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Phylogeography of Southeast Asian flying foxes (Chiroptera: Pteropodidae: *Pteropus*)

By

Susan M. Tsang

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Phylogeography of Southeast Asian flying foxes (Chiroptera: Pteropodidae: *Pteropus*)

By Susan M. Tsang

Advisor: David J. Lohman

Flying foxes (*Pteropus*) are a genus of Old World fruit bats that are important seed dispersers and pollinators for plants native to the 200,000+ islands in Southeast Asia, yet they are some of the most poorly known bats in the world. They comprise some of the largest known bat species, and are morphologically relatively conserved on the genus level. *Pteropus* is the most species-rich genus within Pteropodidae, though the origin for this diversity remains incompletely understood. In Chapter 1, I discuss the importance of *Pteropus* to the ecosystem and as reservoir hosts. In Chapter 2, a molecular phylogeny is presented with *Pteropus* species organized into fewer species groups than recognized from previous research that better reflected the comprehensive dataset. An increase in relative divergence rate was detected within *Pteropus* during the Pliocene that led to rapid radiations in three species groups. Additionally, discordant signals from nuclear and mitochondrial genes suggested incomplete lineage sorting and hybridization were present, likely as a result of the young clade age, low genetic variability, and rapid diversification of the genus. In Chapter 3, using the species tree generated in Chapter 2, I tested biogeographic mechanisms and scenarios that resulted in current distributions of *Pteropus* species using several ancestral area reconstruction methods. Dispersal and founder-event speciation were both important mechanisms through which species expanded into new areas. Wallacea was an integral part of the evolutionary history of *Pteropus*, and likely the region of

origin, a new result uncovered largely a product of the increased taxonomic and geographic sampling. I then used a combination of phylogenetics and population genetics to determine the population connectivity of two commonly studied *Pteropus* hosts that are of interest to the disease ecology community, *P. vampyrus* (Chapter 4) and *P. alecto* (Chapter 5). Host metapopulation dynamics are important for predictions of pathogen diversity, aggressiveness, and transmission. *Pteropus vampyrus* and *P. alecto* highlight differences in management strategies needed and pathogen model predictions. Chapter 6 presents a general discussion regarding these findings and future directions for research.

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CHAPTER 1

An introduction to the ecological and biomedical importance of *Pteropus*

The genus *Pteropus* (Chiroptera: Pteropodidae), commonly referred to as flying foxes, is the most species-rich genus of the Old World fruit bats, comprising 65 of approximately 200 species of the family (Simmons, 2005; Helgen et al., 2009). *Pteropus* species are primarily distributed in Wallacea and the South Pacific, but have diversified throughout the Paleotropics, with species reaching as far as Mauritius and Madagascar off the east coast of Africa, the Ryukyu Islands in Japan, coastal areas of Australia, and the remote islands of Tonga and Samoa in the South Pacific. Since most *Pteropus* are island- or coastal-dwelling species that occur in remote areas (Mickleburgh et al., 1992), relatively little population or natural history data have been collected due to the difficulties in accessing these localities. *Pteropus* species are vital to ecosystem functioning in tropical forests (Fujita & Tuttle, 1991; McConkey & Drake, 2006) and important natural reservoir hosts for zoonotic pathogens (Field et al., 2007). A comprehensive understanding of the evolutionary history of the genus is necessary for development of effective conservation and management actions. *Pteropus* species also exhibit unusual evolutionary phenomena such as gigantism (Gould & MacFadden, 2004; Giannini et al., 2012) and evolutionary rate shifts (Shi et al., 2014) that cannot be fully explained in the absence of a resolved phylogeny. To further explore questions about diversification, biogeography, morphological evolution, and to address the urgency of management needs in Southeast Asia, a comprehensive molecular investigation of the genus *Pteropus* using species tree methods was conducted here so that these questions may be addressed within a phylogenetic framework in the future. The need for a greater understanding of *Pteropus* is felt most acutely in Indonesia,

where—despite the occurrence of a third of all extant *Pteropus* species—few recent studies have been conducted (*e.g.*, Sheherazade & Tsang, 2015).

Ecological Significance of Pteropus

Pteropus species are obligate phytophagous bats—they rely solely on fruits, flowers, leaves, pollen, and seeds for their metabolic needs (Voigt et al., 2011). Pteropodids play a key role in ecosystem functioning as seed dispersers and pollinators of over 300 plant species in approximately 200 genera, which include many canopy and emergent tree species (Fujita & Tuttle, 1991). On remote islands, large flying foxes may be the only animals capable of dispersing medium or large seeds (Wilson & Graham, 1992; McConkey & Drake, 2006). In tropical landscapes, plants generally rely on seed dispersing guilds comprised of both birds and mammals, resulting in low specificity with any host (Meehan et al., 2002). However, as seed size increases, fewer seed dispersers with the necessary gape size remain, and these animals are often species that are most susceptible to anthropogenic disturbance (Corlett, 1998). In the past, *Pteropus* species may have only been one of many of these seed dispersers, but the local extinction of other large-bodied dispersers has led to an increasingly important role in this process for remaining *Pteropus* colonies (Meehan et al., 2002). The need for *Pteropus* to forage over large home ranges promotes gene flow between geographically distant areas, though the extent to which this occurs is poorly known in most *Pteropus* species.

Pteropus bats retain seeds in their guts for more than twelve hours, making it possible for them to carry seeds over large oceanic expanses (Shilton et al., 1999). *Pteropus* also periodically drops seeds in flight, which is critical to reseeding clearings (Corlett, 1998), particularly in fragmented landscapes (Nyhagen et al., 2005). *Pteropus* species are an important part of fortifying a healthy seed bank for forest regeneration (Muscarella & Fleming, 2007), which

previous research has demonstrated after entire island ecosystems have been destroyed by typhoons (Esselstyn et al., 2006) or volcanic eruptions (Shilton & Whittaker, 2009). Figs (genus *Ficus*) and other Moraceae are the most commonly visited plants by *Pteropus* species (Fujita & Tuttle, 1991; Stier & Mildenstein, 2005; Nakamoto et al., 2008).

Pteropus bats have been known to visit chiropterophilous (bat pollinated) flowers of economically important plants, including durian (*Durio zibethinus*) (Jones & Kunz, 2000; Bumrungsri et al., 2008; Kingston, 2008). Chiropterophily is a pollination syndrome in plants characterized by flowers that are generally night-blooming, strongly scented, dull-colored, sturdy, and held away from the foliage (Tschapka & Dressler, 2002). Most *Pteropus*-pollinated plants belong to the Malvaceae and Myrtaceae (Fujita & Tuttle, 1991; Elmqvist et al., 1992; Nyhagen et al., 2005). Plants from these families produce important products such as *kapok* (*Ceiba pentandra*), which is used commonly for its light but resistant fibers and essential oils and various light timbers (Fujita & Tuttle, 1991; Singaravelan et al., 2009).

Taxonomy and Its Implications for Disease Ecology Studies

Bats have been increasingly recognized as natural reservoir hosts for a suite of emerging infectious pathogens (Calisher, 2006; Smith & Wang, 2013). Pteropodids alone are implicated as vectors of some pathogens causing recent pandemics (Wong et al., 2007). Of these, species of *Pteropus* have been identified as potential reservoir hosts of paramyxoviruses, including Hendra and Nipah virus, both of which are highly pathogenic to humans and have high mortality rates in humans (Halpin et al., 2011). Rapid human encroachment into primary forests (Sodhi et al., 2010) and intense bushmeat consumption (Mickleburgh et al., 2009) are not only threats to the persistence of bat populations, but also introduce new avenues for potential transmission. Outbreaks of paramyxoviruses have been previously recorded in both Malaysia (Yob et al.,

2001; Rahman et al., 2010) and Bangladesh (Hsu et al., 2004), where their spread resulted in significant negative consequences for both human health and local economies. Cross-disciplinary approaches to surveillance can suggest where host jumps may occur, which is crucial to early detection and effective control of emerging infectious diseases. As next-generation sequencing becomes more readily available and bioinformatics pipelines become more sophisticated and user-friendly, host genomic data and phylogenies will also be able to answer questions on whether selection on host alleles may have occurred in the past or if pathogens may be locally adapted. The growth of this "applied systematics" approach to emerging infectious diseases requires a deeper look into host evolution.

Despite the importance of understanding bat and bat-borne virus ecology, only a handful of species, such as *Pteropus alecto* and *P. vampyrus*, have been studied beyond initial discovery of novel viruses (Table 1.1). These data were obtained primarily through reactionary investigations following an outbreak (Wang et al., 2008). The few recent surveillance efforts focus solely on virus detection, and consideration of host natural history in disease dynamics has been limited (Chua et al., 2001; Sasaki et al., 2012). Knowing the disease profile (*e.g.* prevalence of infectious diseases, particularly in relation to age cohorts; signs and symptoms of disease) for each bat species can better inform public health agencies in each of the localities that the species occurs, and precautions can be taken accordingly. Understanding host natural history, evolution, and diversity will contribute to public health goals by providing 1) data on bat host species diversity, which can inform models for prediction of where high viral diversity may occur, 2) data on bat host relationships to predict where spillovers may occur, and 3) more accurate population-level relationships between host populations to determine where host and pathogen dispersal routes may occur. These elements can contribute to a more fully realized model of

transmission of zoonotic diseases, such as in studies of rabies in *Eptesicus fuscus* (Big Brown Bats) in North America, which found that life history traits along with long viral incubation periods promoted pathogen persistence (George et al., 2011). However, if there are no host ecological niche models, coalescent models of past histories, or phylogenies available, this level of understanding is impossible, as is presently the case with *Pteropus*.

