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Ecological Speciation and Cryptic Diversity of Coral-Associated Nudibranchs



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# Ecological Speciation and Cryptic Diversity of Coral-Associated Nudibranchs

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in Biology

by

Allison Louise Fritts-Penniman

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ABSTRACT OF THE DISSERTATION

Cryptic Diversity and Ecological Speciation of

Coral-Associated Nudibranchs

by

Allison Louise Fritts-Penniman

Doctor of Philosophy in Biology

University of California, Los Angeles, 2016

Professor Paul Henry Barber, Chair

Although it is commonly assumed that most speciation occurs in allopatry, a growing body of research indicates that natural selection can lead to reproductive isolation and speciation in sympatry. A common mechanism for sympatric speciation is ecological speciation, where diversification is driven by differential selection across ecological niches. New genetic and genomic techniques facilitate the discovery of cryptic ecological divergence, and allow us to examine in detail the relative roles of natural selection and gene flow in speciation, particularly in marine systems, where it is difficult to identify mechanisms for allopatric speciation.

This dissertation combines broad exploratory field sampling with population genetic and phylogenetic methods to tease apart the roles of geographic isolation and natural selection in driving speciation in the Coral Triangle. Using the coral-associated nudibranch

genus *Phestilla* as a model, in chapter 1 we examine the effect of coral host and geography on population divergence in *Phestilla lugubris* and *Phestilla minor*. Phylogenetic analyses and analyses of molecular variance of mitochondrial COI sequences indicate very little population structure in *Phestilla lugubris*, but *Phestilla minor* has diverged across the Sunda Shelf and across two major clades of coral host. In chapter 2 we use population genomics to test whether natural selection plays a larger role in the divergence of sympatric host-separated populations than allopatric populations of *Phestilla minor*. Population genetic analyses of genome-wide single nucleotide polymorphisms show host-driven divergence is stronger than that across the Sunda Shelf, despite having occurred more recently. Genomic scans for selection reveal an important role for natural selection in both geographic and host-associated divergence. Finally, in chapter 3 we broadened our approach to examine the roles of geography and host in diversification of all scleractinian-associated nudibranchs. A phylogenetic tree of COI, 16S, and H3 sequences from scleractinian-associated nudibranchs collected across the Indo-Pacific Ocean reveals three times the number of purported species than previously thought, with evidence for host shifting and geographic divergence multiple times throughout evolutionary history. Combined, these three studies demonstrate that both coral host and geography contribute to diversification both within and between species of scleractinian coral-associated nudibranchs.

The dissertation of Allison Louise Fritts-Penniman is approved.

Patrick Krug

Thomas Bates Smith

Paul Henry Barber, Committee Chair

University of California, Los Angeles

2016

TABLE OF CONTENTS

EPIGRAPH

"*In the end we will conserve only what we love, we will love only what we understand, and we will understand only what we are taught*."

-Baba Dioum

LIST OF TABLES

LIST OF FIGURES

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**Fritts-Penniman, AL**; Mahardika, GN; Barber, PH "Genomic evidence for ecological speciation in coral associated nudibranchs." International Coral Reef Symposium 2016. Hawaii Convention Center, Honolulu, HI. 21 June 2016. Conference oral presentation.

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**Fritts-Penniman, AL**; Mahardika, GN; Barber, PH "Genomic signatures of ecological speciation in the coral associated nudibranch genus *Phestilla*." Evolution 2015. Casa Grande Resort, Guaruja, Brazil. 30 June 2015. Conference oral presentation.

**Fritts-Penniman, AL**; Mahardika, GN; Barber, PH "Genomic signatures of ecological speciation in the coral-associated nudibranch genus *Phestilla*." Gordon Research Conference on Speciation. Four Points Sheraton Hotel, Ventura CA. 16-20 March 20015. Conference poster presentation.

**Fritts-Penniman, AL**; Mahardika, GN.; Barber, PH "Genetic signatures of ecological speciation in a coral-associated nudibranch." Society for Integrative and Comparative Biology Meeting. Hilton Austin Hotel, Austin, TX. 6 January 2014. Conference oral presentation.

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**Fritts-Penniman, AL** "Genetic structure of coral-associated nudibranchs across the Pacific Ocean." Western Society of Naturalists Meeting. Embassy Suites, Seaside, CA. 9 November 2012. Conference poster presentation.

**Publications**

Tsuang D, Leverenz JB, Lopez OL, *et al.* (2012) "GBA mutations increase risk for Lewy body disease with and without Alzheimer disease pathology." Neurology **79**(19): 1944-1950.

# INTRODUCTION

The origins of biological diversity have fascinated people for millennia. Over the past couple of centuries, evolutionary biologists have honed in on the specific processes that produce the diversity of species we see on Earth. Since its "discovery" by Alfred Russel Wallace and Charles Darwin, the role of natural selection in speciation has been heavily debated (Darwin 1859). In the modern synthesis of the 20th century, natural selection was credited for producing much of the differences between species. However, physical isolation resulting from geographic barriers to dispersal was considered essential for populations to become isolated enough for species to form (Hutchinson 1959, Mayr 1963).

While divergence in allopatry is likely the most common mode of speciation (Barraclough & Vogler 2000), there is now mounting theoretical and empirical evidence that sympatric speciation can and does occur (Maynard Smith 1966, Schliewen *et al.* 1994; Bush 1994; Dieckmann & Doebeli 1999; Berlocher & Feder 2002; Bolnick & Fitzpatrick 2007; Gavrilets 2014). It was recognized early on that disruptive selection could potentially lead to isolation without a geographic barrier (Mather 1955, Maynard Smith 1966), and laboratory selection experiments did indeed confirmed this prediction (Thoday & Gibson 1962, 1970). One of the earliest proposed mechanisms for sympatric speciation was "competitive speciation" (Rosenzweig 1978), where the existence of multiple specialized phenotypes reduces competition for the resource base of any one phenotype. Competition between specialized phenotypes and intermediate phenotypes causes disruptive selection, promoting assortative mating, and reproductive isolation. Now the broader term "ecological speciation" is used for any speciation that is initiated by divergent selection for different ecological niches (Schluter 2001; Rundle & Nosil 2005).

Over the past few decades new genetic and genomic techniques have allowed us to identify the processes driving population divergence with much greater detail, because we can now detect and observe the process in action. These techniques have facilitated the discovery of cryptic, ecologically-associated, sympatric divergence, and the topic of ecological speciation has surged in the literature (Hendry 2009). Accompanying this surge is a shift in the focus of speciation studies: instead of distinguishing between allopatric and sympatric speciation, studies now examine the roles of ecology, natural selection, and gene flow in speciation (Orr & Smith 1998). This paradigm shift is especially important for studies of speciation in the sea, where the lack of obvious barriers to gene flow makes it difficult to identify clear mechanisms for allopatric speciation (Palumbi 1994; Rocha *et al.* 2005; Krug 2011; Miglietta *et al.* 2011).

Despite the potential for ecological speciation to be an important contributor to marine biodiversity, research on the topic still lags far behind that in terrestrial and aquatic systems. Ecological speciation was first described in phytophagous insects and aquatic fish (Schliewen *et al.* 1994; Schluter & Nagel 1995; Lu & Bernatchez 1999; Filchak *et al.* 2000). Now examples of ecological speciation span the tree of life, including bacteria (Lassalle *et al.* 2015), plants (Lowry *et al.* 2008; Mitsui *et al.* 2011; Andrew *et al.* 2012; Osborne *et al.* 2013; Papadopulos *et al.* 2013; Roda *et al.* 2013), arachnids (Pekár *et al.* 2012), amphibians (Rice *et al.* 2009), reptiles (Rosenblum & Harmon 2011; Nunes *et al.* 2011; Muñoz *et al.* 2013), and birds (Parchman *et al.* 2006; Milá *et al.* 2009; Ballentine *et al.* 2013). In the ocean, evidence of ecological speciation has been observed in cnidarians (Prada & Hellberg 2013), polychaetes (Maltagliati *et al.* 2004), molluscs (Bierne *et al.* 2003; Faucci *et al.* 2007; Johannesson 2009; Bird *et al.* 2011, Simmonds 2016), crustaceans

(Tsang *et al.* 2009; Hurt *et al.* 2013), fish (Munday *et al.* 2004; Puebla *et al.* 2007; Buonaccorsi *et al.* 2011) and sea snakes (Sanders *et al.* 2013). However, considering the vast biodiversity of marine ecosystems such as the Coral Triangle (Veron *et al.* 2007), where ecological associations are plenty and vicariance is relatively rare (Bowen *et al.* 2013), there are a plethora of opportunities to study ecological speciation in the sea.

With the surge in studies of ecological speciation, it is no longer necessary to define or categorize speciation research based solely on geography (Butlin *et al.* 2008; Harrison 2012). Instead, it is often categorized based on the phylogenetic relationships between ecotypes across different locations (Orr & Smith 1998). In parallel ecological divergence, gene flow is limited between locations, but the same selective pressures lead to the independent evolution of the same ecotypes across locations. For example, in the three-spine stickleback, benthic and limnetic ecomorphs have repeatedly evolved in many different isolated lakes (Rundle *et al.* 2000), and at the interface of marine and freshwater habitats, armored plating is always reduced in freshwater (Colosimo *et al.* 2005). Genomic research has shown the same loci under parallel selection across independent population pairs in stick insects and pea aphids associated with different plant hosts (Soria-Carrasco et al. 2014, Nouhaud et al. 2014). Alternatively, many studies reveal that gene flow is restricted more by ecological divergence than geography, such that individuals from the same habitat are more closely related, regardless of geographic location. Examples of this pattern of divergence include limpets (Bird et al. 2011), rockfish (Narum et al. 2004, Buonaccorsi et al. 2011), and some well-studied phytophagous insects such as the apple maggot fly *Rhagoletis* (Feder *et al.* 2005; Powell *et al.* 2014) and the European corn borer *Ostrina* (Midamegbe *et al.* 2011).

Research on ecological divergence tends to focus on single cases of population-level divergence in its early stages, because once reproductive isolation is complete, subsequent changes can obscure the process through which it first evolved (Orr & Smith 1998; Hendry 2009). While these studies provide important insights into the mechanisms driving ecological divergence, they do not actually demonstrate the evolution of reproductive isolation, and they do not address whether the process of ecological speciation is an important process driving the diversification of life on Earth. Understanding the role of ecological speciation in shaping global biodiversity requires connecting individual examples of ecological divergence to speciation patterns at higher taxonomic levels. This means studying one system, ideally one with high taxonomic diversity, across multiple stages of the speciation continuum, from adaptive divergence with gene flow to fully ecologically isolated species (Hendry 2009; Feder *et al.* 2012). A rare example of this approach in the pea aphid complex, where genetic evidence revealed host-specific populations at different stages of divergence, from host race to purported species (Peccoud *et al.* 2009).

This dissertation uses a multi-stage approach to understand the relative roles of geography and ecology in generating and maintaining species diversity in scleractinian coral-associated nudibranchs. We define coral-associated nudibranchs as nudibranch species that rely completely on a coral host for both food and habitat. The relationship between this unique group of nudibranchs and their coral prey draws a strong parallel to well-studied terrestrial phytophagous insects. The large size of coral colonies combined with the limited mobility of adults results in a tendency for individual nudibranchs to stay the same host coral colony for their entire life, including mating (Krug 2011). This life-history promotes reproductive isolation between populations feeding on different corals, and has

the potential to lead to ecological speciation, possibly in sympatry. Speciation via host shifting has been suggested as a mechanism of diversification in genus *Phestilla* (Faucci *et al.* 2007), but has never been explicitly tested. This dissertation tests for ecological divergence at many taxonomic and geographic scales, from investigating host-associated genomic divergence within a single site, to using genetic evidence to identify cryptic coral-associated nudibranch species throughout the entire Indo-Pacific Ocean.

In chapter 1, I use phylogenetic and population genetic techniques to identify cryptic speciation across the Sunda Shelf, and reveal a new example of ecological divergence with *Phestilla cf. minor* diverging on two difference lineages of co-distributed *Porites* coral hosts. In the Pacific Ocean, *Phestilla cf. minor* individuals are more closely related to those from the same host than those from the same reef, indicating that coral host may act as a stronger barrier to gene flow than geographic distance. In chapter 2, I investigate this host-associated genomic divergence in more detail using genome-wide single nucleotide polymorphisms. I use population genetic analyses to demonstrate that host-driven divergence is stronger than that across the Sunda Shelf, despite having occurred more recently. Genomic scans for selection reveal an important role for natural selection in both geographic and host-associated divergence. Finally, in chapter 3 I broaden my approach to include all scleractinian-associated nudibranchs. I conducted exploratory field sampling and genetic sequencing to discover species with previously unknown host associations and cryptic allopatric speciation throughout the Indo-Pacific Ocean. A phylogenetic tree of all scleractinian-associated nudibranchs that reveals multiple instances of host shifting and geographic divergence throughout evolutionary history. Combined, these three studies demonstrate that both coral host and geography contribute to diversification both within and

between species of scleractinian coral-associated nudibranchs. This research provides a solid foundation to develop this system into a new model for the study of ecological speciation in the sea.

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# CHAPTER 1

## Cryptic ecological and geographic divergence of coral-associated nudibranchs in the Coral Triangle

### Introduction

The Coral Triangle (sensu Veron *et al.* 2007) is the global epicenter of marine biodiversity, and multiple theories seek to explain this pattern (Palumbi 1997; Barber 2009; Bowen *et al.* 2013; Barber & Meyer 2015). The Center of Overlap postulates that divergent Indian and Pacific Ocean taxa overlap in the Coral Triangle where these two oceans meet (Woodland 1983; Hobbs *et al.* 2009). The Center of Accumulation, or the "vortex model," states that species that originate in the peripheral islands of the Indian and Pacific Oceans gradually accumulate in the Coral Triangle (Ladd 1960; Jokiel & Martinelli 1992). The Center of Origin counters that speciation occurs in the Coral Triangle (Ekman 1953; Briggs 1992). Fundamental to all of these theories is the question of where and how speciation occurs in the tropical Indo-West Pacific (IWP).

Speciation studies have historically focused on the geographic context of divergence, attempting to determine whether divergence occurred in geographic isolation (allopatry), across an environmental gradient (parapatry), or with no geographic separation (sympatry) (Coyne & Orr 2004). In marine systems, identifying allopatric mechanisms of population divergence and speciation is challenging because of the lack of obvious geographic barriers (Palumbi 1994). Most marine species have highly dispersive larvae, and the longer a species pelagic larval duration (PLD), the farther it typically disperses (Shanks *et al.* 2003). In some cases dispersal can cover many thousands of miles

(Scheltema 1971, 1988), suggesting that for some species there may be no barriers to dispersal or migration. However, if larval duration is short, or if realized dispersal falls short of its potential, then isolation can result as a function of distance, leading to allopatric speciation (Knowlton & Keller 1986; Siegel *et al.* 2003; Rocha & Bowen 2008; Selkoe & Toonen 2011).

Within the Coral Triangle, there is evidence to support allopatric divergence. Genetic data has revealed fine scale endemism within some Indo-Pacific marine invertebrate species, revealing regional populations isolated enough to be potentially allopatric cryptic species (Meyer *et al.* 2005; Barber *et al.* 2006). Similarly, phylogeographic studies have demonstrated the existence of dispersal barriers within the seemingly continuous seas of the Coral Triangle (Fleminger 1985; McMillan & Palumbi 1995; Lavery *et al.* 1996; Palumbi 1997; Barber & Bellwood 2005; Barber *et al.* 2006, 2011), barriers that could lead to speciation. For example, lowered sea levels during Pleistocene glacial periods exposed the Sunda shelf, creating a land barrier between the Indian and Pacific Oceans that likely resulted in allopatric speciation of many taxa (e.g. Fleminger 1985; Lavery *et al.* 1996), as well as pronounced phylogeographic structure in a wide diversity of marine species including clownfish, stomatopods, giant clams, echinoderms, sea stars, and snails (Williams & Benzie 1998; Nelson *et al.* 2000; Barber *et al.* 2006; Crandall *et al.* 2008; Timm & Kochzius 2008; DeBoer *et al.* 2008, Simmonds 2016). Similarly, concordant patterns in a variety of marine fish and invertebrates indicate that oceanographic processes such as the Halmahera Eddy may also promote isolation and diversification (Barber *et al.* 2011; Carpenter *et al.* 2011), a pattern predicted by physical oceanographic models (Kool *et al.* 2011; Treml *et al.* 2015).

While there is clearly evidence for allopatric divergence in the Coral Triangle, the ephemeral nature of isolation in this region and the fact that many sister species have sympatric ranges suggests that other modes of speciation (e.g. sympatric speciation) could significantly contribute to biodiversity in this region. Research in terrestrial and freshwater systems is increasingly providing examples of speciation in sympatry (Bush 1994; Dres & Mallet 2002; Marques *et al.* 2016), the most striking example being cichlid fishes, which have undergone parallel adaptive radiations producing nearly 1,500 species in the Great Lakes of East Africa (Schliewen *et al.* 1994; Brawand *et al.* 2014).

One mode of sympatric speciation is ecological speciation, where divergent selection for different ecological niches promotes reproductive isolation (Rundle and Nosil 2005, Marie Curie SPECIATION Network 2012). Such isolation frequently comes from host shifting, a process by which lineage divergence and speciation occurs as a result of populations of a parent species adapted to live on specific hosts evolving a preference for a novel host (Via and West 2008). Evidence for host-driven ecological speciation is well-documented in terrestrial systems, particularly in phytophagous insects (Berlocher & Feder 2002; Matsubayashi *et al.* 2010). Classic examples include the apple maggot fly (Filchak *et al.* 2000; Feder *et al.* 2005), pea aphids (Via *et al.* 2000), and the walking stick insect *Timema cristinae* (Nosil *et al.* 2002).

In comparison to the terrestrial realm, ecological speciation in marine ecosystems is relatively unstudied (Krug 2011). Notable examples include *Gobiodon* gobies (Munday *et al.* 2004), the barnacle *Wanella milliporae* (Tsang *et al.* 2009), and the snail *Coralliophila violacea* (Simmonds 2016) all of which have strong associations with coral and potentially have undergone speciation via host shifting. Hundreds of marine species have associations

with coral hosts, in relationships that parallel those of phytophagous insects to their host plants. This coral habitat-driven divergent selection could play a significant role in shaping the remarkable diversity of coral reefs (Rocha *et al.* 2005; Alfaro *et al.* 2007).

Heterobranchs are an extremely diverse group of snails and slugs that includes the order Nudibranchia. There are over 2,700 recognized species of nudibranch (Wägele 2004), with an equal number of undescribed species (Gosliner et al. 2015). Nudibranch diversity peaks in the Coral Triangle, where many taxa have overlapping ranges with their sister species, with little to no evidence for divergence via vicariance (Gosliner & Draheim 1996). Many heterobranchs benefit substantially from strong ecological associations with specific algal or cnidarian taxa (Jensen 1997; Wägele 2004; Krug 2011). For example, many aeolid nudibranchs are so well adapted to their cnidarian host/prey that they can consume stinging nematocysts and retain them for their own defensive use (Wagele & Willan 2000; Putz *et al.* 2010). In other taxa, such as *Phestilla*, species have adapted extraordinary camouflage to stay hidden on their coral hosts.

*Phestilla lugubris* (synonymous with *P. sibogae*) and *P. minor* are widespread aeolid nudibranchs that live and prey upon *Porites* corals across the Indo-Pacific Ocean from Hawaii to Tanzania. *Phestilla* mate on their host corals using internal fertilization, then lay eggs on the coral. After a brief planktonic stage, the length of which varies between species, *Phestilla* larvae use chemical cues from corals to identify and initiate recruitment onto their host coral of choice (Hadfield & Pennington 1990; Hadfield & Koehl 2004), where the metamorphosed larva will spend its entire life. *Phestilla* species have specific associations with disparate coral genera (Faucci *et al.* 2007). Given the lack of allopatric barriers between sister species on different hosts, host-associated divergent selection is a

likely mechanism for speciation, but ecological diversification has never been explicitly tested in this group.

In this paper, we use genetics to test for divergence among populations of *Phestilla lugibris* and *Phestilla minor* in the Coral Triangle. Specifically, we examine for divergence among populations that span well-established phylogeographic provinces, and among populations that live on different *Porites* coral hosts, in an effort to test the relative roles of geographic and ecological barriers to gene flow in population divergence of *Phestilla* nudibranchs in the Coral Triangle.

## Methods

### (a) Sample collection

To test the potential impact of biogeographic barriers on diversification of *Phestilla minor* and *Phestilla lugubris* populations, we collected samples from Indonesian coral reefs (Figure 1.1) spanning the well-documented Indian and Pacific Ocean biogeographic boundary (Fleminger 1985; Lavery *et al.* 1996). We also sampled on either side of the Maluku Sea, a region where the Halmahera Eddy isolates populations of many coral reef invertebrates (Barber *et al.* 2002, 2011). We located *Phestilla* through close inspection of loose coral pieces while using snorkel and SCUBA, and collected individual nudibranchs by hand.

To test for ecological diversification among nudibranchs with different coral hosts, we collected *Phestilla minor* and *Phestilla lugubris* from as many species of *Porites* coral hosts as possible. *Porites* corals are notoriously plastic in appearance, making species

identifications based on morphology extremely difficult (Forsman *et al.* 2009). To genetically confirm perceived differences among hosts, we collected a small sample of tissue from each host for genetic analysis. To abide by the limits of our sampling permits, at some locations we could only sample the first 3 individuals of a given coral morpho-species, and any additional coral hosts were classified by morphology alone.

We photographed all nudibranchs on their host coral in the field prior to collection. Individuals were kept alive for ≤ 6 hours in small vials of seawater until sample processing. We anesthetized and relaxed all specimens in the freezer before transferring them to 95% ethanol for long-term preservation. We recorded the specific coral host for all samples, with as many as 12 nudibranchs coming from one small coral. In total, we collected 250 *Phestilla cf minor* and 66 *Phestilla lugubris* samples from 6 *Porites* morpho-species at 8 locations.

(b) DNA extraction, amplification, and sequencing

We extracted DNA from nudibranch and coral tissue using Qiagen DNEasy Kits and Omega Bio-Tek E.Z.N.A. Mollusc DNA Kits. For nudibranchs, we isolated foot tissue for extraction when possible, but for individuals smaller than 5 mm, we used half the body, and for those smaller than 2 mm we used the entire body. We excluded cerata (the projections of the gut covering the bodies of most aeolid nudibranchs) from the DNA extractions as they often contain recently ingested coral tissue (Rudman 1982). For nudibranchs we amplified a section of the mitochondrial cytochrome oxidase subunit I gene (COI) using the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) under the following PCR conditions: 2 minutes at 94ºC; 35 cycles of 94ºC for 30 seconds, 40ºC for 30 seconds, and 72ºC for 30 seconds; with a final extension of 7 minutes at 72ºC. For coral hosts we amplified a section

of the nuclear histone 2 (H2) coding region using the primers zpH2AH4f (5'-GTGTACTTGGCTGCYGTRCT-3') and zpH4Fr (5'-GACAACCGAGAATGTCCGGT-3') under the following PCR conditions: 2 minutes at 96ºC; 34 cycles of 96ºC for 20 seconds, 58.5ºC for 20 seconds, and 72ºC for 90 seconds; and a final extension of 5 minutes at 72ºC (Tishammer *et al.* unpublished). Amplified DNA was then sequenced in both directions with the ABI 3730 sequencer at UC Berkeley's DNA Sequencing Facility. We assembled and edited all sequences in Geneious version 6.1.7 (Kearse *et al.* 2012), and verified the quality of sequences with successful translation to amino acid protein code and alignment using the MUSCLE algorithm as implemented in Geneious.

(c) Phylogenetic analysis

To test for the presence of phylogenetic structure in the two nudibranch species we generated phylogenetic trees of COI sequences. Sequences from *Phestilla lugubris* and "*P. sp. 1"*, the undescribed sister taxon to *P. minor* (Faucci et al. 2007, Ritson-Williams et al. 2003), were included as outgroups for *P. minor*; for *P. lugubris* we used *P. minor* and *P. sp. 1* as outgroups. To confirm coral host species field identifications we generated a phylogenetic tree of H2 sequences from the sampled *Porites* hosts using a *Porites superfusa* sequence (Tisthammer *et al.* unpublished) as an outgroup. For all three phylogenetic trees we determined the best-fit model of evolution (HKY+I+G for *P. minor*, GTR+I+G for *P. lugubris* and *Porites*) for phylogenetic analysis using the Akaike information criterion (AIC) as implemented in JModelTest (Guindon & Gascuel 2003; Darriba *et al.* 2012). Bayesian analysis was conducted in MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012) as implemented in Geneious. We ran analyses for 1,000,000

generations, sampling every 200 generations, with a 10% burn-in, and assessed support for individual nodes using posterior probabilities. We also conducted a maximum likelihood analysis in PhyML 2.2.0 (Guindon & Gascuel 2003) as implemented in Geneious and assessed support for nodes using proportion out of 1,000 bootstraps.

To assess whether nudibranch divergence was initiated by lowered sea levels during the Pleistocene, we compared approximate divergence time between clades to the timing of glacial cycles (Voris 2000; Lambeck *et al.* 2002). We calculated mean sequence divergence between clades by first calculating pairwise percent sequence identity for all samples in a given pair of clades. We then transformed sequence identity to sequence divergence and averaged across all pairwise divergence estimates between the different clades. We approximated divergence time from sequence divergence using the COI sequence divergence between two teguline gastropods calibrated by the Isthmus of Panama (Hellberg & Vacquier 1999).

To determine whether cryptic nudibranch clades could be analyzed using population genetic techniques or should be considered as separate species, we used Automatic Barcode Gap Detection (ABGD, Puillandre *et al.* 2012). ABGD uses DNA barcodes such as COI to partition samples into putative species based on the barcode gap, which occurs when divergence among individuals within the species is less than that between species. We analyzed *P. minor* and *P. lugubris* sequence alignments using the online tool (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with the default range of 0.001 to 0.1 for prior intraspecific divergence (P) and 1.5 for relative gap width (x), with corrected Jukes-Cantor and Kimura distances as well as simple distances.

To test for ecological divergence, we constructed a contingency table categorizing

nudibranchs by phylogenetic clade and coral host. We then conducted a chi-square test and Fisher's exact test for a statistical association between nudibranch clades and coral hosts. These tests calculate whether nudibranchs in a given clade come from a particular coral host disproportionately more than expected by random sampling. If so, it indicates that phylogenetic clades differ in their host associations, and have likely undergone ecological divergence.

(d) Population genetic analysis

To test for geographic or ecological population structure we analyzed haplotype frequencies using Arlequin 3.5 (Excoffier & Lischer 2010). We used the online file-processing tool FABOX (Villesen 2007) to collapse *Phestilla* sequence alignments into haplotypes. To identify haplotype groups and confirm clades from Bayesian analysis, we created a minimum spanning tree of haplotypes based on pairwise distances using the MINSPNET algorithm as employed in Arlequin. For both nudibranch species we color-coded the haplotype trees by sampling locality, and for *Phestilla minor* also by phylogenetic clade and coral host clades.

To determine the relative strength of geographic or ecological barriers to gene flow, we calculated diversity indices, population pairwise $F_{sts}$, and conducted analyses of molecular variance (AMOVA) in Arlequin, with significance determined with 10,000 random permutations. For *Phestilla minor* we defined populations by both locality and coral host clade, but for *Phestilla lugubris* we defined populations only by sampling locality as sampling was too uneven across hosts. We first ran AMOVA assuming no a priori structure. To test for geographic divergence across the Sunda Shelf we ran AMOVA with two groups;

Indian Ocean (Aceh and Cubadak) and Pacific Ocean (Bali, Komodo, Donggala, Bunaken, Lembeh, and Raja Ampat). Although Bali and Komodo border the Indian Ocean, geographically they are on the opposite side of the Sunda Shelf relative to Aceh and Cubadak, so are included with Pacific populations. To test for population structure across the Maluku Sea we excluded Indian Ocean populations and ran AMOVA with Raja Ampat in a separate group from the other Pacific Ocean populations.

To test for ecological divergence among *Phestilla minor* populations, we ran AMOVA grouping populations from different localities together based on their coral host. To compare the relative influence of coral host and geography on gene flow, we calculated pairwise $F_{st}$ values for populations at the same site but on different coral hosts, and on the same host but at different sites.

## Results

### *Phestilla minor*

We sequenced a total of 658 bp of mtDNA COI from 250 *Phestilla cf minor* specimens. As *Phestilla minor* is known to be part of a putative cryptic species complex (Faucci *et al.* 2007), here we present results from one subclade of that complex, which we refer to as *Phestilla minor*, but our full analysis of all *Phestilla cf minor* specimens is reported in chapter 3. For this study we analyzed COI sequences from 146 *Phestilla minor* specimens (Table 1.1), representing 114 unique haplotypes. Phylogenetic analyses revealed three distinct clades, which we have color-coded as white, black, and grey (Figure 1.2). A minimum spanning tree of the 114 haplotypes shows the same three haplotype

groups (Figure 1.3). The white clade only includes samples from Aceh and Cubadak on the west side of the Sunda Shelf (Figure 1.4), and these sequences are 39 mutational steps (7.02 %) removed from the black and grey clades (Table 1.2, Figure 1.3). The black and gray clades, found east of the Sunda Shelf (Figure 1.4), are separated from each other by a mean of 3.94% sequence divergence or 20 mutational steps (Table 1.2, Figure 1.3). These clades show no association with geography, as they both contain specimens from locations as distant as Bali in the west and Raja Ampat in the east (Figure 1.4, Figure 1.5A).

Based on the molecular clock of 2.4% sequence divergence per million years (Hellberg & Vacquier 1999), using our calculations of mean pairwise sequence divergence between clades (Table 1.2) we estimate that the white clade diverged from the black/grey clade about 2.9 million years ago, and the black and grey clades diverged from each other about 1.6 million years ago. ABGD analyses revealed a barcode gap between genetic distances of 0.02 and 0.04 (Figure S1.1), and prior intraspecific divergence values ranging from 0.01 to 0.04 yielded two purported species separated by the Sunda Shelf: the white clade on one side and the black and grey clades on the other. This result was consistent for Jukes-Cantor and Kimura corrected distances and simple distances.

Genetic diversity is generally high within sampling localities (Table 1.3). Given the strong likelihood that speciation has already occurred across the Sunda Shelf, for population genetic analyses we excluded Indian Ocean populations (e.g. white clade). For Pacific Ocean samples, AMOVA with no a priori structure indicates strong population structure (Table 1.4, $\Phi_{ST} = 0.40$, $p < 0.00001$), with 40.2% of the variation among populations and 59.8% within populations, but this could not be attributed to variation among populations spanning the Maluku Sea (Table 1.4, $\Phi_{CT} = -0.16$, $p = 0.67$): 48.6% of

the variation was among populations within groups (Table 1.4, $\Phi_{SC} = 0.42$, $p < 0.00001$), and 67.2% of the variation within populations (Table 1.4, $\Phi_{ST} = 0.33$, $p < 0.00001$).

Ecological divergence

There is a strong association between the *Phestilla minor* grey clade and the coral *Porites cylindrica* (Figure 1.2, Figure 1.5B). The distinctiveness of the host corals we identified as *Porites cylindrica* was confirmed by phylogenetic analysis of 1,488 bp of nuclear H2 region from 47 *Porites* hosts (Figure 1.6). Morphological variation in *Porites* does not generally correspond well to genetic species identity, but the 9 hosts that were identified as *P. cylindrica* in this study form a highly divergent, monophyletic clade (Figure 1.6, blue). All other *Porites* hosts are dispersed throughout a second, more diverse clade, with no agreement between field identification and genetic identity (Figure 1.6, orange).

