

PHYLOGENETICS AND EVOLUTION OF *OREOGRAMMITIS*, *RADIOGRAMMITIS* AND  
*THEMELIUM* (POLYPODIACEAE)

AND

POPULATION GENETICS OF HAWAIIAN ENDEMIC *OREOGRAMMITIS HOOKERI* AND  
*ADENOPHORUS TRIPINNATIFIDUS*

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## Abstract

Grammitids are a large monophyletic group of primarily epiphytic ferns in Polypodiaceae. Genera *Oreogrammitis*, *Radiogrammitis* and *Themelium* (ORT) formed a clade in grammitid phylogeny where three genera are polyphyletic in the ORT clade. A study was conducted to delineate the phylogenetic relationships of the ORT group by using intensive taxon sampling and with nine genetic markers from both nuclear and chloroplast genomes. Gene sequences were analyzed in maximum likelihood and Bayesian algorithms. Nuclear gene trees and nuclear and plastid gene trees were summarized to construct species trees under multi-species coalescent method in ASTRAL program. All the analyses with higher number of species and data revealed that the three genera are polyphyletic. Thus, taxonomic revisions are suggested. *Oreogrammitis* must be redefined to include *Radiogrammitis* and *Themelium*.

*Oreogrammitis hookeri* is an endemic Hawaiian grammitid fern. It is one of the three Hawai‘i endemic *Oreogrammitis* species. *Oreogrammitis hookeri* can be found in all the major islands in Hawaiian archipelago whereas the other two species have limited distribution. Some populations of *O. hookeri* contain unique alleles and the species may have been undergoing incipient speciation or represent cryptic species complex. Current study was conducted to investigate the population structure of *O. hookeri* with populations sampled from all the major islands except Lanai. Double Digest Restriction Site Associated DNA sequencing (ddRADseq) technique was used to generate genomic data. Analysis of 176 loci in STRUCTURE program showed that there are two genotypes in *O. hookeri*. Populations collected from Kaua‘i and Mauna Loa, Hawai‘i is one genotype and O‘ahu and Kilauea, Hawai‘i is another genotype. Maui and Moloka‘i contain both genotypes. Incipient speciation or existence of cryptic species of *O. hookeri* can be ruled out because Maui-Moloka‘i genetic cluster contain both genotypes thus it

supports the idea that individuals from all sampling localities are part of one interbreeding lineage. Genetic differences among populations could simply be due to random processes such as founder effects although localized adaptation cannot be ruled out.

Genus *Adenophorus* is one of the two endemic fern genera in Hawai'i. The genus consisted of ten species. *Adenophorus tripinnatifidus* is highly variable species. A population study done on *A. tripinnatifidus* showed low levels of interpopulational gene flow when compared to its conspecific, *A. tamariscinus*, species. The present study conducted to explore the genetic structure of *A. tripinnatifidus*. Samples collected from Kaua'i, Maui and Moloka'i. DNA samples of 35 samples from four populations were sequenced using ddRADseq technique. Hundred and forty-four loci were analyzed in STRUCTURE. Two genotypes were recovered. Kaua'i populations are genetically distinct from Maui and Moloka'i populations. Thus, it can be inferred that *A. tripinnatifidus* is undergoing incipient speciation.

# Table of Contents

<b>ACKNOWLEDGMENTS .....</b>	<b>I</b>
<b>ABSTRACT.....</b>	<b>II</b>
<b>TABLE OF CONTENTS .....</b>	<b>IV</b>
<b>LIST OF TABLES .....</b>	<b>VI</b>
<b>LIST OF FIGURES .....</b>	<b>VIII</b>
<b>CHAPTER 1 : LITERATURE REVIEW AND THE DISSERTATION PROPOSAL .....</b>	<b>1</b>
<b>1.1 Literature Review .....</b>	<b>1</b>
1.1.1 Part 1: Phylogeny, Evolution and Biogeography of the Oreogrammitis, Radiogrammitis and Themelium (ORT) Clade.....	1
1.1.2 Part 2: Population Genomics of Endemic Hawaiian <i>Oreogrammitis hookeri</i> and <i>Adenophorus tripinnatifidus</i> .....	11
<b>1.2 Dissertation Proposal.....</b>	<b>19</b>
1.2.1 Part 1: Phylogeny, Evolution and Biogeography of the ORT clade .....	19
1.2.2 Part 2: Population Genomics of Hawaiian Endemic <i>Oreogrammitis hookeri</i> .....	21
1.2.3 Part 3: Population Genomics of <i>Adenophorus tripinnatifidus</i> .....	22
<b>1.3 Reference .....</b>	<b>23</b>
<b>CHAPTER 2 : PHYLOGENY, EVOLUTION AND BIOGEOGRAPHY OF OREOGRAMMITIS, RADIOGRAMMITIS AND THEMELIUM (POLYPODIACEAE) (ORT) CLADE .....</b>	<b>33</b>
<b>2.1 Introduction .....</b>	<b>33</b>
<b>2.2 Materials and Method .....</b>	<b>39</b>
2.2.1 DNA Extraction and Amplification of Nuclear and Plastid Gene Markers.....	39
2.2.2 Sequence Alignment and Phylogenetic Analysis .....	43
<b>2.3 Results.....</b>	<b>48</b>
Phylogenetic Relationships of the ORT Clade Inferred Separately from Nuclear and Plastid Gene Markers .....	48
<b>2.4. Discussion .....</b>	<b>60</b>
<b>2.5 References .....</b>	<b>67</b>

<b>CHAPTER 3 : POPULATION GENETICS OF THE HAWAI‘I ENDEMIC <i>OREOGRAMMITIS HOOKERI</i> (POLYPODIACEAE) .....</b>	<b>75</b>
<b>3.1 Introduction .....</b>	<b>75</b>
<b>3.2 Materials and Method .....</b>	<b>80</b>
3.2.1 Sample collection and total genomic DNA extraction .....	80
3.2.2 ddRADseq DNA Library Preparation .....	83
3.2.3 Sample Pooling and Visualization.....	84
3.2.4 Sequence Assembly and Data Analysis .....	84
3.2.5 Population Genetic Structure Analysis.....	85
<b>3.3 Results.....</b>	<b>88</b>
<b>3.4 Discussion .....</b>	<b>94</b>
<b>3.5 References .....</b>	<b>98</b>
<b>CHAPTER 4 : INVESTIGATION OF POPULATION STRUCTURE OF POLYMORPHIC HAWAIIAN ENDEMIC <i>ADENOPHORUS TRIPINNATIFIDUS</i> (POLYPODIACEAE).....</b>	<b>101</b>
<b>4.1 Introduction .....</b>	<b>101</b>
<b>4.2 Materials and Method .....</b>	<b>104</b>
4.2.1 Sample collection .....	104
4.2.3 Sample Pooling and Visualization.....	109
4.2.4 Sequence Assembly and Data Analysis .....	109
4.2.5 Population Genetic Structure Analysis.....	111
4.2.6 Identification of Best K and Graphical Illustration of Results .....	112
<b>4.3 Results.....</b>	<b>112</b>
4.3.1 Population Structure Analysis .....	112
4.3.2 <i>Pinnae Division of A. tripinnatifidus</i> .....	117
<b>4.4 Discussion .....</b>	<b>119</b>
<b>4.5 References .....</b>	<b>123</b>
<b>CHAPTER 5 : RESEARCH QUESTIONS REVISITED .....</b>	<b>128</b>
Chapter 2:.....	128
Chapter 3:.....	129
Chapter 4:.....	130
<b>References .....</b>	<b>130</b>

## List of Tables

<b>Table 2.1:</b> Number of species and samples used in the study .....	41
<b>Table 2.2:</b> Primers Newly Designed for the Study .....	43
<b>Table 2.3:</b> Genetic Variations of Different Gene Markers, Best Substitution Model and Optimal log likelihoods of Best ML Trees Derived from ML Analysis on IQ-TREE .....	44
<b>Table 2.4:</b> Substitution Model and Partition Found by PartitionFinder and ModelFinder Implemented on IQ-TREE .....	46
<b>Table 2.5:</b> Pair-wise SH values among gene sequences and ML trees .....	46
<b>Table 3.1:</b> <i>Oreogrammitis hookeri</i> collections across the main Hawaiian Islands .....	85
<b>Table 3.2:</b> The number of loci caught ipyrad analysis after application of different filters in RADseq data assembly steps .....	86
<b>Table 4.1:</b> <i>Adenophorus tripinnatifidus</i> collections across the Hawaiian Islands .....	107
<b>Table 4.2:</b> The number of loci caught ipyrad analysis after application of different filters in RADseq data assembly steps .....	113

## List of Figures

<b>Figure 2.1:</b> A: ML phylogram of <i>gapCp</i> analysis from IQ-TREE. B: ML phylogram of <i>pgiC</i> analysis from IQ-TREE .....	53
<b>Figure 2.2:</b> A: ML phylogram of <i>SQD1a</i> analysis from IQ-TREE. B: ML phylogram of <i>IBR3</i> analysis from IQ-TREE. ....	54
<b>Figure 2.3:</b> Species tree inferred using ASTRAL from unrooted ML gene trees ( <i>gapCp</i> , <i>pgiC</i> , <i>SQD1a</i> , <i>IBR3</i> and <i>LEY</i> ) inferred in IQ-TREE. ....	55
<b>Figure 2.4:</b> Plastid concatenated ( <i>trnL-trnF</i> , <i>trnG-trnR</i> , <i>rps4-trnS</i> and <i>rbcL</i> ) ML tree inferred in IQ-TREE.. ....	56
<b>Figure 2.5:</b> The super species tree inferred using ASTRAL from unrooted ML gene trees ( <i>gapCp</i> , <i>pgiC</i> , <i>SQD1a</i> , <i>IBR3</i> , <i>LEY</i> , <i>trnL-trnF</i> , <i>trnG-trnR</i> , <i>rps4-trnS</i> and <i>rbcL</i> ) inferred in ASTRAL.....	57
<b>Figure 3.1:</b> Endemic Hawaiian <i>Oreogrammitis</i> species.....	79
<b>Figure 3.2:</b> <i>Oreogrammitis hookeri</i> s.l. sample collection sites of main Hawaiian Islands (Kaua‘i, O‘ahu, Maui, Moloka‘i and Hawai‘i ). ....	82
<b>Figure 3.3:</b> A scatter plot recovered from DAPC analysis in Adegent package in R shows the number of populations (clusters) and Bayesian Information Criterion (BIC) values.....	89
<b>Figure 3.4:</b> A map of the sampling locations and proportion of each of the 81 individuals of <i>O. hookeri</i> assigned to 2 clusters identified in STRUCTURE.....	90
<b>Figure 3.5:</b> Genetic structure Bar graphs of <i>O. hookeri</i> s.l. generated from DAPC (top panel) STRUCTURE (bottom panel) analyses at $K=2$ from 176 SNP loci. ....	92
<b>Figure 3.6:</b> A plot of the individual densities against the first discriminant function retained show that the greatest proportion of variation lies with it; $K=2$ .....	93
<b>Figure 3.7:</b> Genetic structure Bar graphs of <i>O. hookeri</i> s.l. generated from STRUCTURE analyses at $K=3$ (toppanel) and $K=4$ (bottom panel) from 176 SNP loci.....	95
<b>Figure 3.8:</b> DAPC scatterplots drawn using the Adegent package in R of existing genetic clusters of the best-fit models, $K=3$ (left) and $K=4$ (right). ....	97
<b>Figure 4.1:</b> <i>Adenophorus tripinnatifidus</i> sample collection sites of main Hawaiian Islands (Kaua‘i, Maui, and Moloka‘i).....	106
<b>Figure 4.2:</b> A scatter plot recovered from DAPC analysis in Adegent package in R shows the number of populations (clusters) and Bayesian Information Criterion (BIC) values.....	115

**Figure 4.3:** A plot of the individual densities against the first discriminant function retained show that the isolation of recovered cluster;  $K=2$  ..... 116

**Figure 4.4:** Genetic structure bar graphs of *A. tripinnatifidus* generated from DAPC (top panel) STRUCTURE (bottom panel) analyses at  $K=2$  from 144 SNP loci. .... 118

# Chapter 1 : Literature Review and the Dissertation Proposal

## 1.1 Literature Review

### 1.1.1 Part 1: Phylogeny, Evolution and Biogeography of the Oreogrammitis, Radiogrammitis and Themelium (ORT) Clade

#### 1.1.1.1 *Classification of Ferns*

Ferns are distributed all over the world in both Northern and Southern hemispheres, from the poles to the equator (Moran, 2008). Ferns and lycophytes are seed-free (spore-bearing) vascular plants with alternation of two free-living generations. Those similarities led to consider lycophytes as the closest relative of ferns hence, historically ferns and lycophytes have been lumped into a group called “pteridophytes”. Recent molecular phylogenetics delineates two basic lineages of extant vascular plants: lycophytes (club mosses and relatives) and Euphyllophytes. Euphyllophytes comprise Spermatophytes (seed-bearing plants) and Monilophytes (ferns), and they are more closely related to each other than to Lycophytes (Pryer et al., 2001; Smith et al., 2006). Ferns comprise about 2-5% of the species diversity of Euphyllophytes (Mehltreter, 2008) with about 12,200 known species (Moran, 2008). Approximately 85% of fern species occur in the tropics, especially in cloud forests at mid-elevations and on oceanic islands such as the Hawaiian Islands (Tyron, 1964).

A community-derived classification for extant ferns and lycophytes was introduced by the Pteridophyte Phylogeny Group (PPG) (2016) and it is considered as a modern and comprehensive classification. The most widely used classification for ferns with the advent of molecular phylogenetics are those of Smith et al. (2006, 2008) who organized them into four classes, eleven orders, and 37 families. Christenhusz et al. (2011) and Christenhusz and Chase

(2014) proposed classifications for vascular seed-free plant classes (ferns and lycophytes) based on the classifications of Smith et al. (2006, 2008). Christenhusz et al. (2011) identified 45 families with about 280 genera in their classification. Incorporation of molecular data into phylogenetic analyses helped to resolve some uncertain relationships among fern genera that were unclear based solely on morphological characters (Christenhusz and Chase, 2014). The community-derived classification consisted of 2 classes (Lycopodiopsida and Polypodiopsida). The two classes consisted of ca. 11, 916 species and are classified in 14 orders, 51 families and 337 genera. All the extant ferns are included in the class Polypodiopsida. Four subclasses, 11 orders, 48 families and 319 genera are recognized in Polypodiopsida.

Plastid markers have provided good insights for resolving fern phylogenetic relationships. However, plastid genes alone may not reveal the true relationships among species, especially if there has been a history of hybridization in a lineage leading to reticulate relationships (Rothfels et al., 2013). Abiotic factors, such as wind, are the primary dispersal agents of ferns. It has been hypothesized that the evolution of reproductive isolation is much slower in abiotically dispersed plant taxa such as ferns (Rothfels et al., 2015; Ranker and Sundue, 2015). Slow development of reproductive isolation can lead to a lower number of fern species as well as rampant hybridization. Rothfels et al. (2015) reported an intergeneric hybrid, *×Cystocarpium roskamianum*, between two species of ferns that had diverged approximately 60 million years ago. Apart from hybridization, nearly 50% of ferns are shown to be polyploids in cytological studies, mostly allopolyploids (Schuettpelz et al., 2008; Rothfels et al., 2013). These reasons demonstrate the necessity of using biparentally-inherited markers, such as nuclear gene markers, to unravel diploid progenitors of polyploid species (Schuettpelz et al., 2008). Plastid genes have also been shown to be less effective for interspecific level studies because plastid

genomes have low base substitution rates when compared to nuclear genomes (Sang, 2002; Rothfels et al., 2013). Therefore, the phylogenies inferred from chloroplast DNA may have lower resolution than the phylogenies derived from nuclear data.

#### ***1.1.1.2 Family Polypodiaceae***

The family Polypodiaceae is classified under the class Polypodiopsida, order Polypodiales, by Smith et al. (2006, 2008). Polypodiaceae is a major family of leptosporangiate ferns, primarily epiphytic, and the fourth largest epiphytic family of vascular plants (Smith et al., 2006; Christenhusz and Chase, 2014; Sundue et al., 2014). It diverged from related taxa ca. 55.8 Ma in the Paleotropics and migrated to the Neotropics approximately 43 Ma ago (Schuettpelz and Pryer, 2009; Sundue et al., 2014).

PPG I (2016) treated the family as consisting of 65 genera and ca. 1652 species. Species of this family can be identified by their scaly creeping rhizomes with abaxial (rarely marginal), rounded to elliptic, elongate or acrostichoid exindusiate sori (Christenhusz and Chase, 2014). Six subfamilies were identified within Polypodiaceae. The large group, more than two-thirds (~45%) of the family's species diversity, referred to as "grammitids" are classified under subfamily Grammitidoideae Parris & Sundue (PPG I, 2016). These ferns are called grammitids because of their small and seemingly grass-like appearance and the others. Other species included in the family are "non-grammitid polypods" or often simply as "polypods" (Sundue et al., 2014).

Grammitid ferns are primarily epiphytic and are widely distributed in the tropics and subtropics at high elevations (usually above 1000m) (Sundue, et al., 2014; Ranker et al., 2004). Although grammitids are widely distributed in the Paleotropics, a Neotropical origin is suggested around 30.6 Ma and dispersed primarily via long distance dispersal rather than vicariance

(Sundue et al., 2014). The clade comprises nearly 911 species in 33 genera (PPG I, 2016; Sundue, et al., 2014; Perrie and Parris, 2012; Ranker et al., 2004; Schneider et al., 2004).

Grammitids were once classified as a separate family, Grammitidaceae, due to their distinct morphological characteristics, including the presence of green, spheroid, trilete spores with chlorophyll at maturity, sporangial stalks that are a single cell wide in the middle, and leaves with pluricellular setae and the scales are absent from the leaves (Ranker et al., 2004; Sundue, 2010). Whereas non-grammitid polypods have yellow, reniform, monolete spores, sporangial stalks that are three-cells wide in the middle, and leaves without pluricellular setae and bearing scales. However, both groups possess minute branched hairs (~0.1 mm long) on their leaves providing a unifying characteristic (Parris, 1990; Sundue, 2010). Despite their distinct morphology, the preliminary molecular phylogenetic work based on a single plastid gene, *rbcL*, suggested that grammitids are monophyletic, but nested within Polypodiaceae (Hasebe et al., 1995). Schneider et al. (2004) further supported this claim by providing evidence from three plastid markers namely *rbcL*, *rps4* and *rps4-trnS*. Although monophyly of grammitids is supported by molecular phylogenetic studies (Schneider et al., 2004; Ranker et al., 2004) and distinct morphological traits, grammitids arose within the Polypodiaceae. Thus, by the Grammitidaceae being recognized as a distinct family, the Polypodiaceae becomes paraphyletic (Ranker and Haufler, 2008). Therefore, the former Grammitidaceae is now recognized as a subfamily Grammitidoideae within the Polypodiaceae and informally referred to as “grammitids” (Hirai et al., 2011).

Christenhusz and Chase (2014) stated that generic classification for grammitid ferns has been unstable for the last 20 years and they proposed lumping all grammitids in the genus *Grammitis* s. l. However, recent phylogenetic analyses have found strong support for the monophyly of

many genera that are being recognized as existing or newly named genera. In addition to support from molecular characters, unique suites of morphological traits have been identified for all well supported genera. Almost all of the Neotropical genera, as well as many Paleotropical genera, have been resolved as monophyletic (Parris, 1997 and Sundue et al. 2014). However, a few genera from the Paleotropics, such as *Oreogrammitis* Copel., *Radiogrammitis* Parris, and *Themelium* Parris need additional studies to further resolve the limits and phylogenetic relationships (Vernon and Ranker, 2013; Sundue et al. 2014).

### ***1.1.1.3 Oreogrammitis Copel.***

*Oreogrammitis* Copel. was introduced to include only one species, *O. clemensiae* Copel. (Copeland, 1917). This species is only known from Mount Kinabalu, Sabah, Malaysia (Parris, 2007). It is morphologically similar to *Scleroglossum* Alderwerelt by its simple, narrowly linear laminae, stipes that are not articulated to the rhizome, linear sori occurring in two grooves, lateral veins 1-2 (-3)- forked, and rhizome with pale to red-brown scales. However, Copeland (1917) stated that *Oreogrammitis* differs from *Scleroglossum* by having strictly superficial or even slightly elevated sori rather than sunken. Parris (2007) further added that the sporangia are setose and rhizome scales are glabrous. This species is known only from its scanty type specimen and therefore it was considered as a dubious taxon (Christensen, 1929) until its rediscovery in 1934. R. E. Holttum recollected the species from Mount Kinabalu and, due to its distinctive features, the genus was maintained (Christensen and Holttum, 1934). The genus remained largely problematic until three additional specimens of *O. clemensiae* were collected from Mount Kinabalu in 1992 (Parris, 2007). Copeland (1917) placed the species in a separate genus because of the fused sori, but Parris (1992) observed that the species has different degrees of soral fusion

from young to mature. Other species, such as *Grammitis poeppigiana* (Mett.) Pic. Serm., as do species of *Scleroglossum*, also are occasionally found with fused sori, but it is not closely related to *O. clemensiae*. As a consequence, soral fusion was not identified as a valid character to place it in a monotypic genus. *Scleroglossum* differs from *O. clemensiae* by its rhizome and frond hairs showing no similarity to *O. clemensiae* (Parris, 2007). Therefore, *Oreogrammitis* was maintained as a monotypic genus until Parris (2007) redefined the genus to include ca. 100 species, over 90% of which were previously placed in the polyphyletic *Grammitis sensu lato* (*s.l.*). *Grammitis sensu stricto* (*s.s.*) now only includes species with the black-marginal leaf character found in the type species *G. marginella* (Sw.) Sw. (Ranker et al., 2004).

Presently, ~153 species are recognized in *Oreogrammitis* (Ranker, 2014). Species of *Oreogrammitis* share characteristics similar to *O. clemensiae* (Parris, 2007) as described above, but vary in the rhizome and frond characteristics as described in other related genera.

#### ***1.1.1.4 Radiogrammitis Parris***

*Radiogrammitis* is a recently established genus (Parris, 2007) that comprises ca. 36 primarily or completely Paleotropical species (Ranker, 2010, 2014; Sundue et al. 2014). The type species of the genus is *R. setigera* (Blume) Parris. According to Parris (2007), it was necessary to introduce a new genus for some orphan species which were formally included in *Grammitis s.l.* when she was preparing an account of the Grammitidaceae for the Flora of Peninsular Malaysia. Synapomorphies of the genus were described in terms of prominent vegetative characters, such as rhizomes, hairs, fronds, and reproductive features such as sori and sporangia. The generic name refers to the radial rhizomes present in species and to *Grammitis*, the genus in which these species were formerly placed (Parris, 2007). After the introduction of this genus, several

molecular phylogenetic studies of grammitids have been conducted that included a small number of species of *Radiogrammitis*; these limited data sets do not support the genus as monophyletic (Sundue, 2010; Sundue et al., 2010; Sundue et al. 2014; Ranker, 2014).

#### **1.1.1.5 *Themelium* Parris**

The species of *Themelium* Parris were previously included in *Ctenopteris* Blume ex Kunze. *Ctenopteris* was identified as an artificial taxon based on frond dissection and, therefore, a few species of *Ctenopteris* were redefined and included in new genera such as *Prosaptia* C.Presl and *Themelium*. Currently, nearly 27 species have been identified in *Themelium* (Parris, 1997; Parris, pers. comm.). The type species of *Themelium* is *T. tenuisectum* (Blume) Parris.