Important but Endangered—the Conservation Status of Pteropus

Many *Pteropus* species are threatened by habitat loss, specifically loss of roosting sites in primary forest or mangroves, and are persecuted by farmers as crop pests (IUCN, 2014). Additionally, *Pteropus* are often sought after in the bushmeat trade, either as a form of sustenance or as a traditional remedy (Mickleburgh et al., 2009; Croes, 2012; IUCN, 2014; Sheherazade & Tsang, 2015). Hunting of *Pteropus* often operates at an unsustainable rate, and increases the degree of contact between humans and bats, providing opportunities for pathogens to spread and increasing disease risk (Epstein et al., 2009; Mickleburgh et al., 2009; Harrison et al., 2011).

The entire genus *Pteropus* is listed under the Convention for International Trade of Endangered Species (CITES) Appendix II (CITES, 1989) to provide protection against illegal international trade, though no protection within their native countries. Some Pacific island species have been listed as Appendix I due to overhunting for the commercial bushmeat trade across international borders prior to the 1980's (CITES, 2014): *P. insularis* (now *P. pelagicus*, see Buden et al., 2013), *P. loochoensis*, *P. mariannus*, *P. molossinus*, *P. pelewensis*, *P. pilosus*, *P. samoensis*, *P. tonganus*, *P. ualanus*, and *P. yapensis*. In sum, 85% of the genus is classified by IUCN in a threatened category or Data Deficient (Fig. 1.1) (IUCN, 2014). Several species listed as of Least Concern likely warrant further study because their population trends are either

unknown (*P. neohibernicus*, *P. admiralitatum*), populations appear to be decreasing (*P. hypomelanus*, *P. giganteus*), or their reported population trends represent a combined, global measure of species status without taking recent population crashes or other threats into account. For instance, the death of thousands of *P. alecto* in Australia due to extreme heat waves, as documented recently by Welbergen et al. (2008, 2014), and intensive bushmeat hunting in Sulawesi, Indonesia as documented in Sheherazade & Tsang (2015) threaten different populations locally in a way that may affect their global conservation status.

Conservation Implications of Revised Taxonomy

Due to the lack of recent study and much historical confusion regarding species identities, clarification of taxonomic issues will make conservation management easier for non-taxonomists. The benefits of accurate taxonomic delineation and creation of unambiguous keys to *Pteropus* conservation are immediately apparent. Appendix II species are listed to protect similar-looking species as a way of discouraging illegal wildlife trafficking, and *Pteropus* clearly suffer from being indistinguishable to non-experts due to high levels of morphological similarity. An easily navigable dichotomous key would be invaluable to wildlife management authorities and forestry staff for monitoring illegal trade or local population trends. *Pteropus* bats are charismatic species for public outreach and conservation efforts, but an accurate guide to these species cannot be made without revising the currently flawed taxonomy.

International conservation legislation requires focuses on the rank of species, and an updated understanding of *Pteropus* taxonomy is therefore essential for accurate assessment of conservation priorities. This is a pressing issue as poorly defined species complexes and understudied species will not have access to resources that may help conserve their habitats or populations (Bickford et al., 2007). As human populations continue to grow exponentially and

encroach into natural habitats, new species are as likely to be discovered in a bushmeat market as in a forest (*e.g.* Sanamxay et al., 2013). Despite Southeast Asia being home to multiple biodiversity hotspots and facing dire environmental issues, biodiversity research still lags behind that of other tropical regions and is sorely needed (Sodhi et al., 2010; Wilcove et al., 2013).

In the late nineteenth and early twentieth centuries, each newly discovered island population of *Pteropus* was often given a new name, though many were later synonymized with the species names in use today (Dobson, 1878; Andersen, 1912; Corbet & Hill, 1992). Understudied *Pteropus* populations require further examination, as they may represent multiple species (Almeida et al., 2014), but determination of species limits for most *Pteropus* have not yet incorporated molecular data. Recognition of cryptic taxa as distinct species is critical to the protection of island species, as demonstrated by the recent preventable extinction of *Pipistrellus murrayi* on Christmas Island, Australia (Lumsden, 2009; Martin et al., 2012). Similarly ill-defined species designations exist within *Pteropus*. For instance, there is still discussion as to whether or not *Pteropus melanopogon* should be recognized as distinct from *P. aruensis* and *P. keyensis* (Flannery, 1995; Bergmans, 2001; Simmons, 2005)—a problem that can only be resolved with more research on this species complex. The revised phylogeny presented in this dissertation clarifies relationships among *Pteropus* species and also allows for identification of evolutionarily distinct lineages in support of further conservation action.

To study evolutionary and biogeographic phenomena and provide a framework for other applied studies, my dissertation research addressed these foundational questions about *Pteropus*: 1) What are the evolutionary relationships among species, and do molecular data corroborate previous classifications from morphology; 2) What is the age of the genus *Pteropus*; 3) Are lineages evolving at a uniform rate through time; 4) What mechanisms govern their distribution,

and how does geography affect speciation; 5) Does the inclusion of founder-event speciation affect the performance of models of ancestral area reconstruction; 6) What is the population structure of *P. vampyrus*, and what implications does this have for conservation and management actions; 7) What is the population structure of *P. alecto* and what implications does this have for conservation and management actions.

Table 1.1. List of viruses previously isolated from *Pteropus*. Of these, in-depth studies about host dynamics exist only for *P. alecto*, *P. giganteus*, and *P. vampyrus*.

Virus Name	Bat species	Country	Citation
RNA Viruses			
Flaviviridae			
GBD-V	*P. giganteus*	Bangladesh	Epstein et al., 2010
Japanese encephalitis	*P. alecto*	Australia	van den Hurk et al., 2009
Paramyxoviridae			
Nipah virus	*P. hypomelanus*	Malaysia	Yob et al., 2001
Nipah virus	*P. vampyrus*	Malaysia	Yob *et al.* 2001
Nipah virus	*P. hypomelanus*	Malaysia	Chua et al., 2001
Nipah virus	*P. giganteus*	Bangladesh	Hsu et al., 2004
Nipah virus	*P. lylei*	Cambodia	Reynes et al., 2005
Nipah virus	*P. rufus*	Madagascar	Iehlé et al., 2007
Tioman virus	*P. rufus*	Madagascar	Iehle *et al.* 2007
Tioman virus	*P. hypomelanus*	Malaysia	Chua *et al.* 2001
Hendra virus	*P. alecto*	Australia	Halpin et al., 2000
Hendra virus	*P. poliocephalus*	Australia	Halpin *et al.* 2000
Hendra virus	*P. rufus*	Madagascar	Iehle *et al.* 2007
Menangle	*P. alecto*	Australia	Philbey et al., 2008
Menangle	*P. poliocephalus*	Australia	Philbey *et al.* 2008
Menangle	*P. conspicillatus*	Australia	Philbey *et al.* 2008
Reoviridae			
Pulau virus	*P. hypomelanus*	Malaysia	Pritchard et al., 2006
Broome virus	*P. scapulatus*	Australia	Thalmann et al., 2010
Rhabdoviridae			
Lyssavirus	*P. alecto*	Australia	Gould et al., 1998
Lyssavirus	*P. hypomelanus*	Philippines	Arguin et al., 2002
DNA Viruses			
Adenoviridae			
Mastadenovirus	*P. dasymallus*	Japan	Maeda et al., 2008

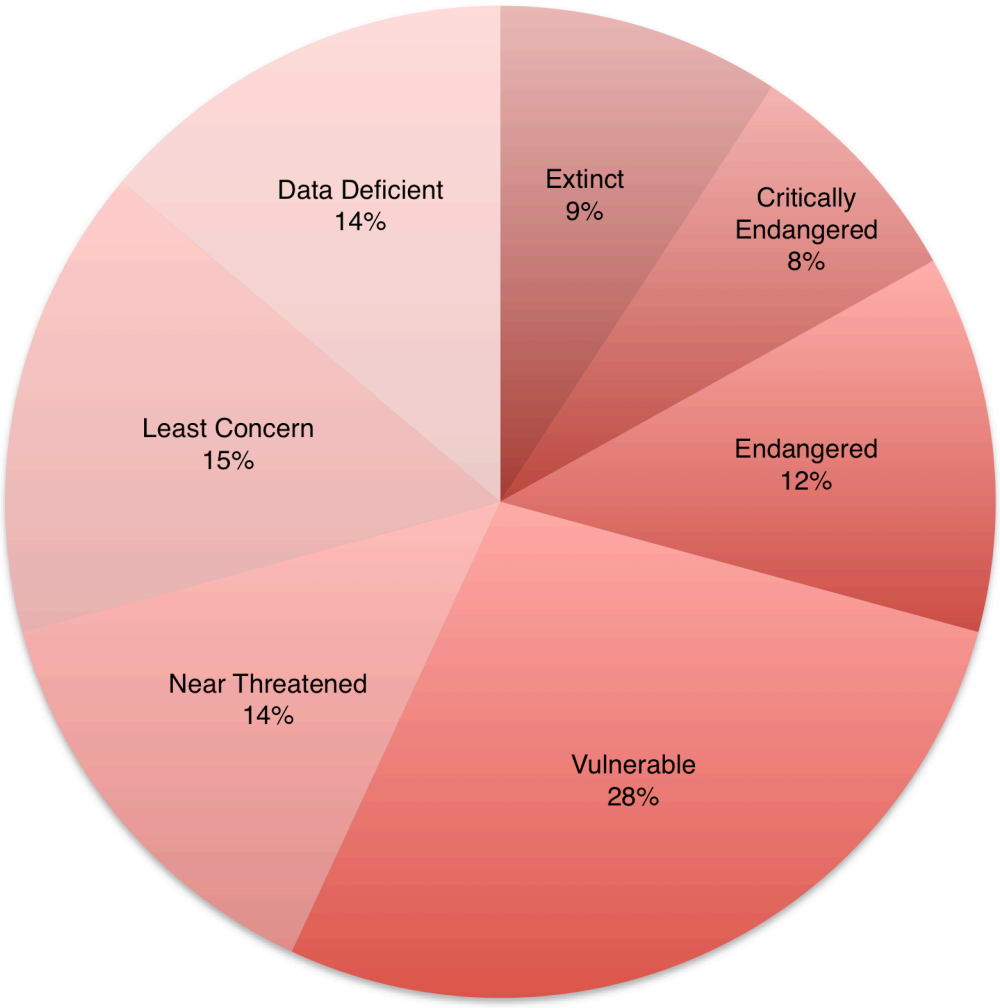


Figure 1.1 IUCN Red List status of *Pteropus* species. This summarizes species and names as they are understood by IUCN (2014) and may not represent taxonomic and nomenclatural changes made from more recent studies.