We constructed a contingency table (Table 1.5) classifying all *Phestilla minor* individuals by clade (grey or black) and by host (*P. cyclindrica* or other *Porites*). Almost all grey clade nudibranchs (14 of 15) were collected from *Porites cylindrica*, and the majority of black clade nudibranchs (102 of 107) were collected from other corals (see bar graphs in Figure 1.2). This extremely skewed distribution of nudibranch clades on specific coral hosts indicates a very significant ecological association between the grey clade and *Porites cylindrica* (Fisher's exact test, $p < 0.0001$; Chi-square = 78.65, $p = 7.4 \times 10^{-19}$).

In AMOVA, we see that 58.1 % of the variation in Pacific Ocean populations of *Phestilla minor* is explained by grouping based on these two coral clades (Table 1.4, $\Phi_{CT} = 0.58$, $p < 0.00001$), with only 6.10 % of the variation among populations within groups (Table 1.4, $\Phi_{SC} = 0.15$, $p < 0.01$), and 35.8% of the variation within populations (Table 1.4,

$\Phi_{ST}$ = 0.64, $p < 0.05$). Pairwise $F_{sts}$ show that within localities, populations found on *Porites cylindrica* are extremely differentiated from populations from other hosts (average pairwise $F_{st}$ = 0.58, Table 1.6). In Bali the differentiation between host clades is not as high as in Raja Ampat and Donggala. While all of the *P. minor* samples from *Porites cylindrica* in Raja Ampat and Donggala fall into the grey nudibranch clade, in Bali they are split between the grey and black clades. The populations from *P. cylindrica* are also quite differentiated from each other (average pairwise $F_{st}$ = 0.51) while populations from the orange host clade are not at all differentiated across the 6 locations (average pairwise $F_{st}$ = 0.00).

*Phestilla lugubris*

We sequenced 648 bp of mtDNA COI from 65 *Phestilla lugubris* specimens, representing 43 unique haplotypes forming a single phylogenetic clade (Figure 1.7) and a comparatively close-knit haplotype network (Figure 1.8). The maximum distance between any two haplotypes in the minimum spanning tree is 5 mutational steps. The most common haplotypes were found in all six locations broadly distributed across Indonesia (Figure 1.8), with no clear geographic pattern.

AMOVA with no a priori groupings showed no evidence of population structure (Table 1.7, $\Phi_{ST}$ = -0.04, p = 0.95). Further AMOVAs indicate that neither the Sunda Shelf (Table 1.7, $\Phi_{CT}$ = 0.01, p = 0.42) or the Maluku Sea (Table 1.7, $\Phi_{CT}$ = -0.04, p = 0.86) act as barriers to gene flow, and diversity within populations explains 100% of the nucleotide variation (Table 1.7).

## Discussion

Allopatric explanations dominate studies of speciation (Barraclough & Vogler 2000; Coyne & Orr 2004), including those focused on the evolution of the Coral Triangle biodiversity hotspot (see Barber *et al.* 2011 and Carpenter *et al.* 2011 for reviews). However, results from sympatric populations of *Phestilla minor* showed clear evidence of ecological divergence. On the Pacific side of the Sunda Shelf, populations of *P. minor* hosted by *Porites cylindrica* exhibit no significant genetic structure over 1750 km of ocean, yet they are strongly divergent from *P. minor* that live on other *Porites* hosts collected from the exact same locality. This result confirms the suggestion by Faucci *et al.* (2007) that host shifting could promote speciation in *Phestilla*, providing us with an exciting new system for the study of ecological speciation in the sea.

In addition to ecological divergence, results also showed clear evidence of geographic isolation of *Phestilla minor* populations on either side of the Sunda Shelf. This pattern is commonly seen in many different marine taxa (Lavery *et al.* 1996; Williams & Benzie 1998; Nelson *et al.* 2000; Barber *et al.* 2006; Crandall *et al.* 2008; Timm & Kochzius 2008; DeBoer *et al.* 2008, Simmonds 2016), and is typically attributed to the exposure of the Sunda Shelf during periods of low sea level (Fleminger 1985). However, there was no evidence for further phylogeographic structure east of the Sunda Shelf, including across the Maluku Sea, a region where strong phylogeographic structure is frequently observed (Barber *et al.* 2011; Carpenter *et al.* 2011).

Despite geography and host having a dramatic impact on *Phestilla minor,* the closely related *Phestilla lugubris* showed no evidence of genetic structure throughout the region. The observance of strong genetic structure in one species, but not in a closely related co-

distributed species is observed in populations of *Nerita* snails that also span the Sunda Shelf (Crandall *et al.* 2007), indicating that closely related species can respond very differently to shared geographic and ecological processes. In *Phestilla*, this difference is most likely the result of differences in pelagic larval duration.

Ecological divergence

Our results suggest that *Phestilla minor* populations are undergoing ecological divergence on their *Porites* coral hosts in sympatry. While many described morphospecies of *Porites* corals are not genetically distinguishable, *Porites cylindrica* is distinct, and *Phestilla minor* populations mirror this differentiation. The relatively high level of divergence between sympatric *Phestilla minor* host races in COI, a putatively neutral marker, indicates that coral host acts as a strong barrier to gene flow, and these populations are on their way towards becoming separate species. This result adds to the slowly growing evidence that ecological speciation may be an important evolutionary driver in the ocean (Rocha *et al.* 2005; Krug 2011; Miglietta *et al.* 2011).

Ecological speciation via adaptation to host is well established in phytophagous insects (e.g. Nosil *et al.* 2002; Feder *et al.* 2005). However, host-driven ecological speciation has only been suggested a few times in marine systems (Munday *et al.* 2004; Faucci *et al.* 2007; Tsang *et al.* 2009; Hurt *et al.* 2013, Simmonds 2016) despite strong parallels between plant-insect and coral-gastropod associations. Both associations involve a parasite on a specific sessile host, and adults mate and lay eggs directly on the host. This direct linkage between habitat preference and mate choice is important for the evolution of reproductive isolation in sympatry (Maynard Smith 1966). Interestingly, insects are less

vagile as larvae and disperse as young adults, while gastropods disperse as larvae, then tend to stay on one host coral colony for their entire adult life (Krug 2011). Research on host-driven speciation in gastropods lags far behind that in insects, so little is known about how this difference in life history may impact ecological divergence.

Given the close proximity of *Porites cylindrica* to the other *Porites* hosts at the collection sites, the barrier to gene flow between nudibranch host races cannot be attributed to geography, and must be maintained by natural selection. This selection could act at any life stage: selection of host, metamorphosis, survival to adulthood, mate selection, or reproductive success. Larvae hatched from *P. cylindrica* may not be able to successfully recruit to or metamorphose on other *Porites* corals, and vice versa. Ritson-Williams *et al.* (2003, 2007, 2009) tested prey choice and metamorphic competence of *Phestilla* species on different corals in the lab. *Phestilla minor* were collected from *Porites* annae, and not surprisingly, larvae had the highest rate of metamorphosis on *P. annae*. However, many larvae did undergo metamorphosis using cues from other corals, including *P. cylindrica*, and even in fresh seawater. It appears that *Phestilla minor* larvae do not require a specific host in order to metamorphose, but they may prefer to settle on a specific host.

Alternatively, selection could act in the form of differential survival. If individuals are unable to survive on the wrong host, a phenomenon known as immigrant inviability (Nosil & Schluter 2011), it is unlikely that adults will ever encounter individuals originating from the other host, and assortative mating will occur by default. This is likely in *Phestilla minor*, given that we collected nudibranchs of all sizes/ages, and very few individuals were found on a coral host mismatched with their genetic clade. It may be that populations are adapted to eat a specific host, and unable to eat the “wrong” host. In fact, in one no-choice

experiment, *P. minor* born on *Porites annae* would only eat *P. annae* (Ritson-Williams *et al.* 2003).

Lastly, if some individuals do survive on the wrong host, selection could act on reproduction. Individuals from different hosts may not be able to mate successfully, or they may not even recognize each other as potential mates. Further experimental work on host choice, the role of corals in metamorphosis, and mate choice is required to identify at which stage natural selection acts.

While natural selection is crucial to the process of ecological speciation, the high level of divergence that we see in a putatively neutral gene indicates that genetic drift is also acting. It appears that ecologically-mediated assortative mating is already limiting gene flow, leading to isolation by adaptation or IBA (Nosil *et al.* 2008; Feder *et al.* 2012). In the absence of gene flow, genetic drift may obscure the signature of natural selection on the genome, and genomic divergence due to IBA will resemble that which results from isolation by distance (IBD). In fact, if host choice is determined by a single trait such as a chemical cue, and the coral hosts are otherwise very similar, the effect of natural selection on the genome could be negligible. More genetic data are needed to determine the relative roles of genetic drift and natural selection in *Phestilla minor* divergence. Fortunately, advances in sequencing have made it possible to generate huge amounts of genomic data efficiently and relatively cheaply. Future studies on this system should use next-generation sequencing data to determine whether natural selection has left a signature on the genome, or if ecological speciation involves more neutral evolution than expected.

Geographic divergence

The divergence of *Phestilla minor* across the Sunda Shelf is consistent with previous phylogeographic studies of marine invertebrates in the Coral Triangle (Lavery *et al.* 1996; Williams & Benzie 1998; Barber *et al.* 2006; DeBoer *et al.* 2008, Simmonds 2016). Divergence in marine taxa across this region is typically believed to result from periods of lowered sea level when the Sunda shelf formed a land barrier between ocean basins (Voris 2000), although coupled bio-physical models suggest that larval dispersal and gene flow could also be limited by ocean currents (Kool *et al.* 2011; Treml *et al.* 2015). The divergence among clades of *P. minor* date to approximately 2.9 million years ago, a date that roughly coincides with the onset of the first Pleistocene glacial cycles (Lambeck *et al.* 2002). We recognize that the molecular clock rate we used may not be accurately calibrated for *Phestilla*. Recent work on marine invertebrates in the Sunda Shelf region has shown that rates of evolution are dependent on the time of calibration, and that in recent history rates may have been elevated by population expansion (Crandall *et al.* 2011). The high divergence observed between *P. minor* clades may be more recent than estimated, but still falls within the time of Pleistocene glaciation. This result suggests that glacial cycles could have promoted diversification in *P. minor* populations spanning the Sunda Shelf, or might have reinforced historical barriers in this region.

An alternative, but not mutually exclusive explanation for this pattern of divergence is that natural selection limits dispersal of *P. minor* across the historical Sunda Shelf barrier. The waters of the eastern Indian Ocean are very different from the South China and Java Seas, and if populations have adapted to these conditions, ecological barriers may remain even though the physical barrier is gone. In certain species of *Echinolittorina* snails in the

Indo-West Pacific, local adaptation to continental or oceanic habitat restricts their distributions (Williams & Reid 2004; Reid *et al.* 2006) and Crandall et al. (2007) suggested that even small ecological differences could result in vastly different phylogeographic structure in gastropod taxa spanning the Sunda Shelf. If *P. minor* larvae can disperse across the Sunda shelf, but are maladapted and unable to recruit successfully, environmental differences could drive diversification or reinforce historical isolation. Future work using ecological niche modeling is required to test the hypothesis of ecological limits to larval dispersal.

In contrast to the highly structured *P. minor*, populations of *P. lugubris* maintain connectivity across all of Indonesia, with no evidence of divergence across well-known biogeographic and phylogeographic breaks (Barber *et al.* 2011; Carpenter *et al.* 2011). This result is similar to marine snails, *Nerita albicilla* and *N. plicata*, where disparate phylogeographic patterns were attributed to differences in habitat requirements: *N. plicata* lives in the high intertidal while *N. albicilla* lives in protected rubble fields (Crandall *et al.* 2007). While *P. minor* and *P. lugubris* have nearly identical habitat requirements, exploiting identical coral hosts in lagoonal environments, they do differ in larval ecology and dispersal ability. Larvae of *P. lugubris* are facultative planktotrophic and metamorphic competence within 5 days, but with food available can remain in the plankton for as long as 42 days (Kempf & Hadfield 1985; Miller & Hadfield 1986; Faucci *et al.* 2007; Ritson-Williams *et al.* 2007, 2009). The larvae of *P. minor* are lecithotrophic and reach metamorphic competence within 3 days, sometimes as fast as 1 day (Faucci *et al.* 2007, Ritson-Williams *et al.* 2007, 2009). The longer PLD of *P. lugubris* may have reestablished gene flow between ocean basins when sea levels rose. Alternatively, *P. lugubris* may have gone extinct on one side of

the Sunda Shelf land bridge, and its current widespread distribution a result of secondary colonization in more recent history, while *P. minor* survived on both sides and underwent allopatric speciation. More genetic information is needed to determine the full demographic history of this panmictic species.

Speciation in the sea

It is well established that geographic limits to dispersal contribute to divergence and speciation in the Coral Triangle (Barber 2009, Carpenter *et al.* 2011, Treml *et al.* 2015). However, allopatric explanations may be insufficient to explain speciation and the incredibly high biodiversity of the region. Neither the Sunda Shelf nor Halmahera Eddy are hard dispersal barriers. The majority of the Coral Triangle region was still open-ocean during Pleistocene glacial maxima (Voris 2000), and periods of lowered sea levels were also ephemeral (Lambek *et al.* 2002). As such, even if populations were isolated during periods of lowered sea level, gene flow should resume once sea levels rise again, potentially erasing diversification in neutral loci that occurred in allopatry. However, ABGD results indicate that these two clades are likely separate species. If environmental differences were sufficiently large on either side of the Sunda Shelf, ecological divergence could reinforce incipient allopatric divergence. The accessibility of genomic tools and marine environmental databases (e.g. Sbrocco & Barber 2013) will make it possible to test this hypothesis in the future.

While ecology may reinforce allopatric divergence, the results of this study clearly show that ecological barriers can also operate in sympatry. Distinct clades of *P. minor* are strongly differentiated by coral host associations despite gene flow being maintained within

these clades across long distances. Gene flow is even maintained across well-known phylogeographic barriers like the Maluku Sea (Barber *et al.* 2006, Barber *et al.* 2011). Thus, the only reasonable explanation for our results is that *P. minor* is undergoing sympatric, ecological divergence throughout the Coral Triangle. Given the prevalence of strong host associations in coral reef environments, it is likely that there are many more examples of ecological divergence in this region, multiplying the potential for diversification in the Coral Triangle. It is important for future marine speciation research to move beyond purely allopatric explanations of diversification and begin to explore the potential role of ecological differences in driving speciation in the sea.

TABLES AND FIGURES

**Table 1.1.** List of populations and sample sizes used for phylogeographic analyses, with coral host as labeled in the field based on visual identification.

| | Host species | *Phestilla lugubris* | *Phestilla minor* |
|---|---|---|---|
| West of Sunda Shelf | | | |
| 1. Aceh | *Porites lobata* | 9 | 7 |
| 2. Cubadak | *Porites lobata* | 7 | 5 |
| | *Porites lutea* | 6 | 9 |
| | *Porites sp.* | | 2 |
| East of Sunda Shelf | | | |
| West of Moluku Sea | | | |
| 3. Bali | *Porites lobata* | 10 | 13 |
| | *Porites lutea* | 2 | 14 |
| | *Porites attenuata* | | 2 |
| | *Porites cylindrica* | | 6 |
| | *Porites sp.* | 1 | 2 |
| 4. Komodo | *Porites lobata* | | 11 |
| | *Porites annae* | | 2 |
| 5. Donggala | *Porites lobata* | 6 | 19 |
| | *Porites cylindrica* | 0 | 10 |
| 6. Bunaken | *Porites lobata* | | 13 |
| | *Porites lutea* | 13 | 4 |
| | *Porites cylindrica* | 2 | 0 |
| 7. Lembeh | *Porites lobata* | 4 | 10 |
| | *Porites lutea* | | 4 |
| East of Moluku Sea | | | |
| 8. Raja Ampat | *Porites lobata* | 5 | 9 |
| | *Porites lutea* | 1 | 1 |
| | *Porites cylindrica* | | 3 |
| **Total** | | 66 | 146 |

**Table 1.2.** *Phestilla minor* divergence time estimates, based on molecular clock of 2.4% sequence divergence per million years (Hellberg and Vacquier 1999).

| | White clade vs. grey/black | Grey vs. black |
|---|---|---|
| Mean sequence identity | 92.98 | 96.06 |
| Mean sequence divergence | 7.02 | 3.94 |
| Divergence time | 2.92 | 1.64 |

**Table 1.3.** Summary statistics by locality for *Phestilla lugubris* and *Phestilla minor*. Haplotype diversity (h), nucleotide diversity ($\pi$), and $\theta_s$ caluculated in Arlequin. One P. minor sample from Komodo (Komo_06.03) was excluded because it belongs to the subspecies found in Aceh and Cubadak.

| | *Phestilla lugubris* | | | | *Phestilla minor* | | | |
|---|---|---|---|---|---|---|---|---|
| Locality | N | h | $\pi$ | $\theta_s$ | N | h | $\pi$ | $\theta_s$ |
| 1. Aceh | 9 | 0.972 | 0.009 | 5.887 | 7 | 1.000 | 0.010 | 3.808 |
| 2. Cubadak | 13 | 0.974 | 0.009 | 7.412 | 16 | 1.000 | 0.009 | 8.137 |
| 3. Bali | 13 | 1.000 | 0.010 | 8.056 | 37 | 0.964 | 0.012 | 15.091 |
| 4. Komodo | | | | | 12 | 1.000 | 0.010 | 8.278 |
| 5. Donggala | 6 | 1.000 | 0.011 | 7.007 | 29 | 0.958 | 0.022 | 13.750 |
| 6. Bunaken | 15 | 0.895 | 0.007 | 5.536 | 17 | 0.993 | 0.008 | 9.170 |
| 7. Lembeh | 4 | 1.000 | 0.008 | 5.455 | 14 | 1.000 | 0.008 | 8.805 |
| 8. Raja Ampat | 6 | 0.867 | 0.007 | 4.818 | 13 | 0.987 | 0.018 | 12.568 |

**Table 1.4.** AMOVA results for *Phestilla minor* Pacific populations. Within localities, all samples collected from *Porites cylindrica* were treated as one population and all samples collected from other hosts are treated as another population. We first ran AMOVA with no a priori groups, then grouped populations east and west of the Maluku Sea, then grouped by coral host. Significant $\Phi$ statistics ($p < 0.05$) shown in bold.

| | Among groups | | | | Among populations within groups | | | | Within populations | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | Var | %Var | $\Phi_{CT}$ | df | Var | %Var | $\Phi_{SC}$ | df | Var | %Var | $\Phi_{ST}$ |
| None | | | | | 8 | 2.07 | 40.2 | | 113 | 3.08 | 59.8 | **0.40** |
| Maluku Sea | 1 | -0.73 | -15.8 | -0.16 | 7 | 2.23 | 48.6 | **0.42** | 113 | 3.08 | 67.2 | **0.33** |
| Coral host | 1 | 5.00 | 58.1 | **0.58** | 7 | 0.53 | 6.10 | **0.15** | 113 | 3.08 | 35.8 | **0.64** |

**Table 1.5.** Contingency table to test association between *Phestilla minor* clades and coral host clades. Fisher's exact test $p < 0.0001$; Chi-square test, $X^2 = 78.65$, $p = 7.4 \times 10^{-19}$.

| Clade | *Porites* (other) | *Porites cylindrica* | Total |
|---|---|---|---|
| *P. minor*– black | 102 | 5 | 107 |
| *P. minor*– grey | 1 | 14 | 15 |
| Total | 103 | 19 | 122 |

**Table 1.6.** Pairwise $F_{sts}$ between populations on different corals at the same locality. Significant $F_{sts}$ ($p < 0.05$) shown in bold.

| | Bali other | Donggala other | Raja Ampat other |
|---|---|---|---|
| Bali cylindrica | 0.161 | | |
| Donggala cylindrica | | **0.739** | |
| Raja Ampat cylindrica | | | **0.838** |

**Table 1.7.** AMOVA results testing no a priori structure, then the Sunda Shelf and the Maluku Sea as barriers to gene flow for *Phestilla lugubris*. No significant $\Phi$ statistics.

| | Between groups | | | | Among populations within groups | | | | Within populations | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | Var. | %Var. | $\Phi_{CT}$ | df | Var. | %Var. | $\Phi_{SC}$ | df | Var. | %Var. | $\Phi_{ST}$ |
| None | - | - | - | - | 6 | -0.11 | -4.16 | - | 59 | 2.84 | 104.2 | -0.04 |
| Sunda Shelf | 1 | 0.03 | 1.06 | 0.01 | 5 | -0.13 | -4.70 | -0.05 | 59 | 2.84 | 103.6 | -0.04 |
| Maluku Sea | 1 | -0.12 | -4.41 | -0.04 | 5 | -0.09 | -3.43 | -0.03 | 59 | 2.84 | 107.8 | -0.08 |

**Figure 1.1.** Sampling localities.


**Figure 1.2.** Bayesian phylogenetic tree for 146 *Phestilla minor* COI sequences. Node values show Bayesian posterior probability on top and maximum likelihood (ML) bootstrap proportion on bottom. Graphs show for each of the three clade the percent of samples collected from each host clade.

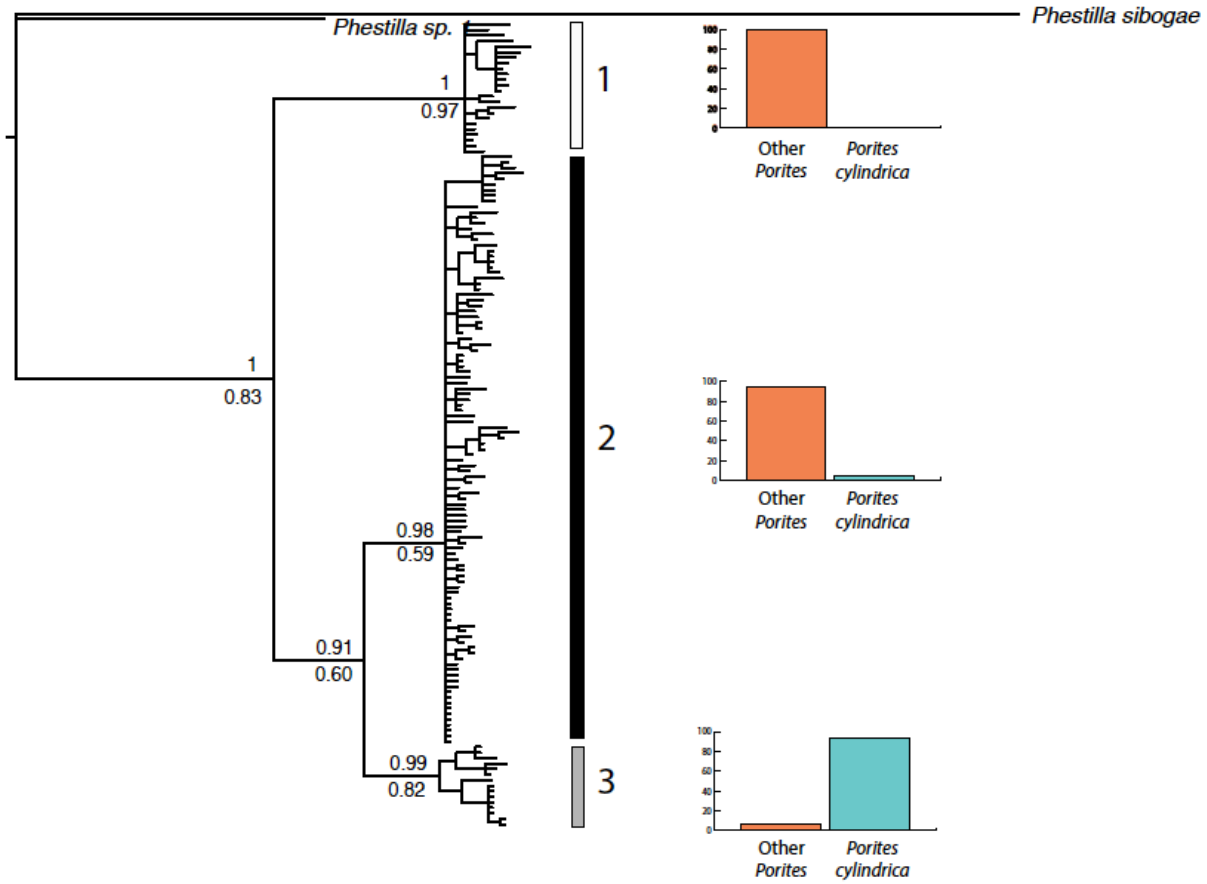


**Figure 1.3.** Minimum spanning tree of 114 *Phestilla minor* COI haplotypes. Node size corresponds to haplotype frequency, ranging from 1 to 10, and line length corresponds to mutational steps between each haplotype. Colors correspond to clades in Figure 2.

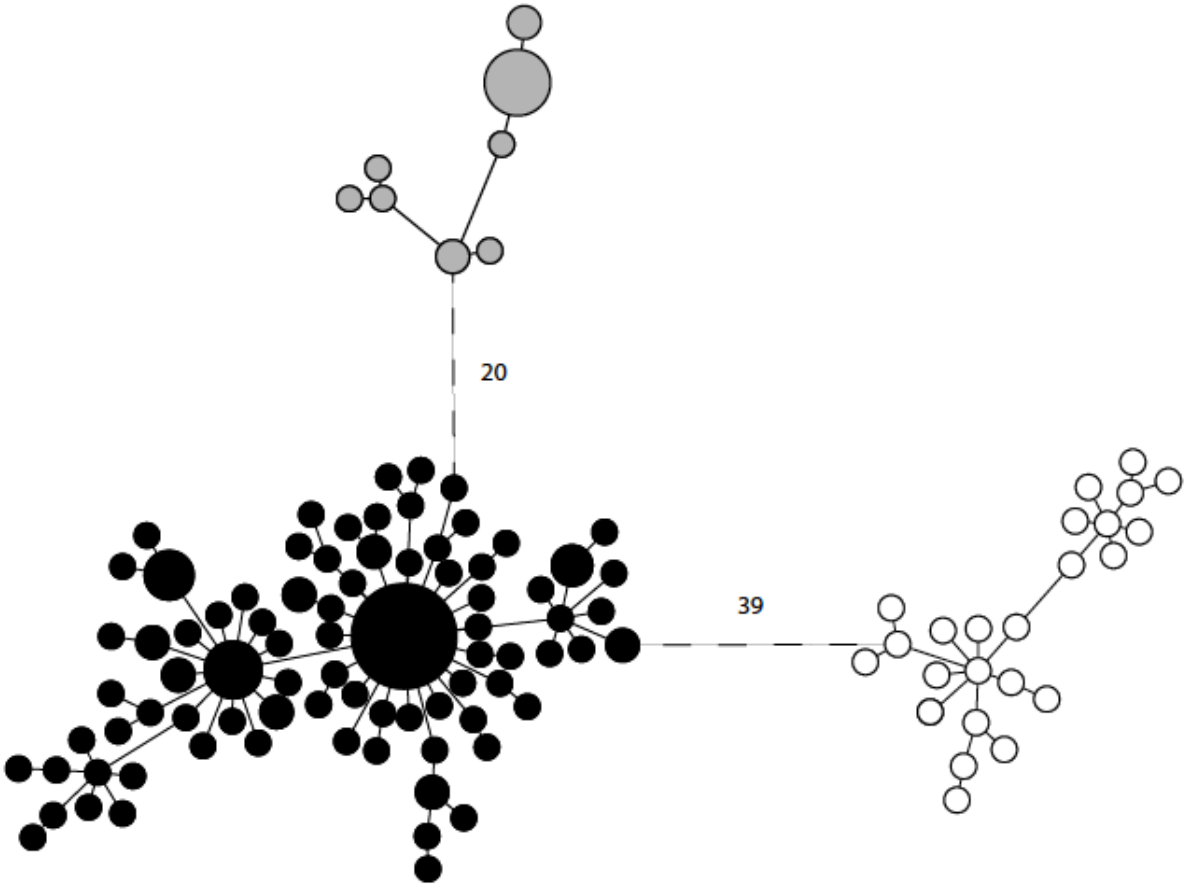
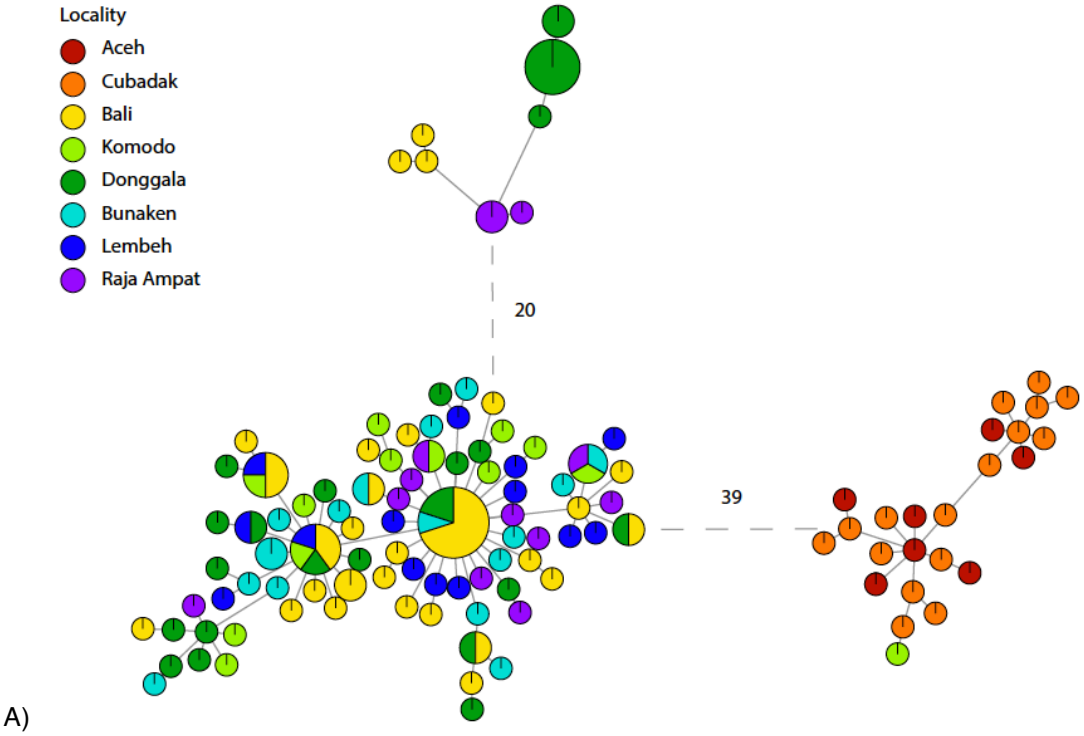
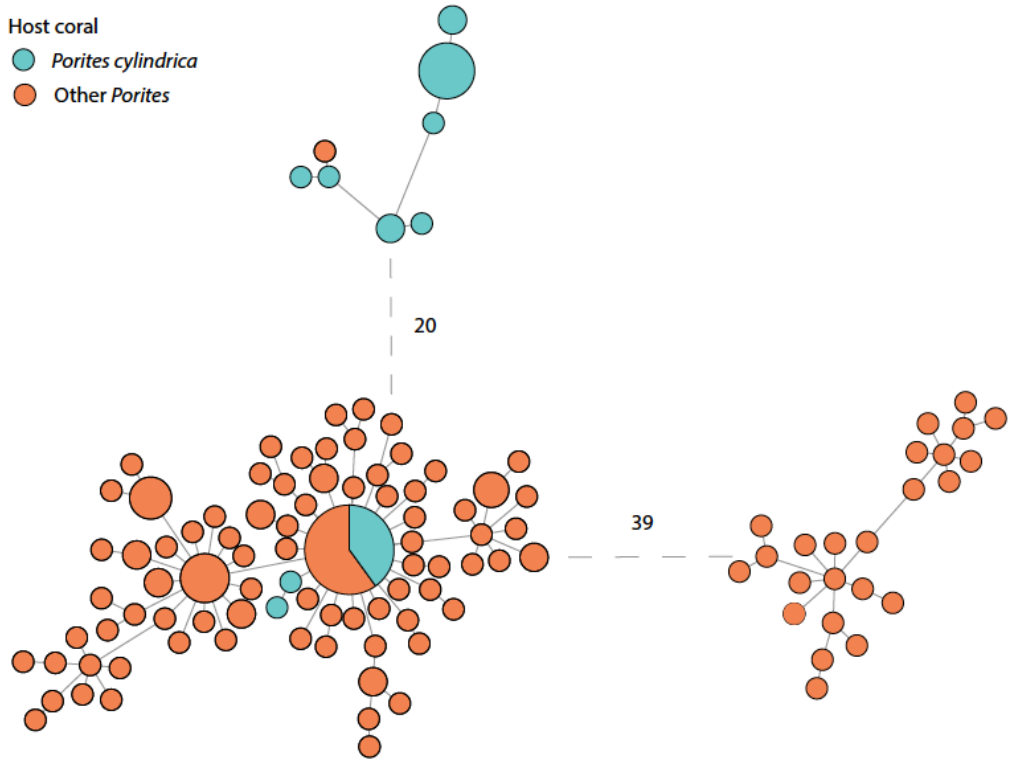


**Figure 1.4.** *Phestilla minor* haplotype map. Pie charts show proportion of haplotypes from each of the three clades shown in Figures 2 and 3. Size of pie charts is proportional to samples size.