*Themelium* shows close affinity to species of *Oreogrammitis* and *Radiogrammitis*. It differs from *Oreogrammitis* and *Radiogrammitis* by having pinnate or bi-pinnate fronds whereas the latter primarily (although not always) have simple leaves. *Themelium* species are always nested with those of *Oreogrammitis* by having dorsiventral rhizomes and glabrous rhizome scales, but species do not possess setose sporangia as in *Oreogrammitis* species. Also, rhizome scales are usually subclathrate to clathrate in *Themelium* in contrast to rhizome scales being usually not clathrate in *Oreogrammitis* (Parris, 2007; Sundue et al., 2014).

#### **1.1.1.6 Current Phylogenetic Status of the genera *Oreogrammitis*, *Radiogrammitis* and *Themelium***

The monophyly of each of the three genera, *Oreogrammitis*, *Radiogrammitis*, and *Themelium*, is questionable due to limited sampling in earlier studies. Sundue et al. (2010, 2014) observed *Oreogrammitis* and *Themelium* species nested within *Radiogrammitis*, forming a clade

(the ORT clade). Those studies were conducted using a limited number of species even though the ORT clade is one of the species-rich groups among grammitids. As currently circumscribed, there are over 200 species (~ 30% of grammitid species diversity) that are probably in the ORT clade. Further, existing phylogenies are constructed exclusively on plastid gene markers.

Current circumscriptions of the focal genera are not consistent with molecular phylogenetic studies. The low resolution of relationships within the ORT clade as currently circumscribed may be due to the poor sampling of the species (Sundue et al. 2014; Ranker, 2014). Phylogenetic studies of grammitid ferns have included only a few species of the ORT clade despite their predominant position among grammitids (Hirai et al. 2011; Sundue et al. 2010 & 2014). Thus, the true phylogenetic relationships among the species of the ORT clade have not been rigorously explored.

The ORT genera have been primarily distinguishing by only a single character of rhizome symmetry, *Radiogrammitis* has radial rhizomes while *Themelium* and *Oreogrammitis* have dorsiventrally flattened rhizomes Parris (1997). The other characters that differentiate the genera from each other are *Radiogrammitis* sometimes lack of scales in contrast to scaly, dorsiventral rhizomes in *Oreogrammitis*. *Themelium* on the other hand is different from *Oreogrammitis* and *Radiogrammitis* by having pinnate or bi-pinnate fronds or sometimes-rigid sclerified axes and reduced laminar tissues. *Themelium* is similar to *Oreogrammitis* in having dorsiventral rhizomes and glabrous rhizome scales but species do not possess setose sporangia as in *Oreogrammitis* species. Also, the rhizome scales are usually subclathrate to clathrate in *Themelium* in contrast to the non-clathrate rhizome scales of *Oreogrammitis*. (Parris, 2007; Sundue et al., 2014). The presence or absence of some of the character states are not consistently

present in the ORT taxa thus, the characters that being used are not distinct enough to make a clear cut among the taxa in *Oreogrammitis Radiogrammitis* and

Major grammitid phylogenetic studies conducted, which include candidates from the ORT clade, so far are based on plastid molecular markers such as *rbcL*, *atpB* genes and intergenic spacer regions. Genetic information provided by those molecular markers may not be adequate to resolve the phylogenetic relationships among the putatively closely related and/or recently diverged taxa of the ORT clade (Sang, 2002, Sundue, 2010, Sundue et al., 2010 and Vernon and Ranker, 2013). Also, the hybridization leading to speciation events are not apparent in phylogenies derived from plastid molecular markers.

Consequently, thorough sampling of the species along with detailed analysis of morphological and molecular data is essential to resolve clades and relationships of the species treated in the ORT clade. A fully resolved phylogenetic hypothesis for grammitids will allow for robust analyses of historical biogeography, rates of diversification, and many other evolutionary issues related to grammitids.

#### ***1.1.1.7 Biogeography of the ORT clade***

Phylogenetic evidence demonstrates a Neotropical origin of grammitids although diversification and endemism of grammitids is much higher in the Paleotropics (Schneider et al., 2004; Ranker, 2014; Sundue et al., 2013; Sundue et al. 2014). Sundue et al. (2013) identified putative dispersal events from the Neotropics to the Paleotropics, but there is no evidence of dispersal from the Paleotropics to the Neotropics. Species of the ORT clade are primarily Paleotropical (Sundue et al., 2013; Ranker, 2010), but biogeographical relationships among species are poorly understood due to the weakly resolved phylogenetic hypotheses. For example,

the origin of the three species of Hawaiian *Oreogrammitis* cannot be determined due to the unresolved phylogeny (Vernon and Ranker, 2013). According to Ranker et al. (2004), Hawaiian *Oreogrammitis* species resemble other taxa from the southwest Pacific and their results support a sister relationship to *O. knutsfordiana* (Baker) Parris, a relationship also supported by Geiger et al. (2007). Vernon and Ranker (2013) pointed out, however, that the exact origin of the Hawaiian *Oreogrammitis* clade cannot be discerned due to the widespread distribution of *O. knutsfordiana*.

Even though the phylogeny of the species of the ORT clade is unclear, the clade shows a sister relationship to *Prosaptia* in several phylogenetic studies. *Prosaptia* is an Old World genus (Ranker, 2014), therefore, we can infer a Paleotropical origin for the species in the ORT clade. Historical biogeographical patterns and processes within this large group, however, are largely unknown necessitating the importance for an extensive systematic study of this group.

#### ***1.1.1.8 Rate of lineage-diversification of the ORT clade***

Grammitids are the most species-rich and diverse group of ferns (Parris, 2009). Since their divergence 31 Ma, the rapid diversification of the lineage has been within the last 8.4 Ma. Although, a Neotropical origin is suggested for grammitids, species-rich genera are found in the Paleotropics (Sundue et al. 2014). Two alternative hypotheses are proposed to explain the elevated rates of speciation in grammitids: acquisition of new morphological features in some taxa can give rise to distinct lineages from its progenitors or the rapid rate of diversification is correlated with shifts into new habitats or as an adaptation (Silvertown, 2004) to changing environmental conditions.

The ORT clade is one of the most species-rich groups and includes ca. 216 species. The ORT clade with its putative sister genus, *Prosaptia*, has shown variation in diversification rates

(Sundue et al. 2014). Sundue et al. (2014) proposed that the increased diversification rates could be due to adaptations to new habitats, but this should be further tested using a more detailed morphological dataset and a more robust phylogeny that includes a greater number of species.

## **1.1.2 Part 2: Population Genomics of Endemic Hawaiian *Oreogrammitis hookeri* and *Adenophorus tripinnatifidus***

### ***1.1.2.1 Hawaiian Fern Flora***

Many plant communities in Hawai'i are richly endowed with ferns. Approximately 15% of native vascular plant species diversity in Hawai'i is due to ferns (Geiger et al. 2007). The ratio (1 fern: 6 angiosperms) is much higher in Hawai'i than on continents (ca. 1:14) (Wagner 1995). The majority of fern colonizers to Hawai'i (48%) appear to have originated from the Indo-Pacific region (Forsberg 1948), but there are species and clades whose ancestors likely came from the New World (12%), boreal regions (5%), and Austral regions (4%). In addition, some species are or their progenitors appear to have been pantropically distributed (21%). The likely origins of many taxa, however, are still obscure (11%). About 110 species (76%) and 140 taxa (84%) of ferns are endemic to the Hawaiian Islands (Vernon and Ranker, 2013; Ranker, 2016), which represent the highest levels of fern endemism of a regional flora in the world (Smith, 1972; Ranker, 1992a). Despite their high endemism and abundance in forest ecosystems, few studies have been carried out so far to understand their phylogeny, biogeography, or population dynamics (Geiger et al., 2013; Ranker, 2016).

Ferns are an important plant group in ecosystems of Hawai'i. Ferns are among the first colonizers of new lava substrates (Moran, 2008). Primary colonizers of lava can eventually build up organic materials and help develop the soil creating favorable sites for later successional plant

species. Ferns are important in nutrient recycling such as from the leaves of tree ferns. For example, the Hawaiian native *Cibotum* spp. have high quantities of nitrogen (N), phosphorous (P) and potassium (K), and increase the nutrient availability for other plants once they shed their leaves (e.g., see Ranker, 2016, and references therein). High N, P, and K are due to disproportionate absorption and retention in tree ferns. This may create a temporary nutrient deficiency for other plants. However, ferns are important in succession of disturbed habitats due to their notable ability for nutrient uptake and sequestration, and their absence can significantly delay this process. On the other hand, some ferns are very vigorous and competitive over others. A good example is thicket-forming ferns such as the Hawaiian native scrambling fern, *Dicranopteris linearis* or *uluhe*. Fern thickets persist for a long time and slow down the successional process by inhibiting seed germination while at the same time minimizing soil erosion (Walker et al., 2010).

Among the endemic ferns, nearly 30% are epiphytic species (Wagner, 1981). Epiphytic ferns form a significant component of understory vascular plant communities in Hawaiian lowland and montane forests (Ranker, 1992, 2016). Like other epiphytes, ferns are important in organic matter recycling and maintaining water balance in forested areas especially in Hawaiian watershed preserves. Epiphytic ferns form microhabitats that can serve as the basis for food webs of arthropods and vertebrates such as birds. Aside from their ecosystem role, they can also be used as indicators of environmental changes and forest disturbances due to their sensitivity to direct sunlight. Because of their abundance and vital ecological role, epiphytic ferns provide a better opportunity to understand the natural history of Hawai'i and to conserve the unique biological diversity of Hawaiian archipelago (Ranker, 1992).

### ***1.1.2.2 Phylogenetics, Biogeography and Population Genetics of Hawaiian Grammitid Ferns***

Approximately 14% of fern species diversity in Hawai'i is represented by grammitids. Grammitid ferns comprise 15 spp. in three genera: *Adenophorus* Gaudich., *Oreogrammitis* (see --) and *Stenogrammitis* Labiak. Species of those genera are primarily epiphytic but may occasionally grow on mossy rocks (Ranker et al., 2003). The origin of Hawaiian grammitid species was hypothesized in several studies (Ranker, et al. 2003; Ranker, et al. 2004; Geiger, et al. 2013), yet it is still obscure mainly due to wide distribution of sister taxa. It was hypothesized that the primary colonizers may have been brought via the jet stream from the Indo-Pacific or in a Hadley Cell shift from the South Pacific (Geiger, et al. 2013).

*Adenophorus* is an endemic genus to the Hawaiian Islands and includes a radiation of ten species. A seminal work on this genus was completed by Ranker et al. (2003). The species of *Adenophorus* are different from related grammitid genera by having putatively unique glandular receptacular paraphyses (Bishop, 1974). Similar paraphyses were also identified in species of *Chrysogrammitis* Parris, *Grammitis rigida* Hombr., and possibly some species of *Ctenopteris* but none of the phylogenetic studies showed those taxa as being related to *Adenophorus*. Two morphologically distinct subgenera were recognized by Bishop (1974): subg. *Adenophorus* and subg. *Oligadenus*. The six species of subg *Adenophorus* include *A. hymenophylloides* (Kaulf.) Hook & Grev., *A. tamariscinus* (Kaulf.) Hook. & Grev., *A. epigaeus* (L. E. Bishop) W. H. Wagner, *A. abietinus* (D. C. Eaton) K. A. Wilson, *A. tripinnatifidus* Gaudich, and *A. tenellus* (Kaulf.) Ranker. These species have 2- to 3- pinnatifid or pinnate-pinnatifid leaves and root buds are absent. The four species of subg. *Oligadenus* include *A. haalilioanus* (Brack.) K. A. Wilson, *A. oahuensis* (Copel.) L. E. Bishop, *A. periens* L. E. Bishop, and *A. pinnatifidus* Gaudich. Subg. *Oligadenus* species have less dissected leaves (simple to pinnatifid) and root buds (Bishop 1974

and Ranker, et al. 2003). Bishop (1974) described *A. tamariscinus* with two varieties: var. *montana* and var. *epigaeus*, but Wagner et al. (1995) treated them as species. Ranker et al. (2003) conducted the first molecular phylogenetic study to assess the relationships among the taxa of the genus using three chloroplast regions (*rbcL*, *atpB*, and the *trnL-trnF* intergenic spacer). Molecular data supported the monophyly of subg. *Adenophorus* but subg. *Oligadenus* was paraphyletic. Similar to Wagner (1995), they also suggested treating varieties of *A. tamariscinus* as distinct species despite their high molecular and morphological similarity which they attribute to recency of divergence from a common ancestor. Further, a Neotropical origin was proposed for *Adenophorus* (Ranker et al., 2003), but further studies are necessary to confirm this.

*Stenogrammitis* is a recently named genus (Labiak, 2010). Species of this genus were previously included in *Lellingeria* A. R. Sm. & R. C. Moran. Morphological and molecular phylogenetic analyses showed that species now included in *Stenogrammitis* form a monophyletic group, hence their recognition as a separate genus from the polyphyletic *Lellingeria* (Labiak, 2010). *Stenogrammitis* differs from *Lellingeria* by possessing linear leaves usually less than 5 mm wide, clathrate iridescent rhizome scales that are glabrous except for a single apical cilium, unbranched veins and only one vein per segment, fertile veins usually with dark sclerenchyma visible beneath the sporangia, and a base chromosome number of  $x=33$  (Labiak 2010). The species of this genus are Pantropical. Ten species are found across Hawai'i, Islands of the South Pacific, Africa, and Madagascar and ca. 14 species are in the Neotropics. One species, *S. saffordii* (Maxon) Labiak, is endemic to the Hawaiian Islands. This species is common in rainforests throughout Hawai'i and the species is characterized by having small, decumbent to erect rhizomes and inconspicuous winged stipes that are clustered on rhizomes. Blades are simple,

linear and lobed. The basal portions of the fronds are sterile, while the fertile 1/4 to 2/3 distal ends have nearly entire margins and abaxial surfaces almost entirely covered with sori (Smith et al. 1991; Palmer, 2003).

As the first study on the population genetics of any epiphytic fern species, Ranker (1992a) examined the genetic diversity of endemic Hawaiian epiphytic ferns using isozyme analyses and subsequently identified conservation needs for those species (Ranker 1992b, 1994, 2016). His studies emphasized the importance of genetic diversity in conspecific populations for the continuation of evolutionary processes even though biological diversity is usually measured in species or higher levels for conservation purposes. Ranker (1992a) analyzed genetic diversity among intra- and inter-island conspecific populations of *A. tamariscinus*, *A. tripinnatifidus*, *A. tenellus* and *O. hookeri* (Brack.) Parris. Several intriguing outcomes were obtained from this study. Genetic variability of the four epiphytic species was generally high when compared to continental outcrossing fern species, and that there was little or no evidence of inter-island population differentiation (Ranker, 2016). *Adenophorus tamariscinus* showed the highest population genetic diversity whereas *O. hookeri* and *A. tripinnatifidus* were the lowest. However, the populations of *O. hookeri* and *A. tripinnatifidus* harbored higher frequencies of island unique alleles.

Population-unique alleles can be important sources of genetic variability and evolution and could be of value in guiding conservation management decisions. For example, Chakraborty et al. (1991) emphasized the significance of unique alleles in admixed populations to examine hereditary characters such as disease frequency differences in populations. In a study to analyze the population structure of Atlantic salmon, a higher frequency of unique alleles in the North American population was found compared to Europe, and it was hypothesized that the observed

differences were due to glacial histories of two continents (King et al., 2001). King et al. (2001) also highlights the importance of identification of genetic variability for evolution and thereby for conservation and management. Similarly, further studies are necessary to examine the genetic diversity and the island unique alleles of *A. tripinnatifidus* and *O. hookeri* using more powerful genetic techniques.

### **1.1.2.3 Adenophorus tripinnatifidus Gaudich.**

*Adenophorus tripinnatifidus* is an extremely morphologically variable species. The fronds can grow as short as 8 cm or as long as 50 cm but usually fronds grow about 12-25 cm long. Typically rhizomes are epigeous on and in moss mats at the base of trees thus potentially never touching the ground. Blades are 2-pinnate-pinnatifid to 3-pinnate. *Adenophorus tripinnatifidus* is highly similar to *A. tamariscinus*, but the former species can be differentiated by slender, long-creeping rhizomes with stipes that are about 1-2 cm apart from each other, and usually with 3-pinnate fronds. The most basal acroscopic pinnules of each pinna can also be divided again (Palmer, 2003).

There are many localized, semi-isolated and some stable forms of *A. tripinnatifidus* (Palmer 2003). For example, plants with more leathery fronds, a stout stipe with winged rachises and costae, basal pinnae that are not reduced in size, and broad ultimate segments with acute tips are found in the Ko‘olau range of O‘ahu (Palmer 2003). The Ko‘olau form of *A. tripinnatifidus* is morphologically different from other populations, therefore it was previously known as *A. hillebrandii* (Hook.) K.A. Wilson. Similarly, the division of basal pinnules of fronds is variable and the degree of frond dissection varies from bipinnate to tripinnate.

Isozyme analysis demonstrated that the genetic diversity of four populations collected across the Hawaiian archipelago have less genetic diversity when compared to *A. tamariscinus* except the Mt. Ka‘ala, O‘ahu population (Ranker 1992a). The mean fixation indices were close to zero suggesting the populations were outcrossing. However, *A. tripinnatifidus* showed generally lower interpopulation gene flow (Ranker, 1992a). Thus, genetic differentiation among interisland populations are higher compared to *A. tamariscinus*. Further, the populations of *A. tripinnatifidus* retained fewer population-unique alleles but the frequency of such alleles was higher than in *A. tamariscinus*.

#### **1.1.2.4 *Oreogrammitis hookeri māku‘e lau li‘i (small-leaved māku‘e)***

*Oreogrammitis* in Hawai‘i is represented by three endemic species. These are *O. hookeri* (Brack.) Parris., *O. baldwinii* (Baker) Parris and *O. forbesiana* (Wagner) Parris. All three species occur in wet forests above 650 m. *Oreogrammitis baldwinii* is endemic to the island of Kaua‘i whereas *O. forbesiana* and *O. hookeri* occur on the main high islands of Kaua‘i, Oahu, Maui, Moloka‘i and Hawai‘i (Palmer, 2003). From personal observations, *O. hookeri* is more abundant on the island of Hawai‘i than on other islands and *O. forbesiana* is common on the islands of Maui and Moloka‘i. *Oreogrammitis forbesiana* possesses intermediate morphological characters of the other two species and it may be represent a fertile hybrid between them (Palmer, 2003).

Ranker’s (1992a) study included four populations of *O. hookeri* from the islands of Maui and Hawai‘i. Those populations contained unique alleles even among the closely located populations in Hawai‘i (Kilauea and Ola‘a). Population differentiation was evident in *O. hookeri* although the individuals are morphologically indistinguishable. Thus, it may be that *O. hookeri* is

not a single species and in fact represents a cryptic species complex at the early stages of evolutionary divergence (i.e., incipient species).

The occurrence of high frequencies of island-unique alleles in populations of *O. hookeri* may be due to separate evolutionary trajectories. Island geography may create barriers to gene flow that give rise to isolated populations. This isolation plays a significant role in the evolution of such populations leading to allopatric speciation or intra-island population divergence. Genetic drift, selection and genetic mutations also shape the microevolutionary processes in isolated groups. This phenomenon has been shown by other groups of organisms such as native Hawaiian leafhoppers (Bennett and O'Grady, 2013).

## 1.2 Dissertation Proposal

### 1.2.1 Part 1: Phylogeny, Evolution and Biogeography of the ORT clade

Resolving phylogenetic relationships among taxa is important in evolutionary biology as well as for other areas of science. In grammitids, except *Oreogrammitis*, the other two genera in the ORT clade are recently introduced, and the genera are distinguished based on limited morphological characters. As described above the congeneric species may have polyphyletic relationships as seen in a few studies conducted so far using an insufficient number of representatives from each genus. Hence, the apparent polyphyly in the clade based on limited sampling and/or the exclusive use of plastid gene markers in previous studies, leads to a number of questions including the validity of each genus in the ORT clade.

In that light, the main objective of this component of the research was to develop a detailed phylogeny of species treated in the ORT clade. This research was the most comprehensive study to examine the generic relationships of the ORT clade. In summary the objective of this study was achieved by using intensive sampling of taxa across the ORT clade and the use of sequence data of novel nuclear gene markers for grammitids along with plastid molecular markers. *Prosaptia* was used as the outgroup which has been supported as the sister to the ORT clade in some phylogenetic studies (e.g., Sundue et al., 2014). The resulting phylogeny was used to infer generic boundaries and relationships and morphological characters correlated with them were identified. Character state evolution, rate of diversification and biogeographical relationships were examined.

**Research questions addressed in this component of the study**

**Question 1:** Are *Radiogrammitis*, *Oreogrammitis* and *Themelium* monophyletic genera? If not, are there well-supported clades that can be characterized by particular suites of molecular and morphological characters?

**Question 2:** Are phylogenies based on analyses of nuclear DNA markers congruent with those based on plastid DNA markers?

**Question 3:** Are the similarities among species due to convergent evolution or due to inheritance from common ancestors?

## **1.2.2 Part 2: Population Genomics of Hawaiian Endemic *Oreogrammitis hookeri***

*Oreogrammitis hookeri* is an endemic species of fern in the Hawaiian Islands. Ranker (1992) showed that even though the inter-island population genetic differentiation was low, certain island populations harbored a significant number of unique alleles. Ranker (1992) hypothesized that *O. hookeri* may represent more than one cryptic species. This seminal work was done using isozyme markers and the current study employed more powerful genomic techniques to test the hypothesis of incipient speciation in *O. hookeri* populations.

The objective of the study was to analyze the genomic diversity among populations of *O. hookeri* from the islands of O'ahu, Hawai'i, Maui, Moloka'i and Kaua'i to identify genetic structure among the populations and islands and determine if cryptic speciation has occurred or if incipient speciation is occurring. Restriction-site associated genomic sequences were obtained through double digest RAD sequencing (ddRADseq) in Illumina Hi-seq2500 platform.

### **Research questions that were addressed in this component of the study**

**Question 1:** Are conspecific populations of *O. hookeri* from different islands diverging from each other?

**Question 2:** Do conspecific populations of *O. hookeri* on each island harbor unique genetic variability, possibly indicating the existence of diverging evolutionary trajectories?

### **1.2.3 Part 3: Population Genomics of *Adenophorus tripinnatifidus***

*Adenophorus tripinnatifidus* is a morphologically highly variable, such that one discrete morphotype was even named a separate species. The current study was conducted to analyze the genomic diversity among populations of *A. tripinnatifidus* to assess levels and patterns of genetic differentiation.

#### **Research questions that were addressed in this component of the study**

**Question 1:** Are conspecific populations of *A. tripinnatifidus* of different islands genetically distinct and, thus, potentially diverging from each other?

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## Chapter 2 : Phylogeny, Evolution and Biogeography of *Oreogrammitis*, *Radiogrammitis* and *Themelium* (Polypodiaceae) (ORT) Clade

### 2.1 Introduction

With the advent of molecular phylogenetics our understanding about fern evolution, biogeography and systematics have been largely increased. Prior to molecular phylogenetic studies the classical fern taxa were based on morphology that led to some artificial taxonomic groupings. The evolutionary relationships were unclear in many traditional taxa. Most of the earlier taxonomic groups were failed to recreate monophyletic groups thereby, those were re-circumscribed in subsequent molecular phylogenetic studies. Polypodiaceae is one such example, which has undergone significant changes in its generic delimitations (Sundue, et al., 2014).