CHAPTER 2

Evolutionary relationships and nomenclatural changes of the genus *Pteropus* based on multilocus molecular evidence and species tree methods

Abstract

This study aims to resolve taxonomic issues and phylogenetic relationships within the genus *Pteropus* using molecular evidence, extensive sampling of Southeast Asian species, and species tree methods for reconstructing a phylogeny. This study includes five species that have never before been included in molecular analyses; their placement in the *Pteropus* phylogeny informs biogeography and conservation research. *Pteropus* species were found to belong to fewer distinct evolutionarily lineages than previously thought and were categorized into the following species groups following the species tree from this study: *personatus*, *pelagicus*, *scapulatus*, *vampyrus*, *temminckii*, *griseus*, and *samoensis*. The genus is only Miocene in age, with many short internodes, suggesting rapid radiations during the Plio-Pleistocene. This agrees with findings from BAMM (Bayesian Analysis of Macroevolutionary Mixtures), which suggest a relative increase within the genus in lineage divergence rates for the *vampyrus*, *temminckii*, *griseus*, and *samoensis* species groups. This species tree is the foundation of the biogeographic work completed for Chapter 3, and will also be used to provide better taxonomic keys for non-experts to use by clarifying some confounding aspects of *Pteropus* relationships. Discordance in nuclear and mitochondrial signals suggest rampant incomplete lineage sorting issues, with some potential cases of hybridization as well in some lineages. However, due to the low genetic variability of *Pteropus*, these issues can only be addressed more rigorously in the future with high throughput sequencing data.

Introduction

Previous Research

Traditionally, the genus *Pteropus* has been split into as many as 17 species groups based on morphological characters including pelage coloration, cranial morphology, and dentition (Andersen, 1912; Corbet & Hill, 1992; Francis, 2008). Andersen's monograph was the last comprehensive treatment of the genus and was based on 956 skins, 444 alcohol specimens, and 1228 skulls, with each species represented by at least one or a series of skulls (with the exception of *P. aruensis*). However, *Pteropus* species often lack unambiguous diagnostic morphological differences, and character variation is insufficient for use in phylogenetic reconstruction (Colgan & Flannery, 1995). In a study including over 4500 phenotypic characters, the Assembling the Tree of Life Mammals Project was able to score 2600 phenotypic characters in one species of *Pteropus* (O'Leary et al., 2013) but only 909 of these vary among the bats sampled, and fewer than 0.05% of these vary among species within the genus *Pteropus*. Even dental morphology, the most commonly used character system of mammalian systematics, is relatively uninformative in pteropodids. Giannini and Simmons (2005) were able to identify only 37 dental characters that vary within Pteropodidae, and most of these are invariant within the genus *Pteropus*.

Species of *Pteropus* are distinguished morphologically by body size measurements (which may overlap among species), qualitative craniodental anatomy, and differences in pelage patterns (*e.g.*, color of the ventral fur and neck ruff) that may also overlap among species (Andersen, 1912; Corbet & Hill, 1992; Flannery, 1995; Patterson & Webala, 2012). Andersen's (1912) species groups, based on this limited subset of morphological information, often make very little sense from a biogeographic perspective. For example, the "*livingstonii*" species group consists of *P. livingstonii*, *P. melanopogon*, *P. aruensis*, and *P. keyensis*. *P. livingstonii* is found

in the Comoros near Madagascar and the other species all form a closely related species complex in the Moluccas in Indonesia (Simmons, 2005). More recent molecular data have resulted in regrouping *P. livingstonii* with another Mascarene species, *P. voeltzkowi*, instead (O'Brien et al., 2009; Almeida et al., 2014). That two geographically distant species are morphologically similar to one another may be an example of convergence within the morphologically conservative *Pteropus* genus and do not reflect real evolutionary lineages. Although neither of these two studies included any *P. melanopogon* specimens, it is unlikely the *P. melanopogon* species complex is sister to *P. livingstonii*. The revised species groups from Almeida et al. (2014) more likely reflect monophyly, but many relationships remain unresolved (see Table 2.1 for old species group designations).

Despite recent advances in genetic and phylogenetic methods, few molecular phylogenies have included *Pteropus* species from multiple biogeographic areas or species groups. Earlier molecular studies were unable to resolve species-level relationships among *Pteropus*, largely due to sparse taxon sampling and a dearth of genes for resolving such a young clade (Giannini & Simmons, 2005; Giannini et al., 2008). A more recent study included short fragments of mitochondrial *cyt-b* from rare or extinct *Pteropus* by using ancient DNA from museum skins along with available Genbank data for a total representation of 50 species (Almeida et al., 2014). In the full nuclear dataset from the same study, only 21 species were included in the final analysis. Almeida et al. (2014) classified *Pteropus* into 13 species groups, many of which were found in the same region of the world, unlike the species groups of Andersen (1912). Their study also provided the first evidence that there may be incomplete lineage sorting issues in *Pteropus* and that the genus had only experienced its most explosive radiations in the past one to two million years.

These data provide a starting point from which to consider the species history of the genus *Pteropus* in the present study. Previous studies fail to appreciate that: 1) gene trees can reflect the species tree and species history, but should not be treated as equivalent to species trees (Maddison, 1997), 2) species should not be represented by a single individual as it may not fully represent species genetic diversity (Edwards & Beerli, 2000; Edwards, 2009), 3) males and females may behave differently, resulting in sex-specific genealogical histories. This may be especially evident in mammals, which are known for sex-based dispersal and philopatry (*e.g.* Weyandt et al., 2005; Lausen et al., 2008; Clare, 2011). Previous studies also failed to take advantage of new advances in statistical analyses that allow for 1) the treatment of each gene as an independently evolving locus, 2) the inclusion of multiple individuals to represent a single species, and 3) incorporation of coalescent methods (Bouckaert et al., 2014).

Table 2.1. List of species groups from this study as compared to those recognized in Andersen (1912) and Almeida et al. (2014). Putative placements based on morphological data are preceded by a question mark; those that are based on previous studies are in parentheses. Names used here are following Simmons (2005), Helgen et al. (2009), and Buden et al. (2013). Species marked with † are extinct. Species groups are similar to those in Almeida et al. (2014) and have been aligned by group names for ease of comparison.

From this study	Almeida et al. (2014)	Andersen (1912)
personatus group	***personatus* group**	***personatus* group**
personatus	? *personatus*	*personatus*
lombocensis		*capistratus*
	***lombocensis* group**	*temminckii*
	lombocensis	
***scapulatus* group**	***scapulatus* group**	***scapulatus* group**
scapulatus	*scapulatus*	*gilliardorum*
		mahaganus
pelagicus group	***pelagicus* group**	*scapulatus*
molossinus	*macrotis*	*woodfordi*
gilliardorum	*woodfordi*	
woodfordi	*mahaganus*	***molossinus* group**
(*pelagicus*)	*gilliardorum*	*lombocensis*
(*macrotis*)	*molossinus*	*molossinus*

(*mahaganus*)	*pelagicus*	*rodricensis*
(*tokudae*†)	*tokudae*†	
***vampyrus* group**	***vampyrus* group**	***vampyrus* group**
caniceps	*pselaphon*	*giganteus*
chrysoproctus	*dasymallus*	*intermedius*
melanopogon	*pumilus*	*lylei*
poliocephalus	*rodricensis*	*vampyrus*
dasymallus	*vampyrus*	
pselaphon	*giganteus*	***caniceps* group**
pumilus	*lylei*	*caniceps*
giganteus	*aldabrensis*	
rufus	*rufus*	***livingstonii* group**
lylei	*seychellensis*	*aruensis*
vampyrus	*niger*	*keyensis*
niger		*melanopogon*
(*livingstonii*)	***livingstonii* group**	*livingstonii*
(*voeltzkowi*)	*livingstonii*	
(*seychellensis*)	*voeltzkowi*	***niger* group**
(*aldabrensis*)		*aldabrensis*
(*rodricensis*)		*niger*
? *subniger*†		*rufus*
? *keyensis*		*seychellensis*
? *aruensis*		*voeltzkowi*
? *argentatus*		
	***poliocephalus* group**	***poliocephalus* group**
	poliocephalus	*macrotis*
		pohlei
		poliocephalus
***samoensis* group**	***samoensis* group**	***samoensis* group**
anetianus	*nitendiensis*	*anetianus*
capistratus	*tuberculatus*	*samoensis*
rayneri	*anetianus*	
samoensis	*fundatus*	***pselaphon* group**
vetulus	*samoensis*	*pelagicus*
(*rennelli*)	*rayneri*	*nitendiensis*
(*cognatus*)	*cognatus*	*pilosus*†
(*nitendiensis*)	*rennelli*	*pselaphon*
(*fundatus*)	? *brunneus*†	*tokudae*†
(*tuberculatus*)	? *pilosus*†	*tonganus*
	? *coxi*†	*tuberculatus*
	? *allenorum*†	*vetulus*