**Figure 1.5.** Minimum spanning tree of 114 *Phestilla minor* COI haplotypes. Node size corresponds to haplotype frequency, ranging from 1 to 10, and line length corresponds to mutational steps between each haplotype. Colors correspond to A) sampling location and B) coral host clade.




A)




B)

**Figure 1.6.** Maximum likelihood tree of 47 H2 sequences from *Porites* hosts of *Phestilla minor* and/or *P. lugubris*. Node values show Bayesian posterior probability on top and ML bootstrap proportion on bottom.

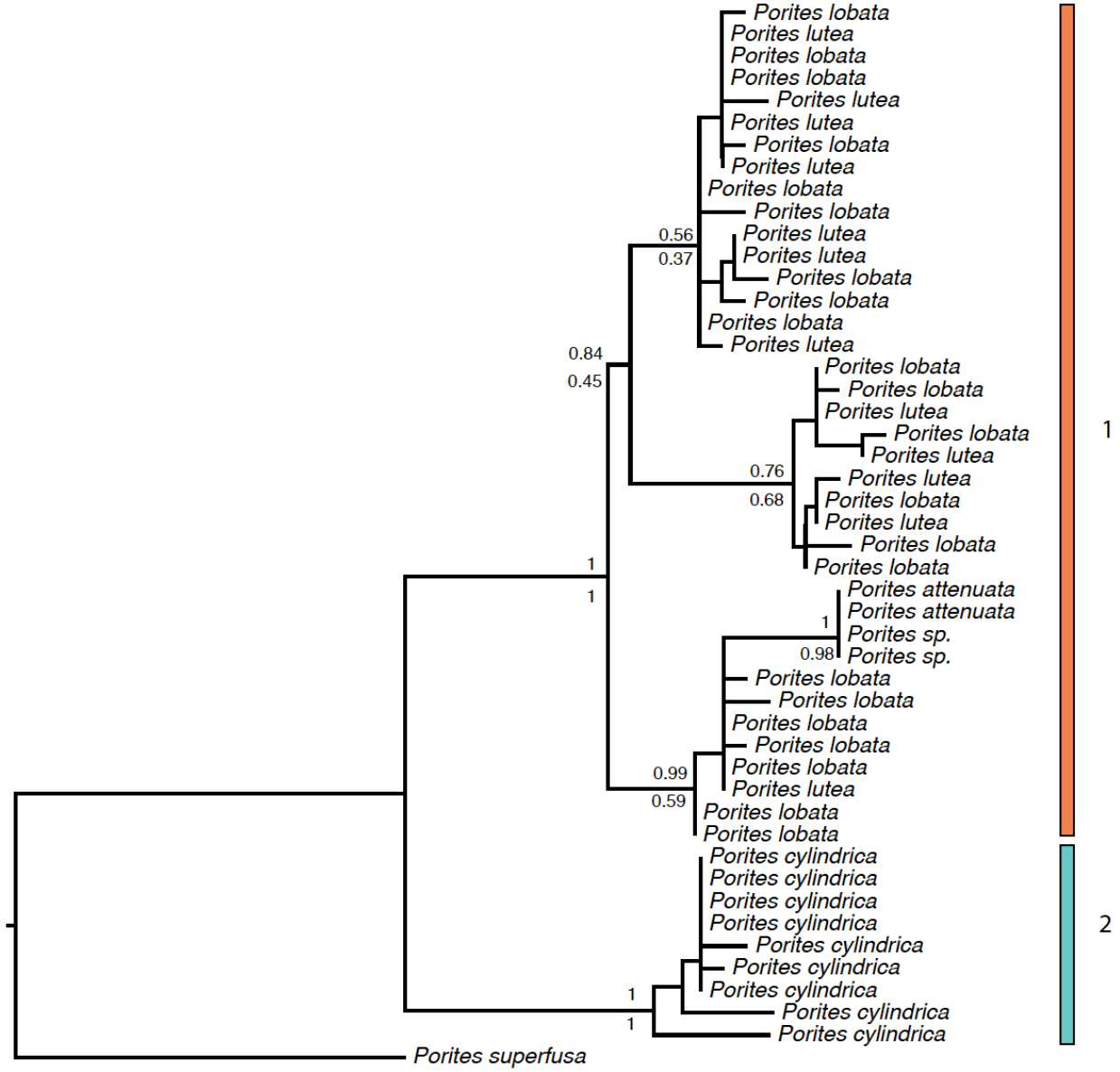


**Figure 1.7.** Bayesian phylogenetic tree of 66 *Phestilla lugubris* COI sequences. Node values show Bayesian posterior probability on top and ML bootstrap proportion on bottom.

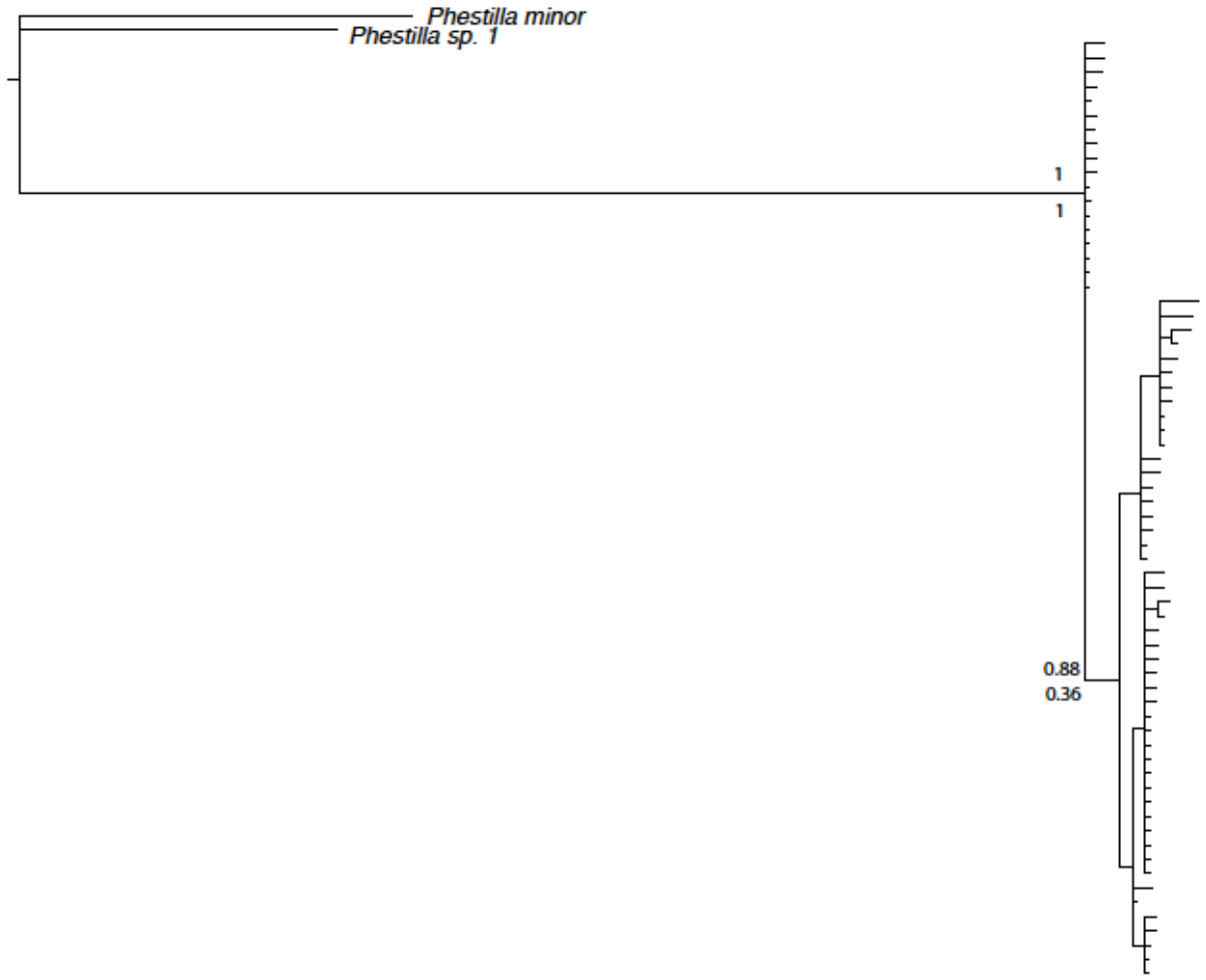
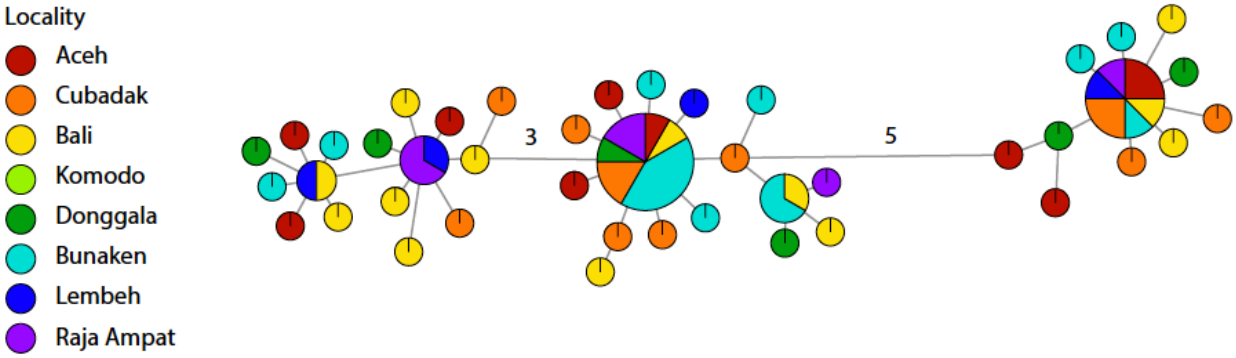
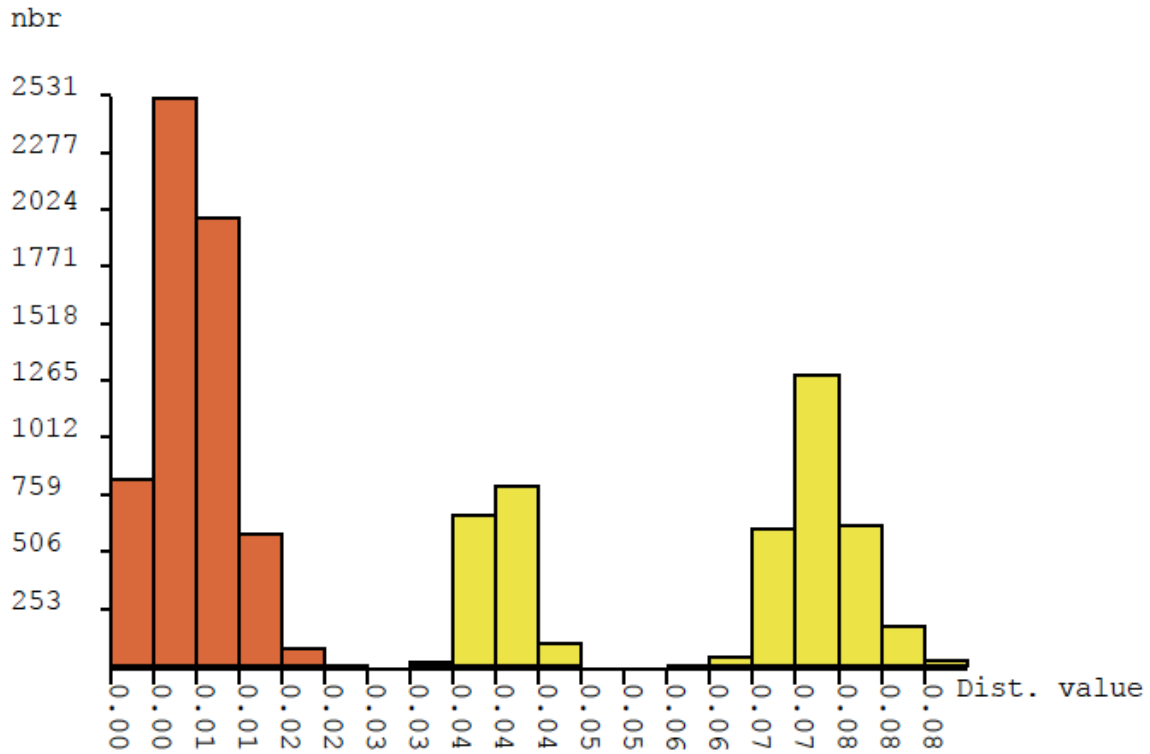



**Figure 1.8.** Minimum spanning tree of 43 *Phestilla lugubris* COI haplotypes. Node size corresponds to haplotype frequency, ranging from 1 to 12, and line length corresponds to mutational steps between each haplotype. Colors correspond to sampling location.



Supplement

**Figure S1.1.** ABGD results for 146 *Phestilla minor* COI sequences. Intraspecific genetic distances shown in orange, interspecific distances shown in yellow.



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# CHAPTER 2

## The relative roles of natural selection and limits to gene flow in genomic divergence of coral-associated nudibranchs

### Introduction

After more than a century of study, speciation remains one of the most intriguing and studied processes in biology. The key to understanding speciation is in understanding the underlying processes driving the evolution of reproductive isolation (Mayr 1963). The most commonly invoked and conceptually simple speciation process is allopatric divergence—reproductive isolation resulting from extended physical isolation of populations (Coyne & Orr 2004). However, a growing body of research is challenging this traditional view of speciation, indicating instead that natural selection can drive population divergence and lead to reproductive isolation in sympatry (Rosenzweig 1978; Schliewen *et al.* 1994; Bush 1994; Dieckmann & Doebeli 1999; Smadja & Butlin 2011; Gavrilets 2014).

One of the most commonly proposed mechanisms for sympatric speciation is ecological speciation, where reproductive isolation and diversification is driven by differential selection between ecological niches (Rundle & Nosil 2005; Schluter 2009). Examples of ecological speciation span the tree of life, ranging from bacteria (Lassalle *et al.* 2015), to plants (Lowry *et al.* 2008; Mitsui *et al.* 2011; Andrew *et al.* 2012; Osborne *et al.* 2013; Papadopulos *et al.* 2013; Roda *et al.* 2013), and vertebrates (Smith 1997; Rundle *et al.* 2000; Parchman *et al.* 2006; Rice *et al.* 2009; Nunes *et al.* 2011; Ballentine *et al.* 2013; Muñoz *et al.* 2013; Sanders *et al.* 2013; Brawand *et al.* 2014).

Our expanded understanding of the relative roles of allopatric and sympatric speciation is largely due to advances in genomic techniques that allow the identification of cryptic sympatric divergence in wild populations (Peccoud *et al.* 2009; Garvin *et al.* 2010). In addition, new theoretical models are helping us understand the relative roles of genetic drift and natural selection through the unique signatures that these different processes leave on the genome (Beaumont & Balding 2004; Via & West 2008; Nosil 2009; Pinho & Hey 2010; Feder *et al.* 2012). Importantly, genome-wide data allow the detection of genetic signals of adaptive divergence when populations are still connected by gene flow, providing new insight into the first steps of ecological speciation, much like phylogeography provides insights on nascent allopatric divergence.

Ecological divergence is often proposed as an early stage of "speciation-with-gene-flow" (Feder *et al.* 2012). However, ecological speciation requires that ecological divergence culminates in reproductive isolation (Orr & Smith 1998; Hendry 2009). Reproductive isolation happens most rapidly when the trait on which natural selection acts directly influences mate choice. For example, in swamp sparrows, the ecological differences between tidal marsh and terrestrial habitats are associated with divergent selection on bill length (Grenier & Greenberg 2005). These changes in bill morphology affect song production, impacting mate recognition (Ballentine *et al.* 2013), and allowing sexual selection to reinforce ecological divergence that may ultimately result in reproductive isolation. Such traits that result from selection based on ecological conditions but also promote nonrandom mating have been dubbed "magic traits" (Thibert-Plante & Gavrilets 2013) as they have the necessary ingredients to promote rapid reproduction isolation and speciation based on ecological differences.

While magic traits may seem rare, under a broad definition they also include extrinsic associations between ecology and mate choice (Servedio *et al.* 2011). Host-parasite relationships are a prime example. When mating is limited to other individuals on the same host, assortative mating automatically accompanies host preference, promoting the evolution of reproductive isolation. Ecological speciation via host range expansion or host shifting has been most commonly demonstrated in phytophagous insects (Hawthorne & Via 2001; Nice *et al.* 2002; Janz & Nylin 2008; Apple *et al.* 2010; Singer & McBride 2010; Midamegbe *et al.* 2011; Powell *et al.* 2014; Soria-Carrasco *et al.* 2014) but also in other taxa such as fungal pathogens (Silva *et al.* 2012), coral-dwelling barnacles (Tsang *et al.* 2009), snails (Simmonds 2016), and nudibranchs (Faucci *et al.* 2007, Fritts-Penniman *et al.* in prep). These latter marine examples all involve adults with low mobility, so wherever larvae initially settle is where they will find a mate.

Of course, host-mediated assortative mating is not enough to cause speciation. Even if mate choice is restricted to other individuals on the same host, if individuals disperse and settle randomly each generation and host choice has no fitness consequence, host races cannot diverge. Divergence can only occur if individuals associated with specific hosts have traits that are better suited to a specific host, leading to differential survival and reproduction on different hosts (Matsubayashi *et al.* 2010). In this case, selection will favor settlement onto the host for which an individual is best equipped, ultimately resulting in ecological speciation.

Key to the process of ecological divergence across hosts is the relative influence of selection and gene flow, which have different effects on the genome. If migration between hosts is initially common, but selection acts on a few traits and genetic loci, adaptive

divergence will precede neutral divergence, and the genes under selection may be identified as highly diverged relative to the background level of gene flow occurring at other loci, resulting in "genomic islands of divergence" (Michel *et al.* 2010). Over time, divergence hitchhiking can cause these islands to grow, so the amount of time under selection greatly influences these genomic signatures (Via 2012). However, if selection simultaneously acts on many genes spread across the genome, divergence hitchhiking may rapidly become genome hitchhiking, where we see "continents" rather than "islands" of divergence (Feder *et al.* 2012), and the overall effect on the genome can resemble that of genetic drift alone. In either case, eventually selection for host preference and the accompanying assortative mating will result in the host races becoming reproductively isolated.

In contrast, if host preference acts to automatically isolate populations, the effect of selection may be negligible, as the lack of gene flow and subsequent genetic drift will promote neutral genetic differentiation across the genome (Smadja & Butlin 2011; Bird *et al.* 2012). For example, the initial mutation that promotes settlement onto a new host could include a preference for or an adaptive advantage on that host, and selection on future generations will already favor host specificity. In this case, adaptive and neutral divergence would occur simultaneously, and speciation could proceed rapidly (Thibert-Plante & Hendry 2010). As such, it is important to have a broad view of genetic differentiation associated with ecological speciation. It is well known that the neutral process of genetic drift contributes greatly to allopatric speciation, and that divergent natural selection is a critical component of ecological speciation (Rundle & Nosil 2005). However, if ecological divergence occurs between discrete microhabitats such as hosts, and this habitat specificity acts as a strong barrier to gene flow from the start, genetic drift could also play an important role in

ecological divergence. Meanwhile, different selective pressures on either side of a geographic barrier are likely to contribute to allopatric divergence. As a result, allopatric and ecological diversification may not be as fundamentally different as the literature suggests (Orr & Smith 1998).

The coral associated nudibranch *Phestilla minor* feeds, lives, and mates upon *Porites* corals in the Indo-Pacific Ocean. Previous work on mitochondrial COI shows that populations collected west and east of the Sunda Shelf are divergent enough to be considered two species, allopatric divergence that likely resulted from geographic isolation during low sea level stands (Fritts-Penniman *et al.* in prep). However, within populations east of the Sunda Shelf, there are two divergent clades of *P. minor* that live on different host coral clades, a pattern consistent with ecological divergence. As such this group of nudibranchs is an ideal system in which to test whether sympatric, ecological divergence leaves a different signature on the genome than allopatric divergence.

In this paper, we examine patterns of genomic divergence between lineages of *Phestilla minor* that are isolated either by geographic barriers or by host. Specifically, we test the hypothesis that natural selection plays a larger role in divergence among host lineages than among geographic lineages. To determine the relative roles of natural selection and neutral evolution in host-driven divergence compared to allopatric divergence, we conducted genome scans independently on population pairs comprised of either a) sympatric populations on different hosts, or b) allopatric populations on the same host. We use outlier tests to show that while allopatric divergence and sympatric ecological divergence can leave similar signatures on the genome, the repeated occurrence of specific loci under selection distinguishes the process of ecological speciation.

Methods

To test the relative importance of geographic and ecological barriers to gene flow in *Phestilla minor*, we collected specimens from 8 localities in Indonesia that span the Sunda Shelf, with Aceh and Cubadak on the west side, and Bali, Komodo, Donggala, Bunaken, Lembeh, and Raja Ampat on the east side (Figure 2.1). Nudibranch specimens were collected by hand, photographed, relaxed on ice, and stored in ethanol or RNALater.

We field identified the *Porites* corals from which we collected nudibranchs. However, because *Porites* corals are notorious for having incomplete species boundaries, we grouped coral hosts into two previously established clades: clade 1- *Porites lobata*, *Porites annae, Porites attenuata, Porites lutea;* clade 2- *Porites cylindrica* (Fritts-Penniman *et al.* in prep). To confirm coral field identifications, we took coral tissue samples to genetically assess the membership of *Porites* samples to each of these two clades.

We extracted DNA from ethanol preserved nudibranchs using Qiagen DNeasy tissue kits and Omega E.Z.N.A. Mollusc DNA kits. We isolated foot tissue for extraction when possible, but for individuals smaller than 5 mm, we used half the body, and for those smaller than 2 mm we used the entire body. We excluded cerata (the projections of the gut covering the bodies of most aeolid nudibranchs) from the DNA extractions as they often contain recently ingested coral tissue (Rudman 1982).

We used reduced representation sequencing to simultaneously discover and genotype single nucleotide polymorphisms (SNPs). Specifically, we used a restriction site-associated (RAD) method developed by Wang *et al.* (2012). RAD sequencing facilitates genome-wide genotyping at a reduced cost through targeted sequencing of short fragments

surrounding recognition sites of a restriction endonuclease. The chosen method is called 2b-RAD for its use of a type IIB restriction enzyme. The enzyme cleaves the DNA upstream and downstream of the recognition site throughout the genome, producing 35 basepair fragments of the same loci across all individuals. These fragments were amplified and tagged with sample-specific barcodes, then random combinations of 12-17 individuals were pooled with normalized concentrations and run in a single lane of the Illumina HiSeq2000, generating approximately 15 million 50 bp single end reads per sample.

We trimmed, assembled, filtered, and genotyped the sequence data using a custom set of Perl scripts written for 2b-RAD by Wang et al. (2012). This protocol simultaneously ascertains and genotypes SNPs, eliminating the risk of ascertainment bias. Without a reference genome, we were concerned about DNA contamination from ingested coral tissue in the small samples from which we extracted DNA from the entire body. To eliminate that risk, we built the de novo reference scaffold using a subset of samples from which the DNA had been extracted only from foot tissue. We filtered loci for: 10x coverage, 30% missing individuals, and repetitive sites, then randomly selected one SNP per RAD tag to avoid linkage. We then used VCFtools (Danecek *et al.* 2011) to remove individuals with higher than 50% missing genotypes. These moderate thresholds ensured that we retained as many informative loci as possible to fully understand patterns of genomic divergence among sampled populations.

To test for genome-wide population structure of *Phestilla minor* among coral hosts and sampling sites we used ADMIXTURE (Alexander *et al.* 2009). ADMIXTURE takes multi-locus allele frequency data and uses a cross validation procedure to predict the number of ancestral populations (K), and for each individual outputs the proportion of its genotypes

derived from each population. This method does not require previous knowledge of the number of populations, and allows for the identification of individuals sampled from a population other than their population of origin.

To estimate the level of overall gene flow between populations we calculated $F_{st}$ (Weir & Cockerham 1984) using SNPrelate (Zheng *et al.* 2012). To visualize the distribution of genetic variation among populations we conducted a principle components analysis (PCA) using SNPrelate. To determine the hierarchy of divergence among the resulting populations, we selected three samples from each population, converted SNP genotypes into a concatenated alignment, and built a phylogenetic tree using the coalescent method of SNAPP (Bryant *et al.* 2012) in BEAST 2.4.3 (Bouckaert *et al.* 2014). We ran SNAPP with the default parameter settings, with an MCMC chain length of 3,000,000, sampling every 1,000$^{th}$ tree. We analyzed the resulting trees with a 10% burn-in, and assessed node support with posterior probability. To tease apart the contributions of neutral and adaptive processes to population structure, we repeated all of these analyses after removing outlier loci, which we detected using the following methods.

To determine the influence of natural selection in the divergence of *Phestilla minor* populations, we tested for genomic signals of adaptive divergence. Because the *Phestilla* SNP data was ascertained randomly from across the genome, several hundred genotyped loci are randomly expected to fall within or near functional genes. Genes under divergent selection between populations will have much stronger differences in allele frequencies, resulting in higher than expected $F_{st}$ values (Beaumont & Balding 2004). SNPs within non-coding DNA that lie adjacent to selected loci are also expected to have higher than expected differentiation due to genetic hitchhiking (Flaxman *et al.* 2013). Therefore, we

analyzed SNP data to calculate the level of gene flow between populations in terms of $F_{st}$ for each locus, and detect outlier loci with $F_{st}$ values significantly different from the rest, providing evidence of strong selection.

We used two programs to determine Fst outliers: BayeScan (Foll & Gaggiotti 2008), which takes a Bayesian approach, and fhetboot (Flanagan & Jones in review), which employs the frequentist (**fdist)** method. We ran BayeScan with the default settings. The prior odds for the neutral model were set to 10, meaning the neutral model was considered to be 10 times more likely than that of selection. We considered a locus to be an outlier if it had a q value less than 0.05, corresponding to a 5% false discovery rate (FDR = 0.05). Outliers with a positive alpha value are under divergent selection, while outliers with a negative alpha value are under balancing selection. To determine the effect of coral host and geographic isolation on overall genomic divergence, we extracted the locus-specific $F_{sts}$ calculated by BayeScan and used a Welch two sample t-test to compare the distribution of $F_{sts}$ across all loci between ecologically and geographically isolated population pairs.

The program fhetboot is a new R package for implementation of the fdist outlier test, which generates 95% and 99% confidence intervals for the neutral model by bootstrapping the $F_{st}$-heterozygosity distribution of each dataset. As recommended by developers, we tested fhetboot with 10 bootstraps and 100 bootstraps, and since we obtained identical results, we proceeded with 10 bootstraps for analyses (Flanagan and Jones in review, or R vignette). We considered a locus to be under divergent selection if it fell outside of the 99% confidence interval and had a positive $F_{st}$. We considered those loci outside the 99% confidence interval with a negative $F_{st}$ to be under balancing selection.

Although selection may act in allopatric divergence, due to geographic variability of ecological conditions we do not expect the same loci to be under selection between different population pairs. In contrast, if selection is associated with coral host clade, we expect the same loci to be under selection across the species range, and should detect the same outlier loci in multiple host-separated population pairs. To test this expectation, we narrowed our dataset to loci detected in two or more population pairs, and counted the number of times recurring outliers were only found in instances of either geographic or coral host divergence. We created a heat map to visualize the degree of outlier overlap between the tested datasets.

Finally, to learn more about the ultimate mechanism of coral-mediated natural selection on *Phestilla minor*, we investigated the potential function of outlier loci that repeatedly occurred in tests of host divergence. We used the online Basic Local Alignment Search Tool (BLAST, NCBI) to search all publically available molluscan genomes for potential matches to our outlier loci (Altschul *et al.* 1990). We also used the command line version of BLAST, blastn, to search for matches in the transcriptome of *Phestilla sp. 1*, which is the sister species to *Phestilla minor*. This transcriptome was sequenced from a sample we collected for phylogenomic analysis (Goodheart *et al.* in prep).

## Results

### SNP filtering

We sequenced 101 individuals, but immediately filtered out 4 under-sequenced individuals that lacked sufficient data for genotyping, leaving 97. 80 of these individuals

were collected from *Porites* clade 1 corals, and 17 from *Porites* clade 2 corals, as determined by previous study (Fritts-Penniman *et al.* in prep). We built the de novo reference using the first 2,000,000 reads each from 8 individuals spanning the geographic range of the samples and both coral hosts. After alignment to the reference and filtering for high quality reads with a minimum coverage of 10x, we obtained genotypes for 1,060,724 polymorphic sites across these 97 individuals. After applying locus and individual filters to the whole dataset, 4,966 loci and 86 individuals remained (Table 2.2, dataset 1).

Overall population structure

ADMIXTURE results from 4,966 SNPs across 86 individuals, yielded the lowest cross-validation (CV) error with three populations (Table 2.3). These three populations are not strictly associated with geography. One cluster is composed of all populations west of the Sunda Shelf, which all live on host clade 1 (Figure 2.2, maroon); a second cluster is composed of those populations east of the Sunda Shelf that live on host clade 1 (Figure 2.2, orange), and the third cluster is all populations east of the Sunda Shelf on host clade 2 (Figure 2.2, blue). Phylogenetic analysis of these three clusters using coalescent methods in SNAPP showed that divergence occurred first across the Sunda Shelf, then across coral hosts on the eastern side (Figure 2.3A). Removing outlier loci (see below) had no impact on population structure (Table 2.3, Figure 2.2C) or phylogenetic results (Figure 2.3B), but a higher proportion of individuals appeared to have admixed ancestry than when outliers were included.