Polypodiaceae is a major family of leptosporangiate ferns, primarily epiphytic but some members occupy diverse habitats including terrestrial, epipetric, rheophytic, climbing and hemiepiphytic (Sundue, 2015). It is the fourth largest epiphytic family of vascular plants (Gentry and Dodson, 1987; Smith et al., 2006; Christenhusz and Chase, 2014; Sundue et al., 2014) and possibly the most species-rich family of ferns (Sundue et al, 2015). Radiation into the epiphytic niche began during the Cenozoic era. Angiosperms occupied the ecosystems once the ferns were dominated and forced them to shift to new habitats (such as epiphytic habitat). Even though the ferns are an ancient plant group, in fact the species rich fern lineages were diversified “in the shadow of angiosperms” (Schneider et al., 2004). Simultaneously Polypodiaceae diverged in the Cenozoic, around 55.8 Ma, in the Paleotropics and colonizers migrated to the Neotropics approximately 43 Ma ago (Schuettpelz and Pryer, 2009; Sundue et al., 2014). As currently circumscribed the family contains about 1,500 species (Sundue et al., 2014).

Scaly creeping rhizomes with abaxial (rarely marginal), rounded to elliptic, elongate or acrostichoid exindusiate sori are the key characteristics of Polypodiaceae (Christenhusz and Chase, 2014). Morphological and molecular phylogenetic studies, however, recognized two evolutionarily distinct groups within the family. Two thirds of the family's species diversity consist of monophyletic "grammitids" and the remaining one third by "non-grammitid polypods or polypods" (Sundue et al., 2014, 2015).

The large group of grammitids is primarily epiphytic and dwarfed. Grammitids were once classified as a separate family, Grammitidaceae, due to their distinct morphological characteristics, including the presence of green (chlorophyllous), spheroid, trilete spores at maturity, sporangial stalks that are a single cell wide in the middle, number of vascular bundles in the petiole reduced from several to one, leaves with pluricellular setae, and the absence of scales (Ranker, et al., 2004 and Sundue, 2010). Conversely polypods possess yellow (non-chlorophyllous), reniform, monolete spores, sporangial stalks that are three-celled wide in the middle, and leaves without pluricellular setae, but bearing scales. Both groups, however, possess minute branched hairs (~0.1 mm long) (Parris, 1990 and Sundue, 2010). Despite distinctive morphological differences between the two groups a pioneering study on fern phylogeny using a single plastid gene, *rbcL*, suggested a close affinity of grammitids to polypods (Polypodiaceae s.s.; Hasebe et al., 1995). Schneider et al. (2004) further supported the claim by providing the evidence from three plastid markers namely *rbcL*, *rps4* and *rps4-trnS*. Additional molecular phylogenetic studies supported a monophyletic grammitid clade that arose within Polypodiaceae (Schneider et al., 2004; Ranker et al., 2004). Hence if former family Grammitidaceae is recognized as a distinct family Polypodiaceae becomes paraphyletic (Ranker and Haufler, 2008), therefore, the grammitids are now recognized within the Polypodiaceae (Hirai et al., 2011).

Phylogenetics, biogeography, and the origin of grammitids have been widely studied in the last two decades. Grammitids are widely distributed in the Paleotropics and the Neotropics at mid to high elevations and probably arose in the Neotropics about 30 Ma from a polypod ancestor (Ranker et al., 2004; Sundue, et al., 2014). Primary colonizers putatively dispersed via long-distance dispersal, rather than vicariance, from the Neotropics to the Paleotropics and massively diversified in the Paleotropics as seen today (Schuettpelez and Pryer, 2009; Sundue et al., 2014). Most currently recognized grammitid genera are either confined to the Neotropics or the Paleotropics except for the genera *Stenogrammitis*, *Ctenopterella*, *Notogrammitis*, and *Grammitis*. Parris (2003) identified two phytogeographic zones of grammitid distribution as the Neotropics-Africa-Madagascar region, which includes the Mascarenes, Seychelles and Comoros, and the Asia-Malesia-Pacific region. The highest diversity is recorded from the latter region, which includes about 500 species and with about 400 species in the former (Ranker et al., 2004; Schneider et al., 2004; Perrie and Parris, 2012; Sundue, et al., 2014).

Generic delimitation of grammitids has been controversial but recent molecular phylogenetic studies have provided robust support for numerous clades, many of which have been recognized as distinct genera. Prior to molecular phylogenetics Tryon and Tryon (1983) identified one genus, four by Parris (1990), 12 by Copeland (1947), 18 and 25 by Parris (2003; 2009, respectively). The controversy of defining genera has been mainly due to the use of such homoplastic characters as blade dissection to delimit generic groups that may obscure the evolutionary relationships among taxa. Later phylogenetically more informative traits such as hydathodes, rhizome symmetry, root insertion that were used to define genera were recognized as homoplastic characters (Kessler et al., 2011). The best example is the *Grammitis* s.l. which is a widely distributed genus ranging from the western Pacific through the Neotropics to Africa,

Madagascar and the Mascarenes. Together with *Grammitis*, *Xiphopteris* and *Ctenopteris* were delimited based on leaf dissection. Those genera were found to be polyphyletic in subsequent works. Thus *Grammitis* s.s. has been redefined to include only the species with distinct black, sclerified leaf margins. The orphaned species of *Grammitis*, *Xiphopteris*, and *Ctenopteris* were classified in *Archigrammitis* Parris (Parris, 2013), *Chrysogrammitis* Parris (Parris 1998), *Dasygrammitis* Parris (Parris, 2007), *Notogrammitis* Parris (Perrie and Parris, 2012), *Radiogrammitis* Parris (Parris, 2007), *Themelium* (T. Moore) Parris (Parris, 1997), *Tomophyllum* (E. Fourn) Parris (Parris, 2007), and *Oreogrammitis* Copel. (Parris, 2007) based on suits of synapomorphies (Sundue et al., 2014). With the use of genetic data for generic delimitations, however, systematists realized that most of the grammitid genera are not monophyletic and the traits that have been used are homoplastic. As a result, many grammitid genera were re-circumscribed (e.g., Kessler et al., 2011; Sundue et al., 2014).

The generic boundaries of the species included in *Oreogrammitis*, *Radiogrammitis*, and *Themelium* remain to be resolved. The species are distributed in the Asia-Malesia-Pacific region and are more or less similar to each other. A study of the global grammitid phylogeny (Sundue et al., 2014) and several other fern studies (e.g., Ranker et al., 2004; Sundue et al., 2010; Hirai et al., 2011) included only a few species from these genera and, thus, have been unable to clearly delimit the phylogenetic relationships among the species. Major grammitid phylogenetic studies conducted, which include some species from focal genera, so far were based on plastid molecular markers such as *rbcL* and *atpB* genes and intergenic spacer regions. Genetic information provided by those molecular markers may not be adequate to resolve the phylogenetic relationships among the putatively closely related and/or recently diverged taxa (Sang, 2002, Sundue, 2010, Sundue et al., 2010, Vernon and Ranker, 2013). Also, the use of homoplasious

characters (Ranker et al., 2004) to define the genera obscure true evolutionary relationships. Sundue et al. (2010 and 2014) demonstrated that *Oreogrammitis* species nested in three places within *Radiogrammitis*. In phylogenetic analyses, the monophyletic *Themelium* group is always nested within the *Radiogrammitis* and *Oreogrammitis* species, which combined form a large clade (here called the ORT clade). The ORT clade represents one the most species-rich groups of grammitids with over 200 species (~ 30% of grammitid species diversity).

*Oreogrammitis* was introduced to include only one species, *O. clemensiae* Copel. (Copeland, 1917). This species is only known from Mount Kinabalu Sabah, Malaysia (Parris, 2007). It is morphologically similar to *Scleroglossum* by its simple, narrowly linear laminae, stipes are not articulated to the rhizome, linear sori occur in two grooves, lateral veins 1-2 (-3)-forked, and rhizome with pale to red-brown scales, but Copeland (1917) stated that *Oreogrammitis* differed from *Scleroglossum* by having strictly superficial or even slightly elevated sori rather than sunken. Parris (2007) further noted that the sporangia are setose and rhizome scales are glabrous. This species is known only from its scanty type specimen and therefore it was considered as a dubious taxon (Christensen, 1929) until 1934. Holttum recollected the species from Mount Kinabalu and, due to its distinctive features, the genus was maintained (Christensen and Holttum, 1934). The genus remained largely problematic until Parris also collected three additional specimens of *O. clemensiae* from Mount Kinabalu in 1992 (Parris, 2007). Copeland (1917) placed the species in a separate genus because of the fused sori, but Parris (1992) observed that the species has different degrees of soral fusion from young to mature. As a consequence, soral fusion was not identified as a valid character to place it in a monotypic genus. Other species, such as *Grammitis poeppigiana* (Mett.) Pic. Serm., occasionally are found with fused sori but were not closely linked to *O. clemensiae*. *Scleroglossum* has fused

sori, but rhizome and frond hairs show no similarity to *O. clemensiae* (Parris, 2007). Therefore, *Oreogrammitis* was maintained as a monotypic genus until Parris (2007) redefined the genus to include ~100 species, including more than 90% of species formally placed in the polyphyletic *Grammitis* s.l. Presently about 153 species are recognized in *Oreogrammitis* (Ranker, 2014) including three Hawaiian species (Vernon and Ranker, 2013).

*Radiogrammitis* comprises ca. 36 species (Sundue et al. 2014). The type species of the genus is *R. setigera* (Blume) Parris. Species in this genus are highly similar to *Oreogrammitis* except in having radial rhizomes and sometimes lack of scales in contrast to scaly, dorsiventral rhizomes. The generic name refers to the radial rhizomes present and to *Grammitis*, in which species were formerly placed (Parris, 2007).

The species of *Themelium* were previously included in *Ctenopteris*. Several species of *Ctenopteris* were redefined and included in new genera such as *Prosaptia* and *Themelium*. Currently, nearly 27 species have been identified in *Themelium* (Parris, 1997; Parris, unpubl.). The type species of *Themelium* is *T. tenuisectum* (Blume) Parris. *Themelium* shows close affinity to *Oreogrammitis* and *Radiogrammitis*. It differs from *Oreogrammitis* and *Radiogrammitis* by having pinnate or bi-pinnate fronds or sometimes-rigid sclerified axes and reduced laminar tissues. *Themelium* is similar to *Oreogrammitis* in having dorsiventral rhizomes and glabrous rhizome scales but species do not possess setose sporangia as in *Oreogrammitis* species. Also, the rhizome scales are usually subclathrate to clathrate in *Themelium* in contrast to the non-clathrate rhizome scales of *Oreogrammitis*. (Parris, 2007; Sundue et al., 2014).

The main objective of the present study was to develop a detailed phylogeny of species treated in the ORT clade and to assess generic boundaries. The study was conducted by using

intensive sampling of taxa across the ORT clade and the use of sequence data of novel nuclear gene markers for grammitids along with plastid molecular markers. Five species of *Prosaptia*, *P. alata*, *P. contigua*, *P. palauensis*, *P. obliquata* and *P. nutans*, were used as the outgroup taxa because *Prosaptia* has been supported as sister to the ORT clade (Sundue et al., 2014).

Several specific questions were addressed in this study. 1. Are *Radiogrammitis*, *Oreogrammitis*, and *Themelium* monophyletic? If not, are there well-supported clades that can be characterized by particular suites of morphological characters? 2. Are phylogenies based on analyses of nuclear DNA markers congruent with those based on plastid DNA markers? 3. Are the similarities among species due to convergent evolution or due to inheritance from shared ancestors?

## **2.2 Materials and Method**

### **2.2.1 DNA Extraction and Amplification of Nuclear and Plastid Gene Markers**

The ingroup included 38, 15, and 5 species of *Oreogrammitis*, *Radiogrammitis*, and *Themelium*, respectively, and those were ca. 25%, 42%, and 18%, respectively, of the species diversity of those genera (Table 2.1). About 35 species included more than one accession as given in Table 2.1. Type specimens *R. setigera* (Blume) Parris and *T. tenuisectum* (Blume) Parris were included in the analysis. *Prosaptia* was selected as the outgroup because it resolved as the sister taxon of large ORT clade in the global grammitid phylogeny of Sundue et al. (2014). Specimen identifications were verified by Barbara Parris (pers. comm.). Total genomic DNA was extracted from silica-dried leaf tissues or leaf fragments following a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987; Morden et al.,

1996). Tissue samples were ground in a buffered solution with 2% CTAB, 100mM Tris-HCl (pH 8.0), 1.4M NaCl, 2% PVP-40, 4mM diethyldithiocarbamic acid and 20mM EDTA, with 0.2%  $\beta$ -mercaptoethanol. Extracted DNA was purified with a phenol-chloroform extraction followed by ethanol precipitation, and the pellet was resuspended in 50  $\mu$ l TE (Sambrook et al., 1982). If a leaf fragment for DNA extraction was <10 mg, a Qiagen DNeasy Plant Mini Kit (QIAGEN Inc., Gaithersburg, MD) was used. Quality and concentration of extractions were assessed with a NanoDrop spectrophotometer (Thermo Fisher, Inc.). Tissue samples for this study were obtained from the Pringle Herbarium at the University of Vermont, Taiwan Forestry Research Institute herbarium, and collectors from China, Vietnam, and Malaysia. Original collection numbers were kept throughout the study. the DNA samples were deposited in the Hawaiian Plant DNA Library (HPDL) (Morden et al., 1996) at the Department of Botany, University of Hawai'i at Mānoa. Previously extracted DNA samples of *Prosaptia* were used.

**Table 2.1:** Number of species and samples used in the study

<b>Genus</b>	<b>No. of species</b>		<b>Total No. of Taxa used</b>		<b>Species Diversity</b>	
	<b>Nuclear</b>	<b>Plastid</b>	<b>Nuclear</b>	<b>Plastid</b>	<b>Nuclear</b>	<b>Plastid</b>
<i>Oreogrammitis</i> 156 spp.	38	50	26	85	25%	32%
<i>Radiogrammitis</i> 31 spp.*	15	24	66	35	42%	77%
<i>Themelium</i> 27 spp.*	5	6	10	14	18%	22%
Total	58	80	102	134		
<b>Outgroup</b>						
<i>Prosaptia</i>	5					

Twenty novel primers (Rothfels et al., 2013) for single-copy nuclear markers were screened with ten species, four from *Oreogrammitis*, three each from *Radiogrammitis* and *Themelium*. Four protein-coding nuclear gene markers (*IBR3*, *SQD1a*, *gapCpSh*, and *pgiC*) were selected based on the quality and nucleotide variability. Gene sequences of *IBR3*, *gapCpSh*, and *pgiC* [*SQD1a* used as given in Rothfels et al. (2013)] were used to design better annealing primers for grammitids and to obtain longer sequence reads (*IBR3S*, *gapCpL* and *pgiCL*). For fragmented and degraded DNA extractions that were not amplified with *pgiCL*, three internal primer pairs were designed in Primer-BLAST tool in NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>). Attempts were made to amplify each DNA sample with *gapCpL* and *pgiCL* primers but if no amplification or poor sequence reads were obtained, *gapCpSh* or *pgiCL* internal primers were used (i.e., *pgiCL* primer combination: *pgiCL\_F* and *pgiC6R*; *pgiC8F* and *pgiCL\_R*; *pgiC6F* and *pgiC8R*). Novel *IBR3S* primers were designed manually by exploring conserved flanking regions (5' and 3') of multiple sequence alignments of *IBR3* sequences (Table 2.2). Additionally, LEY primers were used to amplify *LEAFY* genes. A pair of LEY primers were designed for the focal species. Initial LEY primer sequences were obtained from Chen et al. (2012) and Adjie et al. (2007). Rothfels et al. (2013) thermocycle protocol was followed. Initial PCR reactions were conducted across a temperature gradient to find the optimum primer annealing temperature (Table 2.2). The plastid gene regions of *rbcL* coding region and *rps4-trnS*, *trnG-trnR* and *trnL-trnF* intergenic spacers were PCR amplified following the protocol of Labiak et al. (2010). Samples were PCR amplified in 25 µl volumes under the following conditions: 10-20 ng of DNA, ca. 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1X Taq Polymerase buffer (10 mM Tris-HCL [pH 9.0 at 25°C], 50 mM KCL, and 0.1% Triton X-100 [Promega]), 1.5 mM MgCl<sub>2</sub>, 0.50 mg BSA, 0.2 mM forward and reverse primers (Cronn et al.,

2002), and ca. 1 unit of *Taq* DNA Polymerase (Promega Inc.). Amplified PCR products were purified following the ExoSAP-IT (ThermoFisher Scientific) protocol and sent for sequencing to the Advance Studies in Genomics, Proteomics and Bioinformatics (ASGPB) at the University of Hawai'i at Mānoa. The majority (97) of plastid gene sequences were obtained from the Michael Sundue, University of Vermont, as they were generated for a study of the global phylogeny of grammitid ferns (Sundue et al., 2014). Six sequences downloaded from NCBI GenBank and 56 novel sequences were generated for the study.

### **2.2.2 Sequence Alignment and Phylogenetic Analysis**

Raw sequences were edited and assembled in Geneious 11.0 (Biomatters Ltd., San Francisco, CA). Sequence names were assigned by giving the collection number followed by the taxonomic name. MAAFT plug-in in Geneious was used to align multiple sequences (Kato, 2013). Alignments were manually checked for any ambiguities.

Maximum likelihood (ML) and Bayesian (BI) analyses were conducted separately for nine gene markers. Maximum Likelihood gene trees were constructed in IQ-TREE (Nguyen et al., 2014) implemented in CIBIV, Austria (<http://www.iqtree.org/>). Branch supports of ML trees were inferred by conducting 10,000 replicates of ultrafast bootstrap (UFBoot) analyses (Minh et al., 2013) and 10,000 replicates of SH-aLRT (Shimodaira and Hasegawa, 1999) branch test. ModelFinder (Kalyaanamoorthy, et al., 2017) as implemented in IQ-TREE was used to find the best substitution model for the data (Table 2.3). Three Bayesian analyses were performed for each gene in MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) on the CIPRES portal (Miller et al., 2010).

**Table 2.2:** Primers Newly Designed for the Study

Gene region/marker	Primers pairs (Forward; Reverse)	Sequence (5'→ 3')	PCR program*
<i>IBR3</i>	IBR3S_F IBR3S_R	TTTCAAAGCCGCGTCCCTCACCAAG CCAGATGTGGCGTCGTCGGATGCAAC	63.9:30:35
<i>SQD1a</i>	EMSQD1E1F6, EMSQD1E1R4	GCAAGGGTACHAAGGTHATGATCATAGG GCGTGARTCRTGCACTTTGCTRAGATG	55:120:35
	gapCpL_F gapCpL_R	TGCACMACHAACTGCCTTGCRCCBCTT CCATTYARCTCTGGRAGCACCTTTCC	58.5:60:35
<i>pgiC</i>	pgiCL_F pgiCL_R	TCGTGTCGTTGCTCRCAGCTTCCCC GAGTGTCTGGAATGTTTCATTCCCTSGG	62.6:60:35 **pgiCL_F: pgiC6R 62.9:60:35
	pgiC6F pgiC6R	ACCACAGCTTGACATGACGG ATGGATGGTCCAGGGATGGT	**pgiC8F: pgiCL_R 58.2:60:35
	pgiC8F pgiC8R	CCCTGGACCATCCATACGTC CTTGCGCTCACATTCAACA	**pgiC6F: pgiC8R 58.2:60:35
<i>LEAFY</i>	LEY_F LEY_R	ATGGARATGGGYTTGACTGT TCATCMCCRTCCTCACCAG	55:60:35

\*PCR program showed as annealing temperature (°C): annealing time (Sec): number of cycles

\*\* Primer combination used to amplify *pgiC* gene

**Table 2.3:** Genetic Variations of Different Gene Markers, Best Substitution Model and Optimal log likelihoods of Best ML Trees Derived from ML Analysis on IQ-TREE

Gene/Locus	No. of taxa	Gene matrix (bp)	Optimal Log likelihood (lnL)	Parsimony informative sites	Constant sites	Substitution model ModelFinder
<i>gapCp</i>	53	1066	-4707.73	164 (15%)	700 (66%)	TN+F+G4
<i>pgiC</i>	70	1028	-5261.332	177 (17%)	619 (60%)	TN+F+I
<i>SQD1a</i>	76	564	-1954.713	55 (9%)	446 (79%)	K2P+G4
<i>IBR3</i>	56	530	-2296.172	83 (15%)	369 (69%)	HKY+F+G4
<i>LEAFY</i>	48	882	-4096.416	238 (26%)	527 (60%)	TN+F+G4
nrConcat.*	103	4070	-19703.575	717 (18%)	2657 (65%)	
<i>rps4-trnS</i>	95	575	-5713.9035	255 (44%)	223 (39%)	TVM+F+G4
<i>trnG-trnR</i>	91	1323	-11139.9242	438 (76%)	672 (51%)	TIM+F+I+G4
<i>rbcL</i>	95	1338	-5770.6541	164 (12%)	1019 (76%)	TN+F+I+G4
<i>trnL-trnF</i>	95	419	-3867.4406	167 (40%)	186 (44%)	K3Pu+F+G4
pdConcat**	178	3655	-28731.847	1058 (29%)	1800 (49%)	

Each BI analysis was implemented for five million generations and four chains (one cold, three heated) with unlinked parameters, chain temperature was set to 0.2, and uniform priors were used. The posterior probability was sampled in every 1000 generations and the first 25% discarded as the burn-in set to 0.25. The consensus BI tree was constructed according to 50% majority rule consensus. Tracer v 1.6 (Rambaut, et al., 2014) and AWTY (Wilgenbusch, et al., 2004) were used to examine the convergence of MCMC runs of BI. Gene trees obtained from *LEAFY* were not used in any phylogenetic inference because outgroup taxa were not amplified by LEY primers, but the ML tree was used in species tree estimation. Due to incongruence among nuclear gene trees, tree topologies were compared to each other using the SH test (Shimodaira and Hasegawa, 2001) as implemented in the phangorn package (Schliep, 2011) in the R program. Each gene alignment was compared to its ML consensus tree and optimum log-likelihood was obtained using the optim.pml command. Then, likelihood scores between each pair of gene trees were tested with the SH test using 1000 bootstrap replicates. Taxa that were absent in both gene trees were removed from the alignments and ML analyses were conducted in IQ-TREE using the same parameters given above. Those ML trees were used to compare the different topologies. The Incongruence Length Difference (ILD) (Farris et al., 1994, 1995) test was conducted in PAUP\*4.0a165 (Swofford 2002) (partition homogeneity test) to test the null hypothesis that the five nuclear gene regions were homogenous in terms of phylogenetic information. Invariant sites were removed, and 10,000 replicates were conducted. The ILD test was significant, therefore the null hypothesis was rejected. Because of the highly conservative nature of the ILD test (Darlu and Lecointre, 2002), a combined nuclear dataset was used in downstream analyses and interpreted with care (but the phylogenetic tree not presented).