***temminckii* group**
temminckii

***griseus* group**
alecto
ocularis
hypomelanus
griseus
admiralitatum
conspicillatus
neohibernicus
tonganus
pelewensis
(*speciosus*)
(*mariannus*)
(*ualanus*)
(*pohlei*)

group *incertae sedis*
ornatus
melanotus
howensis
faunulus
brunneus†
pilosus†
coxi†
allenorum†

***vetulus* group**
vetulus

***capistratus* group**
capistratus
ennisae
temminckii

***griseus* group**
hypomelanus
griseus
speciosus
neohibernicus
conspicillatus
alecto
tonganus
ualanus
admiralitatum
pohlei
mariannus
pelewensis
? *howensis*
? *faunulus*

***ornatus* group**
ornatus

group *incertae sedis*
melanotus
melanopogon
keyensis
aruensis
argentatus
caniceps

***chrysoproctus* group**
argentatus
chrysoproctus
cognatus
fundatus
rayneri
rennelli

***mariannus* group**
loochoensis
mariannus
pelewensis
ualanus
yapensis

***alecto* group**
alecto

conspicillatus group
conspicillatus
ocularis

***neohibernicus* group**
neohibernicus

***subniger* group**
admiralitatum
brunneus†
dasymallus
faunulus
griseus
howensis
hypomelanus
ornatus
pumilus
speciosus
subniger

***melanotus* group**
melanotus

Previous studies also did not have access to many species from Southeast Asia, particularly Indonesia, which is a hotspot of *Pteropus* species diversity (Simmons, 2005). As mentioned earlier, approximately one-third of all recognized *Pteropus* species are native to Indonesia (21 of the 65), and 12 are found in no other country (Suyanto, 2001). Besides being home to such a diversity of species, the archipelagic nature of Indonesia has apparently led to a variety of subspecies isolated on remote islands (Andersen, 1912; Corbet & Hill, 1992). Some of these species may be better recognized as full species, pending further scientific review. This includes 9 of 16 subspecies of the *P. hypomelanus* species complex and 6 of 7 subspecies of the widespread *P. vampyrus* (Mickleburgh et al., 1992). In terms of individuals from Indonesia, molecular data are available for only three species: *P. hypomelanus* (O'Brien et al., 2009), *P. griseus*, and *P. lombocensis* (Almeida et al., 2014). Other genetic data available for Indonesian species has come from populations in other countries or biogeographic areas (*e.g. P. alecto* from Australia and *P. vampyrus* from Almeida et al., 2014). Missing data from taxa and populations across such an important and broad area as Indonesia undoubtedly skews phylogenetic and biogeographic inferences (Wiens, 2003).

Synthesis of Population Genetics and Phylogenetics—the Case for Species-Tree Methods

In the past decade, systematists have recognized that synthesis with population genetic theory was necessary for resolving phylogenies that more accurately reflect the true species history. It is now broadly accepted that that species evolve as multiple individuals in a population, and that each locus is an independently evolving entity (Edwards & Beerli, 2000). The need to move away from gene trees towards species tree methods was also necessary for decreasing methodological artifacts and increasing the rigor of models employed in such investigations (Edwards, 2009). Species-tree methods may assist in determining if gene-tree

discordance may be due to lineage sorting, introgression (*e.g.* gene flow, hybridization), differences in mitochondrial or nuclear genetic inheritance, and allow for the incorporation of uncertainty around the data (Drummond & Rambaut, 2007).

It may also be the case that topologies in a phylogeny are largely driven by a single gene and the dominance of the phylogenetic signal by a single locus may not be evident if all data were concatenated (Edwards, 2009). Single-gene species-delimitation methods do not provide sufficient levels of evidence that species boundaries exist, and often overestimate species by splitting them into too many mitochondrial clades (Song et al., 2008; Lohse, 2009). However, the opposite problem exists in African pteropodids, where it has been shown that mitochondrial genes may not be able to distinguish species (Cruaud et al., 2011). Species delimitation requires a suite of independently evolving genes and traits to be accounted for when determining where boundaries exist (Fujita et al., 2012), something that cannot be done with a single marker. DNA barcodes suggest that the biodiversity of Southeast Asia may be underestimated due to the existence of cryptic species that require further study (*e.g.* Lohman et al., 2010). Subsequent collection of data from multiple independently segregating markers increase rigor of species delimitations made on the basis on genetic data, and are necessary to detect lineage sorting and hybridization (Yang & Rannala, 2010). Ideally, identification of species combines multiple lines of evidence (*e.g.*, molecular, morphological, behavioral, or acoustic data), and genealogical data are now commonly only the first step in differentiating closely related species.

Methods

Taxonomic Sampling

The total dataset of 188 individuals included multiple individuals of *Pteropus* species, resulting in 39 ingroup taxa. Individuals from the genera *Macroglossus*, *Rousettus*, *Nyctimene*,

Syconycteris, *Chironax*, *Dobsonia*, and *Acerodon* were included as outgroups to root the tree based on what is known of generic relationships within Pteropodidae (Almeida et al., 2011). Typically, addition of multiple individuals is more effective in increasing the accuracy of species tree estimates than addition of multiple loci for recent divergences (Knowles & Kubatko, 2011). Previous research on *Pteropus* suggests that it is a young clade (Almeida et al. 2014), therefore, where possible, multiple individuals were sampled.

Sample Acquisition

Tissue Loans

Tissue sample loans were obtained from the Lubee Bat Conservancy (LBC), Natural History Museum of Los Angeles County (LACM), Museum Zoologicum Bogoriense (Indonesian Institute of Sciences, MZB), National Museum of the Philippines (NMP), Lee Kong Chian Museum of Natural History (formerly Raffles Museum of Biodiversity Research, Singapore, RMBR), Museum of the North (University of Alaska, Fairbanks, UAF), National Museum of Natural History (Smithsonian Institution, USNM), University of Wisconsin Madison Zoological Museum (UWZM), and Western Australian Museum (WAM). These supplemented loans previously granted to Nancy B. Simmons, Francisca C. Almeida, and Kristofer M. Helgen: American Museum of Natural History (AMNH), Australian Museum (AM), Carnegie Museum of Natural History (CMNH), Field Museum of Natural History (FMNH), Museum of Vertebrate Zoology (University of California, Berkeley, MVZ), Royal Ontario Museum (ROM), and USNM. *Pteropus pselaphon* and *P. dasymallus* specimens from Okinawa could not be exported from Japan and lab work was conducted by Norimasa Sugita at the National Museum of Nature and Science, Tokyo (NSMT).

Field Collection

Few if any tissue samples of *Pteropus* were collected from Indonesia and the Philippines prior to 2012. We therefore conducted extensive fieldwork to fill in geographic and taxonomic gaps (Fig. 2.1). A full list of specimens is provided in Appendix 2.1. Methods were approved by the IACUC committee at City College of New York—CUNY through protocol No. 896.2 to D.J. Lohman and S.M. Tsang. Permits for fieldwork were obtained from the Ministry of Foreign Research and Technology and the Ministry of Forestry in Indonesia, and the Department of Environment and Natural Resources and the Biodiversity Management Bureau in the Philippines. Since all *Pteropus* are protected by CITES, I worked with only CITES-approved institutions in each country to ensure that I could receive tissue samples through the AMNH, which is a CITES-approved institution. Lab work took place at the Cullman and Monell Laboratory at AMNH. Import permits from U.S. Fish and Wildlife Services and Centers for Disease Control and Prevention were obtained prior to specimen transport.