Overall $F_{st}$ between these three populations was 0.231 (Table 2.4). When populations were grouped by host $F_{st}$ was 0.213, and when populations were grouped by

side of the Sunda Shelf $F_{st}$ was 0.194 (Table 2.4). Excluding outliers, $F_{st}$ between the three populations was 0.192; grouped by host $F_{st}$ was 0.188; grouped by side of the Sunda Shelf $F_{st}$ was 0.146 (Table 2.4). In a PCA of the 4,779 biallelic SNPs genotyped in these 86 individuals, the first principle component roughly reflected variation associated with coral host, while the second principle component roughly reflected the variation across the Sunda Shelf (Figure 2.4A). Across both PCs, the variation among individuals in the eastern populations from host clade 1 was remarkably low compared to the others. This group formed a very tight cluster, while the rest of the individuals spread out across these axes. The variation within western populations and host clade 2 populations was not explained by sampling locality (Figure 2.5A). However, excluding outliers, the variation represented in PC 2 in the western population completely disappeared, and the variation within host clade 2 populations clearly was associated with sampling locality (Figure 2.5B).

Signatures of natural selection

We ran BayeScan and fhetboot on the overall dataset grouping populations 2 ways: first by the 3 populations indicated by ADMIXTURE, then subdividing these populations by sampling location and host for a total of 11 populations. With 3 populations, we found 0 outliers using BayeScan and 526 outliers using fhetboot; 495 with negative $F_{sts}$ and 31 with positive $F_{sts}$ (Table 2.5). With 11 populations, BayeScan detected 14 outliers with negative $\alpha$ and 15 with positive $\alpha$ (Figure 2.6). With fhetboot we found 592 outliers with negative $F_{sts}$ and 34 with positive $F_{sts}$ (Table 2.5).

The nature of RAD data is such that the more diverse individuals you include during quality filtering, the lower your overall genotyping rates are, and the more data you must

discard. Therefore, to retain more data and maximize our ability to detect loci under selection, we subdivided the data into smaller datasets, and applied filters independently to each set (Table 2.2). These datasets were chosen so that we could do 4 direct comparisons of ecological and geographic divergence (as demonstrated in Figure 2.7), first across the whole sampling range, then looking specifically at Bali, Donggala, and Raja Ampat.

To examine overall genomic patterns of divergence across coral hosts while controlling known geographic divergence, we analyzed all individuals from the east side of the Sunda shelf, grouped into two populations based on their coral host (Table 2.2, dataset 2A). BayeScan found 0 outliers and fhetboot found 996 negative outliers and 43 positive outliers (Table 2,5, Figure 2.8A). To examine overall genomic patterns of divergence across the Sunda Shelf while controlling for coral host, we analyzed all individuals from host clade 1, with Aceh and Cubadak in one population and Bali, Komodo, Donggala, Bunaken, Lembeh, and Raja Ampat in another (Table 2.2, dataset 2B). BayeScan found 0 outliers and fhetboot found 957 negative outliers and 46 positive outliers (Table 2.5, Figure 2.8B).

We then looked specifically at the localities that had both coral hosts: Bali, Donggala, and Raja Ampat. For each site we compared ecological divergence within that site to allopatric divergence between that site and Cubadak on the other side of the Sunda Shelf (Table 2.2, datasets 3-5). Between host-separated populations in Bali, BayeScan found 0 outliers and fhetboot found 2,723 negative outliers and 112 positive outliers (Table 2.5, Figure 2.8C). Between host clade 1 populations in Bali and Cubadak, BayeScan found 0 outliers and fhetboot found 1,440 negative outliers and 81 positive outliers (Table 2.5, Figure 2.8D). Between host-separated populations in Donggala, BayeScan found 0 outliers and fhetboot found 1,081 negative outliers and 84 positive outliers (Table 2.5, Figure 2.9A).

Between host clade 1 populations in Donggala and Cubadak, BayeScan found 0 outliers and fhetboot found 1,976 negative outliers and 94 positive outliers (Table 2.5, Figure 2.9B). Between host-separated populations in Raja Ampat, BayeScan found 0 outliers and fhetboot found 1,735 negative outliers and 63 positive outliers (Table 2.5, Figure 2.9C). Between host clade 1 populations in Raja Ampat and Cubadak, BayeScan found 0 outliers and fhetboot found 813 negative outliers and 111 positive outliers (Table 2.5, Figure 2.9D).

In addition to testing for outliers, BayeScan outputs mean $F_{st}$ across populations for each locus. We compared the distributions of $F_{sts}$ in corresponding population pairs to gain insight into the relative influence of geographic and ecological isolation on the genome. Overall, differentiation between coral host populations ($F_{st}$ = 0.60) was slightly higher than differentiation between populations on either side of the Sunda Shelf ($F_{st}$ = 0.59, Figure 2.10A, t-test: t = 125.86, df = 14347, p-value < 2.2e-16). The difference between divergence mechanisms is particularly extreme in Donggala, where mean $F_{st}$ was 0.50 between coral hosts and 0.36 across the Sunda Shelf (Figure 2.10C, t = 1018.7, df = 23634, p-value < 2.2e-16). In Raja Ampat, mean $F_{st}$ was 0.37 between coral hosts and 0.31 across the Sunda Shelf (Figure 2.10D, t = -433.48, df = 24121, p-value < 2.2e-16). In Bali, the pattern was reversed, with mean $F_{st}$ of 0.36 between coral hosts and 0.37 across the Sunda Shelf (Figure 2.10B, t = 100.07, df = 33102, p-value < 2.2e-16).

Because we were specifically interested in mechanisms of divergent selection, we looked more closely at the loci detected as positive outliers. Due to the risk of false positives in $F_{sts}$ outlier tests, especially when overall divergence is high (Foll & Gaggiotti 2008), we narrowed positive loci down to those detected in more than one outlier test. The majority of outlier loci are only indicated as outliers in one dataset, but 21 outliers came up in 2 or more

datasets, for a total of 23 occurrences (Table 2.6). Only 2 of these 21 repeatedly occur as outliers between population pairs that are diverging in allopatry, while 12 exclusively occur as outliers between population pairs diverging on different coral hosts. We will refer to these 12 as "host-specific" loci following the model of Egan *et al.* (2008). The other 7 are outliers in tests of both coral host and geographic divergence. For each of the 12 host-specific loci, we used BLAST to explore the potential functions. None of these 36bp sequence tags aligned with the *Phestilla sp.* transcriptome scaffold, but all had partial matches to molluscan genomes in the NCBI database. The majority of loci had a variety of matches with no detectable pattern, but locus number 38622 aligned only with membrane protein mRNAs. Its top match is an alignment of 22 basepairs of a lysosome membrane protein in the California Sea Hare, *Aplysia californica*, at 91% identity. This particular locus is the single most commonly detected outlier, having been identified as an outlier by Bayescan and fhetboot for the full dataset (Table 2.2, set 1), with fhetboot in host-diverging populations throughout the Pacific (Table 2.2, set 2A), and locally between host populations in Raja Ampat (Table 2.2, set 5A).

## Discussion

Multilocus SNP data from *Phestilla minor* populations spanning the Indonesian Archipelago provides evidence for the complex interaction between natural selection and gene flow across both geographic and ecological barriers. Analysis of SNPs ascertained using RAD sequencing from across the genome showed strong population structure across the Sunda Shelf phylogeographic barrier (see Barber et al 2011, Carpenter *et al.* 2011 for reviews), and across two major clades of *Porites* coral hosts (Fritts-Penniman *et al.* in prep).

Geographic and host-associated population structure remained even after removing loci that were indicated by outlier tests to be under selection, showing that both coral host and the Sunda Shelf have led to significant neutral divergence across the genome, and are likely undergoing speciation. While strong neutral divergence is not often reported in the ecological speciation literature, it is expected when divergent selection acts as a generalized barrier to gene flow (Thibert-Plante & Hendry 2010).

Scans for natural selection detected many outliers, the vast majority of which were negative, providing evidence for balancing selection across populations (Beaumont & Balding 2004; Foll & Gaggiotti 2008). However, the existence of some positive outlier loci in all population pairs indicated that there is also divergent selection acting across coral host and across the Sunda Shelf barrier. The same loci were generally not found to be outliers across different population pairs. However, recurring loci were more likely to be host-specific than associated with geography, as has been observed in phytophagous insects (Egan *et al.* 2008; Soria-Carrasco *et al.* 2014). These host-specific loci will provide a starting point for deeper investigations into host-driven adaptation in nudibranchs.

Neutral genomic divergence

Population genetic and phylogenetic analyses of genome-wide SNPs supported previous results from mitochondrial COI that both coral host and the Sunda Shelf act as barriers to gene flow in *Phestilla minor* (Fritts-Penniman *et al.* in prep). Both datasets indicate that the species split first across the Sunda Shelf, and more recently between populations inhabiting the two distinct *Porites* host clades east of the Sunda Shelf. Previous COI sequence data suggested a small degree of mismatch between nudibrach and host

coral clades, or that one nudibranch clade was more generalist, occupying both hosts, while the other clade was limited to host 2 (Fritts-Penniman *et al.* in prep). However, SNPs provide higher resolution data and statistical power for population assignment than traditional markers (Helyar *et al.* 2011). With SNPs, we saw that all individuals collected from host clade 2 do in fact cluster together (Figure 2.2).

Looking only at putative neutral SNPs, a small number of nudibranchs collected from clade 1 host corals showed significant probabilities of membership in multiple nudibranch lineages. In some cases, individual samples had genetic signatures associated with both coral host clades (Figure 2.2C, orange and blue), or allopatric populations from the same coral host (Figure 2.2C, orange and maroon). This pattern could be evidence of either shared ancestral polymorphism or recent gene flow (Hebert *et al.* 2013). For recently diverged sympatric clades, it is difficult to distinguish between these two. For allopatric populations, our phylogenetic results lend some insight into whether admixed individuals have resulted from ongoing gene flow or ancestral polymorphism. We know that the east and west populations diverged first, followed by coral-associated divergence in the east (Figure 2.3). Any shared ancestral polymorphism from before divergence across the Sunda Shelf should be seen throughout all populations, regardless of host. However, zero individuals from host clade 2 show evidence of admixed ancestry with populations west of the Sunda Shelf. Therefore, it is much more likely that the admixture seen between allopatric populations on host clade 1 is due to recent gene flow. The Sunda Shelf is not a hard geographic barrier; many marine invertebrates are well connected by gene flow throughout this entire region, including the congener *Phestilla lugubris* (Carpenter *et al.* 2011, Fritts-Penniman *et al.* in prep).

BayeScan tests of population pairs spanning either the ecological or geographic barrier yielded very high mean $F_{st}$ values (ranging from 0.31 to 0.6) with most loci having an $F_{st}$ close to the mean, and no outliers (Figure 2.10). This is expected when gene flow is low and genetic differentiation has proceeded relatively consistently across the genome, a signature of neutral evolution (Foll & Gaggiotti 2008). Surprisingly, in three out of four comparisons $F_{st}$ values for the vast majority of loci were higher between sympatric host populations than between populations across the Sunda Shelf (Figure 2.10), despite phylogenetic evidence that divergence across the Sunda Shelf occurred first (Figure 2.3). This result aligned with our ADMIXTURE analysis, which showed lower rates of admixture between sympatric different-host populations than between allopatric, same-host populations. If host-driven selection is strong, it may be easier for *Phestilla minor* to cross the Sunda Shelf via pelagic larval dispersal than it is to settle on the wrong host. Such habitat-driven isolation is a key mechanism for ecological speciation (Rundle & Nosil 2005).

Signatures of natural selection

It is clear that *Phestilla minor* is diverging on different coral host lineages. However, to isolate the influence of adaptation to coral hosts from adaptation to local environmental conditions, we compared pairwise genome scans of *P. minor* collected from the same population from different host corals to scans for selection from allopatric *P. minor* populations collected from the same host coral lineage. We detected a handful of outlier loci in BayeScan, and hundreds in fhetboot, especially negative outliers presumed to be under balancing selection (Table 2.5). We expected to see a stronger signal of balancing selection (negative outliers) across allopatric populations that were on the same host, and a stronger

signal of divergent selection (positive outliers) across populations on different hosts. However, there were no discernable trends; both negative and positive outliers were found in similar proportions across both barriers (Table 2.5). However, when we removed outliers from population structure analyses, it had a bigger effect on geographic variation than ecological variation. This is most evident in the PCA, where populations across the Sunda Shelf were distinguished with outliers included, but cluster more closely without outliers, indicating that natural selection plays an important role in divergence across this barrier (Figure 2.4). Within clade 2 coral host populations, no geographic structure is evident with outliers, but without outliers we see some geographic clustering (Figure 2.5). Given the large proportion of negative outliers, this may be indicative of the homogenizing effect of balancing selection across clade 2 coral host populations (Mäkinen *et al.* 2008).

Similar studies found higher rates of outliers between different-host populations than same-host populations, but also had much lower overall $F_{sts}$ between populations (Egan *et al.* 2008; Apple *et al.* 2010). As noted earlier, mean $F_{sts}$ between *Phestilla minor* populations pairs were very high, which can limit the power of an outlier test to identify loci genuinely under divergent selection, especially when sample sizes and population numbers are low (Beaumont & Balding 2004; Foll & Gaggiotti 2008). It is possible that these populations are too divergent to accurately assess the role of natural selection in the process. However, looking only at the distribution of outliers that we found, we see no evidence that natural selection plays a larger role in divergence among sympatric populations from different coral host clades than in allopatric populations from the same coral host clade.

A significant risk of $F_{st}$ outlier detection methods is that loci identified from only one analysis may be false positives, (Beaumont & Balding 2004; Foll & Gaggiotti 2008). Loci

truly under selection across coral hosts or across the Sunda Shelf should, therefore, be identified regardless of the dataset analyzed. In particular, we expected that outliers would overlap between population pairs, because many of our population pairs were not independent; we tested the same barriers at different geographic scales. However, results showed that outlier overlap occurs disproportionately in the tests of coral host divergence, with very few overlapping outliers between tests across the Sunda Shelf (Table 2.6). For example, 8 of the outliers detected as under divergent selection across hosts in Bali (dataset 3A) were also detected as being under divergent selection across hosts throughout all eastern populations, which includes Bali (dataset 2A). In comparison, only 1 outlier that was detected across the Sunda Shelf between Bali and Cubadak (dataset 3B) was also detected when testing this barrier across all sampling sites (dataset 2B). This disproportionate recurrence of outliers provides strong evidence for the consistency of natural selection between coral hosts across the range of *Phestilla* minor, but not across the Sunda Shelf. In similar studies on phytophagous insects, recurring outliers are considered to be candidates for further investigation into the mechanisms of selection (Egan *et al.* 2008; Soria-Carrasco *et al.* 2014). Since we are interested in the specific mechanism of natural selection imposed by *Porites* corals on their nudibranch parasites, we took a closer look at the 12 outliers that overlap exclusively in tests of coral host divergence, the "host-specific" outliers.

There are two main ways in which coral hosts can impose selective pressure on *Phestilla* nudibranchs: host recognition, and host compatibility. *Phestilla* nudibranchs in the water column recognize chemical cues released by corals, which induce them to settle and metamorphose on the coral below (Hadfield & Pennington 1990; Hadfield *et al.* 2006;

Ritson-Williams *et al.* 2007). While host recognition is essential to the evolution of host specificity, in order for host specificity to evolutionarily favorable, there must also be a selective advantage to life on that particular host, i.e., host compatibility. Selection may act on a nudibranchs ability to overcome the coral's defense, in order to live upon and consume the coral tissue. Like all cnidarians, corals use stinging nematocysts for defense, and several nudibranchs have evolved the ability to consume these nematocysts without harm, some even retaining them for their own defensive use in a specialized organ called a cnidosac (Martin 2003; Churchill *et al.* 2014). *Phestilla* nudibranchs do not have cnidosacs, but they are still able to consume nematocysts without harm (Rudman 1981).

We analyzed BLAST results looking for gene matches that could potentially be involved in host recognition or coral-feeding. The most promising hit was a locus for which 22 basepairs aligned at 91% identity with a scaffold from the California Sea Hare, *Aplysia californica,* that is predicted to be a lysosomal membrane protein. Extensive research on the movement of nematocysts through the nudibranch digestive tract indicates that in some aeolid nudibranchs, nematocysts are retained in phagosomes rather than quickly fusing with lysosomes and being digested, as is the normal outcome for victims of phagocytosis (Martin 2003; Greenwood 2009). In *Phestilla*, nematocysts are likely digested more rapidly, since they do not store them for defense, but the mechanism that determines how nematocysts are treated inside the digestive tract is still unknown. If membrane proteins aids in recognition of nematocysts, these could either induce fusion and digestion by the lysosome, or avoidance and retention of the nematocyst within the cerata. This would not be the first time selection on lysosomal activity has been observed in a mollusk. In *Mytilus edulis*, natural selection maintains an allele frequency cline in the *Lap* locus, which encodes an

aminopeptidase found in digestive cell lysosomes, between areas of differing salinity (Koehn *et al.* 1980).

Ecological speciation

Genetic evidence for host-specific clades in *Phestilla minor* strongly suggests that ecological divergence has occurred, and that natural selection played a role in this process. However, the recovery of high level of neutral differentiation precludes determination of whether divergence was initiated with or without gene flow. The ecological speciation literature is full of empirical examples and models of the early stages of ecological divergence-with-gene-flow, in which only loci under selection diverge, and the rest of the genome remains homogenized (Pinho & Hey 2010; Andrew & Rieseberg 2013; Huang *et al.* 2014). However, there are fewer examples demonstrating later stages along the speciation continuum (Nosil & Feder 2012; Powell *et al.* 2013). In *Phestilla minor* we have discovered either a more advanced stage of divergence-with-gene-flow, or a case of secondary contact between host races that evolved in allopatry. These processes are indistinguishable after the fact, because they leave similar signatures on the genome. In allopatric ecological divergence, natural selection and genetic drift act in concert, causing widespread genomic divergence (Orr & Smith 1998). In divergence-with-gene-flow, when multiple loci are under selection, genetic hitchhiking causes differentiation of quite a large proportion of the genome, referred to as genome hitchhiking (Thibert-Plante & Hendry 2010; Flaxman *et al.* 2013). The end result is a genomic architecture that closely resembles that caused by isolation by distance, referred to as isolation by adaptation (Nosil *et al.* 2008; Funk *et al.* 2011).

Evidence for isolation by adaptation has been found in many organisms, from Atlantic cod (Berg *et al.* 2015) to phytophagous walking sticks (Nosil *et al.* 2008) and leaf beetles (Funk *et al.* 2011). Models have shown that a high level of neutral divergence between sympatric populations is particularly likely when divergent selection is acting between two habitats that are distributed in a coarse mosaic over the landscape (Flaxman *et al.* 2012). The current results cannot reject initiation of divergence in allopatry. However, given the fact that these populations currently exist in sympatry, and both host corals have very large, overlapping ranges in a mosaic distribution, we believe that a scenario of isolation by adaptation as a result of divergence-with-gene-flow is more likely than allopatric divergence. Furthermore, coralivorous snails living and feeding on the same *Porites* coral hosts show very similar patterns of ecological differentiation (Simmonds 2016). It is more likely that the similarities between these nudibranchs and snails results from differentiation on hosts, rather than both diverging in allopatry and then expanding ranges independently to yield concordant patterns of host differentiation.

Our research contributes to a growing body literature on ecological speciation in the sea. Recent use of genomic techniques has shown evidence for ecological speciation in coral reef fishes, such as in the genera *Hypoplectrus* (Puebla *et al.* 2014), *Acanthurus* (Gaither *et al.* 2015), and *Haemulon* (Bernal *et al.* 2016). The evolution of host preference and its effect on speciation, have been thoroughly explored in the phytophagous insect literature (Janz & Nylin 2008; Hood *et al.* 2015; McClure *et al.* 2016), but relatively little in marine systems, despite strong parallels (Krug 2011). Given the growing literature on the variable responses of different corals to climate change and ocean acidification (Pandolfi *et al.* 2011), it is becoming more important to recognize the role of specific coral species in

producing and maintaining marine biodiversity. Coral reefs provide a wealth of systems that are yet untapped in the field of ecological speciation (Rocha *et al.* 2005; Krug 2011). We encourage researchers interested in host shifting and host specialization as mechanisms for speciation to turn to the sea for inspiration.

TABLES AND FIGURES

**Table 2.1.** Samples collected and sequenced.

| | Host | |
|---|---|---|
| Location | *Porites* clade 1 | *Porites* clade 2 |
| West of Sunda Shelf | | |
| Aceh | 7 | |
| Cubadak | 12 | |
| East of Sunda Shelf | | |
| Bali | 12 | 5 |
| Komodo | 9 | |
| Donggala | 14 | 9 |
| Bunaken | 14 | |
| Lembeh | 9 | |
| Raja Ampat | 7 | 3 |
| **Total** | 84 | 17 |

**Table 2.2.** Subsets of data independently filtered and analyzed for tests of natural selection.

| Set | Barrier | Populations | Individuals | SNPs |
|---|---|---|---|---|
| 1 | Both | Aceh – host 1 | 5 | |
| | | Cubadak – host 1 | 8 | |
| | | Bali – host 1 | 12 | |
| | | Bali – host 2 | 5 | |
| | | Komodo – host 1 | 9 | |
| | | Donggala – host 1 | 11 | |
| | | Donggala – host 2 | 7 | |
| | | Bunaken – host 1 | 11 | |
| | | Lembeh – host 1 | 9 | |
| | | Raja Ampat – host 1 | 6 | |
| | | Raja Ampat – host 2 | 3 | |
| | | Total | **86** | **4,966** |
| 2A | Host | Bali – host 1 | 12 | |
| | | Bali – host 2 | 5 | |
| | | Komodo – host 1 | 9 | |
| | | Donggala – host 1 | 11 | |
| | | Donggala – host 2 | 6 | |
| | | Bunaken – host 1 | 12 | |
| | | Lembeh – host 1 | 9 | |
| | | Raja Ampat – host 1 | 7 | |
| | | Raja Ampat – host 2 | 3 | |
| | | Total | **74** | **7,429** |

| | | | | |
|---|---|---|---|---|
| 2B | Sunda Shelf | Aceh – host 1<br>Cubadak – host 1<br>Bali – host 1<br>Komodo – host 1<br>Donggala – host 1<br>Bunaken – host 1<br>Lembeh – host 1<br>Raja Ampat – host 1<br>Total | 5<br>8<br>12<br>9<br>11<br>12<br>9<br>7<br>**73** | **7,223** |
| 3A | Host | Bali – host 1<br>Bali – host 2<br>Total | 12<br>5<br>**17** | **18,730** |
| 3B | Sunda Shelf | Bali – host 1<br>Cubadak – host 1<br>Total | 12<br>8<br>**20** | **14,746** |
| 4A | Host | Donggala – host 1<br>Donggala – host 2<br>Total | 11<br>9<br>**20** | **11,645** |
| 4B | Sunda Shelf | Donggala – host 1<br>Cubadak – host 1<br>Total | 11<br>8<br>**19** | **12,567** |
| 5A | Host | Raja Ampat – host 1<br>Raja Ampat – host 2<br>Total | 7<br>3<br>**10** | **12,839** |
| 5B | Sunda Shelf | Raja Ampat – host 1<br>Cubadak – host 1<br>Total | 7<br>9<br>**16** | **11,396** |

**Table 2.3**. ADMIXTURE CV errors for K=1 through K=7, 86 samples across all sampling sites.

| K | All loci | Outliers excluded |
|---|---|---|
| 1 | 0.22176 | 0.19722 |
| 2 | 0.21477 | 0.19310 |
| 3 | 0.21157 | 0.19202 |
| 4 | 0.22174 | 0.21016 |
| 5 | 0.23169 | 0.21322 |
| 6 | 0.23658 | 0.21347 |
| 7 | 0.23217 | 0.20961 |

**Table 2.4.** Weir and Cockerhams's $F_{st}$ calculated in SNPrelate, with and without outlier loci.

| Population groups | $F_{st}$ - all loci | $F_{st}$ - outliers excluded |
|---|---|---|
| ADMIXTURE K = 3 | 0.231 | 0.192 |
| Across Host | 0.213 | 0.188 |
| Across Sunda Shelf | 0.194 | 0.146 |

**Table 2.5.** Outlier test results for all sets of populations. We present the total number of outliers identified, the number of negative outliers (for Bayescan defined as outliers with $\alpha \leq 0$, for fhetboot defined as outliers with $F_{st} \leq 0$), positive outliers (for Bayescan defined as outliers with $\alpha > 1$, for fhetboot defined as outliers with $F_{st} > 1$), and the proportion of each relative to the total number of loci used in each test.

| | | | BayeScan | | | | fhetboot | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Data | Populations # - Barrier | Total loci | - $\alpha$ | Prop. - | + $\alpha$ | prop. + | -$F_{st}$ | Prop. - | +$F_{st}$ | prop. + |
| 1 | 3 - Both | 4,966 | 0 | 0 | 0 | 0 | 495 | 0.010 | 31 | 0.006 |
| 1 | 11 - Both | 4,966 | 14 | 0.003 | 15 | 0.003 | 592 | 0.119 | 34 | 0.007 |
| 2A | 2 - Host | 7,429 | 0 | 0 | 0 | 0 | 996 | 0.134 | 43 | 0.006 |
| 3A | 2 - Host | 18,730 | 0 | 0 | 0 | 0 | 2,723 | 0.145 | 112 | 0.006 |
| 4A | 2 - Host | 11,645 | 0 | 0 | 0 | 0 | 1,081 | 0.093 | 84 | 0.007 |
| 5A | 2 - Host | 12,839 | 0 | 0 | 0 | 0 | 1,735 | 0.135 | 63 | 0.005 |
| 2B | 2 - Geography | 7,223 | 0 | 0 | 0 | 0 | 957 | 0.132 | 46 | 0.006 |
| 3B | 2 - Geography | 14,746 | 0 | 0 | 0 | 0 | 1,440 | 0.098 | 81 | 0.005 |
| 4B | 2 - Geography | 12,567 | 0 | 0 | 0 | 0 | 1,976 | 0.157 | 94 | 0.007 |
| 5B | 2 - Geography | 11,396 | 0 | 0 | 0 | 0 | 813 | 0.071 | 111 | 0.010 |

**Table 2.6.** Heat map showing overlap of outliers between population pairs having a coral host barrier, a geographic barrier, or both. Note that not all datasets are completely independent population pairs. Dataset 1 includes samples from all other datasets; dataset 2A includes samples from 3A, 4A, and 5A; dataset 2B includes samples from 3B, 4B, and 5B.

| | | Barrier | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Both | Coral Host | | | | Geography | | | |
| | Data | 1 | 2A | 3A | 4A | 5A | 2B | 3B | 4B | 5B |
| Both | 1 | | 3 | 1 | | 1 | | 1 | | 2 |
| Coral host | 2A | | | **8** | **2** | **1** | | 1 | | |
| | 3A | | | | **1** | | | | | |
| | 4A | | | | | | | | | |
| | 5A | | | | | | | | | |
| Geography | 2B | | | | | | | | | **1** |
| | 3B | | | | | | | | **1** | |
| | 4B | | | | | | | | | |
| | 5B | | | | | | | | | |

**Figure 2.1.** Sampling localities (same as Figure 1.1).

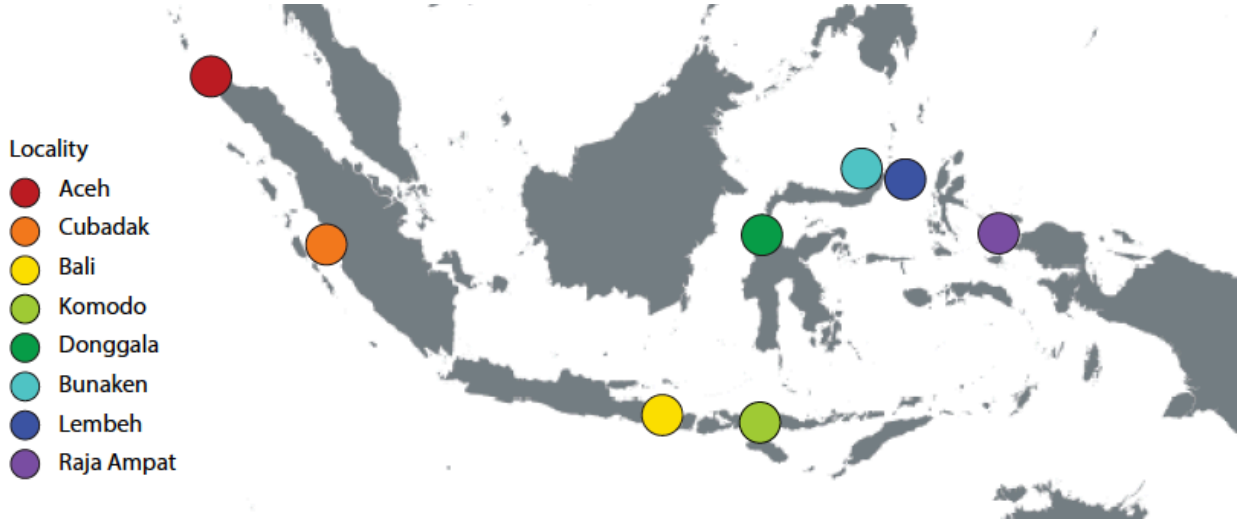


**Figure 2.2.** ADMXITURE results, K = 3. A) samples organized by geographic location, west to east, B) samples organized by location and coral host, C) samples organized by location and coral host, with outlier loci removed.

