen identified by Liu & Wang (2009) has not been definitively linked to the morphology described by Itono (1979) and was not available for morphological comparisons in this study. As such, the phylogenetic group it clusters with under the Micronesian clade cannot be attributed to *A. robusta* Itono; genetic data of Itono's "true" species is required before making further statements about the taxonomy of *"A. robusta"* Liu & Wang. Regardless of this link, however, the clade containing Liu & Wang's sequences is distinct from others in the genus. Pending genetic data of *A. robusta* Itono, there are two possible outcomes for these sequences from Liu & Wang: (1) *"A. robusta"* Liu & Wang specimens and sequences *are* representative of *A. robusta* Itono and therefore accurately named, or (2) these specimens are different from *A. robusta* Itono and constitute a new species. The specimens in this clade should not be identified as *A. robusta* Itono without further review.

Actinotrichia taiwanica

With definitive genetic data and morphological characteristics, the identify of this species as distinct from any others in the genus is clear. Further sampling in Taiwan and surrounding areas will determine the extent of its range.

Actinotrichia sp.

Wiriyadamrikul et al. (2013) preliminarily suggests that this specimen could be a representative of *A. calcea*, but with no good description to go on, this cannot be settled until the type specimen of *A. calcea* is examined. The sequence of *A. sp.*, however, is distinct enough to be supported as a separate species from others in the genus. More sampling in the region will help resolve the status of this specimen.

Actinotrichia lenwoi sp. nov. ("Sp. A")

This species described is from specimens from Pohnpei (21) and Kosrae (26). Further investigation of gametophytic plants is required as only two male gametophytes were found; no female gametophytes were recovered in this study. A preliminary description of the species morphology is given below:

Plants 1 – 6.5 cm high, dichotomously branched every 1 – 21 mm at an angle of 60 – 106° with up to 8 levels of branching on each thallus portion. Axes terete, 200 – 493 μm diameter composed of a cortex 2-3 cells thick with a filamentous medulla. Cortical cell lumens angular, except those from which assimilatory filaments arise, which are round. Assimilatory filament whorls appear either consistently concentric or very sparse throughout thallus. Filaments appear to be deciduous. Diecious. Tetrasporophytic plants develop filaments in patches on thallus with tetraspores arising from the apex of each; occasionally tetraspores form as branches on assimilatory filaments.

Actinotrichia carolinia sp. nov. ("Sp. B")

To date, this species has only been recorded from Chuuk (11) and Ant Atoll, Pohnpei (11). Three male gametophytes were found in the samples, but no female gametophytes were recovered. Further investigation of male structures reproductive structures is required, but a preliminary description of the species morphology is given below:

Plants 0.5 – 7 cm high, dichotomously branched every 2 – 26 mm at an angle 53 – 103°. Axes terete, 259 – 375 μm diameter composed of a cortex 2 – 3 cells thick with filamentous medulla. Assimilatory filaments found in concentric whorls on cortex whorls spaced 113 – 246 μm apart. Filaments deciduous on some specimens. All cortical cell lumens are angular, but lumens of cortical cells without assimilatory filaments are more angular than the lumens of cortical cells from which assimilatory filaments arise. Dioecious. Tetraspores form mainly as branches on assimilatory filaments, occasionally on the ends of assimilatory filaments.

Actinotrichia micronesica sp. nov. ("Sp. C")

Owing its name to a wider geographical range, *A. micronesica* has been found in Guam (3), Chuuk (2), and Pohnpei (1). Despite the fewer number of specimens collected, two female gametophytes and one male gametophyte were recovered. A preliminary description is below:

Plants 1.5 - 4 cm high, dichotomously branched every 2 – 10 mm at an angle 64 - 102°. Axes terete, 376 - 457 μm diameter composed of a cortex 2 – 3 cells thick with filamentous medulla. Assimilatory filaments form concentric whorls on cortex 143 – 278 μm apart and appear to be deciduous Dioecious. Carpogonial branches develop 2 – 3 sterile branches, each consisting of 1 – 2 cells.

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