**Table 2.4:** Substitution Model and Partition Found by PartitionFinder and ModelFinder Implemented on IQ-TREE

Gene matrix		Subset 1 (bp)	Subset 2 (bp)	Subset 3 (bp)
nrConcat.	Gene position	1-557, 2110-3110, 558-1578	1579-2109	3111-3988
	Substitution model	TN+F+G4	TNe+G4	TN+F+I+G4
pConcat.	Gene position	1-575	3239-3655 576-1899	1900-3238
	Substitution model	TVM+F+G4	TVM+F+I+G4	TN+F+I+G4

**Table 2.5:** Pair-wise SH values among gene sequences and ML trees

Optim.pml	Sequence data	ML tree	InL	Difference (InL)	P-value
Fit 1	<i>gapCp</i>	<i>gapCp</i>	-5322.282	0.000	0.4923
Fit 2	<i>SQD1a</i>	<i>SQD1a</i>	-9551.090	4228.808	<0.0000
Fit 1	<i>SQD1a</i>	<i>SQD1a</i>	-1807.833	0.000	0.4825
Fit 2	<i>IBR3</i>	<i>IBR3</i>	-3027.820	1219.987	<0.0000
Fit 1	<i>SQD1a</i>	<i>SQD1a</i>	-1663.753	0.000	0.4913
Fit 2	<i>pgiC</i>	<i>pgiC</i>	-2551.273	887.5197	<0.0000
Fit 1	<i>pgiC</i>	<i>pgiC</i>	-4420.14	0.000	0.4904
Fit 2	<i>IBR3</i>	<i>IBR3</i>	-5546.76	1126.619	<0.0000
Fit 1	<i>gapCp</i>	<i>gapCp</i>	-4701.560	0.000	0.4865
Fit 2	<i>pgiC</i>	<i>pgiC</i>	-6599.997	1898.437	<0.0000
Fit 1	<i>IBR3</i>	<i>IBR3</i>	-5077.053	0.000	0.4865
Fit 2	<i>gapCp</i>	<i>gapCp</i>	-6786.835	1709.782	<0.0000

Multiple gene alignments of nuclear (nrConcat) and chloroplast (pdConcat) gene alignments were concatenated in Geneious. Using AICc in PartitionFinder (Lanfear et al., 2012) in CIPRESS portal, the best partition scheme for each concatenated dataset was estimated (Table 2.4). Consensus ML gene trees were constructed in IQ-TREE given the partition scheme. Best models for each partition were estimated by ModelFinder implemented in IQ-Tree. Resulting tree files were visualized and edited in FigTree v 1.4.3 (Rambaut, 2009) and iTOL (Letunic and Bork, 2019).

Three consensus species trees (nuclear, plastid, and nuclear+plastid) were inferred in ASTRAL v5.6.3 using the ASTRAL-III algorithm (Zhang et al., 2018). Unrooted gene trees (gene trees of *gapCp*, *pgiC*, *SQD1a*, *IBR3*, *LEAFY* and *rbcL*, *trnF-trnL*, *trnG-trnR*, *rps4-trnS* gene trees and all the nine trees) from IQ-TREE analyses were input to ASTRAL to find the optimum species trees. To get a measure on gene tree conflict, the quartet support (proportion of quartets in gene trees that agree with a branch in the species tree) was calculated in addition to the ASTRAL branch supports. ASTRAL also provided the quartet score (QS) and normalized QS (percentage of all quartet trees from the gene trees found in the species tree) to indicate the gene tree conflict. Higher quartet scores indicate less conflict or low levels of incomplete lineage sorting (ILS) of genes. Quartet scores of nrConcat and pdConcat were also estimated in ASTRAL.

## 2.3 Results

### Phylogenetic Relationships of the ORT Clade Inferred Separately from Nuclear and Plastid Gene Markers

#### 2.3.1 Sequence Variations of Nuclear Genes

The *gapCp*, *pgiC*, *SQD1a*, *IBR3*, and *LEAFY* gene alignments were 1066 bp, 1028 bp, 564 bp, 530 bp and 882 bp, respectively (Table 2.3). Among the five nuclear genes, *LEAFY* had the highest number of parsimony informative sites. *SQD1a* was the least variable gene yet the *SQD1a* ML tree had the highest log-likelihood (InL) score. The least number of taxa was recorded in the *LEAFY* alignment. No outgroup taxa were available in this alignment thus the unrooted trees were used in consensus gene analyses.

All the pair-wise comparisons of congruence between nuclear genes were significantly different among genes ( $p < 0.05$ ) but gene concatenation was done. Concatenated (nrConcat) gene alignment consisted of five gene markers and 4070 bp (Table 2.3). Three best-fit partitions were found for the dataset (Table 2.4). The number of parsimony informative characters was about 18% of the total number of characters, more than 2500 sites were constant. The optimal log-likelihood scores of ML trees were about -20,000.

#### 2.3.2 General Trends of Phylogenetic Relationships of the ORT Clade Based on Individual Nuclear Gene Analyses

All the pair-wise comparisons in SH tests showed the phylogenies derived from each nuclear gene analysis were incongruent to each other (Table 2.5). Resulting topologies for ML and Bayesian analyses were highly similar thus only ML trees are shown. All the genes showed

polyphyly of the genera in the ORT clade (Figure 2.1-2.2). Taxa that were used in the study resolved in three major clades (clade 1, 2 and 3), except in *pgiC* analysis, (Figure 2.1-A, 2.2-A and B). The majority of taxa were included in the clade 3, but it was poorly resolved (polytomous) while 1 and 2 clades were small but well resolved. The majority of branches were well supported (>70 SH-aLRT and BB). Some taxa were closely related to each other and consistence in most of the analyses such as Hawaiian endemic *O. hookeri*, *O. forbesiana*, and *O. baldwinii*; *R. havilandii* and *R. beddomeana*; *R. holttumii* and *R. parva*; *O. nana* and *R. membranifolia* and *R. graminella*. Placement of some species was uncertain because multiple accessions showed polyphyly including *R. setigera*, *R. jagoriana*, *O. reinwardtii*, *O. sumatrana*, *O. locellata*, *O. fasciata*, *O. padangensis* and *O. scabristipes*. The placements of two putative hybrids between *O. sumatrana* and *O. longiceps* [*O. sumatrana* x *longiceps* VT541 and 3762 (hereafter referred to as hybrid 1 and hybrid 2, respectively)] were inconsistent among different gene analyses. Hybrid 1 showed a close affinity to *O. archboldii* in more than one gene analyses. Species of *Themelium* and *O. fasciata* clustered (except in *SQD1a* because *O. fasciata* was missing in the alignment) together in most gene analyses. In the *IBR3* analysis, *Themelium* formed a monophyletic group. Phylogenetic relationships of individual nuclear gene analyses are described as below.

**gapCp Analysis:** The majority of internal branches in the phylogenetic tree derived from *gapCp* were well supported. *Oreogrammitis sinohirtella*, *R. hirtella*, and *R. parva* were resolved as sister to the other species of the ORT clade. Four well-supported *Oreogrammitis* clades (> 3 species) were recovered (Figure 2.1-A). *Oreogrammitis reinwardtii* and *O. locellata* and *R. jagoriana* were orphan species because they appeared twice in different places with high branch

support, thus correct placement of the species was uncertain. Hybrid 1 formed a monophyletic group with Hawaiian *Oreogrammitis* species (Figure 2.1-A).

**pgiC Analysis:** Most deeper branches and some species relationships were resolved with strong support. *Oreogrammitis dorsipila* was sister to other ORT species. Also *O. subevenosa*, *R. beddomeana*, and *R. havilandii* diverged from the remaining taxa of the ORT species. Except *R. beddomeana* and *R. havilandii* clade, all the other clades were formed by *Oreogrammitis* species. The placements of *O. sumatrana*, *O. locellata*, *O. fasciata*, *O. reinwardtioides*, *O. archboldii*, and *O. reinwardtii* were uncertain since they appeared multiple places in the phylogeny. Hybrid 2 closely related to *O. longiceps*. *Oreogrammitis locellata* and *O. sumatrana* clade was sister to hybrid 2 and *O. longiceps*. *Themelium yoderi* arose separately from other *Themelium* species (Figure 2.1-B).

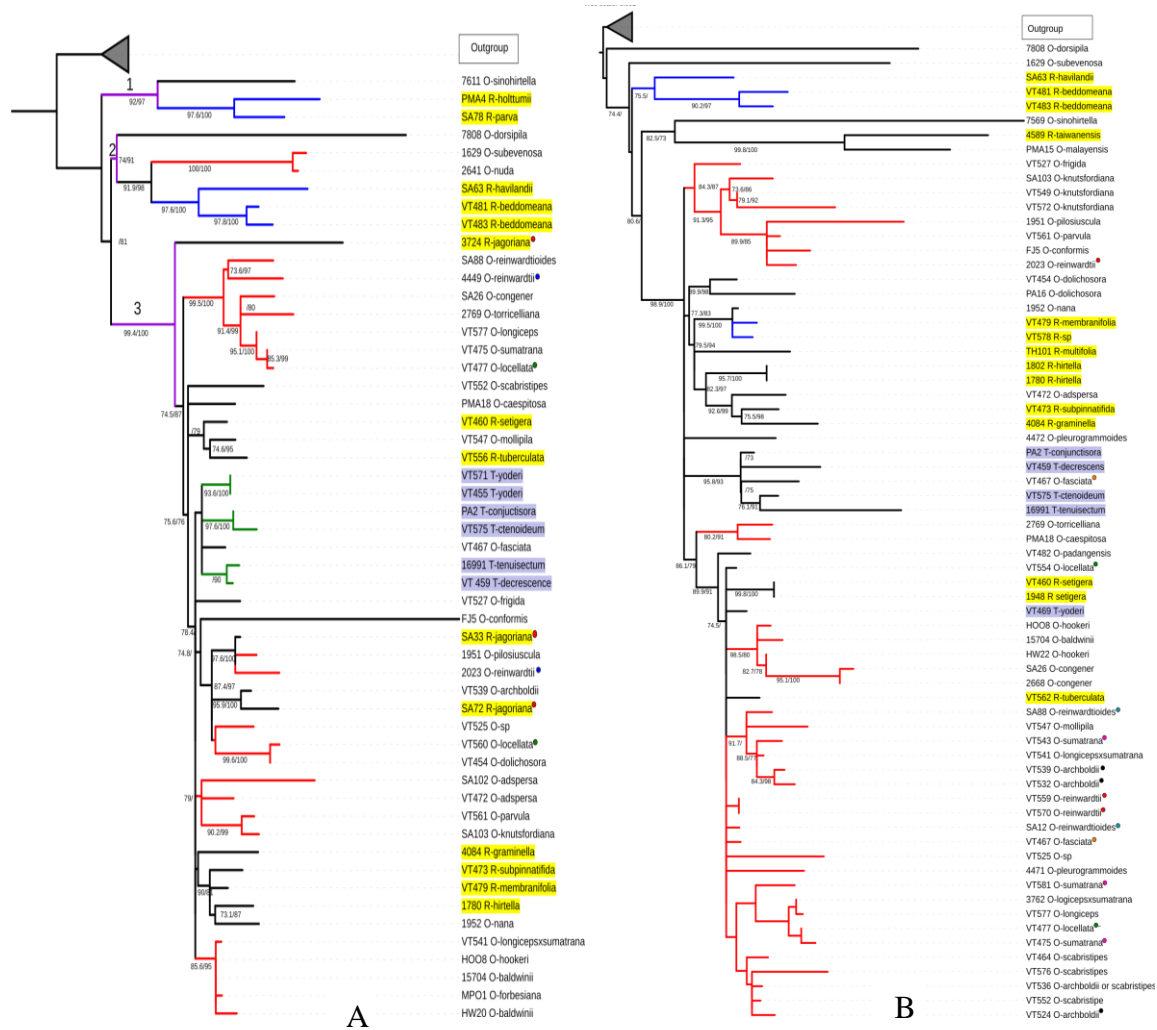
**SQD1a Analysis:** The phylogeny derived from *SQD1a* alignment had the well supported deeper branches except the branch that led to the clade 2 and 3 (Figure 2.2-A). inner two clades. As shown in figure 2.2, the clade 3 inner clade had two sub-clades with largely unresolved relationships. Two *Radiogrammitis* species, *R. havilandii* and *R. beddomeana*, were recovered as sister to all the other ingroup species which was unexpected as in other analyses *Oreogrammitis* species resolved as sister to other ingroup taxa. A few *Oreogrammitis* clades, one *Themelium* (*T. yoderi*, *T. conjunctisora*, and *T. tenuisectum*) and one *Radiogrammitis* (*R. tuberculata* and *R. hirtelloides*) were recovered. Two accessions of each of *R. subpinnatifida* and *O. locellata* arose separately from each other. One *R. subpinnatifida* was sister to four *Themelium* species. *Themelium ctenoideum* and one *T. yoderi* sample appeared separately from its congeneric species (Figure 2.2-A).

**IBR3 Analysis:** The highest number of ORT species were included in the *IBR3* gene matrix. The majority of species relationships were resolved with good branch support, however, polytomous relationships occurred in clade 3 taxa. *Oreogrammitis debilifolia*, *O. mollipila*, *O. neocaledonica*, *O. padangensis*, and two unidentified *Oreogrammitis* species were sister to the remaining species of the ORT clade and formed a well-supported monophyletic group. Eight species of *Oreogrammitis* (e.g., *O. dorsipila*, *O. torricelliana*, *O. sumatrana*, *O. locellata* and *O. knutsfordiana*, *O. mollipila*, *O. archboldii*, and *O. adspersa*) and *R. graminella* were polyphyletic. As in other nuclear gene analyses hybrid 1 did not closely related to *O. archboldii* but *O. archboldii* was in the same clade with hybrid 1. Hybrid 2 showed close affinity to *O. longiceps* as in other gene analyses. Congeneric species of *Themelium* formed a clade (Figure 2.2-B).

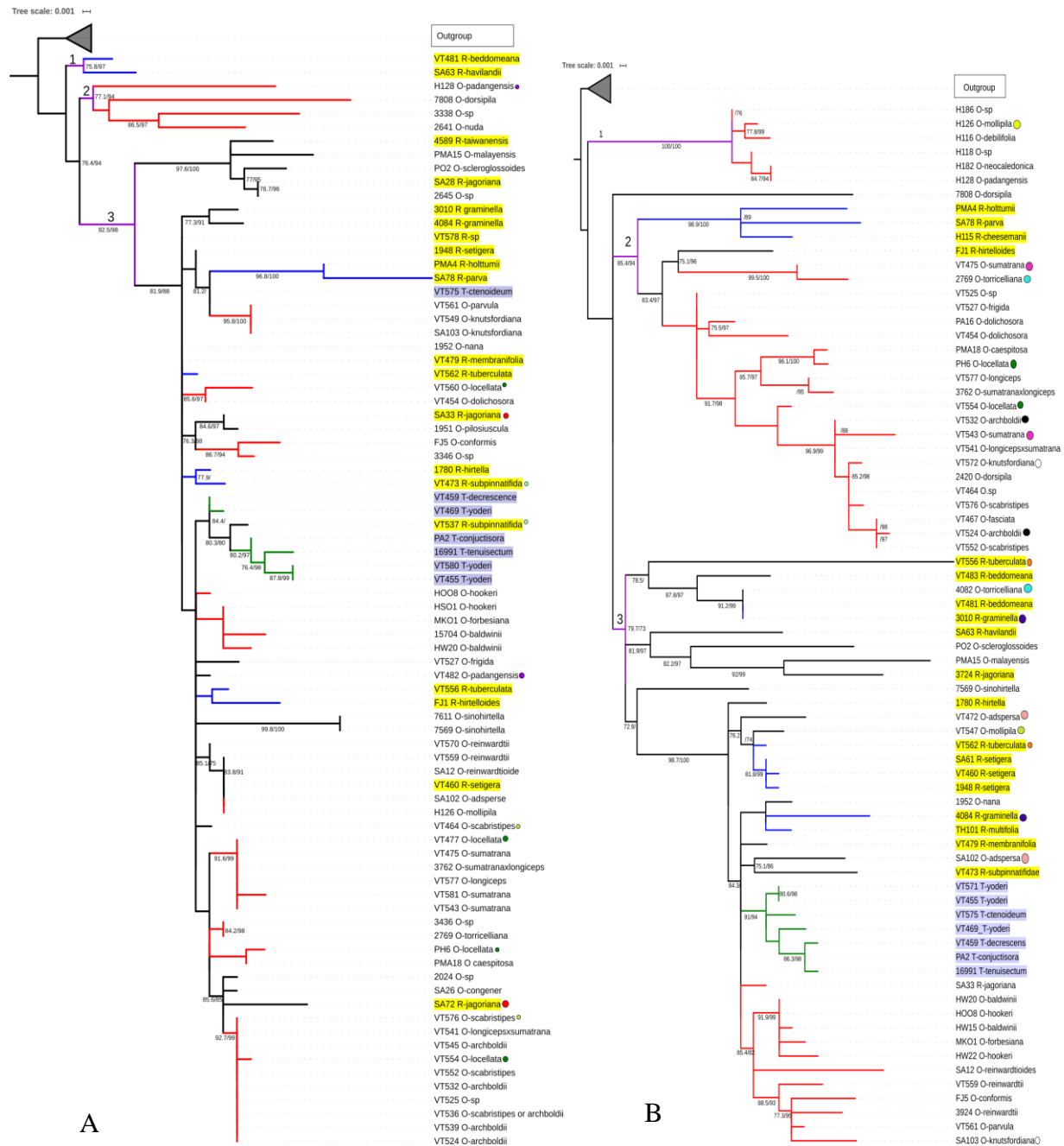
### 2.3.3 Species Tree Inferred from Nuclear Gene Markers

The consensus tree constructed from five gene trees (here after referred to as the nuclear species tree) had a normalized quartet score (QS) of 0.75 and 2,255,412 induced quartet trees from the gene trees were in the species tree. These quartets were 75% of all the quartet trees that could be found in the species tree. The nrConcat ML tree had 1,825,266 quartet trees and it is 61% (normalized QS = 0.61) of all quartet trees found in the nrConcat ML tree. On the nuclear species tree, the majority of internal and terminal branches and taxon relationships were well supported by high local PP (>50%) (Figure 2.3). Two *Oreogrammitis* species (*O. dorsipila* and *O. padangensis*) were resolved as sister to all other ORT species. Except for two monophyletic groups, all the other groups consisted of a mixture of species from different genera. *Radiogrammitis setigera* was polyphyletic in the nuclear species tree but in *IBR3* and *pgiC*

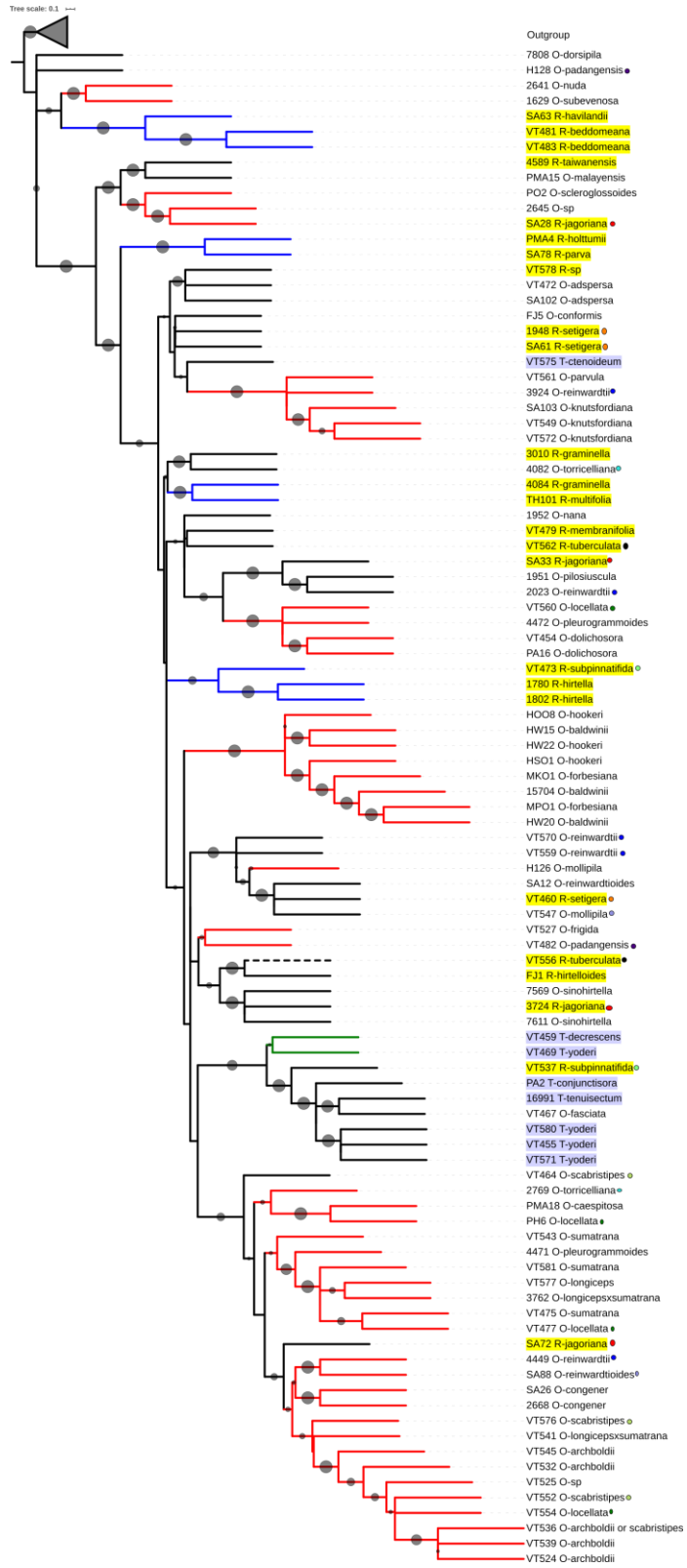
analyses, different accessions of this species showed monophyly. The other polyphyletic species were also present in single gene analyses. Whereas *Oreogrammitis archboldii*, *O. sumatrana*, *O. adspersa* and *O. knutsfordiana* were monophyletic in contrast single gene analyses. Monophyly of *Themelium* not observed. *Oreogrammitis fasciata* and *R. subpinnatifida* showed close relationship to *Themelium* species except *T. ctenoideum* which was arose separately from other *Themelium* species.



**Figure 2.1:** A: ML phylogram of gapCp analysis from IQ-TREE. B: ML phylogram of pgiC analysis from IQ-TREE. Taxon names are formatted as accession number followed by the species name. *Oreogrammitis* spp. non highlighted ; *Radiogrammitis* spp. highlighted in yellow and *Themelium* spp. highlighted in grey. Three main clades 1, 2 and 3 labelled and nodes are colored. *Oreogrammitis* clades in red; *Radiogrammitis* in blue and *Themelium* in green. Colored dots represent uncertain species relationships. Branch support in SH-aLRT/ BB values (>70%).