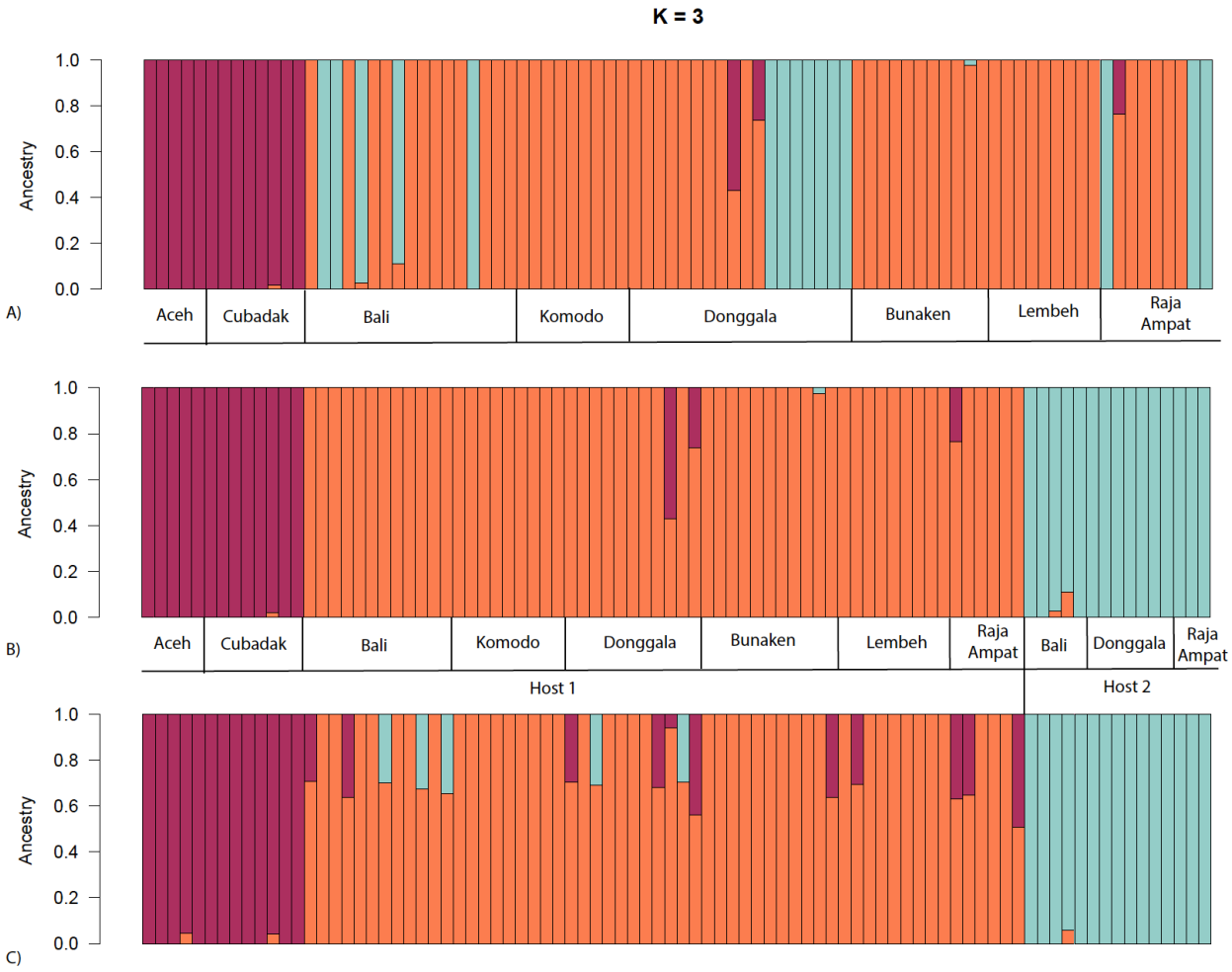
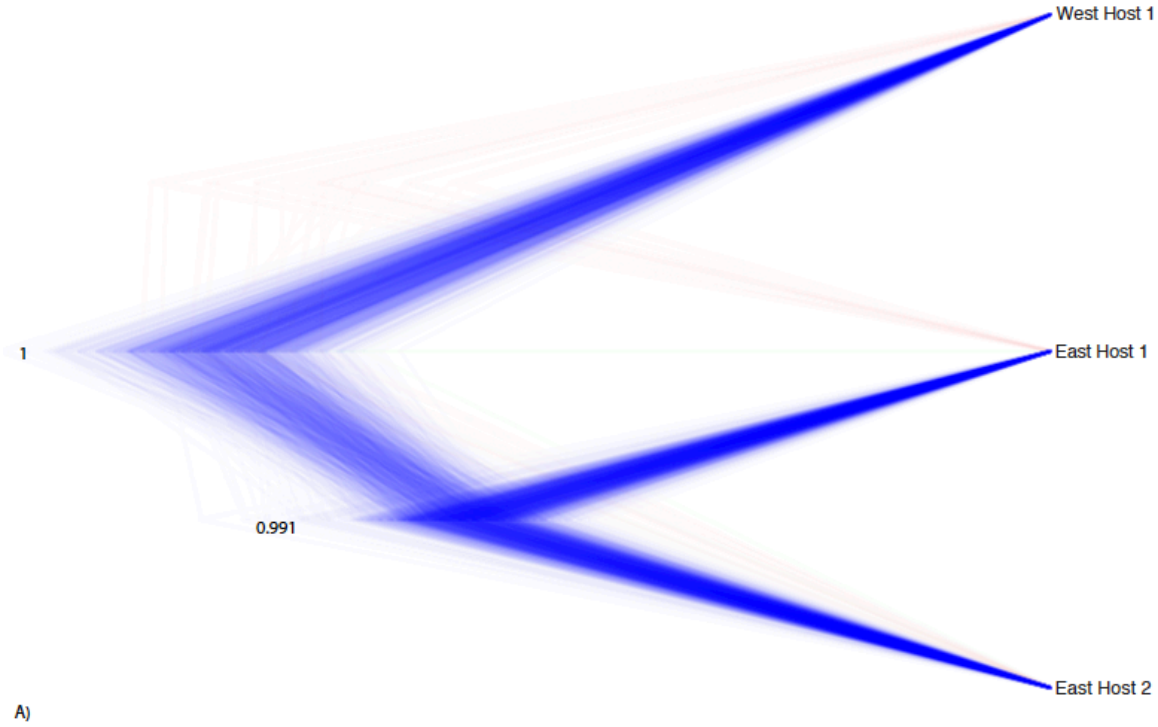
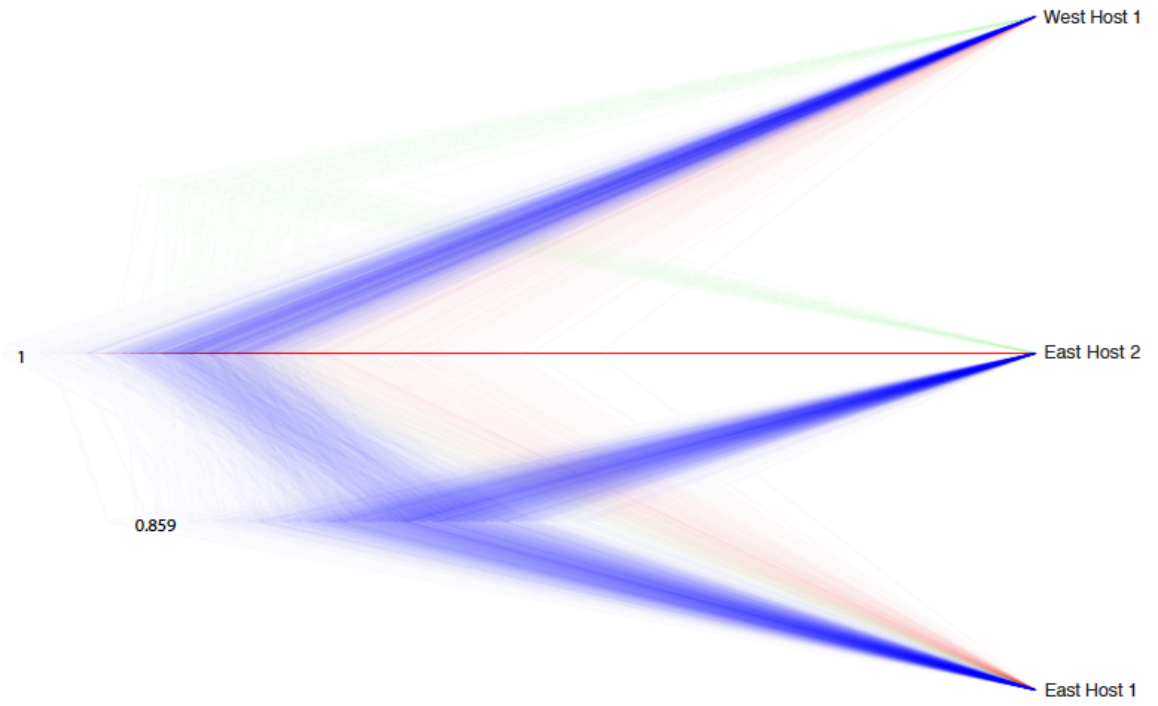


**Figure 2.3.** Phylogenetic tree generated by SNAPP using 3 samples per clade and aligned genotypes for A) all 4,966 SNPs and B) 4,090 non-outlier SNPs.




A)




B)

**Figure 2.4.** PCA of 86 individuals, color coded by ADMIXTURE cluster. A) 4,779 biallelic SNPs. B) 3,990 non-outlier SNPs.

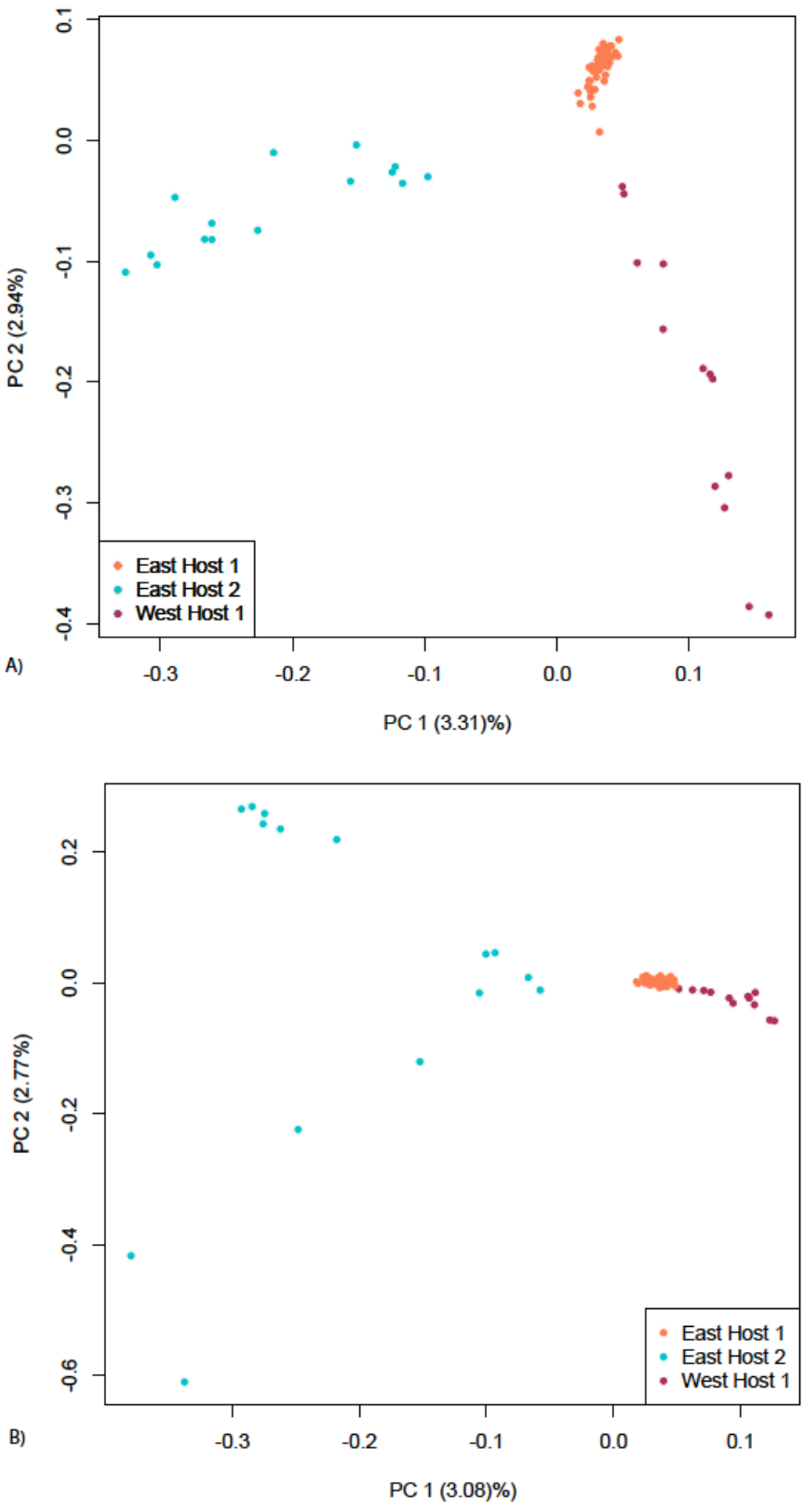
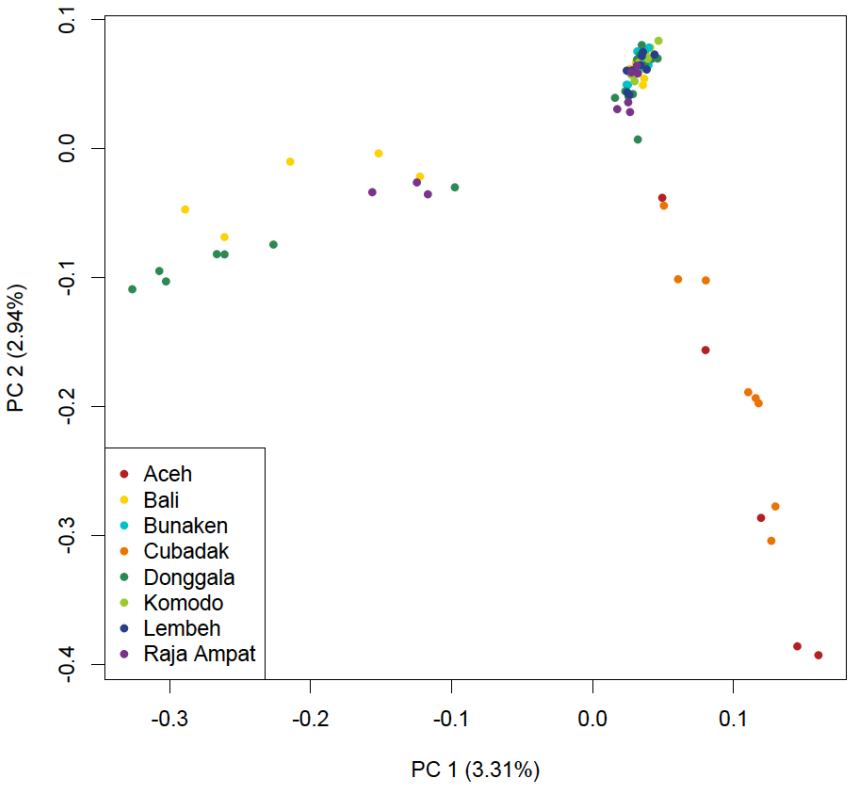


**Figure 2.5.** PCA of 86 individuals, color coded by locality. A) 4,779 biallelic SNPs. B) 3,990 non-outlier SNPs.



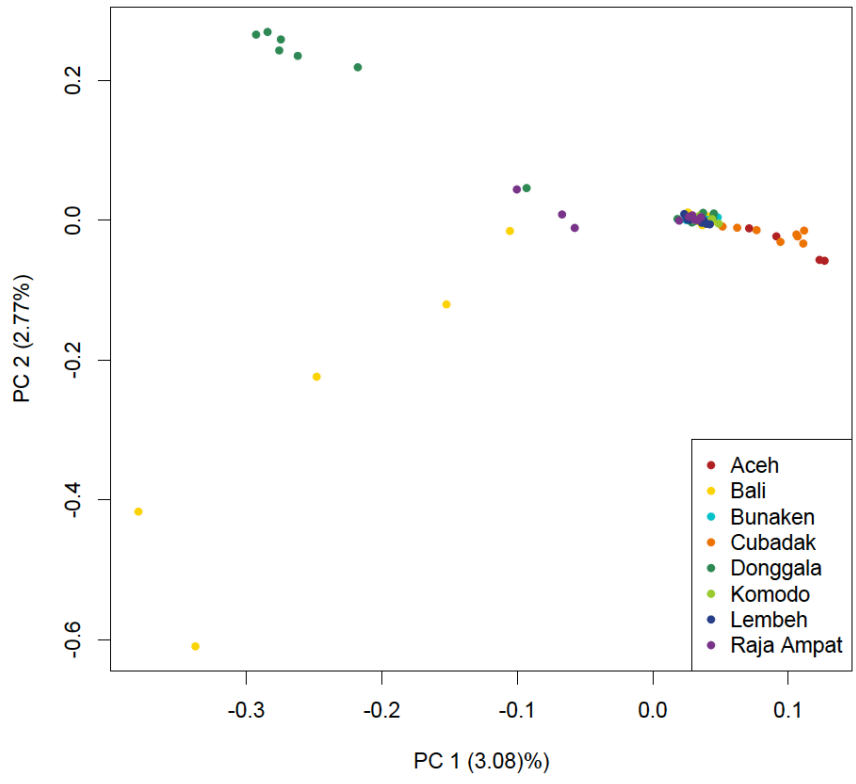



**Figure 2.6.** BayeScan results for full dataset subdivided into 11 populations by locality and host. Vertical line indicates q = 0.05; all loci to the left of that threshold are outliers. Outliers on the upper half of the distribution are under divergent selection; those on the bottom half are under purifying selection.

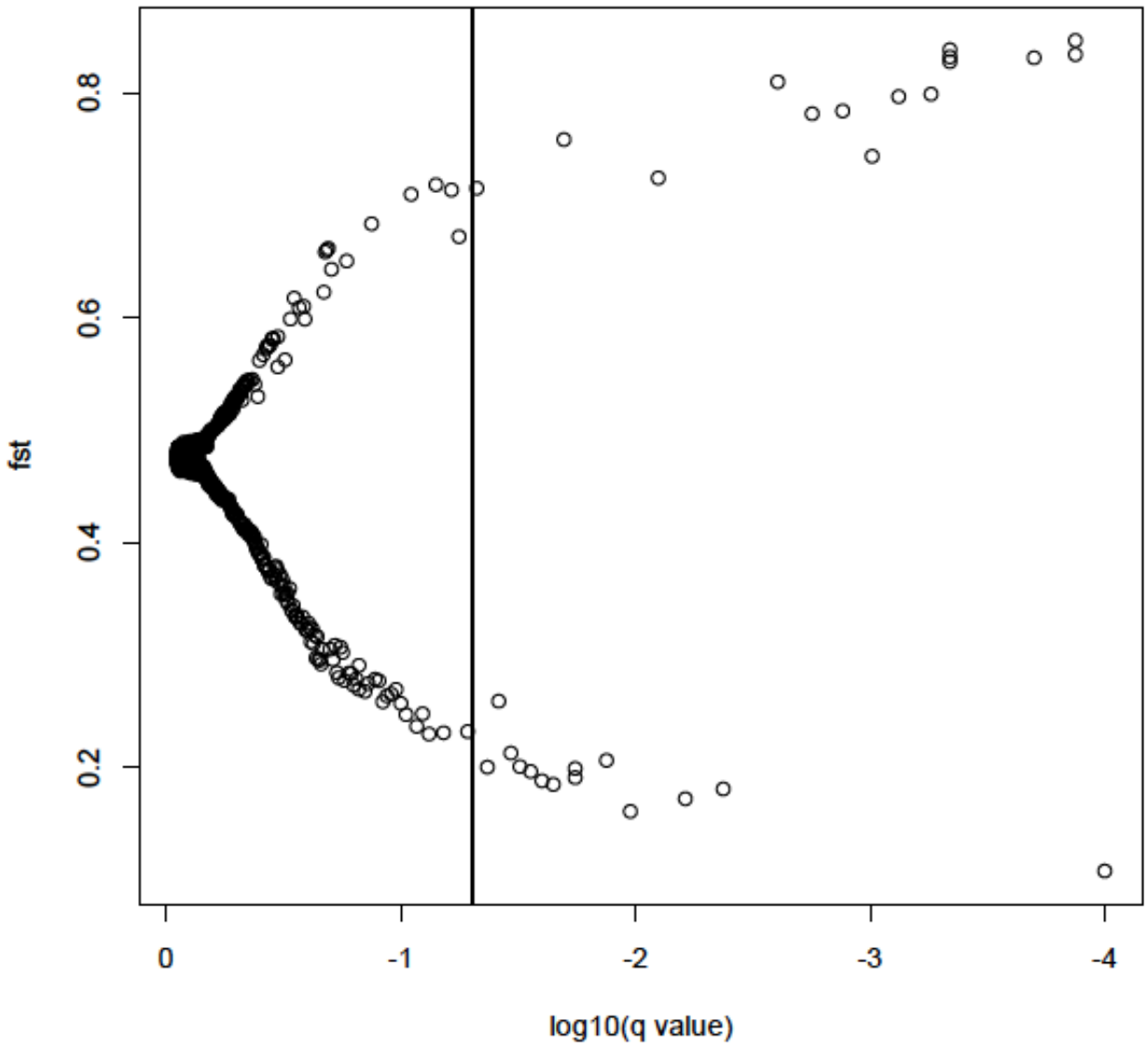


**Figure 2.7.** Schematic diagram of population pairs for comparison of the effect of coral host and allopatry on genomic divergence.

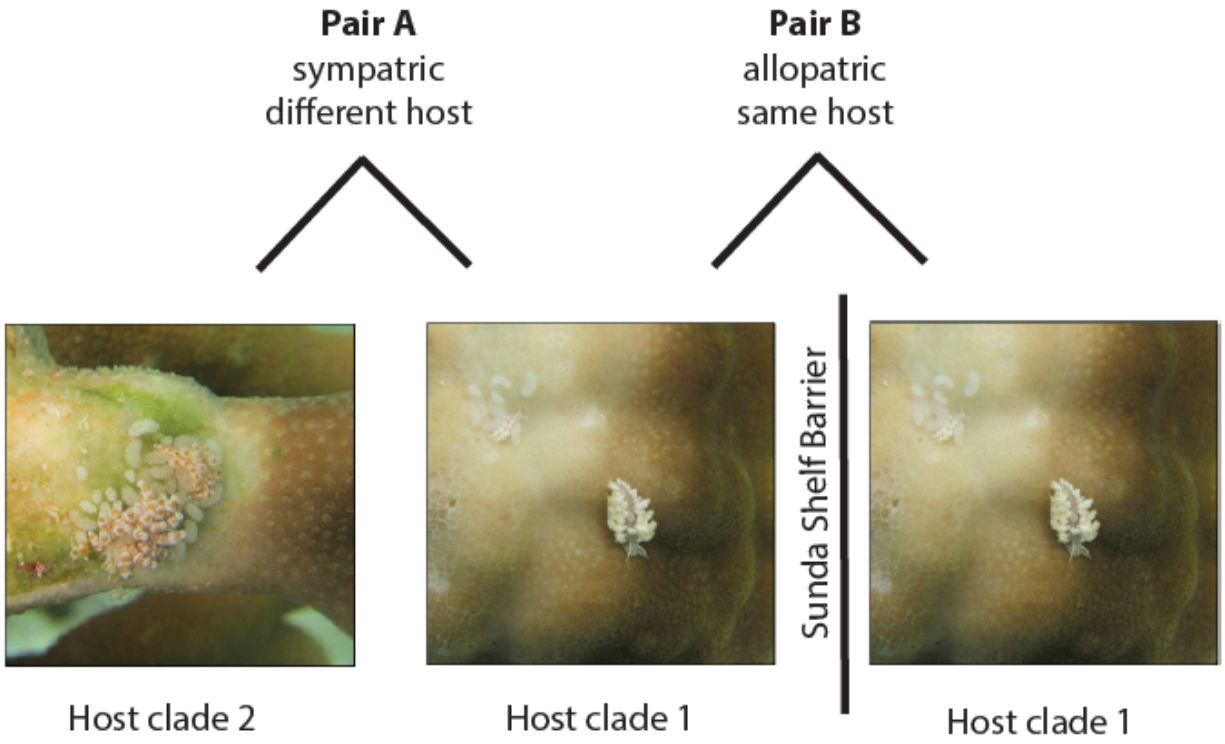


**Figure 2.8.** Results from fhetboot for A) all individuals east of the Sunda Shelf, with populations defined by host coral clade, B) all individuals on host clade 1, with populations defined by side of the Sunda Shelf, C) all individuals in Bali, with populations defined by host coral clade, and D) individuals on host clade 1 from Bali and Cubadak. Outliers were defined as those falling outside the 99% confidence interval, shown in yellow.

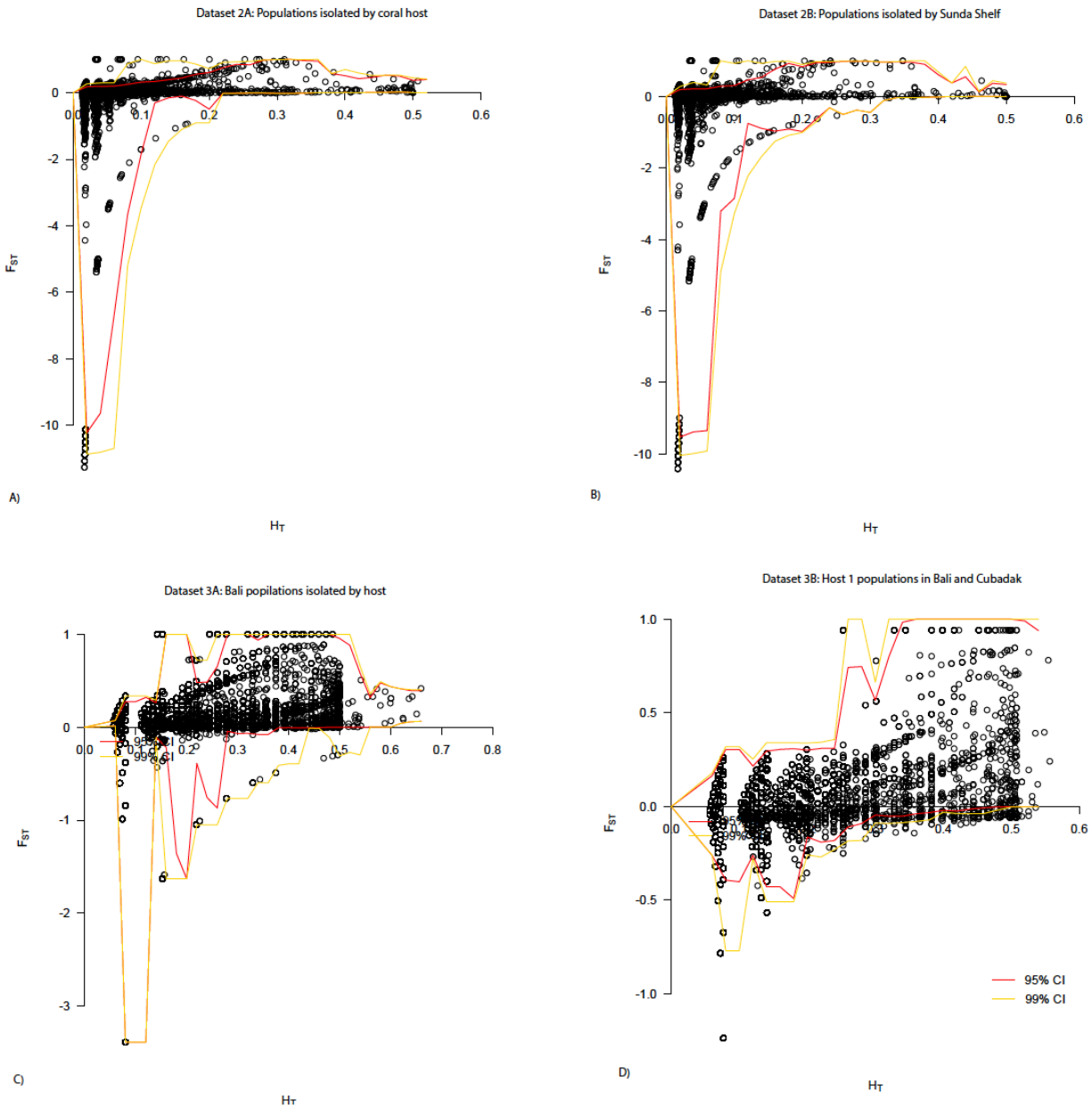


**Figure 2.9.** Results from fhetboot for A) all individuals in Donggala, with populations defined by host coral clade, B) individuals on host clade 1 from Donggala and Cubadak.C) all individuals in Raja Ampat, with populations defined by host coral clade, and D) individuals on host clade 1 from Raja Ampat and Cubadak. Outliers were defined as those falling outside the 99% confidence interval, shown in yellow.

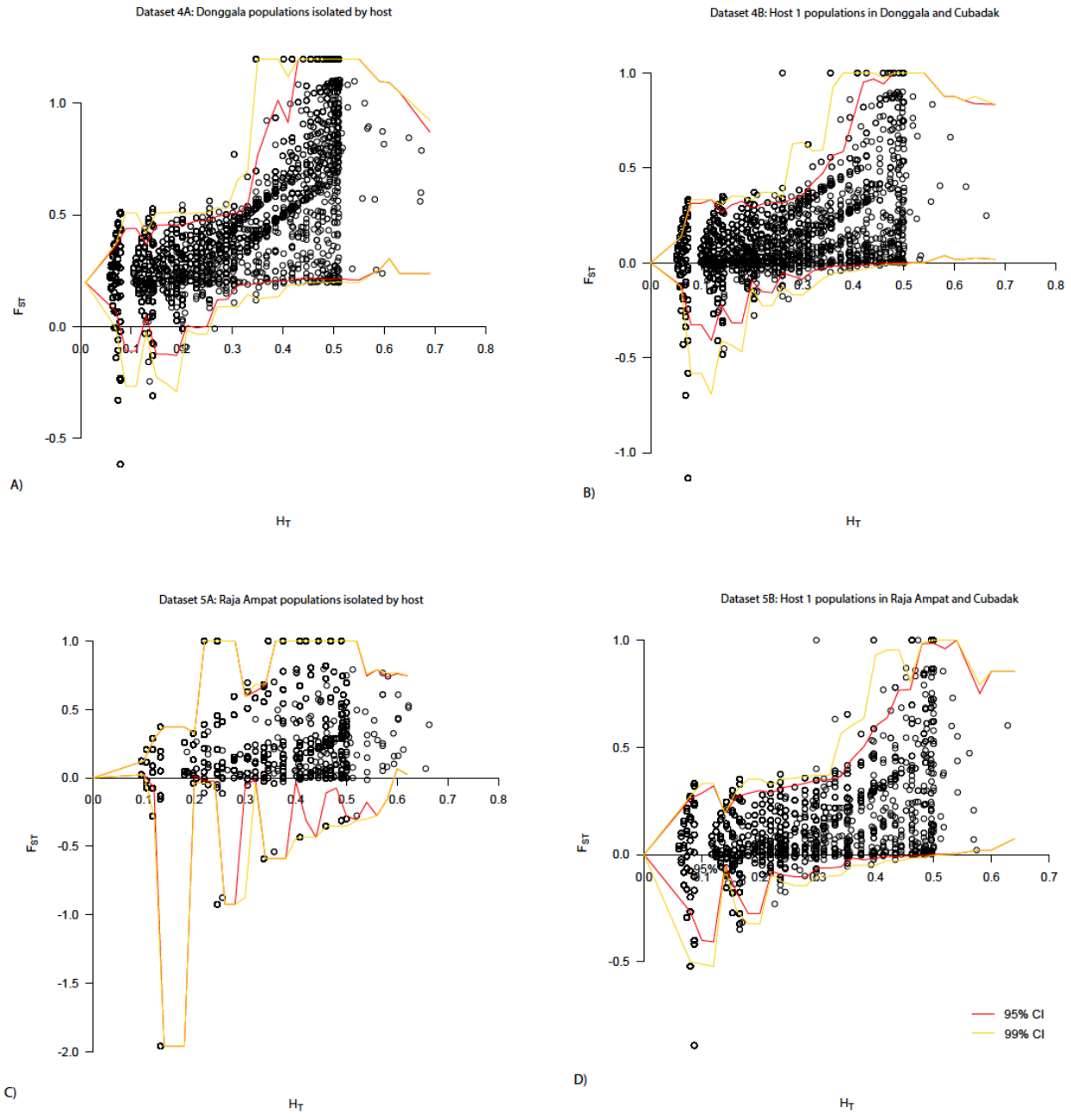
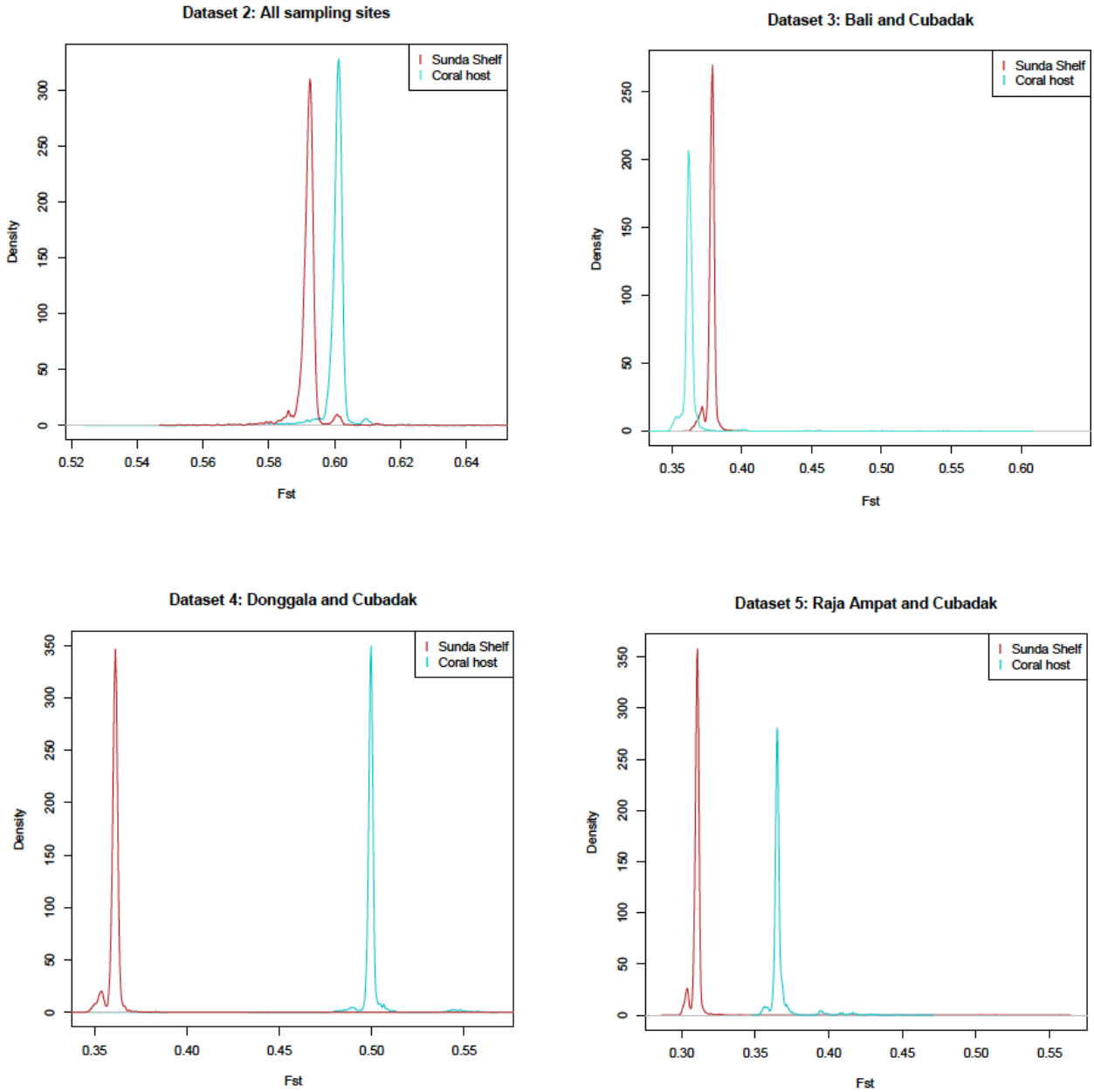


**Figure 2.10.** Distribution of $F_{sts}$ as calculated by BayeScan. Each graph shows the results from two outlier tests: one where populations were defined by coral host clade (blue), one where populations were defined by side of the Sunda Shelf (red). A) Coral host populations east of the Sunda Shelf (blue) and allopatric populations on coral host 1 (red). B) Coral host populations in Bali (blue) and allopatric populations on coral host 1 in Bali and Cubadak (red). C) Coral host populations in Donggala (blue) and allopatric populations on coral host 1 in Donggala and Cubadak (red). D) Coral host populations in Raja Ampat (blue) and allopatric populations on coral host 1 in Raja Ampat and Cubadak (red).