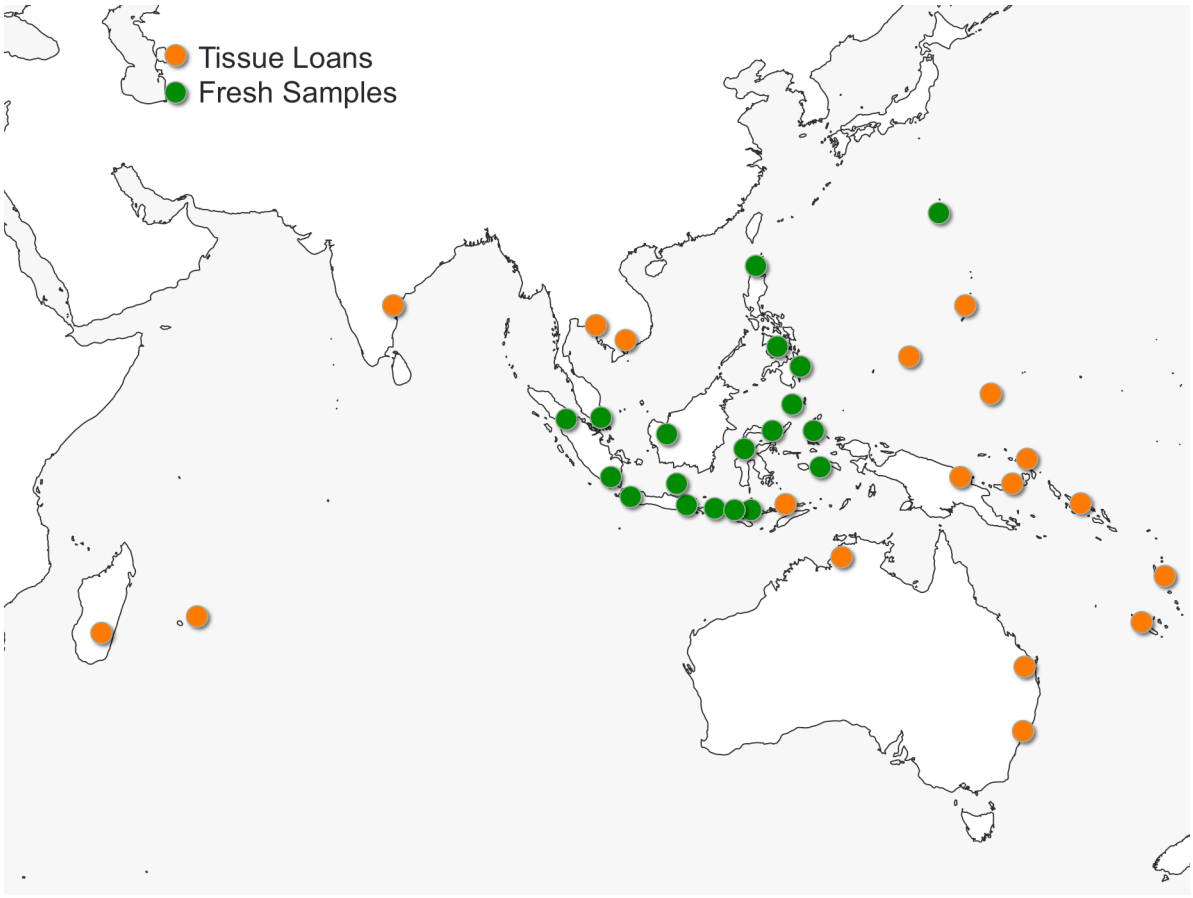


Figure 2.1. Map of *Pteropus* specimens used for this study. Orange dots are museum loans, green dots are fresh tissue samples from recent field expeditions.

Potential roost sites were found by contacting local forestry rangers and researchers for information on if there were any *Pteropus* sighted near the area, with subsequent tracking and confirmation conducted by the field team and myself. Georeferenced locality data for field sites were collected using a Garmin Oregon 550 handheld GPS (available from author upon request). After locating roost sites, the exit trajectory of each flying fox colony was observed to determine optimal locations for mist net placement. Potential nearby foraging sites were located and searched for stands of fruit with telltale marks of bat foraging such as large bites and scratched trees. Canopy mist nets were set up 20 to 30 m above the ground by tying the nets to a pole extending above the highest trees. A 6 m, 9 m, or 12 m net was used depending on the distance

between trees in the flyway. When bats were caught in the mist net, the pulley was immediately lowered to extract the bat and raised again until the desired number of individuals were captured—two males and two females. This sampling goal was influenced by both theoretical and practical considerations. While multiple individuals are necessary, typically, the return in species tree estimation from adding too many individuals diminishes rapidly (Heled & Drummond, 2010). Theory and simulation studies suggest that most genes will coalesce within five coalescent units, meaning five individual intraspecific lineages are sufficient for capturing the species' genetic diversity (Patel et al., 2013). Additionally, too much commotion from setting up mist nets repeatedly may repel bats and may disperse the colony from a roost (*pers. obs.*).

Identification of species in the field followed comparisons of external character measurements and qualitative traits with descriptions and data from original diagnoses including those in Andersen (1912), Corbet & Hill (1992), and Suyanto (2001). Upon returning from the field, species identities were confirmed with additional cranial examinations.

After capture, each bat was placed in a white cloth bag. Holding bags were occasionally sprayed with water to keep bats comfortable while they awaited processing. Bats were weighed using spring scales while in the cloth bag. External morphological features were measured in the field using digital calipers: head and body length (HB), tail length (TAIL), ear length (EAR), forearm length (FA), tibia length (TIB), and hind foot length without claw (HF).

A 4 mm^2 wing punch was taken from both wings of each individual; one was preserved in 95% ethanol and the other in RNAlater. Fecal samples were collected opportunistically from cloth bags. Oral swabs and anal swabs of each individual were taken for viral assays. A dry hair sample from the mantle was taken using iris scissors. All samples were labeled with the same field number. Bat ectoparasites were collected whenever they were encountered and preserved in

95% ethanol for later identification. These included bat flies (Diptera: Nycteribiidae), ticks (Ixodida: Argasidae), and mites (Acari). Duplicate batflies were preserved in RNAlater for later genomic analyses related to their potential as an intraspecific pathogen vector. We also collected environmental samples opportunistically at roost sites where possible for later genomic analyses of viruses that could potentially be transmitted through feces or ejecta (seeds and other half-eaten material spat from the mouth). Other incidentally captured pteropodids were sampled and then released after providing a sugary treat to the bat. Voucher specimens were deposited in museums in the countries of collection (MZB and NMP). At some sites, additional tissue samples were taken from salvaged individuals (*e.g.*, hunting remains, fallen juveniles), but these specimens were not always intact. Tissue samples were deposited at the Ambrose Monell Cryo Collection at the American Museum of Natural History (AMNH) for long-term storage.

Traditional Collecting Methods

At some sites in Indonesia, local laws required the use of traditional methods to capture flying foxes to restrict the number of individuals taken. These laws were in place to prohibit the use of guns to ensure the continued residence of the colony at the site. However, this also meant that we were not permitted to use mist nets. At other sites, such as some offshore mangrove islands, mist net placement was not logistically possible. The following section describes traditional methods used at each site, which supplemented or replaced mist netting of bats due to these limitations. These details were also documented below as they may be of interest to conservation biologists or anthropologists.

Tomoli, Sulawesi Tengah

Pulau Kelelawar is an offshore mangrove island with a large colony of *P. alecto* and *Acerodon celebensis* near the village of Tomoli in Central Sulawesi. The bats move between three

mangrove islands from year to year and the villagers place large bamboo poles (about 6 m in height) around the island the bats are occupying. A monofilament line is drawn loosely between each of these poles and fishing hooks are placed intermittently to catch the bats as they fly to and from the roost. The pole is lowered to bring the line down and the bat is taken. Since we were not allowed to capture bats at this site and this is a regular practice by the villagers, we sampled individuals captured by villagers.

Olibu, Sulawesi Utara

Olibu is a small, remote village nested between the mountains of Northern Sulawesi near Paguyaman. There is a large mangrove island with a mixed colony of *P. alecto* and *A. celebensis*. The locals trade the bats once a week in distant markets and are protective of the bats as a natural resource, even fighting off the army the year prior to our visit when soldiers tried to kill all the bats for food. A large nylon net controlled by a pulley system is set up across the entire width of the entrance of the mangrove (about 25 m). When the bats emerge, they are caught in the nets and brought back to shore. The bats are kept alive in a shed close to the village until they are brought to the market. Since we were not allowed to disturb the bats at their roosting site, we sampled individuals housed in the shed.

Situ Lengkong, Panjalu, Jawa Barat

Situ Lengkong is a large, freshwater catchment lake in the mountains (749 m) in the village of Panjalu in West Java. A colony of *P. vampyrus* lives on a forested island in the middle of the lake, though some individuals were seen occupying trees onshore during the day and perching at the lake shore when exiting at dusk. The lake is a culturally important site—pilgrims visit every day to pray and fetch holy water from the spring on the island. According to local lore, an ancient king brought the water back from Arabia along with Islam, and he is thought to be buried

in the area, though his gravesite is unknown. His son and many other important figures are buried in a cemetery along the route the pilgrims take up to the prayer site. The dense vegetation, varied elevation, and throngs of pilgrims made capture of bats with mist nets difficult. Some local people told us that they captured bats using kites. One or two people would get into a small kayak or stay onshore closest to the island and fly a kite. The kite is flown high into the air with a hook on the line. When a bat nears the kite, the villagers would swing the kite towards the bat to catch it on its wing. The bat then falls into the water and the villagers quickly row over to fetch it. We sampled two *P. vampyrus* through this method. It is also common practice for the local guides to rehabilitate juveniles by raising them as pets if they find fallen from the roost tree, which allowed sampling of wing punches from an additional individual.

Kintamani and Alas Kedaton, Bali

Local religious and cultural beliefs prevented us from taking any vouchered specimens, as Hindus frown upon killing animals. At Kintamani, a coffee plantation farmer had found an orphaned *P. vampyrus* two years ago in a nearby forest and since kept it as a pet. We were permitted to take a wing punch and take feces and ejecta found in its enclosure. At Alas Kedaton,, a colony of *P. vampyrus* is in residence in the forest behind the temple. During the day, a small group of bats descends to a manmade branch about a meter in height made by the locals for their "bat show." The bats allow the locals to handle them and tourists may approach and take photographs. They will even follow simple hand signals and calls in exchange for fruit rewards, with some wearing loose bracelets on their necks and responding to names. At dusk, the bats all return to the tree. The relationship between the people and the bats is symbiotic. As a result, we had permission to only take a single wing punch from each bat.

Cokrowati, Jawa Timur

There is only a single *P. vampyrus* colony of approximately 200 individuals at this site. The villagers believe that the bats are magical, consuming them only occasionally as medicine for respiratory diseases and as a panacea for good health. Only one or two bats are taken under these circumstances, and only by special request of sick people. The night roosting site is atop a small mountain and overlooks a cliff, though the colony splits into two smaller groups during the day. One of the day roosts is near a cemetery, whereas the other is in the middle of farmland, at a mausoleum to the first village chief. The day roosts were at flatter sites than the night roost, but since the trees were too tall at the day roosts for mist nets and we were not permitted to disturb these sites for cultural reasons, capture had to be at the main roost. Hence, I set up a 12 m raised mist net near the roost at an exit point, but difficulties in capture on such steep terrain required the use of kites again. Similar to the method employed at Situ Lengkong, the villagers would run the kite into the bat to bring it down before restraining it. I was able to sample two individuals of *P. vampyrus* using this method.