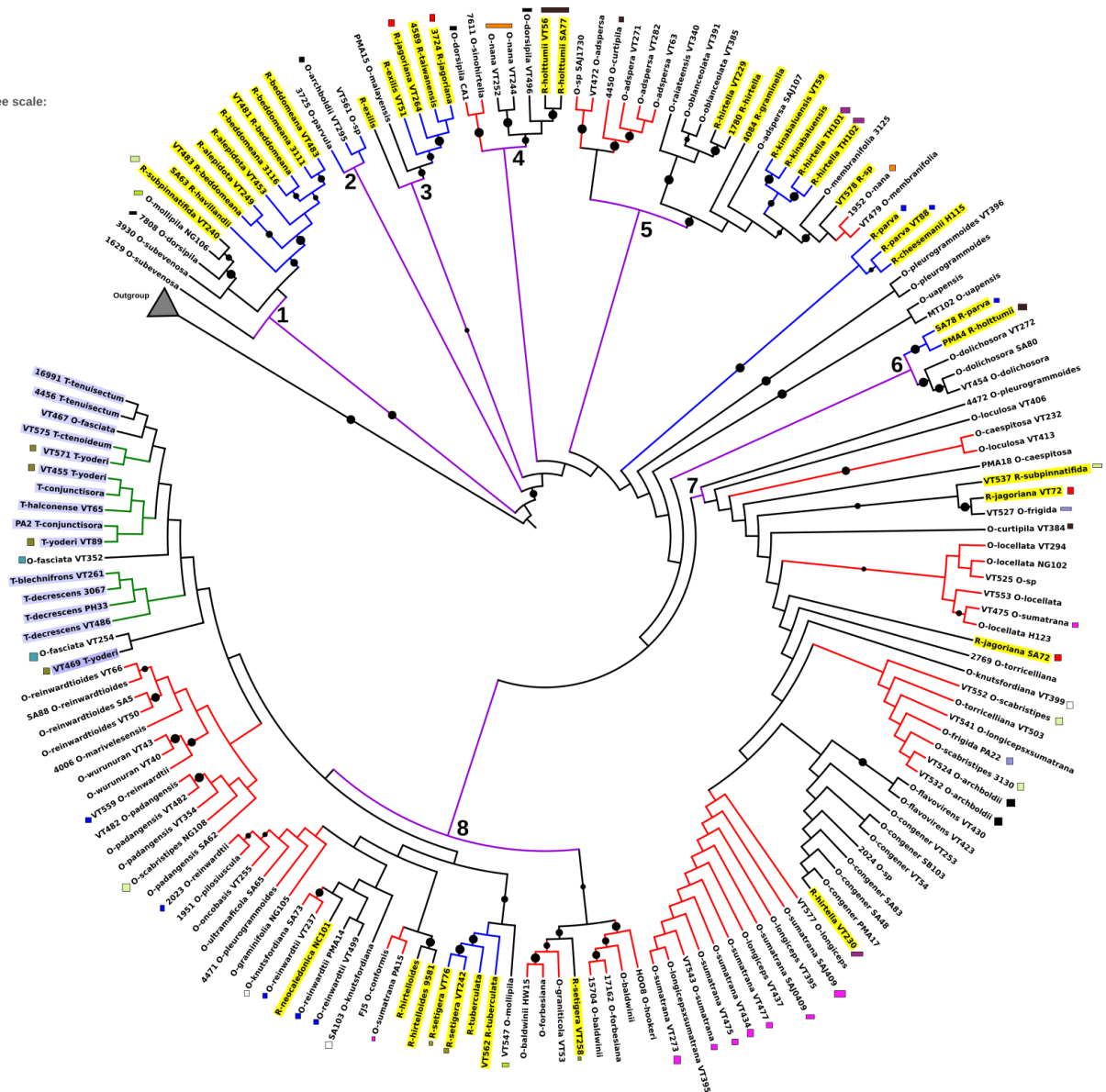


**Figure 2.2:** A: ML phylogram of SQR1a analysis from IQ-TREE. B: ML phylogram of IBR3 analysis from IQ-TREE. Taxon names are formatted as accession number followed by the species name. Oreogrammitis spp. non highlighted; Radiogrammitis spp. highlighted in yellow and Themelium spp. highlighted in grey. Three main clades 1, 2 and 3 labelled and nodes are colored. Oreogrammitis clades in red; Radiogrammitis in blue and Themelium in green. Colored dots represent uncertain species relationships. Branch support in SH-aLRT/ BB values (>70%).



**Figure 2.3:** Species tree inferred using ASTRAL from unrooted ML gene trees (*gapCp*, *pgiC*, *SQD1a*, *IBR3* and *LEY*) inferred in IQ-TREE. Taxon names are formatted as accession number followed by the species name. *Oreogrammitis* spp. not highlighted, *Radiogrammitis* spp. highlighted in yellow and *Themelum* spp. highlighted in blue and green. Colored dots represent uncertain species relationships (polyphyletic). Monophyletic groups (>2 species) in red *Oreogrammitis*; blue *Radiogrammitis* and green *Themelum*. Branch support values are given as bold circles on branch lines in local PP (>50) inferred in ASTRAL. Size of the circle proportionate to the support value.

Tree scale:



**Figure 2.4:** Plastid concatenated (*trnL-trnF*, *trnG-trnR*, *rps4-trnS* and *rbcL*) ML tree inferred in IQ-TREE. Taxon names are formatted as accession number followed by the species name. *Oreogrammitis* spp. not highlighted, *Radiogrammitis* spp. highlighted in yellow and *Themelium* spp. Colored lines and squares represent uncertain species relationships (polyphyletic). Monophyletic groups (>2 species) in red *Oreogrammitis*; blue *Radiogrammitis* and green *Themelium*. Branch support values are given as bold circles on branch lines in BB (>70) inferred in IQ-TREE. Size of the circle proportionate to the support value.



### 2.3.4 Sequence Variation of Plastid Genes

The plastid concatenated (pConcat.) gene alignment of *rps4-trnS*, *trnG-trnR* and *trnL-trnF* intergenic spacers and the *rbcL* gene, was 3655 bp of 178 taxa and 29% of the sites were phylogenetically informative. Genetic variation was higher in intergenic spacers than in nuclear genes (Table 2.1). Sequence alignment matrices of *rbcL* and spacer regions ranged from 400 to 1400 bp. Three partitions were found for pConcat. by PartitionFinder and different evolutionary models were assigned to each partition. Each partition showed rate variation across sites (G) (Table 2.1).

### 2.3.5. Species Tree Inferred from Plastid Gene Markers

Topologies of the pConcat ML tree and the species tree (consensus tree derived from *rps4-trnS*, *trnG-trnR*, *trnL-trnF*, and *rbcL* individual ML trees) were congruent, therefore, only the pConcat ML tree is presented here (Figure 2.3). The species tree had about 9,980,900 quartet trees and it was 81% (normalized QS=0.81) of total (12,223,305) quartet trees in the gene trees. The concatenated tree consisted of ca. 9,519,270 quartet trees. About 78% (normalized QS=0.778) of the total number of quartet trees in gene trees was in the concatenated tree. One to eight well resolved clades (>2 species) were obtained (Figure 2.4). Except the branch that led to clades two to eight, all the other deeper branches of the pConcat ML tree had weaker support (BS >70%) but more terminal branches were well supported. The clade 1 was sister to other ORT species and comprised of four *Oreogrammitis* species and four *Radiogrammitis* species with high branch support. Many *Radiogrammitis* species were monophyletic (see clade 1, 2, 3, 5, 6, and 8 in Figure 2.6) with high branch support except for a several accessions such as *R. subpinnatifida*, *R. jagoriana*, *R. hirtella*. Monophyletic groups of *Oreogrammitis* had lower

branch support. Another putative hybrid (*O. sumatanaxlongiceps*\_VT395; hybrid 3) between *O. sumatrana* and *O. longiceps* was closely related to *O. sumatrana*. Hybrid 1 did not show any relatedness to either putative parents. Three accessions of *O. fasciata* appeared with *Themelium* species in three different places (Figure 2.4).

### **2.3.6. Species Tree Inferred from Nuclear and Plastid Gene Markers**

The species tree inferred from nine gene trees [(here after referred to as super tree) (*rps4-trnS*, *trnG-trnR*, *trnL-trnF*, *rbcL*, *gapCp*, *pgiC*, *SQD1a*, *IBR3* and *LEAFY*)] consisted of 228 taxa (Figure 2.5). About 11,045,000 quartet trees were in the species tree and 77% of the total quartet trees in the species tree. The majority of terminal branches had high local PP. Two species of *Oreogrammitis* and three of *Radiogrammitis* formed a clade that was sister to all other ingroup taxa. Three *Radiogrammitis*, 13 *Oreogrammitis* and two *Themelium* (>2 species) clades were obtained.

Multiple accessions of many ORT species were polyphyletic. *Oreogrammitis hookeri*, *O. forbesiana* and *O. baldwinii* formed a monophyletic group in all nuclear and chloroplast gene analyses but in the super tree two accessions of *O. hookeri* (HW22 and 1116), HW15 *O. baldwinii* and 1321 *O. forbesiana* appeared in two different places in phylogeny separately from the major clade of *O. hookeri*, *O. baldwinii* and *O. forbesiana*. Many species showed polyphyly in total evidence tree (Figure 2.5). The type species of *Radiogrammitis*, *R. setigera*, appeared in three places in the super tree and showed close affinity to *Oreogrammitis* species. *Themelium tenuisectum*, type species of *Themelium*, arose in two places with *T. blechnifrons*, *T. decrescens* and *T. conjunctisora*. *Themelium yoderi* (VT469) appeared separately as in a few other analyses.

Large *O. longiceps*, *O. sumatrana* and hybrid 2 and 3 clade was recovered in super tree as in chloroplast and nuclear species trees. Also, some *O. archboldii* and *O. scabristipes* accessions closely related in this analysis as in others. Hybrid 1 was related to *O. frigida* and recovered in the same clade with *O. achboldii* (placement of hybrid 1 is uncertain but in many analyses, it showed closely affinity to *O. archboldii*).

## **2.4. Discussion**

### ***2.4.1 Phylogenetic Analysis of Nuclear and Plastid genes***

Internal nodes were well resolved and supported in the most nuclear gene phylogenies. while inner clades, clade 2 and 3 were less resolved and mostly polytomous suggesting that the gene markers used in the study are slowly evolving when compared to the rate of species diversification. The species relationships were highly incongruent in nuclear gene analyses indicating that the underlying evolutionary trajectories are different in each nuclear gene that were used in this study. Thus, the combined analysis of all the gene matrices were not applicable to interpret phylogenetics of the ORT genera. Therefore, the nrConcat analysis were not given in detail here.

The incongruency among gene trees is a common phenomenon. The gene tree discordance can be due to several factors, such as analytical factors (the choice of optimality criteria, taxon sampling and the predictions formulated about sequence evolution), biological factors include use of non-orthologous genes due to lineage sorting, hidden paralogy and horizontal gene transfer. Biological factors further include the stochastic error occurred due to sampling bias towards length of genes and use of genes without enough phylogenetic signal. These biological factors are beside the three main mechanisms, hybridization, natural selection

and recombination that cause incongruency. Further, incongruency can occur due to sequence alignments as it can misinterpret the true homology of data and the heterotachy (evolutionary rate of a given site) are the main causes of gene tree incongruence (Som, 2014). As a result, incomplete lineage sorting (ILS) was taken into consideration when constructing species tree. The five individual unrooted ML nuclear gene trees were combined to find the species tree under multi-locus coalescent model (Mirarab et al., 2014). Incomplete lineage sorting of genes may cause highly incongruent phylogenies, but ILS was not apparent among the genes (quartet score was high in the nuclear species tree) that were used in this study in contrast to the slowly evolving nature of the genes (Yu et al., 2013). Therefore, the incongruency can be due to other factors like lack of enough phylogenetic signal in the genes used in the study. This has been seen in gene phylogenies as some of the relationships were unresolved and poorly supported. Besides, the hybridization among species such as hybrid 2 and historical hybrids such as *O. sumatrana* (Parris, 1984) may have played an important role in shaping gene phylogenies.

In contrast, chloroplast phylogenies were well resolved and resulting topologies were highly similar to each other. Quartet scores were relatively high for both the pConcat and the species tree obtained from ASTRAL, thus ILS is not evident in the chloroplast markers. Chloroplast genes undergo similar fates of evolution due to their linked nature, and homogeneity between gene trees is expected (Vogl et al., 2003). Nuclear genes generally provide better resolution of species relationships since nuclear loci are biparentally inherited along with expected high rates of intraspecific gene flow (Yu et al., 2013), but it is important to select fast evolving genes especially when the speciation rates are high. The ORT clade has shown high rates of diversification (unpublished data), thus selection of suitable gene markers is important. A good candidate gene for future examination may be *LEAFY* which showed high variability

compared to the other nuclear genes. *LEAFY* is analogous to the floral-development genes in *Arabidopsis* and *Anthurium* (Shepherd et al. 2008).

#### **2.4.2 Orphaned Species**

Species level relationships of the ORT taxa were not inferred with high confidence in this study albeit all analyses confirmed the non-monophyly of some species. Multiple accessions of some species that were examined, such as *O. locellata*, *O. sumatrana*, *O. reinwardtii* and *R. setigera*, appeared in different places of the phylogeny, therefore the species relationships were uncertain. Such uncertainty could be due to some accessions being incorrectly identified. Most of the sample identity was confirmed by grammitid taxonomists. There were 17 uncertain species and about 25 accessions of those species were included in the study. Some of them recovered with the correctly identified species while confirming the identity (e.g. VT543 and VT581 *O. sumatrana* species identity was not confirmed but recovered with other *O. sumatrana* accessions). The incorrect identification may have been due to the close resemblance of unrelated species due to the presence of cryptic species complexes (Bauret et al., 2017). The unrelated species may have convergently evolve to resemble another species as it is difficult to discern the species identity.

*Oreogrammitis fasciata* (described below) showed close affinity to *Themelium* in all the phylogenetic analyses. Unlike other *Themelium* species *O. fasciata* has simple leaves. It is recommended to include *O. fasciata* in *Themelium* if the genus is continuing to exist.

### **2.4.3 Taxonomic Identity of Putative Hybrids**

The current study included three putative hybrids between *O. sumatrana* and *O. longiceps*. Only two (hybrid 2 and 3) showed close relatedness to their either putative parents in all analyses. While hybrid 1 showed close affinity to *O. archboldii* in majority of phylogenetic analyses. Thus, hybrid 1 may not be a hybrid of *O. sumatrana* and *O. longiceps* but may have possess similar characteristics led to mistakenly identify as a hybrid. Parris (1984) stated that the *O. sumatrana* can be a stabilized hybrid between unknown *Oreogrammitis* species or *O. torricelliana* and *O. longiceps*. This can be true as *O. sumatrana* was consistently form clade with *O. longiceps* but only in a few occasions (the super species tree, *gapCp*) the focal species arose with *O. torricelliana*.

*Oreogrammitis forbesiana* has intermediate characteristics (sori position on fronds and hairiness) of *O. hookeri* and *O. baldwinii* and considered as a hybrid of the later species (Palmer, 2003). Current study did not support the hybrid nature of *O. forbesiana* in any phylogenetic analyses.

### **2.4.4 The Phylogenetics of the ORT clade**

All individual gene, gene concatenation and species tree analyses revealed that the ORT clades are polyphyletic. Polyphyly in the ORT clade among the recognized genera may similarly have been due to convergent evolution of genetically unrelated taxa in each genus. The genera have been in part distinguished by only the single character of rhizome symmetry.

*Radiogrammitis* is delimited by having radial rhizomes whereas *Oreogrammitis* and *Themelium* have dorsiventral rhizomes. Rhizome symmetry has been widely used in grammitids to identify related genera. For example, the large tropical Asian genera *Tomophyllum*, *Dasygrammitis*,

*Scleroglossum*, *Micropolypodium*, *Xiphopterella*, and *Calymmodon* share radial rhizomes and their relatedness is apparent in a grammitid phylogeny based on five chloroplast markers (Sundue et al., 2014). By contrast, radial rhizome symmetry is not monophyletic in the overall grammitid phylogeny having arisen independently several times from dorsiventral rhizomes (Ranker et al., 2004; Sundue, 2010; Sundue et al., 2010, 2014). Thus, radial rhizomes are homoplastic among grammitids. It has been hypothesized that the radial rhizomes were derived to explore more habitats that are not easily occupied by other epiphytes (Sundue et al., 2015). Radial rhizomes are sub-erect rhizomes as leaves are arranged helically, whereas dorsiventral rhizomes are erect with dorsal leaves (Sundue et al., 2014, 2015). Hence, rhizome symmetry character states are not always homologous characters. Even though, rhizome symmetry is useful for distinguish genera, but not suitable for defining phylogenetic relationships.

A second characteristic used to distinguish genera in the ORT clade is that of rhizome scales; scales are absent in numerous species of *Radiogrammitis* and present in *Oreogrammitis* (Parris, 2007). Absence of rhizome scales, however, is not a consistent trait across all *Radiogrammitis* species. For instance, *R. cheesemanii*, *R. parva*, and *R. taiwanensis* all possess rhizome scales ([www.efloras.org](http://www.efloras.org)). *Radiogrammitis parva* and *R. cheesemanii* are closely related to each other but *R. taiwanensis* is distantly related to those species (Figure 2.8) hence, this trait is highly variable and inconsistent even within the genus.

This study also demonstrated that *Themelium* species arise within the clade of *Oreogrammitis* and *Radiogrammitis*. In almost all the analyses, *Themelium* species form a cohesive group although are often paraphyletic with one or two species of *Oreogrammitis* included. In particular, *O. fasciata* was closely related to the *Themelium* species. Species of both genera share dorsiventral rhizomes and glabrous rhizome scales. Unlike *Oreogrammitis*,

*Themelium* does not have setose sporangia, and rhizome scales are usually subclathrate to clathrate. Frond dissection in *Themelium* is pinnate (bipinnate in *T. bipinnatifidus*). In contrast, *Oreogrammitis* fronds are usually simple (rarely pinnatifid or pinnate), this being the main character that differentiates *Themelium* from *Oreogrammitis*. Leaf dissection was used to define generic boundaries within grammitids. As recently recognized, leaf dissection is not homologous across the grammitid clade. Some genera, such as *Ctenopteris*, *Grammitis s.l.*, and *Terpsichore*, define on the leaf dissection. Because of the homoplasy of leaf dissection character, the aforementioned genera were identified as paraphyletic groups. Subsequently, those were circumscribed to include only monophyletic taxa (Ranker et al., 2004; Sundue, 2010).

*Oreogrammitis* is a paraphyletic with *Radiogrammitis* and *Themelium*. Previous studies of these genera used only a few samples of each for comparison and the results suggested that at least *Themelium* was monophyletic (Sundue et al., 2014). However, the present study was able to robustly demonstrate paraphyly in these three genera due to the much higher number of taxa sampled, nearly 50% of total species diversity of the genera, and the use of nine genetic markers including from both nuclear and chloroplast genomes. As such, it is the recommendation here to combine both *Radiogrammitis* and *Themelium* into *Oreogrammitis* since *Oreogrammitis* has nomenclatural priority over other two (first named in 1917 compared to 2007 for *Radiogrammitis* and 1997 for *Themelium*). This study suggests that *Radiogrammitis* is an artificial taxon based on the presence of radial rhizomes and species of *Radiogrammitis* were associated randomly within *Oreogrammitis* in phylogenetic analyses and multiple accessions of the type species, *R. setigera*, were also polyphyletic. *Themelium* as largely monophyletic but arose within *Oreogrammitis* and as such must be recognized as *Oreogrammitis*.

. The morphological definition of *Oreogrammitis* must be revised to include previously recognized *Radiogrammitis* and *Themelium* species. Recircumscription of the ORT clade is important as it will provide better resolution within the grammitid phylogeny.

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## Chapter 3 : Population Genetics of the Hawai'i Endemic *Oreogrammitis hookeri* (Polypodiaceae)

### 3.1 Introduction

The Hawaiian Islands exhibit one of the highest levels of vascular plant endemism in the world. Ecosystems of Hawai'i are dominated by angiosperms (approx. 80% of the species) and about 89% of them are endemic. In contrast, about 74% of ferns and lycophytes (pteridophytes) are endemic to the islands (Geiger and Ranker, 2005; Geiger et al., 2007; Baldwin and Wagner, 2010; Ranker, 2016).

Hawai'i is unique in its formation and it is remotely isolated from other land masses on Earth (Sakai et al., 2002). The archipelago was formed due to the volcanic activity of a stationary hot spot in the middle of the Pacific tectonic plate, which is moving northwestward. Hence, the oldest islands are located in the northwestern end of the chain whereas the larger and younger islands are in the southeast (Neill and Trewick, 2008). Apart from its volcanic origin, these islands are the most isolated large archipelago on Earth being about 4000 km from the closest continent, North America, thus limiting the colonization of organisms that are incapable of long-distance dispersal (Ranker, 2003, 2016; Sakai et al., 2002; Geiger et al., 2013). Limited colonization events to the islands provide more temporal and spatial opportunity for existing species to evolve in newly available habitats. Hawai'i's landscape harbors diverse habitats in a small area allowing organisms to radiate into many forms or species (Sakai et al., 2002). Further, natural and anthropogenic habitat alterations increase the rate of plant extinction in Hawai'i (Aradhya et al., 1991). Hence, it is recognized as one of the 25 biodiversity hotspots and the State with the highest rate of plant and animal extinction in the USA (Sakai et al., 2002).

The Hawaiian biota provides remarkable examples and insights of evolution, speciation and endemism. For example, Craddock (2000) stated that Hawai‘i is an evolutionist’s paradise. The endemism accompanied by intriguing patterns of evolution make the Hawaiian flora an excellent study system. There are well-known examples of species radiations after single colonization events. The Hawaiian silversword alliance (Asteraceae) comprises 33 species in three endemic genera (*Argyroxiphium*, *Dubautia* and *Wilkesia*) (Baldwin et al., 1991; Blonder et al., 2016). The Hawaiian lobeliads (Campanulaceae) represent the largest adaptive radiation event in Hawai‘i with 136 species in six genera (Sporck-Koehler et al., 2015). Hawaiian *Bidens* (Asteraceae) includes 19 species and eight subspecies (Helenurm and Ganders, 1985; Knope et al., 2012) with more morphological variation than is present among more than 200 other species in the genus worldwide. The Hawaiian endemic mints (Lamiaceae) comprise 60 species in three genera, *Haplostachys*, *Phyllostegia* and *Stenogyne* (Lindqvist et al., 2005). Similarly, Hawaiian *Pittosporum* (Pittosporaceae), *Tetramolopium* (Asteraceae), and *Schiedea* (Caryophyllaceae) are other well-studied plant taxa radiated to form many species in this insular environment.

Species produced in adaptive radiations show diverse morphological features and occupy a wide range of habitats making them easy to identify among congeneric species (Knope et al., 2012). Thus, these distinct characters are highly useful for taxonomic and conservation purposes. In contrast, some plant conspecific populations show different morphological forms under different conditions. The best example is *Metrosideros polymorpha* Gaud. (Myrtaceae), locally known as “Ohi‘a lehua”. It is the dominant tree species of Hawai‘i colonizing new lava flows as a pioneer tree species and a forest dominant in climax rainforests. As its name applies, this taxon has many morphological forms associated with altitudinal gradients and age of the substrate (Stemmermann, 1983; Aradhya et al., 1993; Stacy et al., 2014; Izuno et al., 2017).

On the other hand, some conspecific populations represent cryptic species or incipient speciation events that are morphologically indistinguishable from each other (Liu et al., 2018). The phenomenon of cryptic speciation appears to be much more common among Hawaiian insects than among plants. For example, an endemic katydid in the genus *Banza* underwent cryptic speciation on O'ahu and Hawai'i island (Shapiro et al., 2006), two sympatric species of swordtail crickets on the island of Hawai'i reflect cryptic speciation (Mendelson and Shaw, 2002), and cryptic species exist in Hawaiian thrips (Rebijith et al., 2014). Cryptic species are also found in the red alga, *Gibsmithia hawaiiensis*, endemic to the islands, where five have been identified (Gabriel et al., 2016).

Population divergence and speciation are temporal processes (e.g., Bacon et al., 2012). With adequate time, some populations may diverge from other conspecific populations due to the generation of reproductive barriers and differential adaptation into different habitats. Such diverging lineages may or may not be morphologically distinguishable from each other (Aleixandre et al., 2013). Among island biotas, identification of species boundaries is daunting when compared to continental regions because most islands are recently formed (Carlquist, 1974). Younger islands provide more habitats, but less time to speciate resulting in incomplete lineage sorting among taxa. Species delimitation of those populations can be difficult and commonly represent cryptic species complexes or incipient species.

Grammitid ferns (Polypodiaceae) comprise approximately 15% of Hawaiian fern species. There are about 15 endemic species of Hawaiian grammitids in three genera: *Adenophorus* Gaudich., *Oreogrammitis* Copel., and *Stenogrammitis* Labiak. Species of these genera are primarily epiphytic but may occasionally grow on mossy rocks (Ranker et al., 2003). Molecular phylogenetic studies provide evidence of independent colonization events for each of the three

Hawaiian generic clades (Ranker et al., 2003, 2004; Geiger et al., 2007), but the exact origin of each lineage is still obscure.