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# CHAPTER 3

## The roles of host and geography in promoting species diversity in coral-associated nudibranchs

### Introduction

The relative importance of natural selection and geographic barriers in the process of speciation is one of the most enduring questions in evolutionary biology (Mayr 1942, Mayr 1963, Maynard Smith 1966, Coyne & Orr 2004). After decades of debate, we now know that natural selection can drive speciation without geographic barriers (Thoday & Gibson 1970; Dieckmann & Doebeli 1999; Schluter 2001), but we still do not know how important this process is in driving the diversification of life on Earth.

Ecological speciation, where disruptive selection favoring two different ecological niches leads to reproductive isolation, has recently swept through the literature as a mechanism for sympatric speciation in every branch of the tree of life (Rundle & Nosil 2005; Schluter 2009). One mechanism for ecological speciation is when selection on traits associated with resource use causes isolation between populations using different resources (Schluter 2001). Adaptive resource specialization or resource shifts may be important drivers of diversification in large taxonomic groups such as fish (Litsios *et al.* 2012; Burress 2014), insects (Nyman *et al.* 2007; Kjellberg & Proffit 2016), crustaceans (Hurt *et al.* 2013; Malay & Michonneau 2014), and mollusks (Wägele 2004; Krug 2011).

Although ecological speciation can happen through a variety of mechanisms, such as adaptation to trophic niche (Lu & Bernatchez 1999; Fan *et al.* 2012), or across environmental gradients (Lowry *et al.* 2008; Bird *et al.* 2011), one of the most commonly

proposed mechanisms is through host shifting. In insects, many studies have shown that specialization of phytophagous insects on their host plants, and subsequent host shifts, are a driver of ecological speciation (Berlocher & Feder 2002; Hébert *et al.* 2016). Other research on plant-host systems has focused on host range expansion and contraction, in which a species colonizes new hosts, then as selection favors specialization, fragments into multiple specialized species (Janz *et al.* 2006; McClure *et al.* 2016).

Like plants, corals play host to a variety of taxa, including algae, barnacles, polychaete worms, and gastropods (Faucci *et al.* 2007; Tsang *et al.* 2009; Malay & Michonneau 2014; Willette *et al.* 2015; Parkinson *et al.* 2016). The obligate relationship between these organisms and their coral hosts (Hadfield & Pennington 1990) draws a strong parallel to the well-studied terrestrial phytophagous insects. Therefore, if ecological speciation is a driver of speciation in phytophagous plants, it is likely a driver of speciation in coral-associated taxa as well. However, research on ecological speciation on coral reefs lags far behind that in terrestrial and aquatic systems (Rocha *et al.* 2005; Miglietta *et al.* 2011). Instead, most studies still seek geographic explanations for species diversity, even when there are no obvious geographic barriers to promote divergence (Briggs 2005; Barber *et al.* 2006). As a result, we still have an incomplete understanding of diversification processes in the marine environment, particularly biodiversity hotspots like the Coral Triangle, the world's most biodiverse marine ecosystem (Roberts *et al.* 2002).

Aeolid nudibranchs are an extremely diverse group of sea slugs known for specialization on cnidarian prey (McDonald & Nybakken 1997; Carmona *et al.* 2013). While most aeolids specialize on hydroids or anemones, some are known to live and feed on specific scleractinian corals (Rudman 1981a, 1982; Faucci *et al.* 2007). As planktonic

larvae, a chemical cue produced by the coral induces nudibranchs to settle and metamorphose (Hadfield & Pennington 1990). The large size of coral colonies combined with the limited mobility of adult nudibranchs results in a tendency for individual nudibranchs to stay on one host colony for their entire life, including mating (Krug 2011). This life-history could promote reproductive isolation between populations feeding on different corals, and has the potential to lead to ecological speciation, possibly in sympatry. In fact, phylogenetic analysis suggests that host shifting has occurred at least twice in *Phestilla* (Faucci *et al.* 2007) and previous work revealed cryptic adaptive divergence between sympatric populations of *Phestilla minor* on different *Porites* host species (Fritts-Penniman *et al.* in prep). Combined, these results suggest that diversification in this group may be driven by ecological speciation.

A significant challenge in understanding the role of host-shifting in the diversification of corallivorous nudibranchs is that nudibranch taxonomy is lagging, with species being discovered more frequently than taxonomists can describe them. For example, the newest field guide to Indo-Pacific nudibranchs and sea slugs contains as many described and undescribed species (Gosliner *et al.* 2015). To understand the processes contributing to diversification, we must first have accurate and comprehensive taxonomic and phylogenetic information.

Corallivory has been reported in 8 nudibranch species: *Cuthona poritophages, Pinufius rebus,* and six species in the genus *Phestilla,* 2 of which are yet undescribed. Recent work suggests that all of these species may actually belong to one clade, implying that scleractinian-feeding has only evolved once (Gosliner *et al.* in review). However, the lack of systematic study precludes strong conclusions about the phylogenetic relationships

within this unique group of nudibranch, and how diversification may be associated with specialization on different coral hosts.

The aims of this study are to gain insight into the evolutionary history of coral-host association in nudibranchs and to assess the relative roles of host shifting and geography in diversification of coral-associated nudibranchs. Toward these aims we a) conducted exploratory field sampling to discover species with previously unknown host associations, b) used genetic sequencing to detect cryptic species, and c) built a phylogenetic tree of all scleractinian-associated nudibranchs to determine how frequently and where host shifting has occurred throughout evolutionary history.

## Methods

### *Sample collection and DNA extraction*

To maximize taxonomic diversity of coral hosts, we focused our collections on Indonesia because it spans the Coral Triangle, the region with the highest coral species richness in the world (Veron *et al.* 2007). We also collected in Hawaii because it is well known for high levels of endemism for marine species (Alison Kay & Palumbi 1987). While *Phestilla* is most commonly observed on *Porites*, Faucci *et al.* (2007) indicate that they are found on at least two other host genera. As such, to discover potential novel host shifts, we surveyed a wide range of coral taxa, more than are known to host *Phestilla*.

*Phestilla* are notoriously difficult to find on large coral heads, but are commonly found on the undersides of small unattached or broken corals. We located *Phestilla* through close inspection of loose coral pieces while using snorkel and SCUBA, and collected

individual nudibranchs by hand. We photographed all nudibranchs on their host coral in the field prior to collection. Individuals were kept alive for ≤ 6 hours in small vials of seawater until sample processing. We anesthetized and relaxed all specimens in the freezer before transferring them to 95% ethanol for long-term preservation. We recorded the specific coral host for all samples, with as many as 12 nudibranchs coming from one small coral. For all nudibranchs, we recorded the host coral from which it was collected. Because *Porites* taxonomy is problematic (Forsman *et al.* 2009) and species are morphologically plastic, in Indonesia we collected host coral tissues from *Porites* hosting *Phestilla* nudibranchs to genetically confirm perceived differences among hosts. In Hawaii our permits prohibited the collection coral tissue, so we used local coral sequences from a collaborator at University of Hawaii (Tisthammer *et al.* unpublished).

In addition to field collected samples, we also received 3 *Phestilla* specimens from the Los Angeles County Museum of Natural History: 1 each from Clipperton Isles, Guam, and Mexico. These samples had no host information, but we included them as part of our search for cryptic diversity. The sample from Mexico was of particular interest because we thought it might be the species *Phestilla panamica*, the only coral-associated nudibranch described in the Eastern Pacific.

We extracted DNA from all nudibranchs and corals using Qiagen DNEasy Kits and Omega Bio-Tek E.Z.N.A. Mollusc DNA Kits. For nudibranchs we isolated foot tissue for extraction when possible, but for individuals smaller than 5 mm, we used half the body, and for those smaller than 2 mm we used the entire body. We excluded cerata (the projections of the gut covering the bodies of most aeolid nudibranchs) from the DNA extractions as they often contain recently ingested coral tissue (Rudman 1982).

*Standard Molecular Markers*

We sequenced two mitochondrial (cytochrome oxidase subunit I, or COI, and the ribosomal RNA gene 16S) and one nuclear gene (histone 3, or H3) that are commonly used for gastropod systematics and phylogenetics (Faucci *et al.* 2007; Moore & Gosliner 2011; Ornelas-Gatdula *et al.* 2011; Carmona *et al.* 2013; Cooke *et al.* 2014). We supplemented the dataset with previously published sequences from Genbank (Benson *et al.* 2005; Faucci *et al.* 2007; Carmona *et al.* 2013).

To identify cryptic species and clades, we first amplified COI from all 406 nudibranchs using the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994) under the following PCR conditions: 2 minutes at 94ºC; 35 cycles of 94ºC for 30 seconds, 40ºC for 30 seconds, and 72ºC for 30 seconds; with a final extension of 7 minutes at 72ºC. Based on the COI results we chose a subset of 132 samples to amplify 16S using the primers 16sar-L and 16sbr-H (Palumbi *et al.* 2002) with the same PCR settings as COI but with an annealing temperature of 50ºC. We then chose an even smaller subset with a just a few representatives from each mitochondrial clade to amplify H3, using the primers HexAF and HexAR (Colgan *et al.* 1998) and the following PCR conditions: 3 minutes at 94ºC; 35 cycles of 94ºC for 35 seconds, 50ºC for 60 seconds, and 72ºC for 75 seconds; with a final extension of 2 minutes at 72ºC. For coral hosts we amplified a section of the nuclear histone 2 (H2) coding region using the primers zpH2AH4f (5'-GTGTACTTGGCTGCYGTRCT-3') and zpH4Fr (5'-GACAACCGAGAATGTCCGGT-3') under the following PCR conditions: 2 minutes at 96ºC; 34 cycles of 96ºC for 20 seconds, 58.5ºC for 20 seconds, and 72ºC for 90 seconds; and a final extension of 5 minutes at 72ºC

(Tisthammer *et al.* unpublished). We sequenced amplified DNA in both directions with the ABI 3730 sequencer at UC Berkeley's DNA Sequencing Facility.

*Phylogenetic analyses*

We assembled and edited all Sanger sequences in Geneious 6.1.7 (Kearse *et al.* 2012) and verified the quality of sequences with successful translation to amino acid protein code. We combined these sequences with 55 additional *Phestilla* COI and 44 16S sequences from GenBank published by Faucci *et al.* (2007) and Wecker *et al.* (2015), and 3 COI, 16S, and H3 outgroup sequences from Carmona *et al.* (2013). We aligned *Porites* H2 sequences from the Indonesian corals we sampled with sequences from Hawaiian *Porites lobata, Porites compressa, Porites evermanni*, with one *Porites superfusa* sequence as an outgroup (Tisthammer *et al.* unpublished). We aligned sequence data using the MUSCLE algorithm as implemented in Geneious, and created concatenated alignments of first COI+16S, then all three genes in Text Wrangler. We used the Akaike information criterion in Jmodeltest (Guindon & Gascuel 2003; Darriba *et al.* 2012) to determine the best model of evolution for each gene.

We conducted Bayesian phylogenetic analyses in MrBayes 3.2.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al.* 2012) as implemented in Geneious 6.1.7, using the partitioned option for the multi-gene alignments. We ran analyses for 1,000,000 generations, sampling every 200 generations, with a 10% burn-in, and assessed support for individual nodes using posterior probabilities. We conducted maximum likelihood analyses using RAxML (Stamatakis 2014) with rapid bootstrapping using the XSEDE system on the CIPRES web portal (Miller *et al.* 2010), and assessed support for nodes using proportion

out of 1,000 bootstraps. To assess the impact the addition of each gene had on node support in the phylogenetic tree, we ran MrBayes and RAxML again on each gene set using only the 41 samples for which we had all three genes sequenced.

To determine which clades might be considered separate species, we used Automatic Barcode Gap Detection (ABGD, Puillandre *et al.* 2012). ABGD uses DNA barcodes such as COI to partition samples into putative species based on the barcode gap, which occurs when divergence among individuals within the species is less than that between species. We analyzed the COI and COI+16S sequence alignments (excluding outgroups) using the online tool (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with the default range of 0.001 to 0.1 for prior intraspecific divergence (P) and 1.5 for relative gap width (x), with corrected Jukes-Cantor and Kimura distances as well as simple distances. We analyzed the COI+16S+H3 alignment with the same settings except for a relative gap width of 1.

To determine whether geographic or ecological isolation are important for creating and maintaining species boundaries, we used the partitioning of COI haplotypes by ABGD to assign all 439 specimens to a clade, then classified each clade based on a) host coral to the species or genus level, and b) the known geographic range. We then compared the overlap of coral host range and geographic range to see which factor was most often a source of isolation for nudibranch species.

## Results

In total we collected 404 nudibranchs living on 9 species of coral host from 8 localities broadly distributed across Indonesia, and on 4 host species from 7 sites around

the island of Oahu, Hawaii (Table 3.1). Most notable among our sampling efforts was the discovery of 3 morphologically distinct species, living on 3 different coral hosts new to *Phestilla.* Photographs taken in the field show that these nudibranchs have astounding camouflage with their preferred coral host (Figure 3.1, new species in B, E, F). SEM images from select *Phestilla* specimens show evidence for divergence of the primary feeding apparatus, the radula (Figure 3.2), which is known to vary with prey type, and is a classic trait used in gastropod taxonomy (Radwin & Wells 1968; Rudman 1981b; Jensen 1993).

We sequenced 589 bp of COI in 383 individuals, 398 bp of 16S in 132 individuals, and 328 bp of H3 in 38 individuals, and combined them with sequences from GenBank. We also received a COI sequence from a nudibranch found by M. Massimo on a *Montipora* coral in an aquarium in Italy, bringing the total to 439 individuals (Table 3.2). ABGD analysis of all 439 COI sequences revealed a barcode gap between genetic distances of 0.05 and 0.06 (Figure 3.3A), and 19 putative species with prior intraspecific divergence from 0.02-0.06, which is similar to intraspecific divergence levels seen previously in this genus (Faucci *et al.* 2007). Samples collected as either *Phestilla minor* or *Phestilla sp. 1* formed 7 of these 19 species. A representative subset of the samples is shown in Figure 3.4, with the same topology as the tree containing all 439 individuals (Supplementary Figure S3.1). This tree shows a deep division between 2 main clades: the *Phestilla minor*-like species complex in the top clade, and all other species in the bottom clade. Included in this bottom clade are the only two species of scleractinian-feeding nudibranchs that are not currently classified as *Phestilla*: *Cuthona poritophages* (Rudman 1979) and *Pinufius rebus* (Marcus and Marcus 1960). Color-coding the tree by coral host shows that all genus-level host shifts, and a couple species-level shifts, have occurred in this bottom clade.

ABGD analysis of 179 concatenated COI and 16S sequences revealed a barcode gap between genetic distances of 0.03 and 0.05 (Figure 3.3B). Corresponding to this barcode gap, a prior intraspecific distance of 0.0359 or 0.0599 shows 17 putative species, with the first 3 COI clades grouped together into 1 species. All 19 clades seen on the COI tree were also recovered in the COI+16S tree, but the deeper nodes are unresolved (Figure 3.5). It is unclear where *Cuthona poritophages* and *Pinufius rebus* fit in relation to *Phestilla*, but *Cuthona sp. 35* comes out as an outgroup rather than clustering with *Cuthona poritophages* within the broader coral-feeding clade.

We were missing H3 sequences for 4 lineages from the COI+16S tree (7, 14, 15, 18) because these lineages were composed entirely of samples from Faucci *et al.* (2007) who did not sequence H3. We included these samples in the 3 gene phylogenetic analyses by coding H3 as missing data for 7 individuals across these 4 clades. ABGD analysis of this dataset did not show a clear barcode gap (Figure 3.3C), but this method does not perform well on datasets with fewer than 3 individuals per species (Puillandre *et al.* 2012). The recovered tree shows all 19 lineages with similar topology to the previous trees, but differs in the placement of the non-Phestilla species: *Cuthona poritophages* now falls outside of *Phestilla*, and *Pinufius rebus* now clusters with the upper clade, sister to the *Phestilla minor* species complex (Figure 3.6). However, this differs slightly in the maximum likelihood tree, which again places *Cuthona poritophages* within *Phestilla* (Figure 3.7).

When we limited analyses to the 41 samples in 15 lineages that were sequenced at all 3 genes, the topology of the 3 gene tree (Figure 3.8) had higher support values and was better resolved than trees built with either COI (Figure 3.9) or COI+16S (Figure 3.10). Of the 19 putative species identified by ABGD in COI, not a single one is hosted by more than one

genus of coral, and 11 of 19 were only collected from a single coral host species (Table 3.3). Of the remaining 8 species, 7 are found on more than 1 *Porites* species, and 1 is found on more than one *Tubastrea* species. Of the 11 species that appear to have specialized on a single host species, 6 of them (clades 7, 8, 9, 10, 14, 15) are the only nudibranch species known to live on the host (Table 3.3). *Phestilla sp.* (clade 8) occurred only on *Montipora porites*, *Phestilla* sp. (clade 9) only on *Pavona explanulata*, *Phestilla* sp., (clade 10) only on *Pavona decussata, Phestilla* melanobrachia (clade 13) only on *Tubastrea coccina, Phestilla sp. 2,* (clade 14) *only on Goniopora fruticose*, and *Phestilla sp. 2*, (clade 15) only on *Goniopora djiboutiensis*. The other 5 overlap in host use with other nudibranch species, including some of their closest relatives (Table 3.3). Geographically, 5 species are known to occur in both the Indian and Pacific Oceans, 1 species only occurs in the Indian Ocean, and the remaining 13 occur in the Pacific Ocean. However, *Phestilla cf. minor* clade 4 *and Pinufius rebus* both have well-supported sub-clades showing divergence between the Indian and Pacific sides of the Sunda Shelf. Within the Pacific, 8 putative species (3, 7, 8, 9, 10, 14, 15, and 18) have only been sampled from one location each, but they occur in sympatry with other closely related species.

Phylogenetic analyses of H2 for all *Porites* hosts revealed very little genetic divergence of perceived morphotypes, and thus little confidence in differences among species, with the exception of *Porites cylindrica* and *Porites evermanni* (Figure 3.11). Two major clades are shown, one of which is composed entirely of *P. cylindrica* (clade 2). Some corals identified in the field as *P. cylindrica* did sort into clade 1 with *Porites attenuata*, and were likely misidentified. However, *Phestilla* species do accurately distinguish between these coral clades. We have already shown that clades 1A and 1B have undergone

divergence based on host membership to these coral clades (Fritts-Penniman *et al.* in prep). Importantly, the two nudibranch clades we have identified that are specific to *P. cylindrica* only occur on clade 2 corals. No individuals from *Phestilla cf. minor* clade 1 were collected from the misidentified *Porites cylindrica* corals; their hosts, which we also presented in Figure 1.6 (chapter 1), are highlighted here in color. Similarly, the individuals from clade 12 were also collected from clade 2 *P. cylindrica*. Only clade 4, which does not show any host-based divergence, was collected from the clade 1 "*P. cylindrica."* We cannot be confident about other potential examples of host specificity on *Porites* species, such as seen in clades 3 and 7, because these nudibranch clades are poorly sampled, and the coral species (*Porites annae* and *P. rus*) have not been confirmed to be genetically distinct.

## Discussion

Phylogenetic analyses reveal high levels of phyletic diversity within the genus *Phestilla,* uncovering 17-19 distinct evolutionary lineages from only 6 named species, indicating that there may be multiple cryptic species in this genus. While deeper tree topology varied among analyses, the clade assignment for any given individual is identical across all 3 genes. Given the high support at the tips in the COI dataset, COI is an appropriate barcode for clade assignment, and can be used to detect cryptic diversity in this group. Cryptic diversity was particularly high in *P. minor,* which contained up to 7 cryptic lineages. Previous phylogeographic research has recovered cryptic lineages in a wide variety of marine molluscs throughout the Coral Triangle and Indo-Pacific Region (Meyer *et al.* 2005; Crandall *et al.* 2007; DeBoer *et al.* 2008; Cheng *et al.* 2014), with cryptic diversity strongly tied to geography. However, while some of these *Phestilla* lineages are associated

with geography, many are associated with different coral hosts, suggesting that geography and coral host and play a significant role in the evolutionary history of this genus.

The recovery of potentially cryptic species that is equal or greater than the number of described species of *Phestilla* is not unusual, as there are hundreds of undescribed species of nudibranchs (Gosliner *et al.* 2015). What is remarkable is that *Phestilla* is known largely from *Porites* corals, and we discovered 3 completely new species in the Coral Triangle that live on corals not previously unknown to host *Phestilla*. Each of these three lineages was found on a single species of coral (*Montipora porites*, *Pavona decussata*, or *Pavona explanulata)*, and have unique morphological traits showing adaptation to life on these corals. These ecological associations add to a growing body of evidence that suggests ecological divergence is likely an important process in shaping patterns of evolution within *Phestilla* nudibranchs (Faucci *et al.* 2007, Fritts-Penniman *et al.* in prep).

Geographic divergence

Our results show two main clades of scleractinian-associated nudibranchs, with very different patterns of geographic and ecological divergence. One clade, comprised of *P. minor* and *Pinufius rebus,* is restricted to *Porites* hosts throughout the Indian and Pacific Oceans. Within this group, the primary driver of divergence appears to be geographic isolation. Within *Pinufius rebus* (clade 19), there are two clades representing the Pacific and Indian Ocean respectively. Similarly, clade 2 of *Phestilla cf. minor* from the Indian Ocean is sister to clade 1 from the Pacific Ocean, and clade 4 of *P. cf. minor* is partitioned based on Indian and Pacific Ocean localities. The diversification of Pacific and Indian Ocean populations of marine taxa is a well known pattern attributed to historical sea level changes,

when the Sunda Shelf was exposed and created a land barrier between the two oceans (Voris 2000). Many species, such as stomatopods (Barber *et al.* 2006), snails (Crandall *et al.* 2007, Simmonds 2016), sea stars (Crandall *et al.* 2008), and giant clams (DeBoer *et al.* 2008) show evidence of allopatric divergence across this barrier. Our previous work on genomic divergence between *Phestilla cf. minor* clades 1 and 2 indicated that both neutral and adaptive processes contribute to divergence across this geographic barrier (Fritts-Penniman *et al.* in prep).

Similarly, *Phestilla cf. minor* populations in Hawaii are also very distinct (Figure 3.6). Lineages 5 and 6 are composed mostly of samples from Oahu, with just one individual from Bunaken included in lineage 5 and a few individuals from Guam and Raja Ampat in lineage 6 (Table 3.1). In *Phestilla cf. lugubris* there is also support for a distinct Hawaiian clade which include specimens collected as *Phestilla sibogae* (Faucci *et al.* 2007), although it has not diverged enough to qualify as a distinct species in ABGD (Figure 3.5). The taxonomic validity of *P. sibogae* has been under question due to lack of differentiation from *P. lugubris*, the two of which were thought to occur in sympatry (Faucci *et al.* 2007). Results from this study suggest that Hawaiian populations may comprise a weakly diverged endemic lineage, despite the fact that samples collected from Mexico and French Polynesia were not genetically distinct. Regional endemism is a common finding in the Hawaiian islands, and has been observed in other nudibranch genera (Gosliner 2002). Many Hawaiian marine invertebrate species (Alison Kay & Palumbi 1987) and coral reef fishes (Hourigan & Reese 1987) have formed due to isolation on the periphery of a broader Indo-West-Pacific species range. This process of peripatric speciation is thought to be one of the driving sources of biodiversity in the Coral Triangle, where species that formed on the periphery eventually

accumulate (Fitzpatrick *et al.* 2011; Cowman & Bellwood 2013). These instances of geographic divergence fit the classic models of allopatric and peripatric speciation (Coyne & Orr 2004). Well known geographic processes act to limit gene flow among populations, over time leading to reproductive isolation and speciation. Given the repeated occurrence of geographic lineages within *Porites*-associated nudibranchs, this is clearly an important mechanism for diversification.

Ecological divergence

While the *Phestilla cf. minor* and *Pinufius rebus* clade are found exclusively on *Porites* and show divergence among Pacific and Indian Oceans, the remainder of *Phestilla* occur exclusively in the Pacific Ocean and appear to have undergone many host shifts, occurring on a diversity of coral hosts. Given the widespread use of *Porites* as a host throughout the tree, *Porites* was most likely the ancestral host. This ancestral state is maintained in *Phestilla lugubris*, which utilizes a wide range of *Porites* species as hosts. However, the remaining 7 lineages live on single species of *Porites.* Unfortunately, with the exception of *Phestilla melanobrachia*, each of these purported species was only collected from a single location, so we do not have information about their geographic ranges. However, their coral hosts are all broadly co-distributed in the Coral Triangle (Veron *et al.* 2016), and in our experience, *Phestilla* are found wherever there hosts are present. Given the lack of any obvious geographic barriers to drive divergence of these species, it is likely that host shifting or specialization, rather than allopatric divergence, is the mechanism for speciation.

One of the challenges of studying speciation is that we rarely get to witness it in action, and we often need to make inferences about processes that happened in the distant past (Losos & Glor 2003). By observing host-associated ecological divergence that is recent and ongoing, we gain confidence that this process is likely to have contributed to divergence of *Phestilla* in the past. In depth study of *Phestilla cf. minor* demonstrates ongoing divergence on *Porites cylindrica*, here shown as clade 1B, which has diverged from clade 1A (Fritts-Penniman *et al.* in prep). This ecologically isolated clade does not yet represent a monophyletic group in analyses using very few genes, but genome-wide SNPs show very strong differentiation (Fritts-Penniman *et al.* in prep). This pattern is observed in *P. lugubris* as well (clades 11A and 11B).

The majority of host-shifting and host specialization scientific literature focuses on plant-insect systems (Berlocher & Feder 2002; Janz & Nylin 2008; Matsubayashi *et al.* 2010). In phytophagous insects, the evolution of host use is driven by both phylogenetic relatedness and geographic range overlap of host plants (Janz & Nylin 2008; Calatayud *et al.* 2016). Closely related insects tend to feed on closely related plants, but can sometimes shift to unrelated, but nearby, host plants (López-Vaamonde *et al.* 2003; Calatayud *et al.* 2016). We see similar patterns in *Phestilla*. No single lineage feeds on more than one genus of coral, and the relationships among coral hosts are roughly mirrored in the relationships among their nudibranch parasites. For example, the two lineages that feed on *Goniopora* corals are sister taxa, as are the two that feed on *Pavona* corals. Looking at broader taxonomic levels, *Porites* and *Goniopora* are both genera in the family Poritidae and *Tubastrea* is in their sister family, Dendrophylliidae (Kitahara *et al.* 2010). Similarly, the nudibranch lineages feeding on *Tubastrea* and *Goniopora* are sister taxa, and share a

common ancestor with *Phestilla lugubris* on *Porites*. Acroporidae, the coral family containing Montipora, and Agariciidae the family of Pavona, are also closely related; as are their nudibranch parasites (Kitahara *et al.* 2010). Parallel relationships between host and parasites, such as these, are seen throughout the tree of life (López-Vaamonde *et al.* 2003; Janz & Nylin 2008; Calatayud *et al.* 2016).

Ecological divergence has now been observed several times in marine gastropods. The corallivorous snail *Coralliophila radula* has also demonstrated divergence across *Porites* hosts in the Indo-Pacific (Simmonds 2016). The snail *Littorina saxatilis* has become a model system for adaptive divergence with its two ecotypes that are adapted to different zones of the rocky intertidal (Hollander *et al.* 2015; Ravinet *et al.* 2015). In the nudibranch *Pteraeolidia ianthina*, cryptic species were identified having symbiotic relationships with different photosynthetic *Symbiodinum* dinoflagellates (Wilson & Burghardt 2015). In Sacoglossan sea slugs, specific associations with algal prey and host switching have been indicated as potential drivers of speciation (Jensen 1997). A number of additional examples have been reviewed thoroughly by Krug (2011), highlighting the potential for future work on ecological speciation in the sea.