Pematang Gedung, Kalimantan Barat

The *P. vampyrus* colony was located at the center of a system of meandering rivers surrounding very dense mangrove forests. The water levels of the river vary as the tide levels changed throughout the course of the day, and it was deemed impossible to enter the mangrove forest to check nets after dusk because the water would have risen too high and there was the possibility of conflict with crocodiles. The colony was located on the far side of the mangrove forest, which could not be reached by boat. Instead, one of the locals traversed the mangrove forest alone, so as to not disturb the colony, and used a slingshot to fell the flying foxes. I was able to sample two individuals of *P. vampyrus* using this method.

Laboratory Methods

I extracted DNA from fresh tissue samples using a QIAgen DNEasy Blood & Tissue Extraction Kit. Some museum loans were from samples that were not preserved using best practices (*e.g.*, kept in unrefrigerated, dilute ethanol for several years). These samples were extracted in a fume hood dedicated to ancient DNA extractions to decrease the possibility of contamination using a modified protocol of the QIAgen DNEasy Extraction Kit with 1X PBS to increase yield, decrease PCR inhibitors, and prevent contamination. I used variable autosomal markers with highly conserved priming sites for this study: ATP7A, PLCB4, BDNF (Eick et al., 2005); STAT5A (Piaggio & Perkins, 2005); RAG-1, RAG-2 (Giannini et al., 2008). I also included COPS7A-4, a variable mammalian intron with a conserved priming site (Igea et al., 2010). Two variable mitochondrial markers commonly employed in vertebrate phylogenetics were also sequenced: *cyt-b* (Kocher et al., 1989) and D-loop (Brown et al., 2011). These standard chiropteran loci were included to facilitate use of the data in future higher-level phylogenetic analyses. The combination of all loci resulted in 7626 bp per specimen of genetic data for analyses. Thermal cycle protocols for each gene were as follows: 35 cycles of initial denaturation at 95º C for X min, annealing for 30 s, extension at 72º C for 2 min; then a final extension at 72º C for 3 min. Annealing temperatures for each primer are listed in Table 2.2. Successfully amplified PCR products were cleaned using ExoSAP or a vacuum manifold. Products were run on an Applied Biosystems 3730xl automated sequencer. Genes were aligned using Geneious 5.4.3 and MAFFT 7.0 (Katoh & Standley, 2013).

Table 2.2. Primers and annealing temperatures for each of the genes used for species tree analysis. Annealing temperatures are based on optimization experiments for *Pteropus* from this study and may vary for different genera. Primers on the first line are forward primers; primers on the second line are reverse primers.

Gene	T_A (°C)	Primers	Size
ATP7A	52	TCCCTGGACAATCAAGAAGC	671
		AAGGTAGCATCAAATCCCATGT	
BDNF	55	CATCCTTTTCCTTACTATGGTT	558
		TTCCAGTGCCTTTTGTCTATG	
cyt-b (part 1)	49	CGAAGCTTGATATGAAAAACCATCGTTG	1140
		AGTGGRTTRGCTGGTGTRTARTTGTC	
cyt-b (part 2)	49	CATGAGGACAAATATCATTCTGAGG	
		TCTTCATTTYWGGTTTACAAGAC	
D-loop	55	GCTGAGGTTCTACTTAAACT	420
		GAGATGTCTTATTTAAGGGG	
FGB7	55	CCACAACRGCATGTTCTTCAGCAC	595
		GTATCTGCCATTTGGATTGGCTGC	
PLCB4	55	GTGAAATTGGAAGCCGAGAT	309
		CACCAAGCTCATTTACTTGTGA	
PSMB8	52	CCACTCAGGGACTGGAAGAA	854
		TCGGACCCTGGACACTACA	
RAG-1	55	GCTTTGATGGACATGGAAGAAGACAT	1058
		GAGCCATCCCTCTCAATAATTTCAGG	
RAG-2	55	GATTCCTGCTAYCTYCCTCCTCT	747
		CCCATGTTGCTTCCAAACCATA	
STAT5A	55	CTGCTCATCAACAAGCCCGA	493
		GGCTTCAGGTTCCACAGGTTGC	

Analytical Methods

Haplotypes for each nuclear gene were estimated probabilistically using PHASE 2.0 (Gowri-Shankar & Rattray, 2007) for 20,000 iterations for 10 runs. The program jmodeltest2 (Darriba et al., 2012) selected appropriate genetic substitution models using a corrected Akaike Information Criterion (AICc; Table 2.3) for use in maximum likelihood and Bayesian phylogenetic reconstructions. Trees of the total taxon set of 188 individuals, including outgroups, were inferred using a partitioned MrBayes 3.2 analysis (Ronquist & Huelsenbeck, 2003),

discarding the first 25% of trees as burn-in. MCMC runs from multiple chains of MrBayes were tested for topological convergence using Are We There Yet? (Nylander et al., 2008).

Table 2.3. Models of evolution for each gene. Model selection was based on AICc scores. The two mitochondrial genes were tested separately in jmodeltest2 since it is known that D-loop broadly evolves at much more rapid rate than *cyt-b* in bats. Best models did not vary greatly except for *cyt-b* and PLCB4.

Gene	AIC	AICc	BIC
ATP7A	GTR+G	HKY+G	TrN+G
BDNF	TrN+G	K80+G	K80+G
COP7A4	TrN+G	TrN+G	TrNef+G
cyt-b	GTR+G	JC	GTR+G
D-loop	TrN+G	TrN+G	TrN+G
FGB7	TVM+G	HKY+G	HKY+G
PLCB4	GTR+G	JC	GTR+G
PSMB8	TIM2+G	K80+G	TrNef+G
RAG1	TIM2+G	TIM2ef+G	TrNef+G
RAG2	TVM+G	TPM1+G	TPM1+G
STAT5A	TIM3+G	K80+G	TPM3+G

To compare results using different reconstruction methods, trees were inferred with the same dataset using TNT (Goloboff et al., 2008) and RAxML-VI-HPC (Stamatakis, 2006) for maximum parsimony and maximum likelihood methods, respectively. Individual gene trees for each locus were run prior to the species tree run in both RAxML and MrBayes to detect potential anomalies in the dataset and ensure potential lineage sorting issues or introgression would be detected. The final species tree was estimated with *BEAST implemented in BEAST 2 (Bouckaert et al., 2014) with a reduced taxon set of 104 individuals that excluded specimens lacking sequence data for some markers (with the exception that one sample of *P. niger* was included despite missing data, as it was the only representative of its species). The tree model utilized a relaxed clock birth-death model. In the case of the widespread species, *P. hypomelanus* and *P. alecto*, populations (some of which corresponded to known subspecies) were treated as distinct operational taxonomic units (OTUs). Gene flow between species units violates the

species delimitation methods' assumption of no introgression, therefore, the mitochondrial data could not be used in species tree estimates. Markov chain performance for species trees was verified using Tracer (to ensure ESS values above 200, appropriate level of burn-in, and chain convergence). This required combining multiple *BEAST runs with the same parameters, for a final completed run of over 2 billion generations.

There are insufficient fossil data to calibrate the tree near the crown clades of Pteropodidae. Instead, the species tree was calibrated using secondary calibrations from Almeida et al. (2014), a study which used *cyt-b* (the only gene with a substitution rate estimated from fossil splits of *Myotis nattereri* and *M. schaubi* at 6 mya and *M. daubentonii* and *M. bechsteinii* at 5 mya) under a relaxed clock model implemented in BEAST to estimate the divergence times for *Pteropus* clades. The divergence times used for the present study were the *Acerodon-Pteropus* split ($\mu = 8.01$ mya, $\sigma = 1.2$) and two local calibrations that corresponded to clades found in earlier Bayesian runs (*P. gilliardorum-P. woodfordi*, $\mu = 0.93$ mya, $\sigma = 0.1$; and a "widespread species" clade consisting mostly of the *vampyrus* species group, $\mu = 4.44$ mya, $\sigma = 0.2$). These splits were chosen since they were the most consistently recovered nodes from all tree reconstruction methods.

Discordance between markers was examined in PhyloNet (Yu et al., 2012, 2014a) to determine whether incomplete lineage sorting or hybridization was to blame for conflicting signal. PhyloNet uses established methods for minimizing deep coalescence (Maddison & Knowles, 2006) by using gene trees along with sequence data to test for both incomplete lineage sorting and hybridization in reticulate phylogenetic networks. I tested for discordance in two separate species trees: one estimated from the nuclear data only and one from the combined nuclear and mitochondrial data.

Species limits were defined following the Metapopulation Lineage Species Concept (MLSC, de Queiroz, 2005), which states that each independently evolving metapopulation lineage is a species. Almeida et al. (2014) suggests that there are potential incomplete lineage sorting and hybridization issues, meaning that reciprocal monophyly may not be the best indicator of species limits. The MLSC recognizes that sister species may be on a continuum of differentiation and may not have all the characteristics that other species concept require to delimit species. This species concept recognizes the potential for incomplete lineage sorting to occur and does not rely on monophyly as a criterion for defining species, making it compatible with species tree methods (Edwards, 2009). Species designations were validated using Bayesian species delimitation methods implemented in BPP 2.2 (Yang & Rannala, 2010) and Brownie (O'Meara, 2009). Additional morphological, biogeographic, or behavioral information was taken into account when considering what are the diagnosable characters to consider something a full species. Each of these two analyses was conducted three times with different starting seeds to confirm results. BPP 2.2 was run for 1,000,000 generations with a sampling frequency of 5 discarding the first 25,000 generations as burn-in. The *BEAST species tree was used as the guide tree and species delimitation was set to 1 (rjMCMC species delimitation). A rjMCMC analysis treats the mixture components as a model parameter instead of assuming they are fixed. The algorithm was set to 0, and with a fine-tuning parameter (ε) of 10 since low ε values may result in poor mixing (*e.g.*, cannot move between models) in large datasets. Species distinctiveness was further confirmed based on available morphological measurements and qualitative external characteristics. For the Brownie analysis input, gene trees were generated from the MrBayes runs and converted to ultrametric trees using the R package *ape*, for input into Brownie. A heuristic search was run for 100,000 generations. This method assumes that the

topology of the most probable gene tree agrees with the species tree and aims to minimize intraspecific genetic discordance, and its results must be treated cautiously, since Bayesian and ML analyses identified conflicting signals among gene trees.