Ranker (1992) examined the population genetic diversity of four endemic grammitid fern species using isozyme analyses, including *A. tamariscinus* (Kaulf.) Hook. & Grev., *A. tripinnatifidus* Gaudich., *A. tenellus* (Kaulf) Ranker (syn. *Grammitis tenella* Kaulf.), and *O. hookeri* (Brack.) Parris (syn. *Grammitis hookeri* (Brack.) Copel.). Genetic variability of all four species was high compared to vascular plants in general with mostly little or no evidence of inter-island population differentiation suggesting that the species were highly outcrossing. The single island populations of *O. hookeri*, however, had relatively high frequencies of island-unique alleles suggesting that they were possibly at the early stages of evolutionary divergence (that is, incipient species). *Oreogrammitis hookeri* population differentiation was evident even among some of the closely located populations on Hawai'i Island (Kīlauea and Ola'a) (Ranker, 1992) even though the populations were morphologically indistinguishable.

Proximity of islands promotes the wide distribution of ferns across the islands because ferns are well known for their capability to produce large numbers of small dust-like spores that can disperse successfully over a broader geographic area (Rose and Dassler, 2017). Hence, ferns are a highly outcrossing group of plants when compared to seed plants. As a result of their high dispersal ability that would tend to inhibit the genetic divergence of populations, a slower rate of speciation can be seen in ferns; therefore, generic and specific endemism of ferns are relatively low compared with angiosperms (Smith, 1972; Ranker and Sundue, 2015).



**Figure 3.1:** Endemic Hawaiian *Oreogrammitis* species. A, & B, *O. hookeri*: A, Sori position and hairs: B, habitat and growth habit: C, *O. baldwini* sori position: D, *O. forbesiana* sori position. Photo Credits, C, KenWood, PTBG

Population genetic diversity of ferns is important to study. High genetic variation among populations provides the opportunity for species evolution and to implement conservation measures (Luan et al., 2006). Also, studies on population differentiation may provide evidence of incipient speciation or formation of cryptic species. Ranker (1992) suggested that the Hawaiian endemic *O. hookeri* may harbor at least two cryptic species.

The objective of the present study was to analyze the genetic diversity among populations of *O. hookeri* from the islands of Kaua‘i, O‘ahu, Maui, Moloka‘i, and Hawai‘i to identify genetic structure among the populations and islands and assess if cryptic speciation has occurred or if incipient speciation is occurring.

## **3.2 Materials and Method**

### **3.2.1 Sample collection and total genomic DNA extraction**

Sample collection of *O. hookeri* was attempted on all the main Hawaiian Islands except Lāna‘i. I visited sampling locations identified by Ranker (1994) and found new locations by making observations of herbarium records at Bernice Pauahi Bishop Museum (BISH), National Tropical Botanical Garden Herbarium (PTBG), and Joseph F. Rock Herbarium (HAW). The number of populations collected from each island varied (Figure 3.2, Table 3.1). Only one population was found on each of the islands of Kaua‘i, O‘ahu, and Moloka‘i, two from Maui, and five from Hawai‘i. Plant samples were cleaned, air dried, and stored at 4°C until DNA was extracted. One individual was collected for DNA extraction from each colony (e.g., host plant) to avoid clonally propagated plants. The number of individuals sampled from each population varied and depended on the population size at each location (Table 3.1). If the plants were abundant and easily located, plants were collected from 20–30 different colonies. Whereas if the

plants were rare and difficult to locate, one individual from each colony (even though the colonies were in close proximity) was collected (typically 5–10 samples). Conversely, if the plants were abundant, individuals were collected ca. 50 m apart (20–30 samples). Geo-location data were recorded, and voucher specimens were deposited at HAW or PTBG (Table 3.1).

Total genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987; Morden et al., 1996) from one or two fresh leaves per plant. Fronds were ground in a buffered solution with 2% CTAB, 100mM Tris-HCl (pH 8.0), 1.4M NaCl, 2% PVP-40, 4mM diethyldithiocarbamic acid, and 20mM EDTA, with 0.2%  $\beta$ -mercaptoethanol. Extracted DNA was purified with a phenol-chloroform extraction followed by ethanol precipitation, and the pellet was resuspended in 50  $\mu$ l TE (Sambrook et al., 1989). Concentration of extractions were assessed with a NanoDrop spectrophotometer (Thermo Fisher, Inc.). DNA extractions were accessioned into the Hawaiian Plant DNA Library (Morden et al., 1996) (Table 3.1).



**Figure 3.2:** *Oreogrammitis hookeri* s.l. sample collection sites of main Hawaiian Islands (Kaua'i, O'ahu, Maui, Moloka'i and Hawai'i ). Collection sites are demarcated by red dots.

### 3.2.2 ddRADseq DNA Library Preparation

DNA extractions from 97 accessions of *O. hookeri* representing 11 different localities were used for the study. The DNA samples were selected based on the availability of DNA extractions and the quality and quantity of the DNA extractions (Table 3.1). DNA samples were diluted using sterilized distilled water to 30–40 ng/μL prior to library preparation.

Libraries for individual samples were prepared following Parchman et al. (2011, 2012). Reduced representation genomic ddRAD DNA libraries were prepared by digesting genomic DNA with the restriction enzymes *EcoRI* and *MseI* (NEB, Inc.). *EcoRI* and *MseI* adaptors were annealed to restriction-digested reactions using a ligase enzyme. The *EcoRI* adaptor contained a barcoded identifier [terminating with Cytosine (C)] *EcoRI* end of the fragment (*EcoRI* side: 5'-CTCTTTCCTACACGACGCTCTTCCGATCT + 10 bp barcode + C). Similarly, an identifier (minus the barcode) terminating with a Guanine (G) were ligated to the *MseI* side (*MseI* side: GCAGAAGACGGCATAACGAGCTCTTCCGATCT + G). Restriction-ligation reactions were diluted with water and PCR amplified with the Illumina Universal PCR primers (Forward: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACA  
CGACGCTCTTCCGATCT; Reverse: CAAGCAGAAGACGGCATAACGAGCTCTTCCGATCT) (Illumina, Inc.) using iproof high-fidelity DNA polymerase (New England Biolabs). Separate PCR products representing each individual were then combined into a cocktail containing all individuals, primers, and PCR reagents, the cocktail was divided into two duplicates, and the PCR was repeated for each duplicate. The duplicate PCR products were then pooled together. This was done to minimize the stochastic variation in PCR amplification as described in Parchman et al. (2011, 2012 and therein). An extra PCR step was conducted to convert single-stranded template from the previous

PCR reaction to double stranded DNA.

### **3.2.3 Sample Pooling and Visualization**

The Agilent 2100 Bioanalyser system (Agilent Inc.) at the Center for Integrated Biosystems at Utah State University was used for sizing, quantification, and quality control of DNA. Pooled samples were run on the Blue Pippin automated size selection platform (Sage Science, Beverly, MA). Amplified products in the 350 to 450 bp size range were selected and extracted for sequencing at the Illumina Sequencing facility at University of Wisconsin Madison Biotechnology Center on the HiSeq2500 sequencing platform.

**Table 3.1:** *Oreogrammitis hookeri* collections across the main Hawaiian Islands

Collection ID of studied samples	HPDL*	Location	Herbarium	Total No. of samples	No. of samples used	No. of retained samples by ipyrad
KMO1, 4, 6, 9, 11, 12, 14	9889, 9892, 9894, 9897, 9899, 9900, 9902 9904 - 9908	Mohini, Kauai	PTBG	20	11	10
KMO16 - 19						
OWO1, 2, 5, 9, 11, 12, 14, 15	9710, 9711, 9714, 9709, 9705, 9706, 9708, 9709 N/A	Wainae kai**, O'ahu	HAW	20	11	10
OKO1_3, 2, 3		Kaala Natural Area**, Oahu		9		
MWO1, 2, 4, 8, 10, 13, 15, 17, 20, 21, 22	9663, 9664, 9666, 9670, 9673, 9677, 9679, 9682, 9685, 9686, 9688	Waikamoi, Maui		25	11	11
MPO1, 2, 14, 19	9826, 9827, 9843 9850	Puu kukui, Maui		4	4	3
MKO10, 17, 1, 19 MKO22, 18	9911 - 9914 9916, 9917	Kamakou, Molokai		22	5	2
HSO1_2, 1_4, 1_6, 1_7, 2_1, 2_2, 2, 3_1, 3_3, 3_5, 4,	8835, 8837, 8839, 8840, 8842, 8843, 8841, 8844, 8847, 8849, 8860	Saddle Road, Hawaii		20	11	10
HOO2, 4, 7, 9, 12, 13, 18, 19, 23, 26, 29	8852, 8854, 8857, 8859, 8862, 8863, 8868, 8869, 8873, 8876, 8879	Olaa, Hawaii		29	11	8
HPO3, 2, 5, 6, 9 -11, 13, 15 - 17	9828, 9827, 9830, 9831, 9835, 9836, 9838, 9730, 9732, 9733, 9734	Puu makai, Hawaii		18	11	8
HKO1 - 7 HKO9	9862 -9869 9871	Kahauale'a, Hawaii		9	9	8
HVL1, 3, 5, 6, 8, 9, 11	9872, 9874, 9876, 9877, 9879, 9880, 9882	Large pit crater***, Volcanic National Park (VNP), Hawaii		11	7	7
HVS1 - 6	9883-9888	Small pit crater***, VNP, Hawaii		6	6	4

\*Hawaiian Plant DNA Library

\*\*Waianae Kai Forest reserve and Kaala Natural Area preserve are continuous forest area

\*\*\*Large and small pit craters are located close to each other. Treated as two populations for this study.

### 3.2.4 Sequence Assembly and Data Analysis

ddRADseq raw data files (Illumina FASTQ output files) were assembled and analyzed using ipyrad v. 0.7.28 (<http://ipyrad.readthedocs.io>). A parameter file with threshold values was set to complete the following seven sequential steps in the ipyrad workflow. Parameters were slightly modified from the default values to best suit the focal species. The ipyrad analysis was carried out separately in the High-Performance Computing (HPC) clusters at the University of Hawai‘i at Mānoa and Utah State University. This process was completed twice and samples with counts less than 17 reads were discarded.

1) *Demultiplexing/loading fastq files* – Barcode sequences of 97 samples were used to demultiplex the sequence reads.

2) *Filtering/editing reads* – Low quality base calls ( $Q < 20$ ) were filtered from fastq data files changed into “N”s and reads with more than the number of allowed “N”s (4) were discarded. Sequence reads greater than the 33 phred Qscore threshold were included in downstream analyses and others were removed. Adapter and primer sequences were also trimmed in this step.

3) *Clustering/mapping reads within samples and alignment* – The selected reads from the previous step were. Within-sample clusters were generated using USEARCH (Edgar, 2010). The resulting data were *de novo* assembled using VSearch (Enns et al., 1990). For each sample, sequences were clustered by similarity (here  $>90\%$ ) and aligned using MUSCLE (Edgar, 2004).

4) *Joint estimation of heterozygosity and error rate* – Sequencing error rate and heterozygosity across clusters were estimated. Maximum number of heterozygotes allowed was four in consensus.

5) *Consensus base calling and filtering* – Clustered reads were used to estimate consensus allele

sequences.

6) *Clustering/mapping reads among samples and alignments* – Consensus sequences across all the samples clustered. Clusters representing putative RAD loci shared among samples were aligned with MUSCLE. If heterozygous alleles (allowed frequency=0.5) appeared in a set of samples, they were discarded assuming that they were paralogs and more likely to be shared across multiple samples rather than ancestral polymorphisms.

7) *Filtering and formatting output files* – Twenty maximum alleles/SNP at each locus were filtered and output files were generated in several different formats.

The above process was carried out twice and the samples with counts of less than 17 were discarded because number of non-missing loci per sample is higher above count 17.

### **3.2.5 Population Genetic Structure Analysis**

The program STRUCTURE 2.3.4. (Pritchard et al., 2000) was used to estimate the genetic clusters of samples selected from ipyrad analysis. Ipyrad output file .ustr was used for the STRUCTURE analysis. STRUCTURE is a Bayesian, model-based algorithm used to cluster individuals to populations based on their allele frequencies. The number of clusters ( $K$ ) can be defined by the user and assumes Hardy-Weinberg equilibrium within clusters (Hubisz et al., 2009). The multi-locus data (.ustr file from ipyrad analysis) were analyzed to determine the population structure of *O. hookeri*. Options for the admixture model and the correlated allele frequencies between populations were chosen as recommended by Falush et al., (2003). Default values were set for all the other parameters. A burn-in length of 1,000,000 followed by MCMC run length 5,000,000 was used; 50 runs were carried out for  $K$ -values ranging from 2-9.

**Table 3.2:** The number of loci caught ipyrad analysis after application of different filters in RADseq data assembly steps

<b>Filter</b>	<b>Total filters</b>	<b>Applied order</b>	<b>Retained loci</b>
Total prefiltered loci	175229	0	175229
Filtered by remove duplicates	7234	7234	167995
Filtered by maximum indels	5910	5910	162085
Filtered by maximum SNPs	5903	388	161697
Filtered by maximum shared heterozygosity	181	65	161632
Filtered by minimum sample	173575	161030	602
Filtered by maximum alleles	47863	346	256
Total filtered loci	256	0	256

To further test the natural genetic clusters of the selected polymorphic loci, Discriminate Analysis of Principal Coordinates (DAPC) (Ivandic et al., 2002) in *adegenet* 2.0.0 (Jombart 2008; Jombart et al., 2010) in R software (R Development Core Team, 2009) was used. This method was used because it is a combination of Principal Component Analysis and Principal Coordinates Analysis. In DAPC analysis, variance between groups is maximized while the variance within groups or clusters is minimized. The optimum number of clusters were found by testing a range of  $K$  values ( $K=2$  to  $K=9$ ). Those different  $K$  values were then compared using the Bayesian Information Criterion (BIC). The lowest BIC value was selected as the best  $K$ .

### ***3.2.6 Identification of Best $K$ and Graphical Illustration of Results***

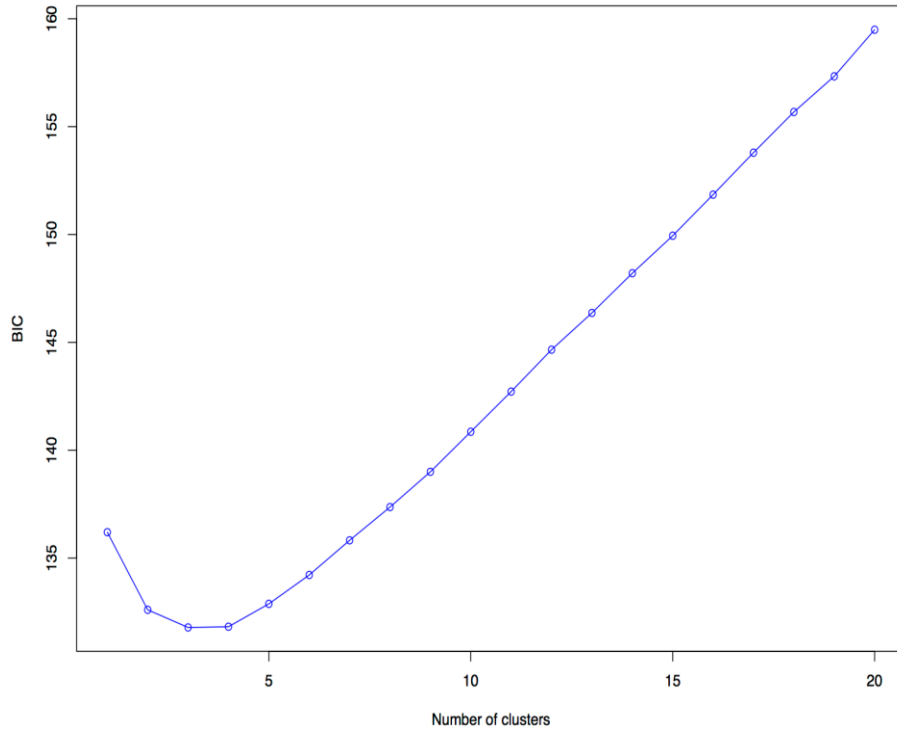
Clustering Markov Packager Across  $K$  (CLUMPAK; available from [clumpak.tau.ac.lk](http://clumpak.tau.ac.lk)) was run to summarize membership coefficient matrices (Q-matrices) that arose from runs within and across  $K$  values of STRUCTURE analyses. Runs were clustered and averaged by CLUMPAK using the Markov clustering algorithm. The most likely  $K$  was generated in CLUMPAK using the Evanno method (Evanno et al., 2005) and the Pritchard method (Pritchard et al., 2000). DISTRUCT (Rosenberg 2004; available within CLUMPAK) was utilized for graphical display of the results. The Q-matrices were also summarized in STRUCTURE HARVESTER (available at <http://taylor0.biology.ucla.edu/structureHarvester/>) for the easier detection of genetic groups that best fit the data (Earl and vonHoldt, 2012).

DAPC analysis used the built-in functions to summarize and visualize data. Scatterplots were obtained for different BIC values at each cluster and optimum  $K$  values were plotted in scatterplots of the first and second linear discriminant axes of DAPC.

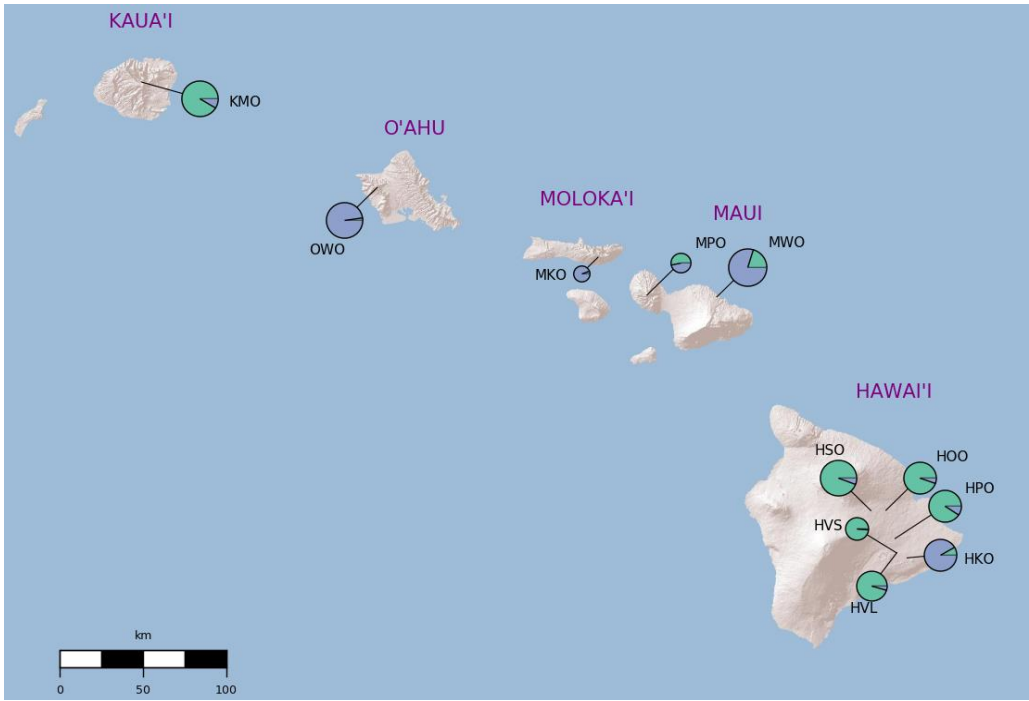
### 3.3 Results

Initial analysis of data from RAD-seq yielded about 175,230 loci. After application of several filters in ipyrad analysis, 256 loci were recovered (Table 3.2). Out of 256 loci, 176 loci were used for STRUCTURE and DAPC analyses. Those were a subset of loci randomly selected with one variable site per locus. Of the 87 individuals examined, six individuals had reads of 17 or less, thus 81 individuals of *O. hookeri* were retained for genetic analysis.

The distribution of  $\Delta K$  across values from  $K=2$  to  $K=9$  were examined in STRUCTURE HARVESTER. Results indicated that the best fit partition for the data was  $K=3$  as it had the highest mean likelihood from STRUCTURE output. The DAPC outputs were further analyzed using the Bayesian Information Criterion (BIC), and these showed that the best  $K$  values were  $K=3$  or  $K=4$  (Figure 3.3). However, CLUMPAK analyses of the Q matrices from STRUCTURE analysis,  $K=2$  resulted in a single mode (referred as major mode). At  $K=2$  all the 50 replicates of STRUCTURE runs were included in the major mode. Whereas at  $K=3$  and  $K=4$  two modes, major and minor, were given (35 of the 50 runs and 23 of 50 runs, respectively) as a consequence of discrepancies between the replicates.



**Figure 3.3:** A scatter plot recovered from DAPC analysis in Adegent package in R shows the number of populations (clusters) and Bayesian Information Criterion (BIC) values. The best number of clusters correspond to the lowest BIC value;  $K=3$  and  $K=4$

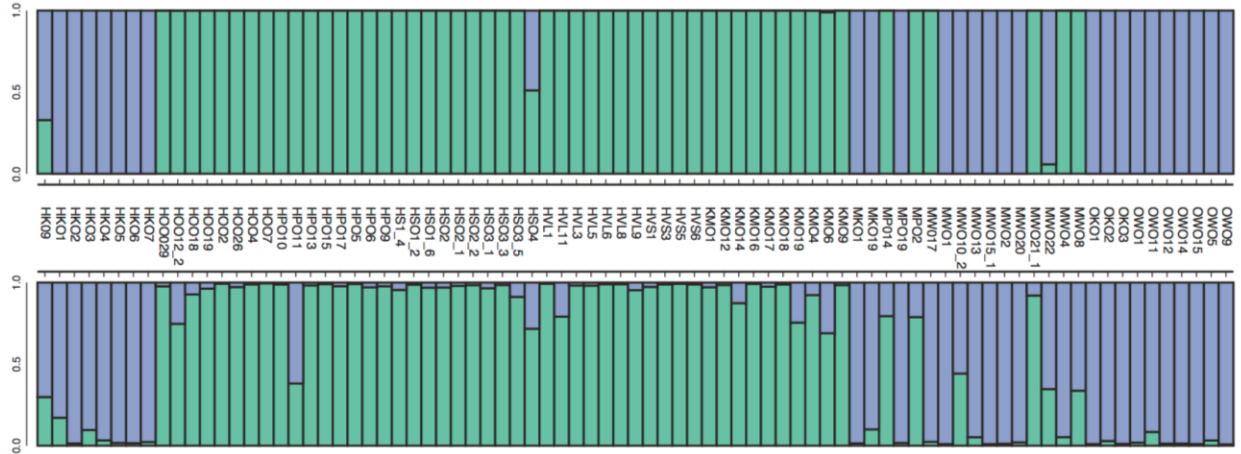


**Figure 3.4:** A map of the sampling locations and proportion of each of the 81 individuals of *O. hookeri* assigned to 2 clusters identified in STRUCTURE. The pie charts showing the allele frequency of each cluster in the population. KMO: Kaua'i Mohihi; OWO: O'ahu Waianae; MKO: Moloka'i Kamakou; MPO: Maui Puu kukui; MWO: Maui Waikamoi; HSO: Hawai'i Saddle Road; HOO: Hawai'i O'laa; HPO: Hawai'i Puu Makala; HKO: Hawai'i Kahauale'a; HVL & HVS: Hawai'i Volcano National Park Large and Small pit craters

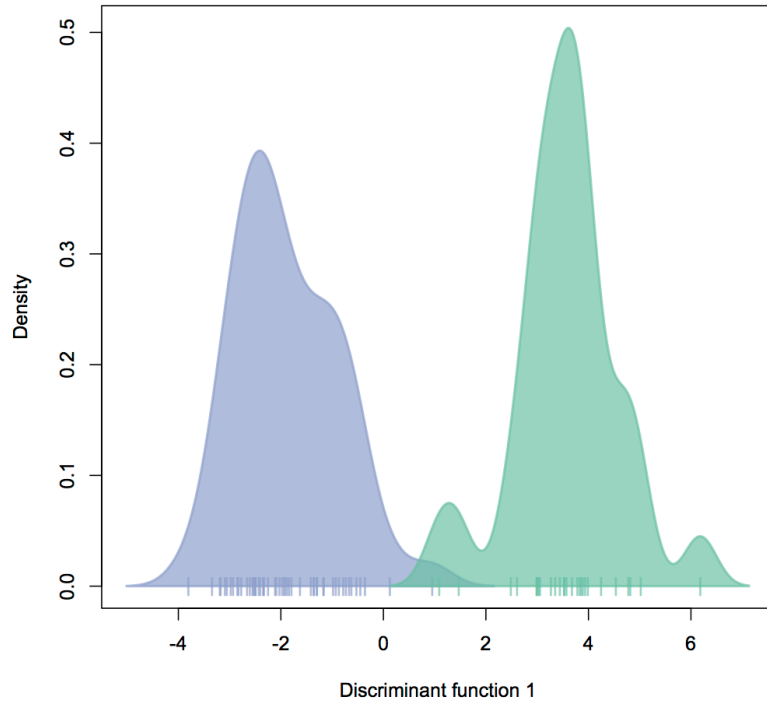
In  $K=2$ , the samples from the O‘ahu population and the Kahauale‘a population on Hawai‘i Island formed one cluster. The second cluster was formed by the individuals collected from Kaua‘i and other locations on Hawai‘i Island. In the case of Hawai‘i, *O. hookeri* samples collected from adjacent localities clustered separately from each other (Figure 3.4). In DAPC analysis, membership probabilities were 1.0 (or exclusive assignment of individuals to either cluster) for most of the samples whereas STRUCTURE showed admixture (or lower individual assignment), but most individuals were clearly assigned to each cluster (Figure 3.5). Samples collected from Maui and Moloka‘i belonged to one or the other cluster. Discrimination of individuals into two groups was also observed when density of individuals was plotted against one discriminant function (Figure 3.6).