Host shifting versus host range expansion and specialization

One interpretation of our results is that the ancestral host for all scleractinian-feeding nudibranchs is *Porites*, the most common host genus, with shifts to *Montipora*, *Goniopora*, *Tubastrea*, and *Pavona* corals at different points in evolutionary history. However, we have already noted that these shifts have not occurred randomly across the genus; they are all concentrated in one clade, where each branch is found on a different host genus or species

(Figure 3.4). However, theory suggests that when a resource is abundant, interspecific competition is not high enough to drive a resource shift (Futuyma & Moreno 1988) and in the Coral Triangle, *Porites* corals are very abundant. An alternative hypothesis is that the ancestor was more general in its coral host preferences, and what we see here is the evolution of specialization. This has been described as the Oscillation Hypothesis in the phytophagous plant literature (Janz & Nylin 2008; Hamm & Fordyce 2015).

The difference between a model of pure host shifting and that of oscillation between generalist and specialist states is subtle, but key to understanding the exact mechanism of speciation. In the oscillation hypothesis, species expand their host range to be more generalist, then over time becomes segmented into numerous species with a specific host preference. Evolution from generalism to specialization is commonly observed in phytophagous plants (Janz *et al.* 2006; Janz & Nylin 2008). In *Porites*-associated *Phestilla*, we see a number of putative specialist clades that are sister to *Porites* generalists, supporting host range expansion and contraction as the mechanism of divergence. However, we also see many putative species that occur on completely different genera of corals, and there are no clades that occur on more than one genus. While we cannot be certain about the ancestral host state, the lack of any multi-host-genus generalist nudibranch species indicates that these species underwent true host shifts.

Adaptations to coral host

Ecological speciation increases morphological diversity as well as cryptic species diversity in this group. Species on different coral host genera are very divergent morphologically, while geographic variants, such as the cryptic clades within *Phestilla cf.*

*minor,* tend to be more similar. While all coral-associated nudibranchs have some mechanism of camouflage with their coral, in certain species it is more complex than in others. *Phestilla* species that feed/live on *Porites*, *Goniopora*, and *Montipora* corals all have the same mechanism of camouflage: take on the color of your coral prey by being essentially transparent, with ingested coral tissue stored in the cerata (Figure 3.6 A, B, C). *Pinufius rebus*, which live on *Porites*, and *Phestilla* living on *Pavona* and *Tubastrea,* corals, blend in not only with the color, but the texture of their coral host (Figure 3.6 D, E, F).

Not only is it important for coral-associated nudibranchs to be cryptic, but it is also critical that they consume coral tissue without being stung by nematocysts (Martin 2003). Feeding on nematocysts may be facilitated by specialized functions of digestive cells, as suggested by previous work on genomic signatures of adaptation in *Phestilla* (Fritts-Penniman *et al.* in prep). Also involved in coral feeding are the jaws and radula. As the only hard parts in the body in nudibranchs, these structures are commonly used for taxonomy, and links have been made between radular morphology and feeding function in other sea slugs (Jensen 1993). However, hard corals are a unique prey type, and not enough data has been collected specifically from scleractinian-feeding nudibranchs to connect form to function. We are beginning to collect these data now (Figure 3.2), and will pursue this question in future work.

Cryptic speciation and systematics

The existence of at 17-19 scleractinian coral-associated nudibranch species, 7 of which are cryptic clades within what is currently identified as *Phestilla minor,* has important implications for nudibranch taxonomy. The use of genetic tools in marine invertebrate

phylogeography and systematics has revealed that species diversity can be many times more than thought based on external morphology (Knowlton 1993; Meyer *et al.* 2005; Barber & Boyce 2006; Gosliner & Fahey 2011; Chen & Hare 2011; Pola *et al.* 2012; Krug *et al.* 2013). In nudibranchs, internal structures such as radulae and jaws can be divergent between externally cryptic species, and do exhibit interspecific differences in *Phestilla*. However, with no external indicators of speciation, it is difficult to know when to look inside. Only broad geographic sampling and genetic testing are reliable indicators of cryptic speciation in sea slugs. Recent molecular work on the nudibranch genus *Notobryon* (Pola *et al.* 2012) and the slug genera *Elysia* and *Plakobranchus* (Krug *et al.* 2013) have uncovered many cryptic species. Similarly, broad ecological sampling is necessary to find new ecological variants. Even when species are morphologically distinct from each other, such as those living on *Pavona* corals, they are still very cryptic relative to their environment. By sampling across large geographic and ecological scales, our dataset combined with that of Faucci *et al.* (2007) has multiplied the number of known scleractinian coral-associated nudibranch species by 3. There are currently about 1,500 known species of nudibranchs in the Coral Triangle (Gosliner *et al.* 2015). If this scale of sampling was repeated on every single one of them, the number of nudibranch species could increase to as many as 4,500.

Future directions

Understanding the role of adaptation to coral host in the diversification of coral-associated nudibranchs in a very large task, and there is much work still to be done. Our sampling of the two non-*Phestilla* scleractinian-associated nudibranchs, *Cuthona*

*poritophages* and *Pinufius rebus*, places them within the *Phestilla* clade, corroborating the evidence that feeding on scleractinian corals has evolved only once in nudibranchs (Gosliner *et al.* in review). This result has interesting implications for our future work comparing the evolutionary history of nudibranch association with both scleractinian and soft corals, and the contribution that each has made to overall nudibranch diversity. However, the relative placement of *Pinufius*, *Cuthona*, and *Phestilla* in the tree is still inconsistent with 3 genes. Additional data are necessary to determine their true evolutionary relationships.

Given how many cryptic species we discovered in this study, it is likely that there are still more undiscovered scleractinian-associated nudibranchs. Our first priority must be to find and include all coral-feeding nudibranchs in further analyses. We will continue this work with an even larger dataset, including the addition of genome-wide loci that are already in development. In addition to better resolving the deeper nodes of the tree, using more loci sequenced from across the genome will enable us to better assess the impact of host shifts on evolutionary rates of divergence. Lastly, we will apply more robust species delimitation techniques, and take an integrative approach that considers both genetic and morphological data, such as that recently applied in sea slugs (Krug *et al.* 2013). Once all scleractinian-associated nudibranchs are accounted for in the phylogenetic tree, we will be able to use the tree to test hypotheses of ecological and allopatric speciation, and gain deeper understanding of the processes generating diversity in this group.

TABLES AND FIGURES

**Table 3.1.** Sample collections in Indonesia and Hawaii.

| Species (field ID) | COI clade | Location | Coral (field ID) | N |
|---|---|---|---|---|
| *Phestilla cf. minor* | 1 | Bali | *Porites annae* | 1 |
| | | | *Porites attenuata* | 2 |
| | | | *Porites cylindrica* | 6 |
| | | | *Porites lobata* | 14 |
| | | | *Porites lutea* | 14 |
| | | | *Porites sp.* | 2 |
| | | Bunaken | *Porites lobata* | 13 |
| | | | *Porites lutea* | 4 |
| | | Donggala | *Porites cylindrica* | 10 |
| | | | *Porites lobata* | 19 |
| | | Komodo | *Porites annae* | 2 |
| | | | *Porites lobata* | 10 |
| | | Lembeh | *Porites lobata* | 10 |
| | | | *Porites lutea* | 4 |
| | | Raja Ampat | *Porites cylindrica* | 3 |
| | | | *Porites lobata* | 10 |
| | | | *Porites lutea* | 1 |
| | 2 | Aceh | *Porites lobata* | 7 |
| | | Cubadak | *Porites lobata* | 5 |
| | | | *Porites lutea* | 9 |
| | | | *Porites sp.* | 2 |
| | | Komodo | *Porites lobata* | 1 |
| | 3 | Bali | *Porites annae* | 1 |
| | 4 | Aceh | *Porites lobata* | 1 |
| | | Bali | *Porites annae* | 1 |
| | | | *Porites attenuata* | 1 |
| | | | *Porites cylindrica* | 1 |
| | | | *Porites lobata* | 28 |
| | | Bunaken | *Porites cylindrica* | 37 |
| | | | *Porites lobata* | 27 |
| | | Raja Ampat | *Porites attenuata* | 1 |
| | | | *Porites lobata* | 1 |
| | 5 | Bunaken | *Porites cylindrica* | 1 |
| | 6 | Oahu | *Porites lutea* | 21 |
| | | Raja Ampat | *Porites lobata* | 1 |
| *Phestilla new species* | 8 | Bali | *Montipora porites* | 10 |
| *Phestilla new species* | 9 | Bali | *Pavona explanulata* | 2 |
| *Phestilla new species* | 10 | Bunaken | *Pavona decussata* | 1 |

| | | | | |
|---|---|---|---|---|
| *Phestilla lugubris* | 11 | Aceh | *Porites lobata* | 9 |
| | | Bali | *Porites lobata* | 10 |
| | | | *Porites lutea* | 2 |
| | | | *Porites sp.* | 1 |
| | | Bunaken | *Porites lobata* | 14 |
| | | | *Porites cylindrica* | 2 |
| | | Cubadak | *Porites lobata* | 7 |
| | | | *Porites lutea* | 6 |
| | | Donggala | *Porites lobata* | 6 |
| | | Komodo | *Porites lobata* | 1 |
| | | Lembeh | *Porites lobata* | 4 |
| | | Oahu | *Porites compressa* | 7 |
| | | | *Porites lutea* | 1 |
| | | | *Porites lobata* | 2 |
| | | Raja Ampat | *Porites lobata* | 5 |
| | | | *Porites lutea* | 1 |
| *Phestilla cf. lugubris* | 12 | Bali | *Porites cylindrica* | 2 |
| | | Bunaken | *Porites cylindrica* | 1 |
| *Phestilla melanobrachia* | 13 | Oahu | *Tubastrea coccinea* | 1 |
| *Cuthona poritophages* | 16 | Aceh | *Porites lobata* | 2 |
| | | Bunaken | *Porites lobata* | 2 |
| | | | *Porites lutea* | 2 |
| *Cuthona poritophages* | 17 | Bunaken | *Porites lutea* | 1 |
| | | Raja Ampat | *Porites lutea* | 2 |
| *Pinufius rebus* | 19 | Bunaken | *Porites lobata* | 2 |
| | | Cubadak | *Porites lobata* | 1 |
| | | Lembeh | *Porites lobata* | 2 |
| Total | | | | 380 |

**Table 3.2.** Samples acquired from Los Angeles County Natural History Museum (LACNHM) and GenBank.

| Species (field ID) | Clade | Location | Coral (field ID) | N | Source |
|---|---|---|---|---|---|
| *Phestilla cf. minor* | 1 | Palau | *Porites lutea* | 2 | Faucci et al. 2007 |
| | 5 | Oahu | *Porites compressa* | 3 | |
| | 6 | Guam | *Porites annae* | 5 | |
| *Phestilla lugubris* | 11 | Clipperton Isles | *unknown* | 1 | LACNHM |
| | | Guam | *Porites rus* | 3 | Faucci et al. 2007 |
| | | | *unknown* | 1 | LACNHM |
| | | Mexico | *unknown* | 1 | |
| | | Moorea | *Porites rus* | 1 | Wecker et al. 2015 |
| *Phestilla melanobrachia* | 13 | Guam | *Tubastrea coccinea* | 3 | Faucci et al. 2007 |
| | | Oahu | *Tubastrea coccinea* | 3 | |
| | | Palau | *Tubastrea micrantha* | 3 | |
| *Phestilla minor II* | 16 | Palau | *Porites lutea* | 1 | |
| | 18 | Palau | *Porites lutea* | 2 | |
| *Phestilla new species* | 8 | Aquarium in Italy | *Montipora porites* | 1 | Personal correspon-dence |
| *Phestilla sibogae* | 11 | French Frigate Shoals | *Porites compressa* | 1 | Faucci et al. 2007 |
| | | Guam | *Porites lutea* | 3 | |
| | | Oahu | *Porites compressa* | 3 | |
| | | Palau | *Porites cylindrica* | 2 | |
| | | | *Porites rus* | 2 | |
| *Phestilla sp. 1* | 1 | Palau | *Porites cylindrica* | 4 | |
| | 4 | Guam | *Porites cylindrica* | 3 | |
| | 7 | Palau | *Porites rus* | 4 | |
| *Phestilla sp. 2* | 14 | Guam | *Goniopora fruticosa* | 3 | |
| | 15 | Palau | *Goniopora djiboutiensis* | 1 | |
| *Cuthona sp. 35* | | | | 1 | Carmona et al. 2013 |
| *Cuthona sp. A* | | | | 1 | |
| *Phyllodesmium horridum* | | | | 1 | |
| Total | | | | 59 | |

**Table 3.3.** Coral hosts and geographic range of 19 putative *Phestilla* species, based on this dataset alone. Number indicates the number of individuals from that *Phestilla* clade found on that coral.

| Sampled from | Phestilla clade | Coral host | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Pacific | 1 | | | | | | 3 | 2 | | 23 | 76 | 25 | | 2 | | |
| Indian | 2 | | | | | | | | | | 13 | 9 | | 2 | | |
| Bali | 3 | | | | | | 1 | | | | | | | | | |
| Indo-Pacific | 4 | | | | | | 1 | 2 | | 41 | 57 | | | | | |
| Pacific | 5 | | | | | | | | 3 | 1 | | | | | | |
| Pacific | 6 | | | | | 5 | | | | | 1 | 21 | | | | |
| Palau | 7 | | | | | | | | | | | | 4 | | | |
| Bali | 8 | | | 11 | | | | | | | | | | | | |
| Bali | 9 | | | | | 2 | | | | | | | | | | |
| Bunaken | 10 | | | | 1 | | | | | | | | | | | |
| Indo-Pacific | 11 | | | | | | | | 11 | 4 | 56 | 13 | 6 | 1 | | |
| Pacific | 12 | | | | | | | | | 4 | | | | | | |
| Pacific | 13 | | | | | | | | | | | | | | 7 | 3 |
| Guam | 14 | | 3 | | | | | | | | | | | | | |
| Palau | 15 | 1 | | | | | | | | | | | | | | |
| Indo-Pacific | 16 | | | | | | | | | | 2 | 3 | | | | |
| Indo-Pacific | 17 | | | | | | | | | | | 3 | | | | |
| Palau | 18 | | | | | | | | | | | 2 | | | | |
| Indo-Pacific | 19 | | | | | | | | | | 5 | | | | | |

Coral hosts:

1. *Goniopora djiboutiensis*
2. *Goniopora fruticosa*
3. *Montipora porites*
4. *Pavona decussata*
5. *Pavona explanulata*
6. *Porites annae*
7. *Porites attenuata*
8. *Porites compressa*
9. *Porites cylindrica*
10. *Porites lobata*
11. *Porites lutea*
12. *Porites rus*
13. *Porites sp. (unknown)*
14. *Tubastrea coccinea*
15. *Tubastrea micrantha*

**Figure 3.1.** Cryptic coloration and texture of *Phestilla* species: A) Clade 1: *Phestilla cf minor* on *Porites lobata* B) Clade 8: *Phestilla sp.* collected from *Montipora porites*, C) Clade 16: *Cuthona poritophages* on *Porites lobata*, D) Clade 19: *Pinufius rebus* on *Porites lobata*, E) Clade 9: *Phestilla sp.* collected from *Pavona explanulata*, F) Clade 10 *Phestilla sp.* collected from *Pavona decussata.*

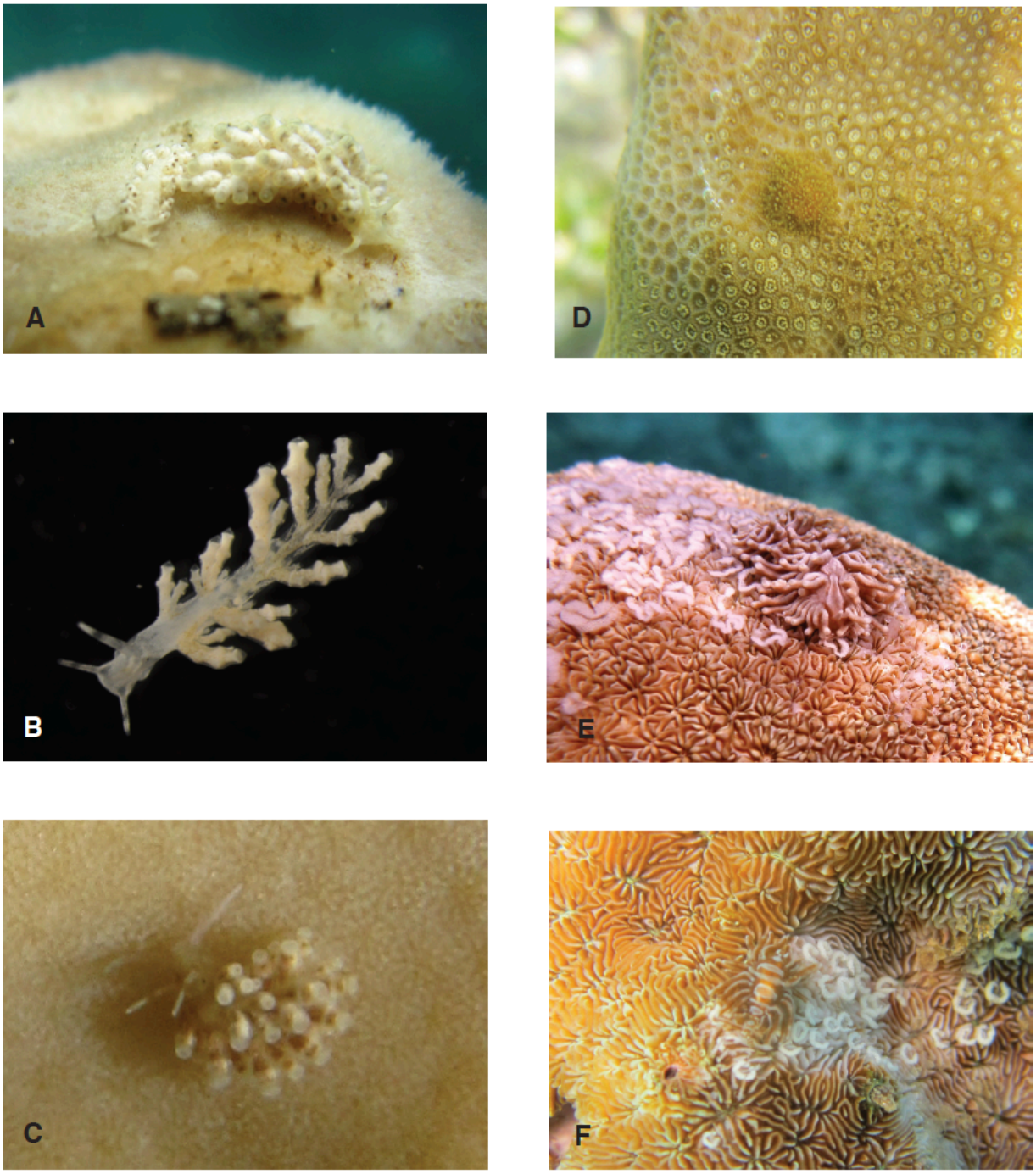


**Figure 3.2.** SEM images of radulae from A) Clade 16: *Cuthona poritophages* collected from *Porites lobata*, B) Clade 8: *Phestilla sp.* collected from *Montipora porites*, C) Clade 12: *Phestilla cf lugubris* collected from Porites cylindrica, D) Clade 4: *Phestilla cf. minor* collected from *Porites cylindrica*, E) Clade 13: *Phestilla melanobrachia* collected from *Tubastrea coccinea.*

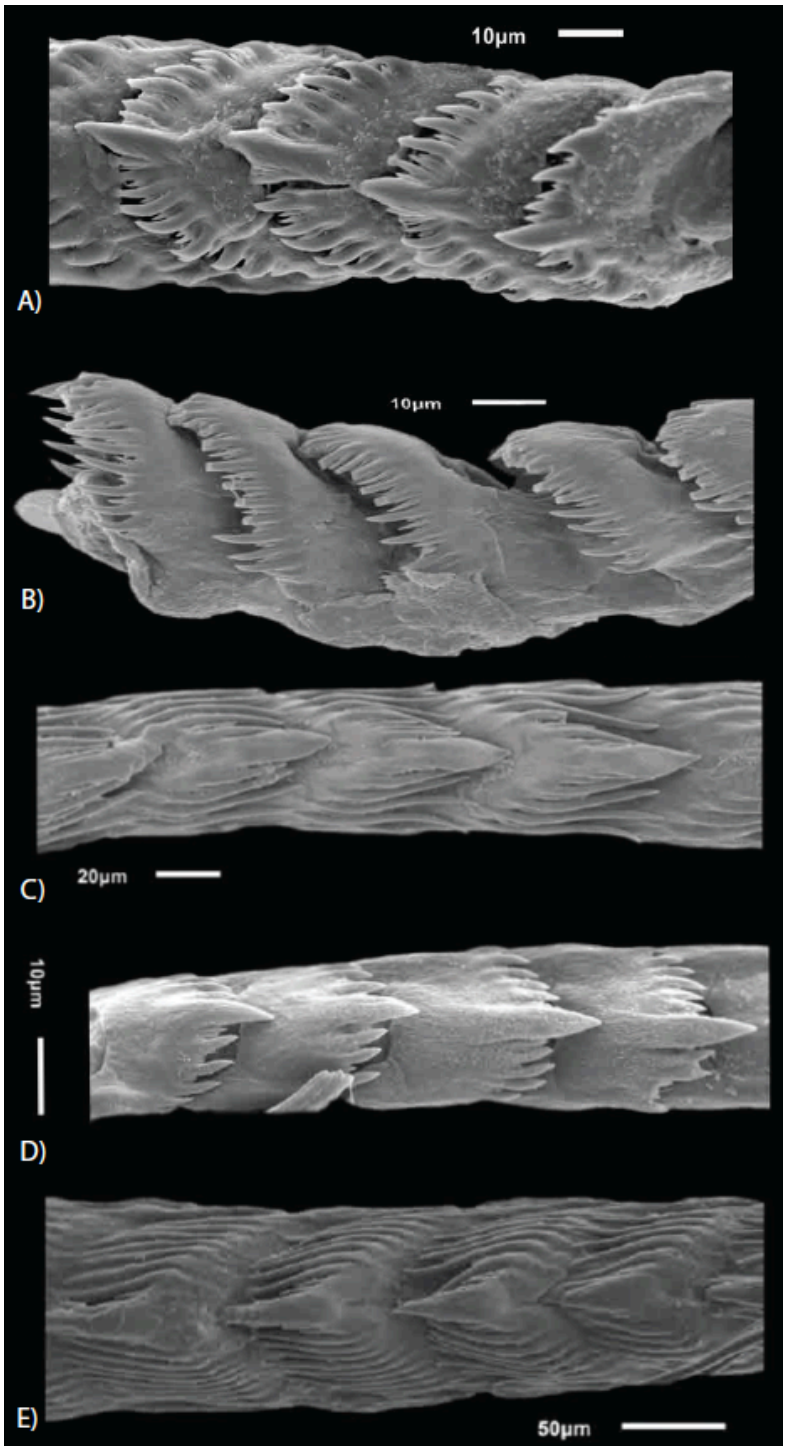


**Figure 3.3.** Jukes-Cantor genetic distances calculated with Automatic Barcode Gap Discovery for A) 439 COI sequences, B) 179 concatenated COI+16S sequences, and C) 48 concatenated COI+16S+H3 sequences. Presumed intraspecific divergence shown in orange, interspecific divergence in yellow.

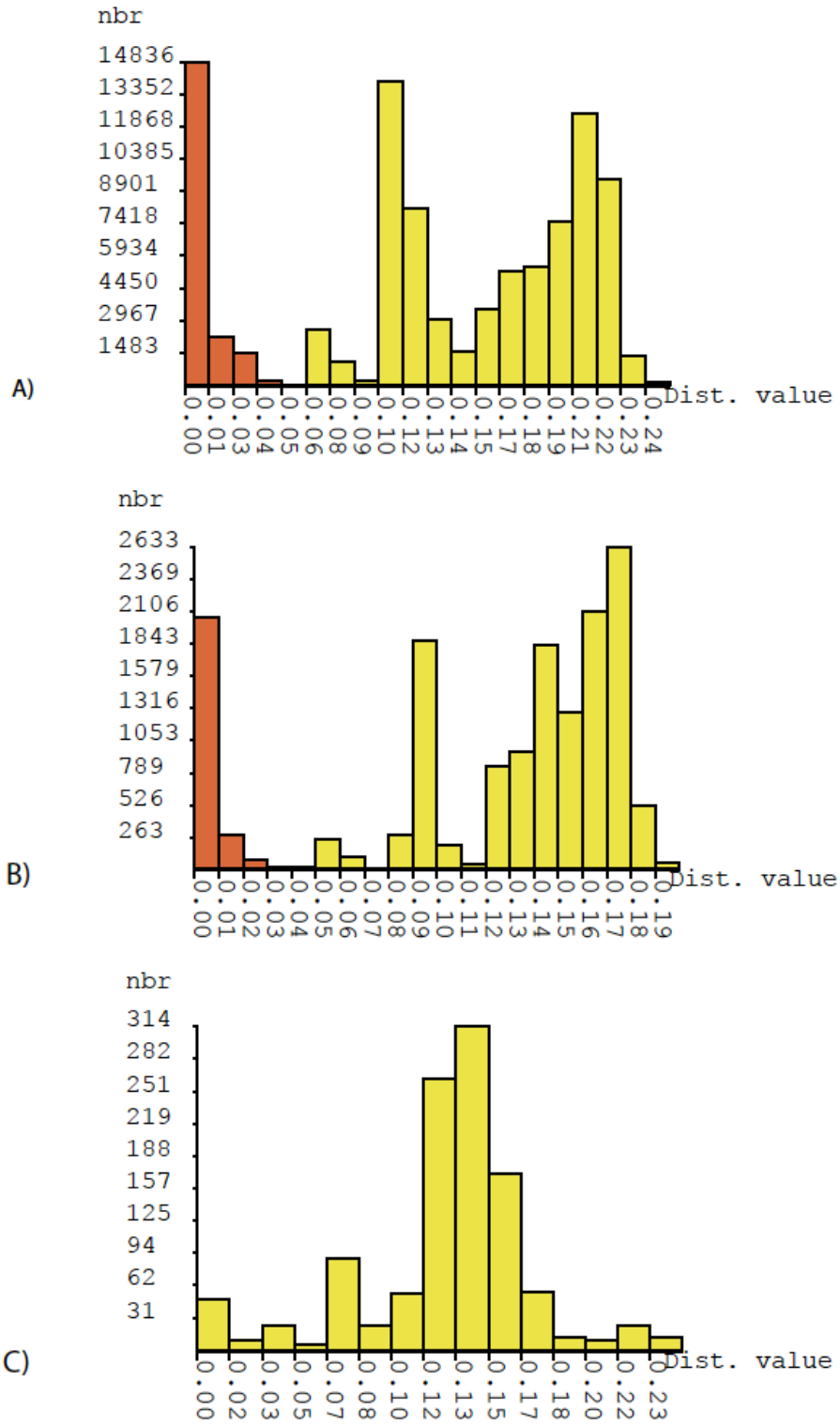


**Figure 3.4.** Bayesian tree of 88 COI sequences. Node labels show posterior probability. Clades color-coded by host species, or genus for those that occur on more than one species. Black bars indicate the two major clades: all *Phestilla minor*-like species in the top clade, and all other species in the bottom clade.

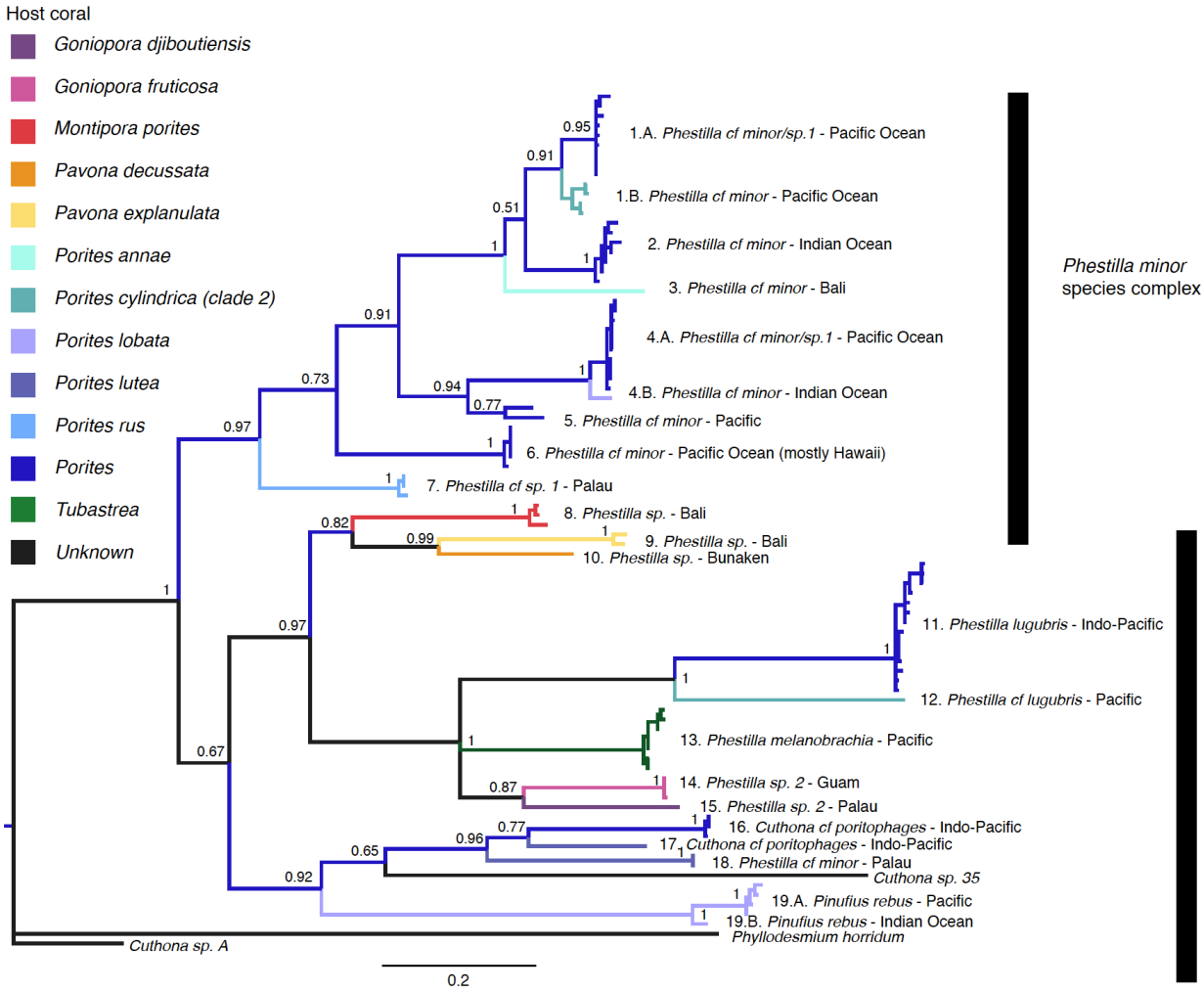


**Figure 3.5.** Bayesian tree of 71 concatenated mitochondrial COI and 16S sequences. Node labels show posterior probability. Clades color-coded by host species, or genus for those that occur on more than one species.