Of all pteropodid genera, *Pteropus* exhibits unusually high species diversity, especially in Wallacea and the South Pacific (Corbet & Hill, 1992; Flannery, 1995). Despite being species rich, *Pteropus* species exhibit relatively low morphological variability at the genus level, leading to a prediction of a higher rate of diversification than expected. To test this hypothesis, I implemented diversification analyses in BAMM (Rabosky et al., 2014), a rjMCMC parameter estimation method to detect branch-specific diversification rate shifts. Starting parameters were estimated using the R package BAMMtools. Additional specimens of both ingroup and outgroup species were included to reach a minimum of seventy species required to increase inferential accuracy. The results of BAMM analyses of the 70-taxon tree were compared to the *BEAST species tree; they were topologically similar. Therefore, the ranked results presented here the four most credible rate shift scenarios generated by analyzing the *BEAST species tree. Analyses of trait evolution were not implemented in BAMM since morphological data for some species represented by loaned tissue samples were not available. The analysis was simulated for 10 million generations with a sampling frequency of 10,000, discarding the first 10% as burn-in. Multiple rate shift configurations were compared using BAMMtools to calculate posterior odds ratios and rank all potential models by posterior probabilities. Comparisons of the null model (no rate shift) to alternative models of rate shifts were compared using Bayes factors.

Results and Discussion

Topologies of the Bayesian and ML inferred gene trees (Appendix 2.2) generally agreed with one another, along with the reconstructed species tree (Fig. 2.2), with a few notable exceptions

that are discussed in greater detail in the species-level findings section below. All putative species were verified as separate species by BPP 2.2 except for *P. yapensis* and *P. ennisae* (discussed further below). Concatenated datasets methods were unable to provide rigorous support for some of these contentious nodes, likely due to the conflicting phylogenetic signals and/or low genetic variation. Notable differences in topology for each species group are discussed below in separate sections below. Nuclear markers generally agreed with one another. However, mitochondrial data produced a substantially different topology from any nuclear gene (Fig. 2.3). Nucleotide diversity of nuclear genes was generally low, and this faint phylogenetic signal was unable to resolve a completely bifurcating tree; several polytomies remain. There was little mitochondrial genetic variability within species, but considerable distance between species. There was an order of magnitude of difference in the nucleotide diversity of the nuclear and mitochondrial genes (Table 2.4). Divergence time estimates in the species tree had low support and the 95% range of credible time estimates for each node were generally overlapping. The relatively recent, late Miocene age of the genus (Almeida et al., 2014), its subsequent rapid radiation, lack of fossil calibrations, and low genetic variability complicate efforts to estimate a time-calibrated phylogeny. Divergence time estimates should therefore be interpreted with caution.

Table 2.4. Nucleotide diversity (π) of nuclear and mitochondrial genes.

Gene	π
mitochondrial	
cyt-b	0.09148
D-loop	0.41537
nuclear	
RAG-1	0.01156
RAG-2	0.01227
STAT5A	0.02984
PLCB4	0.01393
BDNF	0.00353
FGB7	0.01608
PSMB8	0.01469
COPS7A4	0.01921
ATP7A	0.00740

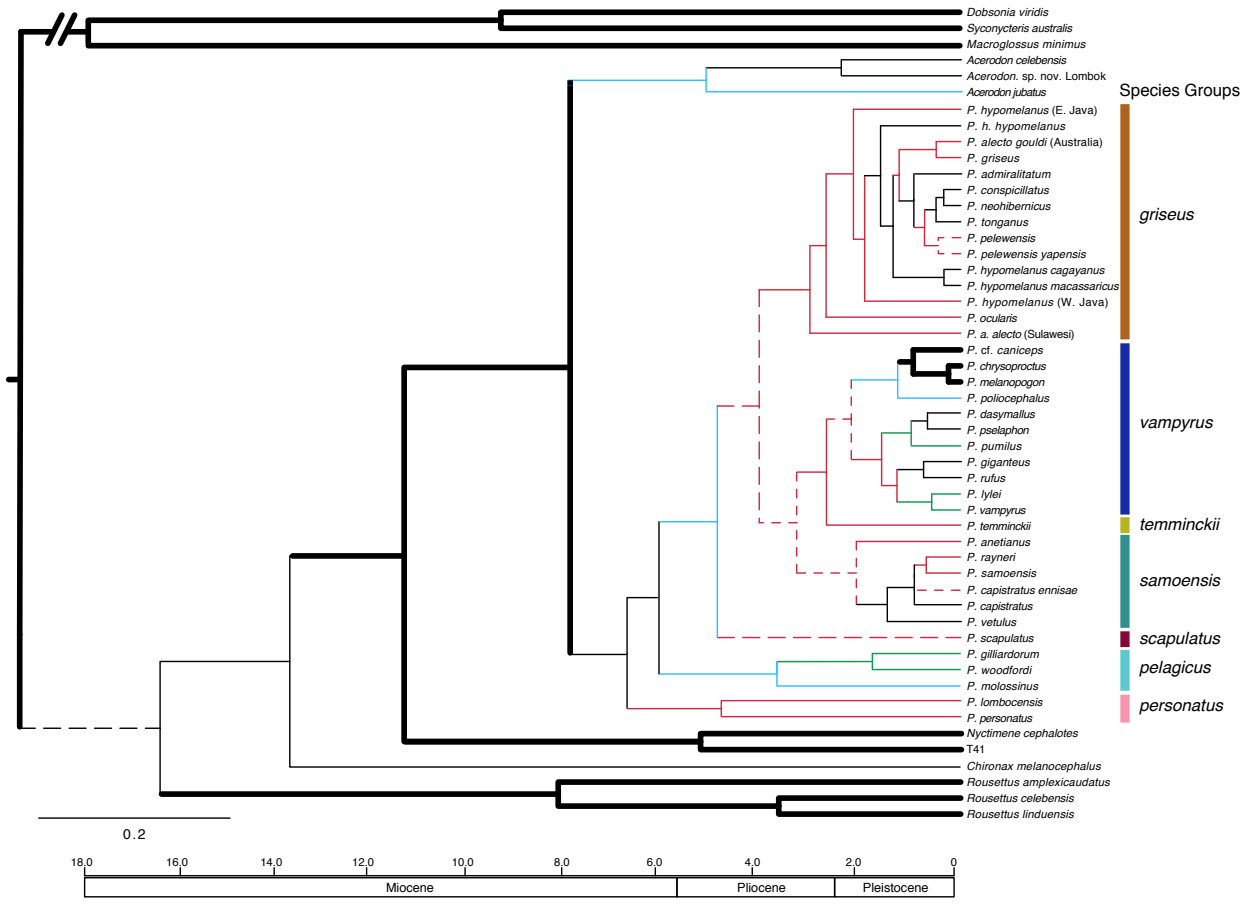
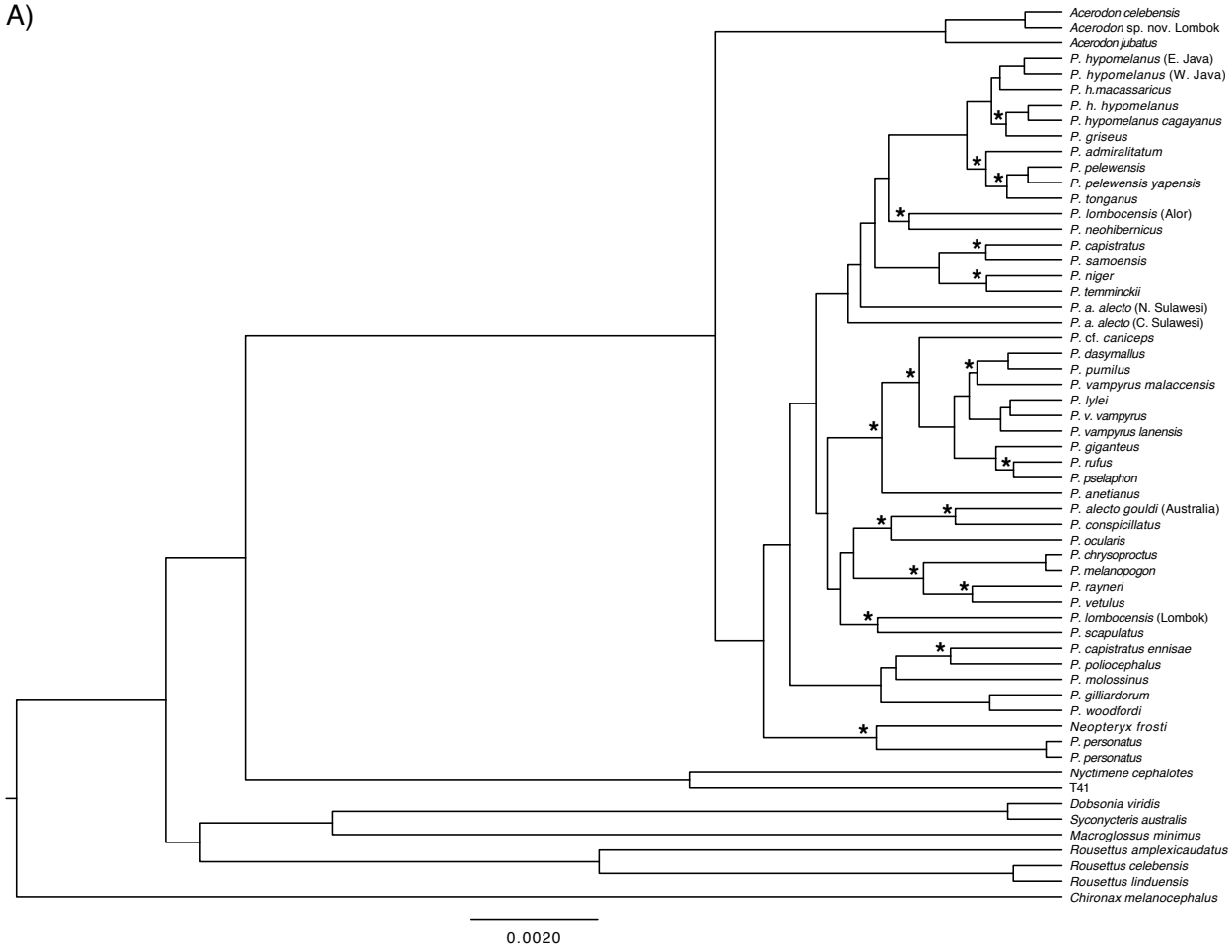