When the  $K$  values were increased to  $K=3$  and  $K=4$ , clustering patterns were obscured (Figure 3.7). Clustering occurred within the same sample while major clusters (O‘ahu and

Kahauale‘a; Kaua‘i and Hawai‘i) remained as in  $K=2$  except in  $K=4$  where O‘ahu and Kahauale‘a were separated from each other (Figure 3.7). But the two discriminant functions clearly showed the assignment of genetically related groups of individuals for  $K=3$  and  $K=4$  separately (Figure 3.8).



**Figure 3.5:** Genetic structure Bar graphs of *O. hookeri* s.l. generated from DAPC (top panel) STRUCTURE (bottom panel) analyses at  $K=2$  from 176 SNP loci. Each of the 81 individuals genotyped in this study represented by a single vertical bar (x-axis) partitioned into two colored segments which represents the individual’s probability of belonging to one of two ( $K=2$ ) groups. “Group 1” is represented in green, while “group 2” is represented in blue. General localities are given along the top of the DAPC bar graph. KMO: Kaua’i Mohihi *Oreogrammitis*; OWO: O’ahu Waianae *Oreogrammitis*; MKO: Moloka’i Kamakou *Oreogrammitis*; MPO: Maui Pu kukui *Oreogrammitis*; MWO: Maui Waikamoi *Oreogrammitis*; HSO: Hawai’i Saddle Road *Oreogrammitis*; HOO: Hawai’i Olaa *Oreogrammitis*; HPO: Hawai’i Pu Makala *Oreogrammitis*; HKO: Hawai’i Kahauale’a *Oreogrammitis*; HVL & HVS: Hawai’i Volcano National Park Large pit crater and Small pit crater

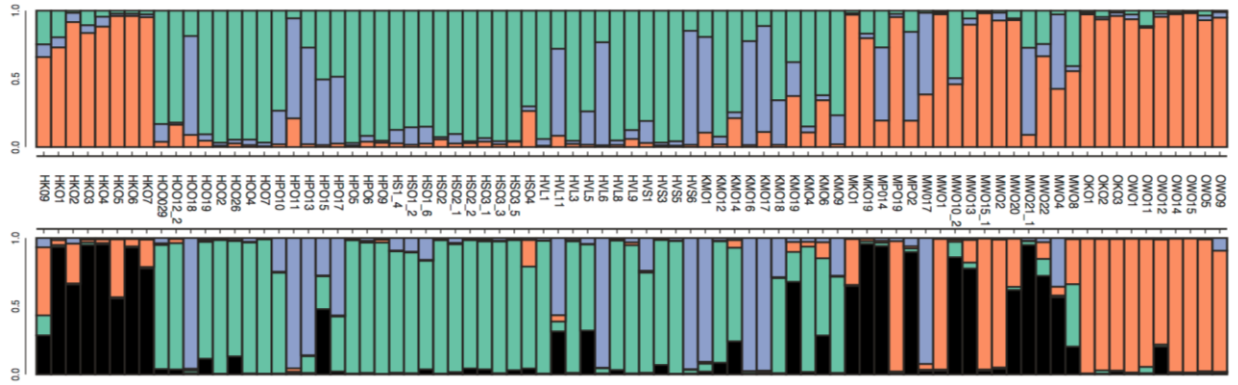


**Figure 3.6:** A plot of the individual densities against the first discriminant function retained show that the greatest proportion of variation lies with it;  $K=2$

### 3.4 Discussion

For the current study, I implemented the RADseq approach that discovered a large number of SNPs across the 81 *O. hookeri* samples. The number of SNP loci derived here was less compared to some other ddRADseq studies such as Gompert et al. (2014), Lemon and Wolf, (2018), and Rowe et al. (2018). This could be partly due to *de novo* assembly (absence of a reference genome) of short reads (Lischer and Shimizu, 2017) and therefore many loci were discarded. Also, stringent filtering of loci in different steps of bioinformatic pipelines may remove informative loci from the final output.

The number of genetic units of *O. hookeri* was determined by two approaches. STRUCTURE analysis (Pritchard et al., 2000) indicated that there were three genotypic units while DAPC suggested that three or four source populations were equally likely in the data. Novembre (2016) stated that setting a meaningful number of populations ( $K$  values) is difficult in STRUCTURE analyses. Thus, others have suggested using a range of  $K$  values (e.g., Prichard et al., 2000; Falush et al., 2003) to obtain a reasonable interpretation and inference of population structure, but Novembre (2016) also noted that increasing the  $K$  value adds more parameters that causes overfitting of data. This phenomenon has been observed here with the estimated  $K$  values from STRUCTURE and DAPC. When values of  $K=3$  and  $K=4$  were tested, not all the replicates converged to the same output and therefore formed major and minor modes and the resulting bar graphs that were generated were unable to form clear resolution of the data. In contrast, when  $K=2$  clusters were examined, a more robust identification of individuals was obtained.

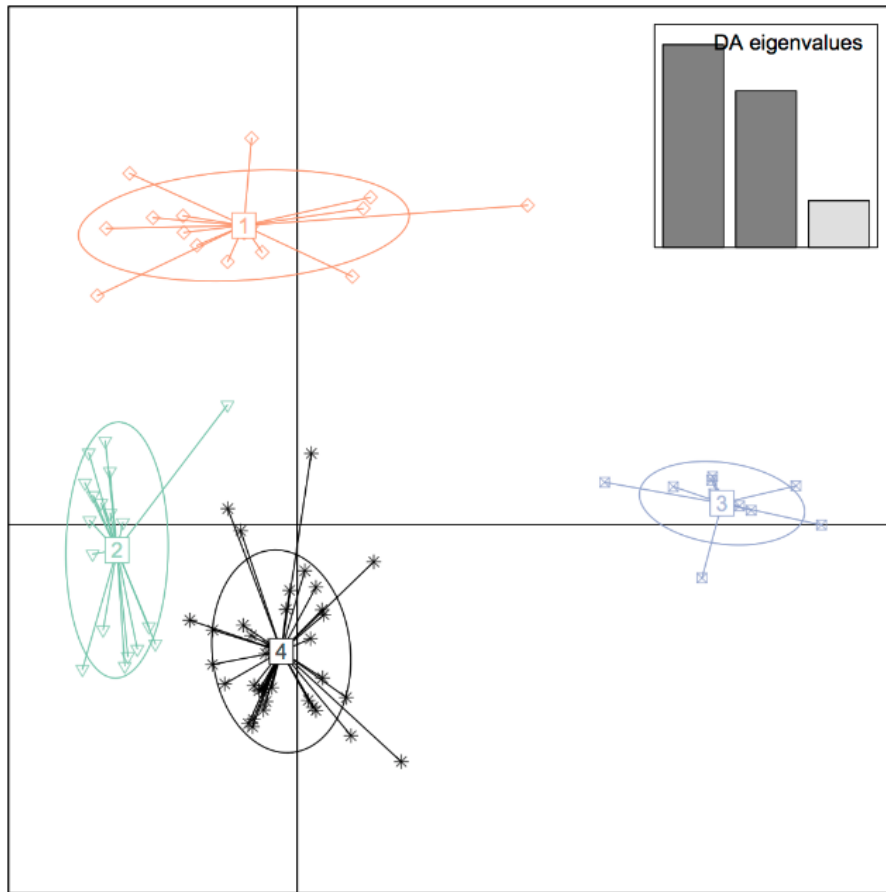


**Figure 3.7:** Genetic structure Bar graphs of *O. hookeri* s.l. generated from STRUCTURE analyses at  $K=3$  (toppanel) and  $K=4$  (bottom panel) from 176 SNP loci. Each of the 81 individuals genotyped in this study represented by a single vertical bar (x-axis) partitioned into colored segments which represents the individual's probability of belonging to one of two ( $K=3$  or  $K=4$ ) groups. Color scheme represents the clustering within individuals rather than forming separate clusters. General localities are given along the top of the  $K=3$  bar graph. KMO: Kaua'i Mohihi *Oreogrammitis*; OWO: O'ahu Waianae *Oreogrammitis*; MKO: Moloka'i Kamakou *Oreogrammitis*; MPO: Maui Pu kukui *Oreogrammitis*; MWO: Maui Waikamoi *Oreogrammitis*; HSO: Hawai'i Saddle Road *Oreogrammitis*; HOO: Hawai'i O'ahu *Oreogrammitis*; HPO: Hawai'i Pu Makala *Oreogrammitis*; HKO: Hawai'i Kahauale'a *Oreogrammitis*; HVL & HVS: Hawai'i Volcano National Park Large pit crater and Small pit crater

The present study most clearly indicates that *O. hookeri* consists of at least two genotypes. The samples collected from O‘ahu and the Kahauale‘a population on Hawai‘i Island are clearly distinct from Kaua‘i and other localities on Hawai‘i Island. Individuals collected from the islands of Maui and Moloka‘i had individuals with mixed genotypes.

Genetic distinctiveness of the Kahauale‘a population on Kīlauea was observed by Ranker (1992). He found that that population was distinct from two nearby populations on Mauna Loa (Figure 3.8), showing significant differences as estimated by  $F_{st}$  values. Ranker (1992) observed seven alleles unique to Hawai‘i Island, and the Kahauale‘a population uniquely possessed six of those seven alleles.

Thus, Ranker (1992) hypothesized that the Kahauale‘a population was diverging from other populations on Hawai‘i and that localized divergence might be occurring possibly indicating incipient speciation. Divergence of nearby populations is rare among ferns as they are usually highly outcrossing and thus gene flow among close populations is typically high. By including new samples from additional islands, however, the present study has shown that the Kahauale‘a population is not unique, with possibly the same genotype occurring on O‘ahu. In addition, the Moloka‘i and Maui populations are admixtures of the O‘ahu-Kahauale‘a and the Kaua‘i-Mauna Loa genotypes, thus supporting the idea that individuals from all sampling localities are part of one interbreeding lineage. Genetic differences among populations could simply be due to random processes such as founder effects although localized adaptation cannot be ruled out.



**Figure 3.8:** DAPC scatterplots drawn using the Adegnet package in R of existing genetic clusters of the best-fit models,  $K=3$  (left) and  $K=4$  (right). Dots represent individuals, with colors denoting sampling origin and inclusion of 95% inertia ellipses.

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## Chapter 4 : Investigation of Population Structure of Polymorphic Hawaiian endemic *Adenophorus tripinnatifidus* (Polypodiaceae)

### 4.1 Introduction

*Adenophorus* is one of three grammitid fern genera in Hawaiian Islands. Grammitids (Polypodiaceae) are a species-rich monophyletic group of mostly tropical ferns. Others are the widespread genera *Oreogrammitis* Copel. (three Hawaiian endemic species) and *Stenogrammitis* Labiak (one Hawaiian endemic species). The members of the genus *Adenophorus* are characterized by having putatively unique glandular, receptacular paraphyses (Bishop, 1974, Ranker et al. 2003). According to Parris (1998) and Ranker et al. (2003), the paraphyses typically comprise a uniseriate column of 2 to several cells with a much enlarged glandular, apical cell. Ten species are currently recognized: *A. abietinus* (D. C. Eaton) K. A. Wilson, *A. epigaeus* (L.E. Bishop) W. H. Wagner, *A. haalilioanus* (Brack.) K. A. Wilson, *A. hymenophylloides* (Kaulf.) Hook. & Gray, *A. oahuensis* (Copel.) L.E. Bishop, *A. periens* L.E. Bishop, *A. pinnatifidus* Gaudich., *A. tamariscinus* (Kaulf.) Hook. & Gray, *A. tenellus* (Kaulf.) T. A. Ranker and *A. tripinnatifidus* Gaudich. (including *A. hillebrandii* (Hook.) K. A. Wilson ) (Wagner, 1995; Ranker, 2003; Snow, 2011).

*Adenophorus* is an exceptional genus because it is one of two fern genera (along with *Cibotium* Kaulf.) with multiple endemic Hawaiian species following radiations derived via putative single colonization events (Schneider et al. 2004b). Other genera that have more than one endemic species to Hawai‘i originated from multiple colonizing ancestors (Ranker, 2016). The historical biogeography of *Adenophorus* is uncertain, but a Neotropical origin is suggested from molecular phylogenetic studies (Geiger et al. 2007, Sundue et al. 2014, Vernon and Ranker, 2013, Ranker, 2016) that supports a sister relationship to a Neotropical clade. The ancestors of

*Adenophorus* might have come to the islands via the trade winds or a storm system (Ranker et al. 2003). Further, biogeography of *Adenophorus* is fascinating since nearly 60% of Hawaiian fern and lycophyte flora originated in the Asian-Paleotropical region whereas only about 14% of ancestors dispersed from the Americas/Neotropics (Forberg, 1948; Geiger et al. 2007; Vernon and Ranker, 2013; Ranker, 2016).

Sundue et al. (2014) conducted a study on global phylogeny and biogeography of grammitid ferns that included several *Adenophorus* species. They estimated that the *Adenophorus* clade diverged from its sister lineage about 22.5 million years ago (Ma). This find is consistent with that of Clague (1996) and Price and Clague (2002) who hypothesized that colonizers were not able to establish on the Hawaiian Islands during an interval from 33 to 23 Ma.

A few studies have been completed examining populations of *Adenophorus* species. Ranker (1994) surveyed allozymic variability in *A. periens*, a species that is a rainforest dwelling and an extremely rare (and probably now extinct) epiphyte. His study revealed that individuals in the Kahauale‘a population, Hawai‘i Island (the only population known at that time) had high genetic variability despite their rarity and small population size. Factors that may be attributed to such high genetic diversity are unknown as there was no gene flow due to the absence of conspecific populations.

Ranker (1992) conducted a similar study on four endemic Hawaiian epiphytic fern species that included *A. tamariscinus* (Kaulf.) Hook. and Grev. and *A. tripinnatifidus* Gaudich. Genetic variability of populations of these species was high; *A. tamariscinus* exhibited more variability than *A. tripinnatifidus*. Interpopulation differentiation was not prominent among populations of *A. tamariscinus* suggesting high levels of gene flow. In contrast, *Adenophorus*

*tripinnatifidus* populations appeared to be undergoing early stages of evolutionary divergence or incipient speciation (Ranker 1992), suggesting this may be a real-time example of species radiation in a remote insular setting.

*Adenophorus tripinnatifidus* is reported to occur on all major high islands: Kaua‘i, O‘ahu, Maui, Moloka‘i, Lana‘i and Hawai‘i (Bishop, 1974, Palmer 2003). It is usually restricted to extremely wet forests at upper elevations between 600–1950 m (Ranker, 1994, Palmer, 2003). Morphologically, *A. tripinnatifidus* is a highly variable species even though it is restricted to very specific habitats. These plants are usually epigeous growing with moss mats at the base of trees and often misidentified as *A. tamariscinus*. However, *A. tripinnatifidus* can be distinguished by its slender, long-creeping rhizomes and usually 3-pinnate fronds (Palmer 2003). Pinnae division is extremely variable from 2-pinnate with no division of acroscopic basal pinnules to fully 3-pinnate out to the last pinnule. Those different morphotypes are localized, semi-localized, and seemingly stabilized (Palmer, 2003). Among them, a highly different population from Ko‘olau Range on O‘ahu Island was identified as *A. hillebrandii*. Plants of the Ko‘olau population have more leathery fronds, stout stipes, and winged rachises and costae, basal pinnae are not reduced in size and the ultimate segments are broad with acute tips (Palmer, 2003).

The presence of morphological variations in species, such as *A. tripinnatifidus*, provokes evolutionary studies that may delimit species boundaries. Prior to molecular genetic research, morphological data were solely used to define species and for many taxa their exclusive use continues today (Wiens and Penkrot, 2002) although these traits can be misleading. As an example, the Hawaiian *Bidens* L. (Asteraceae) populations are highly variable therefore, Shreff identified 43 species and more than 20 varieties whereas Ganders and Nagata recognized only 19 species and eight subspecies. In contrast to its morphological variation, the genetic

differentiation among taxa are very little. Therefore, presence of genetic groupings within the species is questionable in comparison to morphological groups (Helenurm and Ganders, 1985).

The current study was conducted to investigate the population genetic structure of *A. tripinnatifidus* and thereby to determine whether the conspecific populations of *A. tripinnatifidus* of different islands are diverging from each other.

## **4.2 Materials and Method**

### **4.2.1 Sample collection**

Sample collection of *A. tripinnatifidus* was attempted on all the main Hawaiian Islands except Lānaʻi. Initial sampling locations were identified from the literature, observation of herbarium records at the Bishop Museum (BISH) and the Joseph F. Rock Herbarium, University of Hawaiʻi at Mānoa (HAW) (Figure 4.1, Table 4.1). Also, expeditions were conducted in suitable locations which were not previously sampled (Figure 4.1, Table 4.1). I was able to collect two populations from Kauaʻi (20 samples from each location) and one each from Maui (14 samples) and Molokaʻi (eight samples). The species was not found on the islands of Oʻahu and Hawaiʻi despite personal knowledge of other botanists and the use of historical records from herbarium collections. When collecting samples, precautions were taken not to collect multiple individuals from the same colony (host or substrate) for DNA extraction to avoid clonally propagated plants. Plant samples were cleaned, air dried and stored at 4°C until DNA was extracted. The number of individuals sampled from each population varied and depending on the population size at each location (Table 4.1). If the plants were abundant and easily located, 20–30 plants were collected randomly from different colonies; if the plants were rare and difficult to find, one individual were collected from every colony and considered as a small population.

Geo-location data were recorded, and voucher specimens (2–3 plants per population) representing each population were deposited at HAW (all islands except Kaua‘i) and National Tropical Botanical Garden Herbarium (PTBG; Kaua‘i collections only) (Table 4.1).

Total genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987; Morden et al., 1996). One to two fresh leaves were used from each sample to extract DNA. Fronds were ground in a buffered solution with 2% CTAB, 100mM Tris-HCl (pH 8.0), 1.4M NaCl, 2% PVP-40, 4mM diethyldithiocarbamic acid and 20mM EDTA, with 0.2%  $\beta$ -mercaptaethanol. Extracted DNA was purified with a phenol-chloroform extraction followed by ethanol precipitation, and the pellet resuspended in 50  $\mu$ l TE (Sambrook et al., 1982). Quality and concentration of extractions were assessed with a NanoDrop spectrophotometer (Thermo Fisher, Inc.). DNA extractions were accessioned into the Hawaiian Plant DNA Library (HPDL; Morden et al., 1996) (Table 4.1).



**Figure 4.1:** *Adenophorus tripinnatifidus* sample collection sites of main Hawaiian Islands (Kaua‘i, Maui, and Moloka‘i). Collection sites are demacated by yellow dots.

**Table 4.1:** *Adenophorus tripinnatifidus* collections across the Hawaiian Islands

Collection ID of studied samples	HPDL*	Location	Herbarium	Voucher No.	Total No. of samples	No. of samples used	No. of retained samples by ipyrad
KHA1, 5, 8, 10, 12, 16, 20, 22, 24, 28, 18		HonopuValley, Kauai	NTBG	K.R. Wood 17147	29	11	10
KNA2, 3, 6, 9, 13, 16, 19, 21, 23, 26, 29,	9584, 9585, 9588, 9591, 9595, 9598, 9601, 9603, 9605, 9608, 9611	Nualolo (Upper), Kauai	NTBG	K. R. Wood 17132	29	11	9
MWA2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 14	9690, 9691, 9692, 9693, 9694, 9695, 9697, 9698, 9699, 9700, 9702	Waikamoi, Maui	HAW		14	11	10
MKA1, 3, 4, 5, 6, 7	9921, 9920, 9924, 9919, 9923, 9918,	Kamakou, Molokai	HAW		10	6	4

#### 4.2.2 Preparation of ddRADseq DNA Libraries

DNA extractions from 39 accessions of *A. tripinnatifidus* representing different localities/populations were used for the study. DNA samples were diluted using sterilized distilled water to 30–40 ng/μL prior to library preparation.

Libraries for individual samples were prepared following Parchman et al. (2011, 2012). Reduced representation genomic ddRAD DNA libraries were prepared by digesting genomic DNA with the restriction enzymes *EcoRI* and *MseI* (NEB, Inc.). *EcoRI* and *MseI* adapters were annealed to restriction-digested reactions using ligase enzyme. The *EcoRI* adaptor contained a barcoded identifier (terminating with a Cytosine) at the *EcoRI* end of the fragment (*EcoRI* side: 5'-CTCTTTCCCTACACGACGCTCTTCCGATCT + 10 bp barcode + C). Similarly, an identifier (minus the barcode) terminating with a Guanine were ligated to the *MseI* side (*MseI* side: GCAGAAGACGGCATAACGAGCTCTTCCGATCT + G). Restriction-ligation reactions were then PCR amplified with the Illumina Universal PCR primers (Forward: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACA CGACGCTCTTCCGATCT; Reverse: CAAGCAGAAGACGGCATAACGAGCTCTTCCGATCT) (Illumina, Inc.) using iproof high-fidelity DNA polymerase (New England Biolabs). Separate PCR products representing each individual were then combined into a cocktail containing all individuals, primers, and PCR reagents. The cocktail was divided into two duplicates, and the PCR repeated for each duplicate. The duplicate PCR products were then pooled together. This was done to minimize the stochastic variation in PCR amplification as described in Parchman et al. (2011, 2012). An extra PCR step was conducted to convert single-stranded template from the previous PCR reaction to double stranded DNA.

### 4.2.3 Sample Pooling and Visualization

Pooled PCR products (15  $\mu$ L) were visualized on an ~1% agarose gel to identify size range of the concentrated amplified product compared to a size standard. An Agilent 2100 Bioanalyser system (Agilent Inc.) at the Center for Integrated Biosystems at Utah State University was used for sizing, quantification, and quality control of DNA. Pooled samples were run on the Blue Pippin automated size selection platform (Sage Science, Beverly, MA). Amplified products in the 350 to 450 bp size range were selected and extracted for sequencing at the Illumina Sequencing facility at University of Wisconsin Madison Biotechnology Center on the HiSeq2500 sequencing platform.