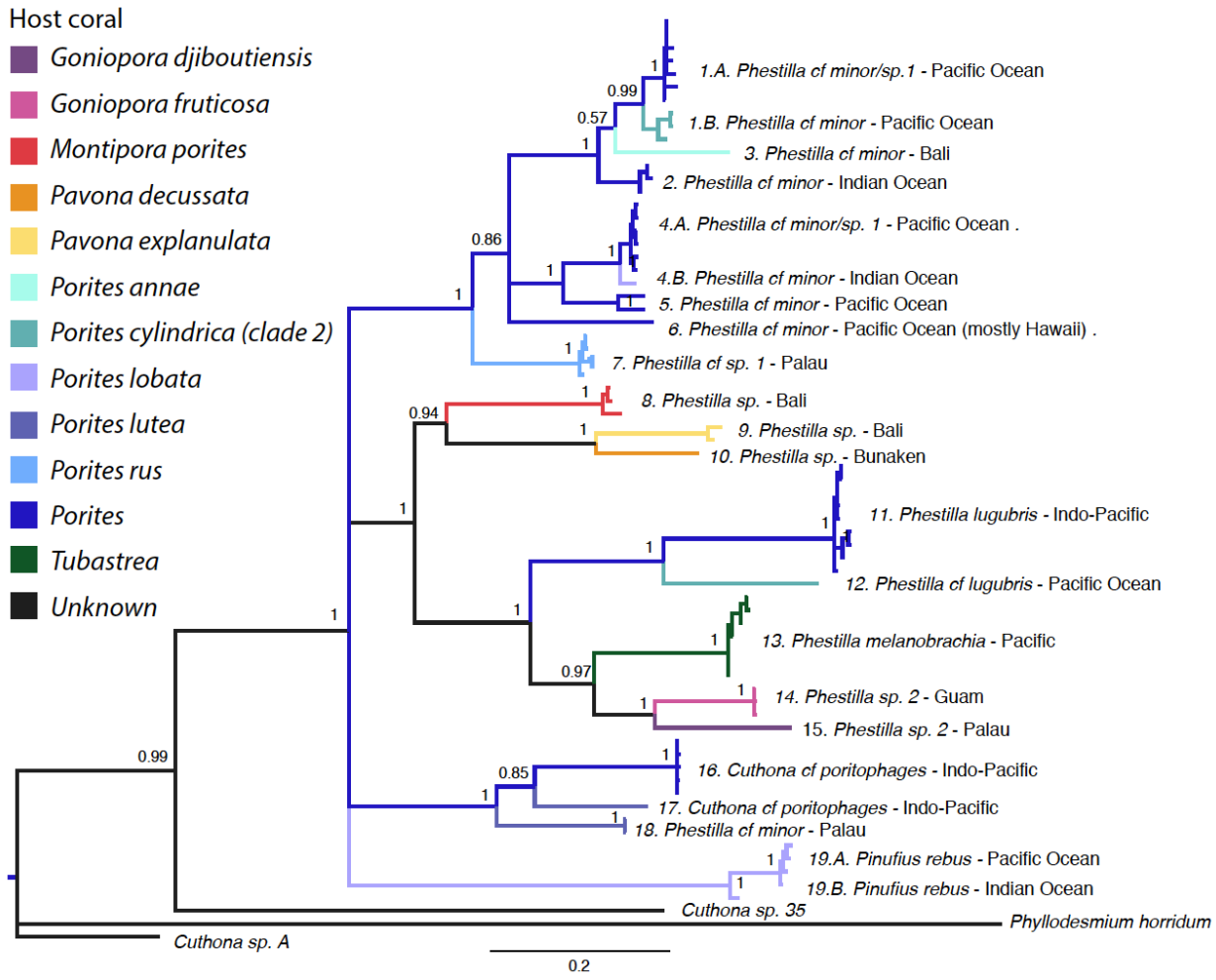


**Figure 3.6.** Bayesian tree of 48 concatenated COI, 16S, and H3 sequences. Node labels show posterior probability. Clades color-coded by host species, or genus for those that occur on more than one species. Black bars highlight the different placement of *Pinufius rebus* and *Cuthona poritophages* relative to the mitochondrial gene trees.

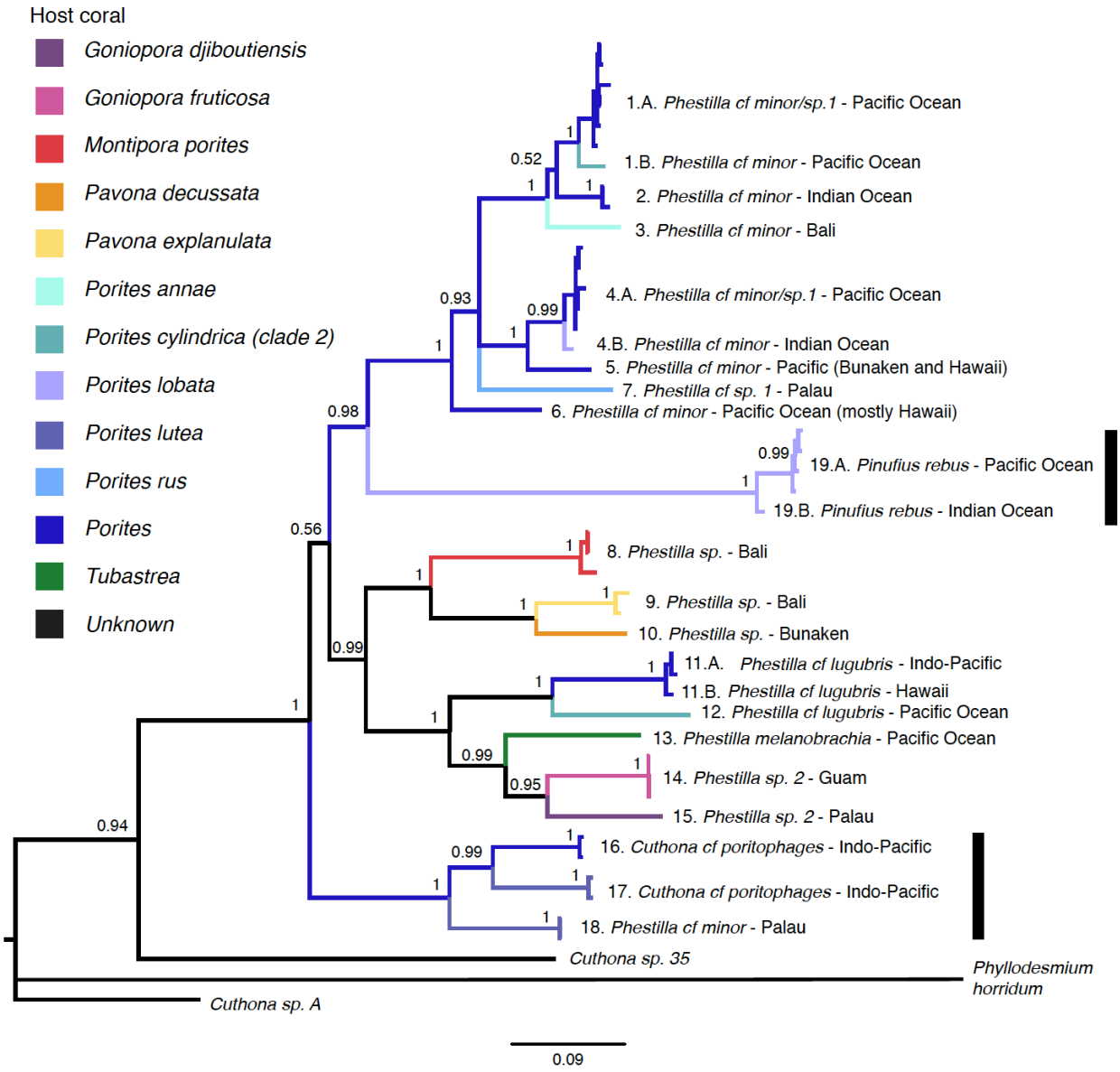


**Figure 3.7.** Maximum likelihood tree of 48 concatenated COI, 16S, and H3 sequences. Node labels show bootstrap proportion. Clades color-coded by host species, or genus for those that occur on more than one species. Black bar highlights the placement of *Cuthona poritophages* inside the clade rather than as an outgroup, as it is in the Bayesian tree.

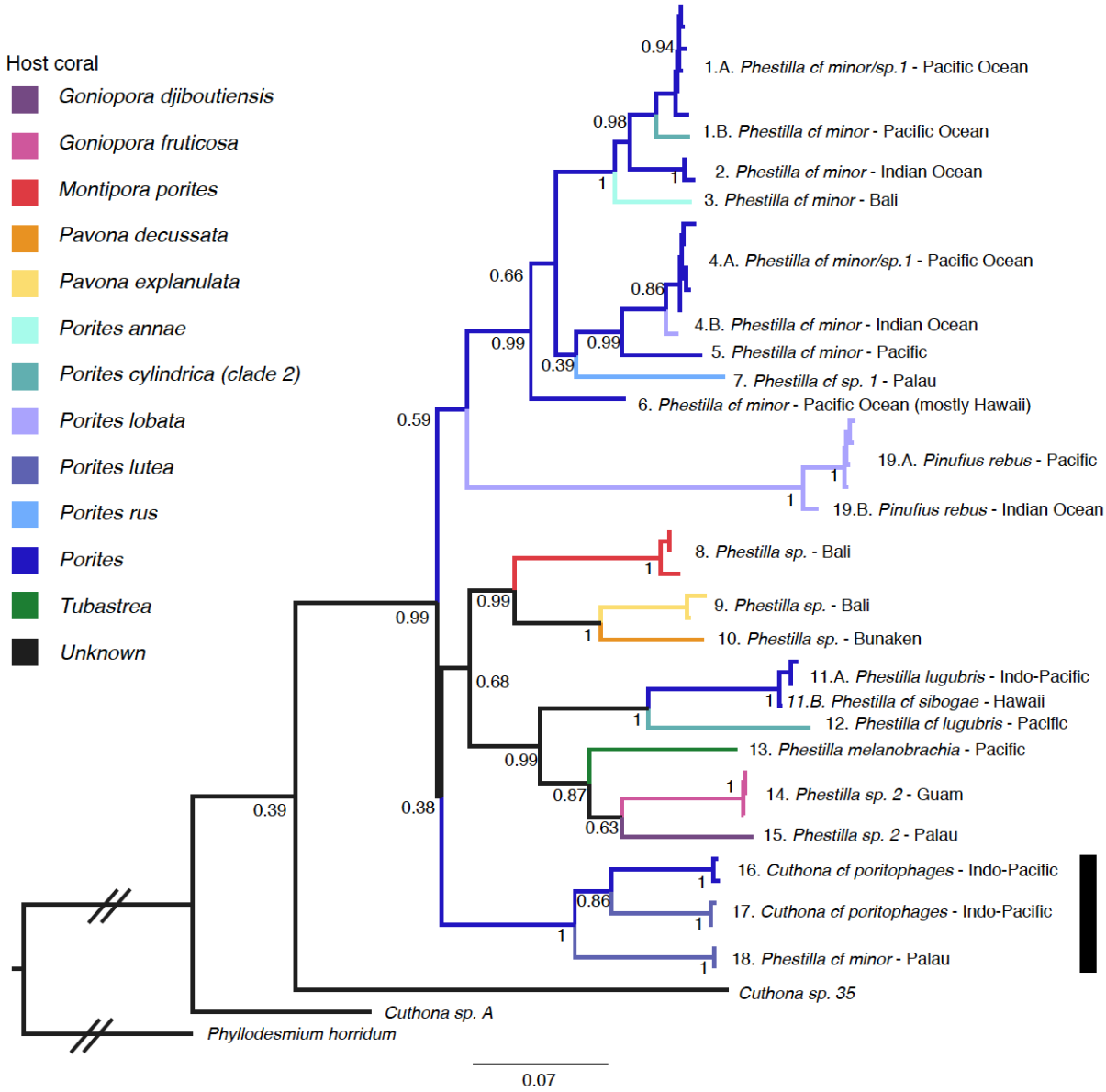


**Figure 3.8.** Bayesian tree of 41 concatenated COI, 16S, and H3 sequences, using only samples with all 3 genes sequenced, which excludes clades 7, 14, 15, and 18. Node labels show posterior probability on top and maximum likelihood on bottom. Clades color-coded by host species, or genus for those that occur on more than one species.

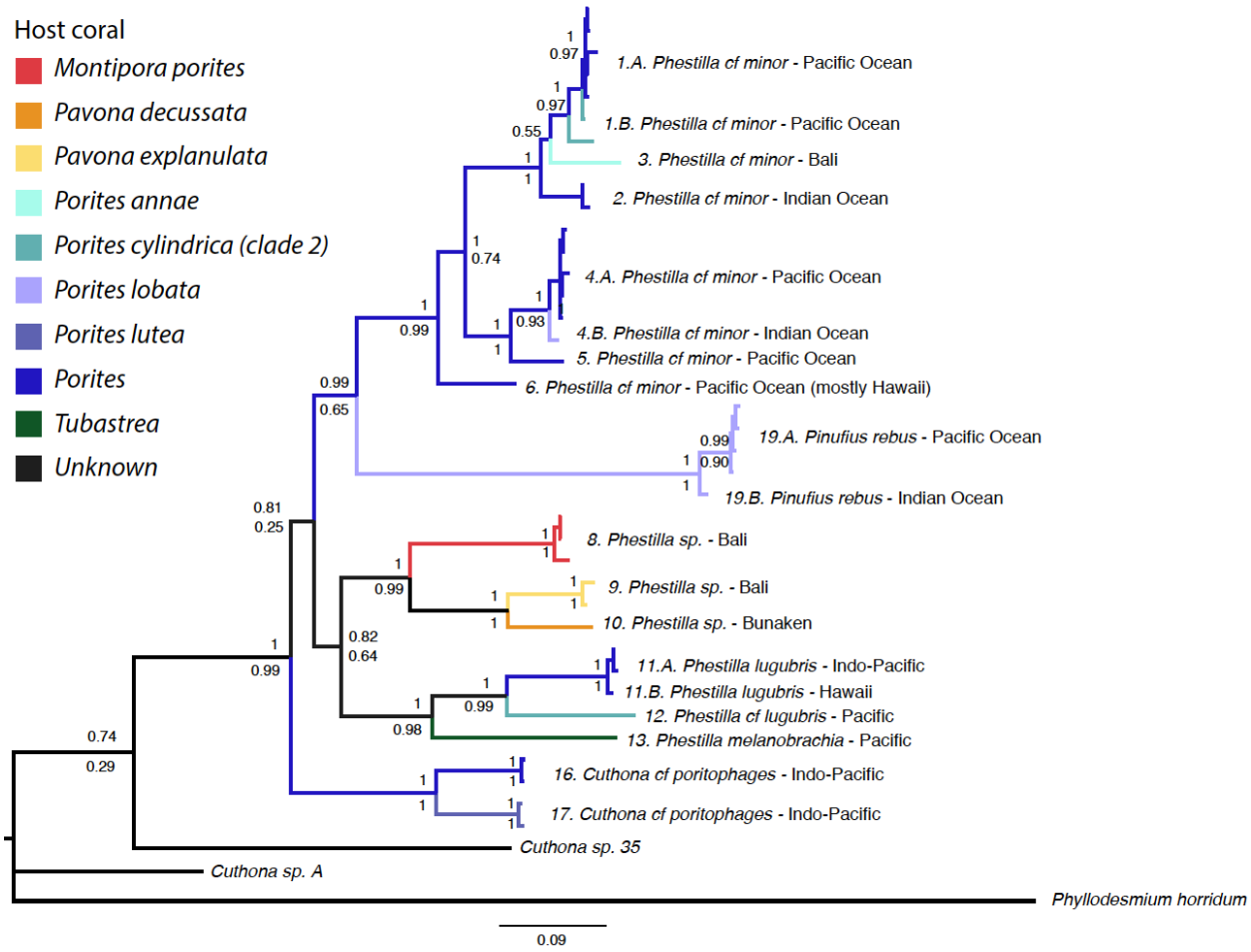


**Figure 3.9.** Bayesian tree of 41 COI sequences, using only samples with all 3 genes sequenced, which excludes clades 7, 14, 15, and 18. Node labels show posterior probability on top and maximum likelihood on bottom. Clades color-coded by host species, or genus for those that occur on more than one species.

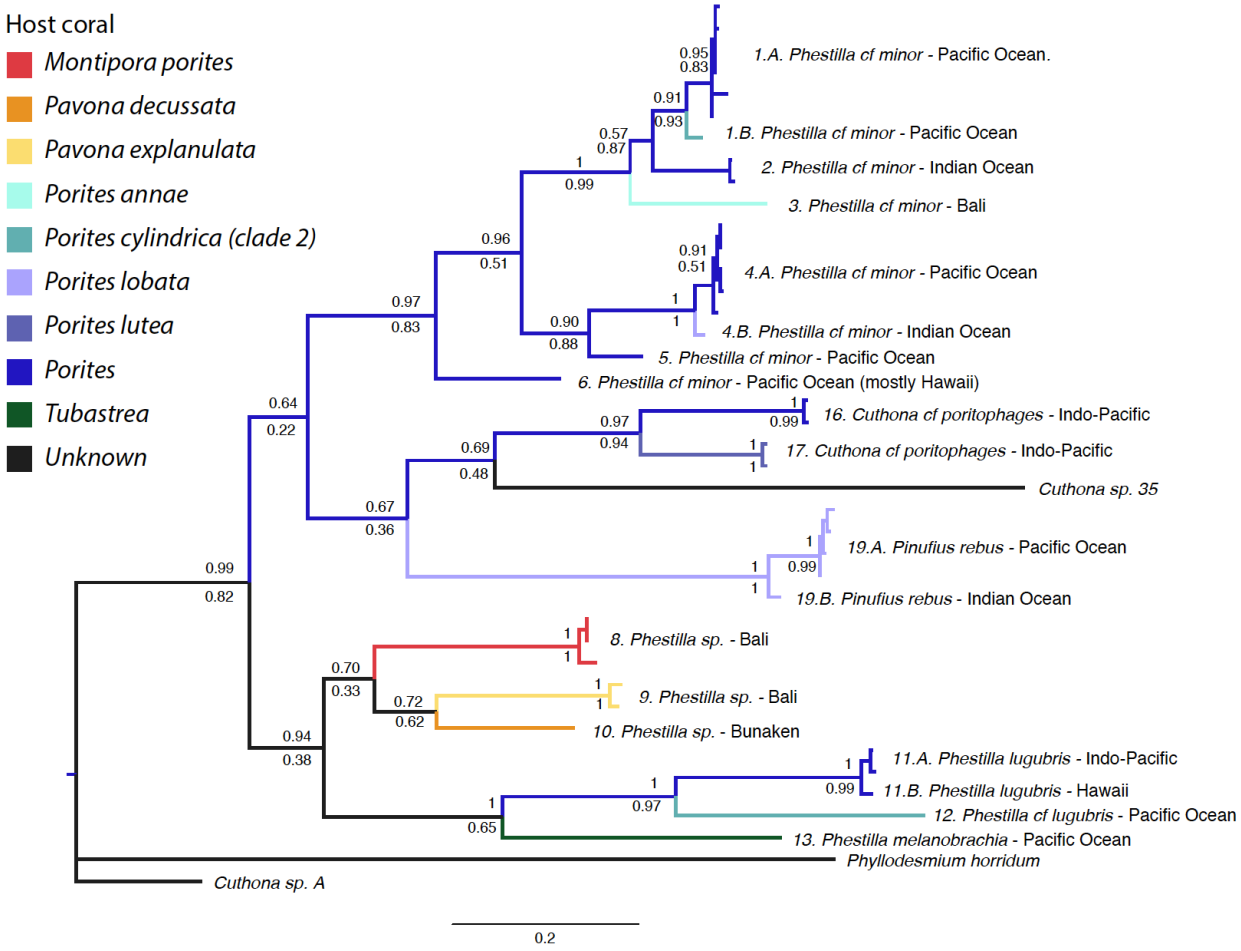


**Figure 3.10.** Bayesian tree of 41 concatenated mitochondrial COI and 16S sequences, using only samples with all 3 genes sequenced, which excludes clades 7, 14, 15, and 18. Node labels show posterior probability on top and maximum likelihood on bottom. Clades color-coded by host species, or genus for those that occur on more than one species.

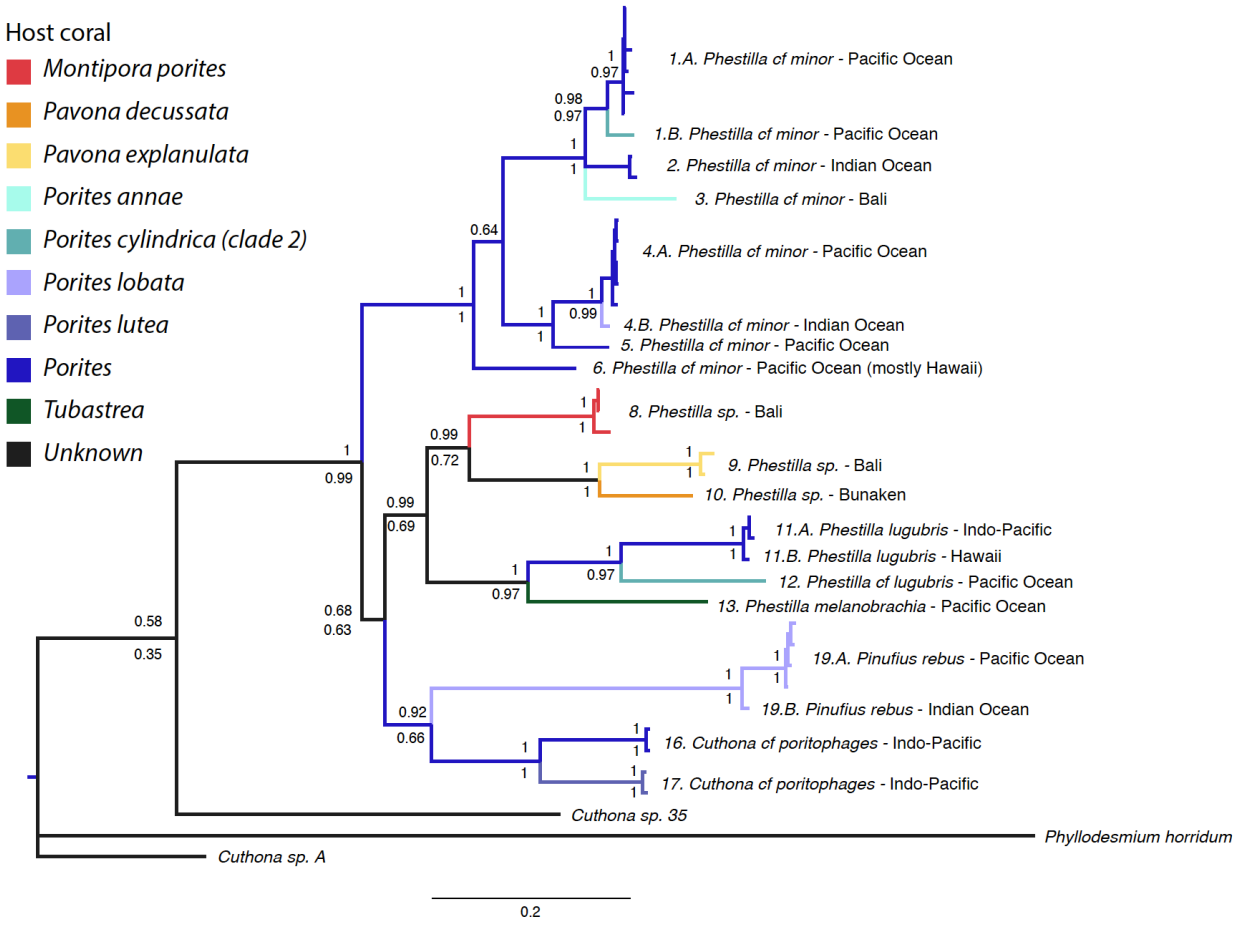
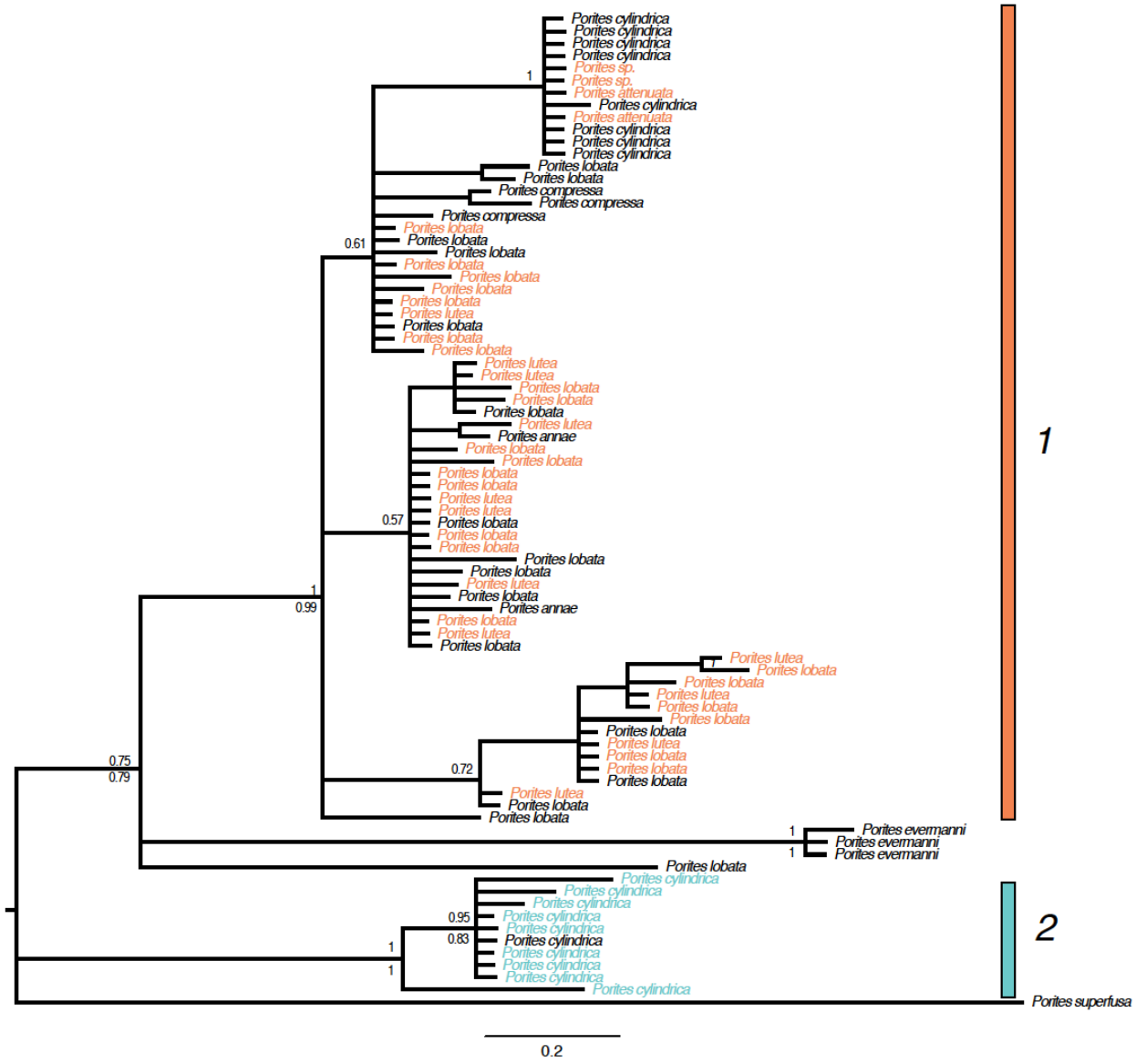
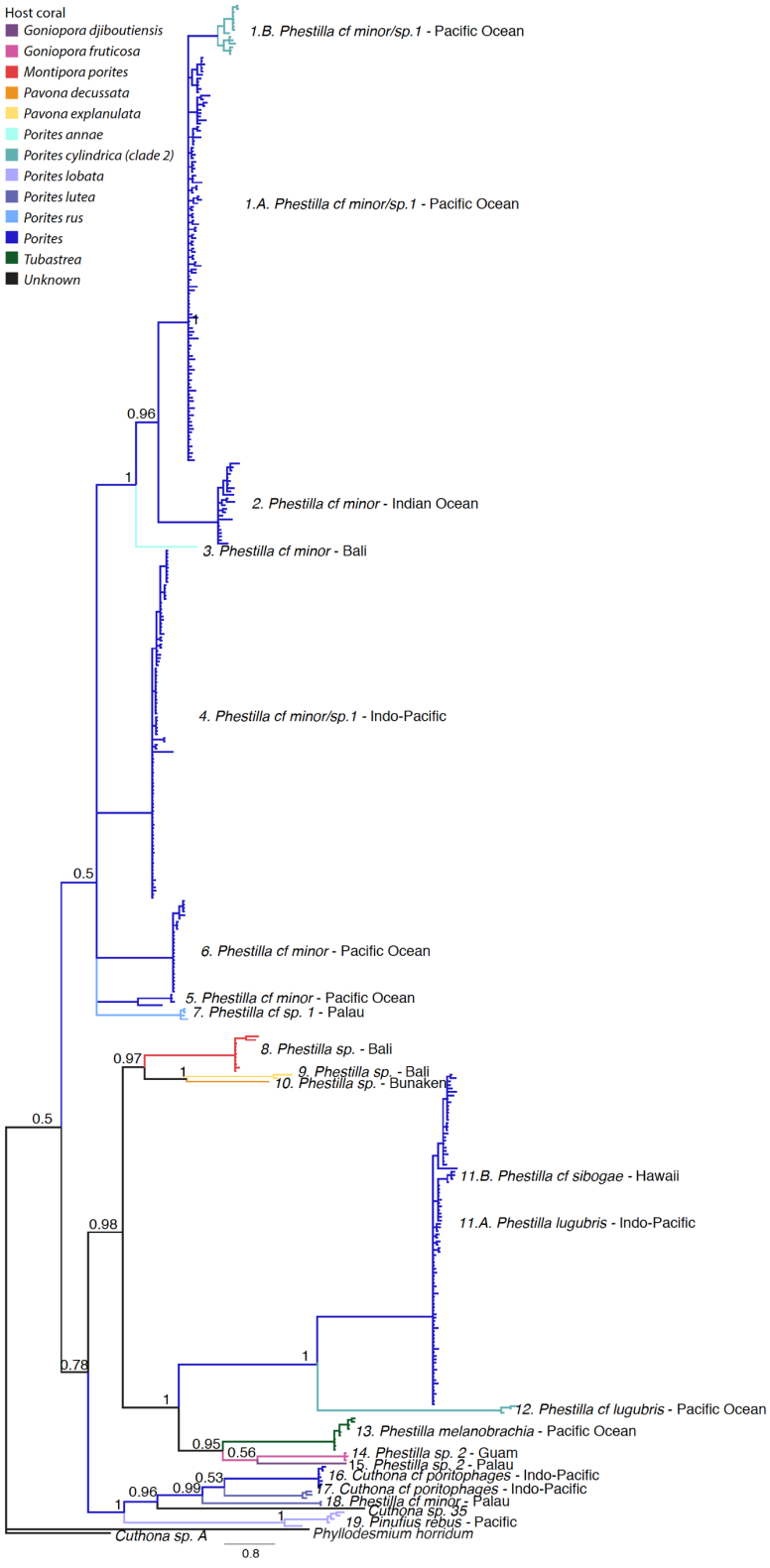


**Figure 3.11.** Bayesian tree of 81 *Porites* H2 sequences. Node labels show posterior probability on top and maximum likelihood on bottom. Corals from Figure 1.6 highlighted.



Supplement

**Figure S3.1.** Bayesian tree of 439 COI sequences. Node labels show posterior probability.



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