### 4.2.4 Sequence Assembly and Data Analysis

ddRADseq raw data files (Illumina FASTQ output files) were assembled and analyzed using ipyrad v. 0.7.28 (<http://ipyrad.readthedocs.io>). A parameter file with threshold values was set to carry out the following seven sequential steps in ipyrad workflow. Parameters were slightly modified from the default values to best suit the focal species. The ipyrad analysis was carried out in the High-Performance Computing (HPC) clusters at the University of Hawai‘i at Mānoa and at Utah State University. This process was carried out twice and the samples with counts less than 17 reads were discarded.

1) *Demultiplexing/loading fastq files* – Barcode sequences of 39 samples were provided as a separate file to demultiplex the data.

2) *Filtering/editing reads* – Low quality base calls ( $Q < 20$ ) were filtered from fastq data files. Low quality base calls were changed into “N”s and reads with more than the number of allowed “N”s (4) were discarded. Sequence reads greater than the 33 phred Qscore threshold were

included in downstream analyses and others were removed. Adapter and primer sequences were also trimmed in this step.

3) *Clustering/mapping reads within samples and alignment* – The selected reads from the previous step were dereplicated and I recorded the number of times each selected read was observed. Within-sample clusters were generated using USEARCH (Edgar, 2010). The resulting data were *de novo* assembled using VSearch (Enns et al. 1990). For each sample, sequences were clustered by similarity (here >90%) and aligned using MUSCLE (Edgar, 2004).

4) *Joint estimation of heterozygosity and error rate* – Sequencing error rate and heterozygosity across clusters were estimated. Maximum heterozygotes allowed was four in consensus.

5) *Consensus base calling and filtering* – Clustered reads were used to estimate consensus allele sequences.

6) *Clustering/mapping reads among samples and alignments* – Consensus sequences across all the samples were clustered. Clusters representing putative RAD loci shared among samples were aligned with MUSCLE. If heterozygous genes with a frequency  $\geq 0.5$  appeared in a set of samples, they were discarded assuming that they are paralogs and more likely to be shared across multiple samples rather than ancestral polymorphisms.

7) *Filtering and formatting output files* – 20 maximum alleles/SNP at each locus were filtered and output files were generated in several different formats.

The above process was carried out twice and the samples with counts of less than 20 reads were discarded.

#### 4.2.5 Population Genetic Structure Analysis

The program STRUCTURE 2.3.4. (Pritchard et al. 2000) was used to estimate the genetic clusters of samples selected from ipyrad analysis. The ipyrad output file .ustr was used for the STRUCTURE analysis. STRUCTURE is a Bayesian, model-based algorithm used to cluster individuals to populations based on their allele frequencies. The number of clusters ( $K$ ) can be defined by the user and assumes Hardy-Weinberg equilibrium within clusters (Hubisz et al. 2009). The multi-locus data (.ustr file from ipyrad analysis) were analyzed to determine the population structure of *A. tripinnatifidus*. Options for the admixture model and the correlated allele frequencies between populations were chosen as recommended by Falush et al. (2003). Default values were set for all the other parameters. A burn-in length of 1,000,000 followed by a MCMC run length of 5,000,000 was used; 50 runs were carried out for  $K$ -values ranging from 2-5.

To further test the inferred genetic clusters of the selected polymorphic loci, Discriminate Analysis of Principle Coordinates (DAPC) (Ivandic et al. 2002) was used in *adegenet* 2.0.0 (Jombart 2008; Jombart et al. 2010) in R software (R Development Core Team, 2009). DAPC analysis is a combination of Principle Component Analysis and Principle Coordinates Analysis. In DAPC analysis, variance between groups is maximized while the variance within groups or clusters is minimized. In order to find the optimum number of clusters, a range of  $K$  values must be tested. Those different  $K$  values are then compared using the Bayesian Information Criterion (BIC). The best  $K$  has the lowest BIC value.

## 4.2.6 Identification of Best $K$ and Graphical Illustration of Results

Clustering Markov Packager Across  $K$  (CLUMPAK; available from [clumpak.tau.ac.il](http://clumpak.tau.ac.il)) was run to summarize membership coefficient matrices (Q-matrices) that arose from runs within and across  $K$  values of STRUCTURE analyses. Runs were clustered and averaged by CLUMPAK using the Markov clustering algorithm. The best or most likely  $K$  was generated in CLUMPAK using the Evanno method (Evanno et al. 2005) and the Pritchard method (Pritchard et al. 2000). DISTRUCT (Rosenberg 2004; available within CLUMPAK) was utilized for graphical display of the results. The Q-matrices were also summarized in STRUCTURE HARVESTER (available at <http://taylor0.biology.ucla.edu/structureHarvester/>) for the easier detection of genetics groups that best fit the data (Earl and vonHoldt, 2012).

DAPC analysis used the built-in functions to summarize and visualize data. Scatterplots were obtained for different BIC values at each cluster and optimum  $K$  values were plotted in scatterplots of the first and second linear discriminant axes of DAPC.

## 4.3 Results

### 4.3.1 Population Structure Analysis

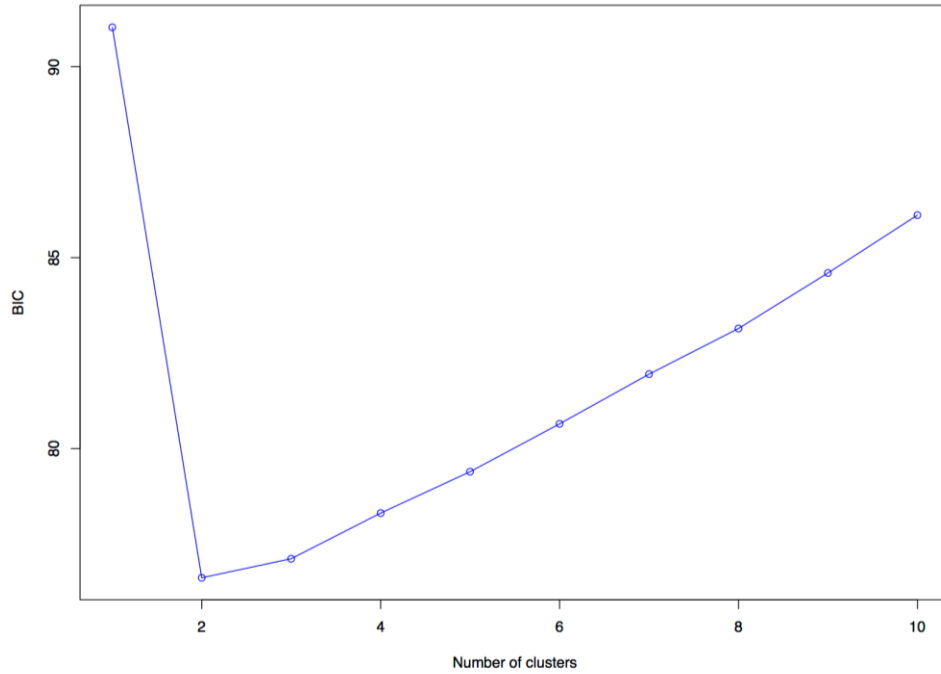
About 98,000 loci were recovered before filtering for duplicates, maximum indels, maximum SNPs, and maximum shared heterozygotes (Table 4.2). A total of 225 loci were retained after filtration and, of those, 144 SNP loci were used for STRUCTURE and DAPC analyses. After removing samples with less than 20 reads, 35 *A. tripinnatifidus* samples representing the four populations were retained for genetic analysis (Table 4.1).

**Table 4.2:** The number of loci caught ipyrad analysis after application of different filters in RADseq data assembly steps

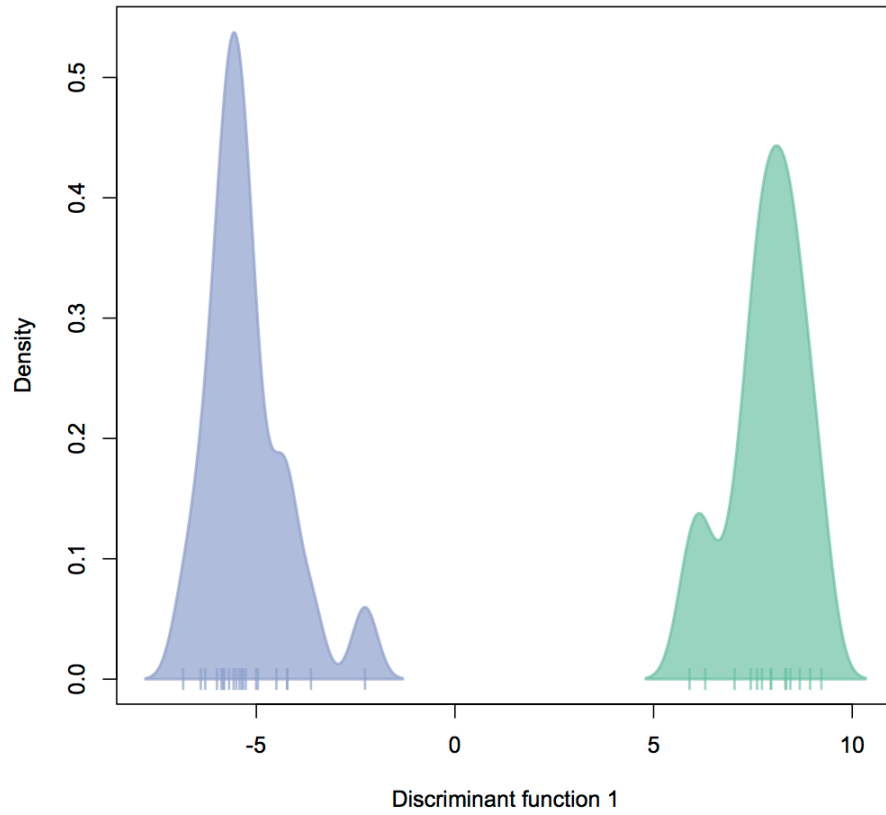
<b>Filter</b>	<b>Total filters</b>	<b>Applied order</b>	<b>Retained loci</b>
Total prefiltered loci	97971	0	97971
Filtered by remove duplicates	4615	4615	93356
Filtered by maximum indels	1183	1183	92173
Filtered by maximum SNPs	3075	150	92023
Filtered by maximum shared heterozygosity	119	43	91980
Filtered by minimum sample	96519	91481	499
Filtered by maximum alleles	18639	274	225
Total filtered loci	225	0	225

The distribution of  $\Delta K$  across values ranging from  $K=2$  to  $K=5$  indicated that the best fit partition for the data was  $K=2$ . The number of optimum genotypic groups ( $K=2$ ) in the data were in agreement in both STRUCTURE HARVESTER and Bayesian Information Criterion (BIC) in DAPC (Figure 4.2). CLUMPAK analysis of the average individual membership coefficients of STRUCTURE output demonstrated that all 50 replicate runs at  $K=2$  to  $K=5$  were inclusive resulting in four major modes for the analysis. However, because both DAPC and STRUCTURE analyses indicated  $K=2$  as the best value and CLUMPAK analysis included  $K=2$  in its range, this value is recognized as most optimal.

At  $K=2$ , samples collected from the same island (Kaua‘i) were clearly differentiated from those collected on the adjacent islands of Maui and Moloka‘i. The genetic clusters were distinct, and the individuals were exclusively assigned to each cluster with 1.0 membership coefficient (Figure 4.4). The DAPC analysis (Figure 4.3) shows clear distinctiveness of the two genetic clusters when individuals are plotted against discriminant function 1. No admixture genotypes within individuals was evident among the two island groups, Kaua‘i or Maui/Moloka‘i.



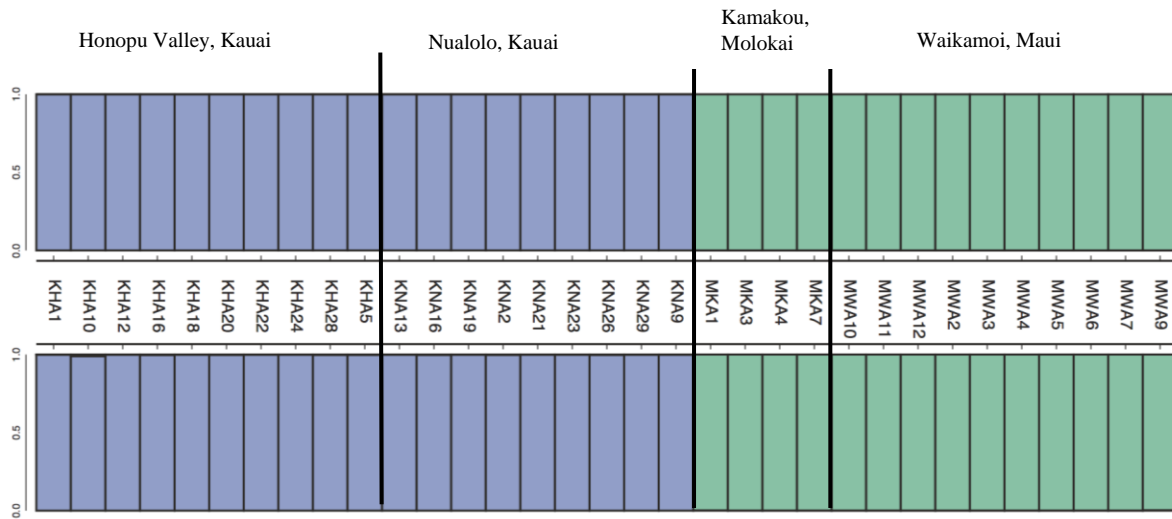
**Figure 4.2:** A scatter plot recovered from DAPC analysis in Adegent package in R shows the number of populations (clusters) and Bayesian Information Criterion (BIC) values. The best number of clusters correspond to the lowest BIC value;  $K=2$



**Figure 4.3:** A plot of the individual densities against the first discriminant function retained show that the isolation of recovered cluster;  $K=2$

### ***4.3.2 Pinnae Division of A. tripinnatifidus***

Samples collected for the study and the herbarium collections from the islands of Kaua‘i, Maui and Moloka‘i were carefully examined for the pinnae division trait. Pinnae division was variable even among the collections of same islands. As per the personal observations, no pattern of pinnae division was observed that may be exclusive to certain islands or populations. Some individuals were 2-pinnate and some were 3-pinnate. Several individuals were observed with highly divided acroscopic basal pinnules (Palmer, 2003). Furthermore, the rhizome characters (thin, long-creeping) were common to all populations. However, there was no single characteristic that would distinguish populations of different islands.



**Figure 4.4:** Genetic structure bar graphs of *A. tripinnatifidus* generated from DAPC (top panel) STRUCTURE (bottom panel) analyses at  $K=2$  from 144 SNP loci. Each of the 35 individuals genotyped in this study represented by a single vertical bar (x-axis) partitioned into two colored segments which represents the individual's probability of belonging to one of two ( $K=2$ ) groups. "Group 1" is represented in green, while "group 2" is represented in blue. General localities are given along the top of the DAPC bar graph. KHA: Kaua'i Honopu Valley *Adenophorus*; KNA: Kaua'i Nualolo *Adenophorus*; MKA: Moloka'i Kamakou *Adenophorus*; MWA: Maui Waikamoi *Adenophorus*

## 4.4 Discussion

Two genotypic groups were obtained for *A. tripinnatifidus* representing different island populations. Kaua‘i populations formed a distinct cluster from those of Maui Nui (historically including the current islands of Maui, Moloka‘i, Lāna‘i and Kaho‘olawe; Price and Clague 2002). The allocation of individuals to each cluster was exclusive and no admixture of genotypes was observed, a pattern consistent for both STRUCTURE and DAPC analyses. Thus, it is possible that *A. tripinnatifidus* populations on different islands are diverging from each other and may, in fact, represent at least two incipient species. Incipient diverging lineages can be seen in many taxa that are actively evolving (McGlaughlin and Friar, 2011). Previous studies have shown the occurrence of incipient speciation among several lineages of Hawaiian taxa (Shaw, 2002; Manier, 2004; Gamble et al., 2008; Mulcahy, 2008).

Isozyme markers have been previously used to examine population genetics of *A. tripinnatifidus* across the Hawaiian Islands (Ranker 1992). He found that at least two island-unique alleles (private alleles), except for the Hawai‘i Island, were present in populations and hypothesized that different island populations are undergoing incipient speciation (Ranker 1992). However, populations within islands are not diverging, likely due to gene flow among them. These findings are consistent with those found here in that the Kaua‘i populations have diverged from those on Maui and Moloka‘i.

Distance among islands is playing a role in evolution of *A. tripinnatifidus*. However, in terms of fern microevolution, distance has not commonly been as a significant factor shaping speciation processes among Hawaiian ferns. Ranker (2016) reported that single-island endemism of both ferns and lycophytes occurs only in 28 taxa (15%) whereas the level among Hawaiian angiosperms is 80% (Wagner et al. 1999). *Adenophorus* is one of the few Hawaiian fern genera

that has undergone a radiation among species (Palmer 2003). Yet, distribution of *Adenophorus* species follow the pattern of other Hawaiian ferns with only two of the nine known from only a single island (Palmer 2003; Ranker, 2003). Unlike seeds or fruits of angiosperms, fern spores are dust-like with high dispersal ability and this is likely the main contributing factor limiting single-island endemism (Ranker, 2016). Yet, the present study demonstrated that the *A. tripinnatifidus* populations from different islands are genetically diverging from each other, even though they are only a few hundred kilometers apart from each other. The closest distance between Kaua‘i and the Maui Nui complex is ca. 150 km (Baird et al., 2009). Populations on O‘ahu may have provided a bridge from Kaua‘i to Maui Nui. No populations were found on O‘ahu which emphasizes its rarity (or possibly extirpation) on that island. Absence of any populations on Oahu may further increase the differentiation of Kaua‘i and Maui Nui populations in the future.

Another factor leading to increased divergence among island populations of *A. tripinnatifidus* may be linked to the spore morphology of grammitids. Grammitid ferns bear chlorophyllous spores, and it has been shown that chlorophyll-containing spores have limited viability compared to nonchlorophyll-containing spores (Pence, 2000). Low viability of chlorophyllous spores may have restricted their ability to survive after dispersing to other islands and thus promoting the separation of populations and possibly speciation.

Ecological isolation of conspecific populations can lead to different evolutionary trajectories. *Adenophorous oahuensis* and *A. haalilioanus* are morphologically very similar species. Both are found in the Ko‘olau Mountains of O‘ahu to which *A. oahuensis* is endemic; *A. haalilioanus* is found on O‘ahu and Kaua‘i. The species both occur in wet forests, but are ecologically allopatric. *Adenophorus oahuensis* is found only at elevations between 300 m and 550 m elevations and utilizes many exotic and native woody plant hosts. In contrast, *A.*

*haalilioanus* occurs above 600 m elevation on is primarily found on native *Kadua* sp. (Rubiaceae) and less often *Metrosideros polymorpha* Gaud. hosts (Palmer, 2003; Ranker et al., 2003). Phylogenetic analyses showed that *A. oahuensis* and *A. haalilioanus* are recently diverged sister species (Ranker et al., 2003). A similar evolutionary fate may be acting upon the *A. tripinnatifidus* populations on three islands. Kaua‘i is an older island, ca. 4.7 Ma old while Maui is ca. 1.5 Ma and Moloka‘i is 2 Ma (Price and Elliott-Fisk, 2004). Due to island erosion and subsidence, the older islands are lower than much younger islands (Price and Elliott-Fisk, 2004). Therefore, *A. tripinnatifidus* populations residing on each island must adapt to the prevailing ecological conditions (partly shaped by the topography and age of the island) which could result in different evolutionary trajectories ultimately resulting in new species.

#### **4.4.1 Morphological Variation**

Fronds of *A. tripinnatifidus* are morphologically highly variable. Pinnae among plants display a range of variation from occasionally 2-pinnate with the acroscopic basal pinnule not further divided to most plants being 3-pinnate at least on the acroscopic basal pinnule or 3-pinnate from basal to apical pinnules (Palmer, 2003). This pinnule variation has been examined among the island populations collected here and among herbarium collections. There is no evidence of a subpopulation genotypic differentiation associated with pinnule variation and a quantitative study is recommended to address this claim statistically. Similarly, there is no evidence from examination of herbarium collections that would suggest a link to population divergence correlated with this trait. Hence, all evidence supports this being a variable trait, possibly plastic, within populations.

The morphological variation within *A. tripinnatifidus* could have occurred as a response to ecological adaptation or else within rapidly evolving insular taxa showing morphological differences prior to the formation of sufficient genetic variation (McGlaughlin and Friar, 2011). Morphologically distinct forms of *A. tripinnatifidus* in the Ko‘olau Mountain Range of O‘ahu was previously recognized as *A. hillebrandii*. No Ko‘olau populations were found that could be included in this study, and therefore the genetic basis of its morphological distinctiveness cannot be addressed. Hence, future studies to rediscover the Ko‘olau populations of *A. tripinnatifidus* and examine the genetic diversity therein are recommended.

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## Chapter 5 : Research Questions Revisited

The following research questions were formulated at the beginning based on the research and literature on taxonomic treatment of *Oreogrammitis*, *Radiogrammitis* and *Themelium*; and the population genetics of *O. hookeri* and *Adenophorus tripinnatifidus*. The following conclusions are drawn from the study.

### Chapter 2:

**Question 1:** Are *Radiogrammitis*, *Oreogrammitis* and *Themelium* monophyletic genera? If not, are there well-supported clades that can be characterized by particular suites of molecular and morphological characters?

*Oreogrammitis*, *Radiogrammitis* and *Themelium* are polyphyletic genera.

*Radiogrammitis* and *Themelium* nested within *Oreogrammitis*. Most of the *Themelium* species and *O. fasciata* nested together. *Radiogrammitis* on the other hand appeared different places on the phylogeny. Some species of three genera showed species level polyphyly. This can be due to the incorrect identification of species and the existence of cryptic species. It was difficult to characterize the recovered clades with particular suits of morphological or molecular characters. It is recommended to redefine *Oreogrammitis* to include *Radiogrammitis* and *Themelium*.

**Question 2:** Are phylogenies based on analyses of nuclear DNA markers congruent with those based on plastid DNA markers?

The phylogenies are not congruent but there are a few clades that were common in both phylogenies. The incongruency can be due to hybrid speciation and the evolutionary trajectories undergone by the different genomes.

**Question 3:** Are the similarities among species due to convergent evolution or due to inheritance from common ancestors?

The similarities among species of same genus are not due to inheritance from common ancestors. The main characters that were used to define the genera are not homologous in grammitids. The similarities occurred as a consequence of convergent evolution of unrelated species of the focal genera.

### **Chapter 3:**

**Question 1:** Are conspecific populations of *O. hookeri* from different islands diverging from each other?

The current study showed that the conspecific populations of *O. hookeri* are not diverging from each other. Populations are not undergoing incipient speciation. Genetic differences among populations could simply be due to random processes such as founder effects although localized adaptation cannot be ruled out.

**Question 2:** Do conspecific populations of *O. hookeri* on each island harbor unique genetic variability, possibly indicating the existence of diverging evolutionary trajectories?

The current study was not able to identify unique alleles in different populations but, the study was able to show that the populations are not diverging due to the presence of mixed genotypes in Maui and Moloka'i.

## Chapter 4:

**Question 1:** Are conspecific populations of *A. tripinnatifidus* of different islands genetically distinct and, thus, potentially diverging from each other?

*Adenophorus tripinnatifidus* populations on Kaua‘i are diverging from Maui and Moloka‘i populations. *Adenophorus tripinnatifidus* populations on each island must adapt to the prevailing ecological conditions (partly shaped by the topography and age of the island) which could result in different evolutionary trajectories ultimately resulting in new species.

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