Figure 2.2. Species tree of the genus *Pteropus* reconstructed using BEAST2, simulated for 2 billion generations with 25% burn-in. Species groups are listed on the right. Thick black lines represent well-supported nodes from all analyses (posterior probabilities ≥ 0.9 from BEAST and MrBayes analyses, bootstrap values ≥ 70 from RAxML and TNT analyses). Thin black lines represent nodes well supported by the species tree and Bayesian analyses only. Green lines represent nodes well supported by the species tree, Bayesian, and ML analyses only. Blue lines represent nodes well supported by the species tree, Bayesian, and MP analyses only. Red lines represent nodes well supported by the species tree only. Dashed black lines represent nodes that are well supported by ML and MP analyses only. Dashed red lines represent nodes that have low support values from all analyses. Timescale should be used with caution, as divergence estimates were based on secondary calibrations and were not well supported by the BEAST analysis.

A)


Acerodon celebensis
Acerodon sp. nov. Lombok
Acerodon jubatus
P. hypomelanus (E. Java)
P. hypomelanus (W. Java)
P. h.macassaricus
P. h. hypomelanus
P. hypomelanus cagayanus
P. griseus
P. admiralitatum
P. pelewensis
P. pelewensis yapensis
P. tonganus
P. lombocensis (Alor)
P. neohibernicus
P. capistratus
P. samoensis
P. niger
P. temminckii
P. a. alecto (N. Sulawesi)
P. a. alecto (C. Sulawesi)
P. cf. caniceps
P. dasymallus
P. pumilus
P. vampyrus malaccensis
P. lylei
P. v. vampyrus
P. vampyrus lanensis
P. giganteus
P. rufus
P. pselaphon
P. anetianus
P. alecto gouldi (Australia)
P. conspicillatus
P. ocularis
P. chrysoproctus
P. melanopogon
P. rayneri
P. vetulus
P. lombocensis (Lombok)
P. scapulatus
P. capistratus ennisae
P. poliocephalus
P. molossinus
P. gilliardorum
P. woodfordi
Neopteryx frosti
P. personatus
P. personatus
Nyctimene cephalotes
T41
Dobsonia viridis
Syconycteris australis
Macroglossus minimus
Rousettus amplexicaudatus
Rousettus celebensis
Rousettus linduensis
Chironax melanocephalus
0.0020

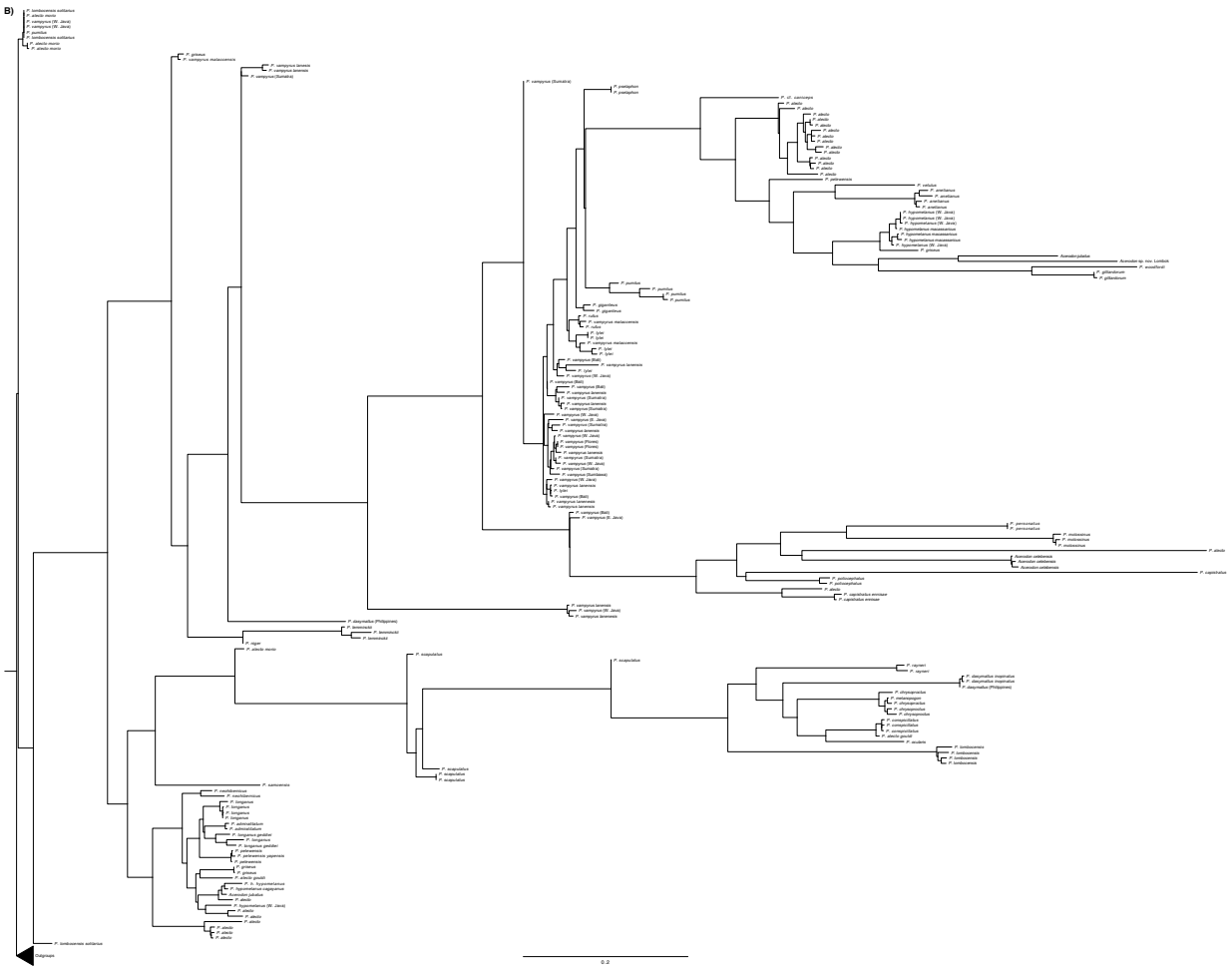


Figure 2.3 Different topologies produced from mitochondrial data using different reconstruction methods. A) Species tree from BEAST2 including mitochondrial data. Asterisks mark significant departures from nuclear data only species tree. B) Mitochondrial data only tree inferred from RAxML. Outgroups were condensed due to high level of divergence. Mitochondrial gene trees from MrBayes and TNT were similar and omitted for clarity. Gene trees had low intraspecific variability with long branches between species, resulting in the *vampyrus* species group taxa collapsing into a large polytomy in both the Bayesian and MP analyses. Paraphyly in gene trees often correspond to where there were significant departures in the species tree with mitochondrial data.

Discordant signals between the mitochondrial and nuclear data may be caused by hybridization or incomplete lineage sorting. The greater number of changes in the mitochondrial data can overwhelm the nuclear signal, thereby biasing the topology of the species tree that utilized both types of molecular data. Based on the PhyloNet analysis, all genes had evidence of incomplete lineage sorting, however, different populations of Australian *Pteropus* may also have hybridized recently. This will be tested more rigorously in Chapter 5. The young age of *Pteropus* (*e.g.* shallow divergences) and colonial nature of most species likely resulted in large ancestral effective population sizes, which increases the likelihood of incomplete lineage sorting. Species designations were generally supported by BPP analyses, though Brownie was unable to differentiate conventionally recognized species: *Pteropus* was divided into just three major groups (*vampyrus*, *samoensis*, and *griseus*). Brownie works by attempting to minimize intraspecific genetic structure, which might explain its poor resolving power with this dataset. Markers analyzed in this study had such a low degree of variation that further minimizing genetic structure resulted in loss of signal.

Species-level findings

By increasing the number of species sampled, the species group organization of *Pteropus* results in far fewer species groups than suggested by Almeida et al. (2014), where 7 out of 13 species groups were monotypic. Species groups in the present study were monophyletic groupings designated using unifying morphological, ecological, and behavioral characteristics, with only a small handful of exceptions in large species groups. These species groups are: *personatus*, *pelagicus*, *scapulatus*, *vampyrus*, *temminckii*, *griseus*, and *samoensis* (Table 2.1). Each of these groups is named for the oldest species name in each group. Support values reported in the text are the posterior probabilities from the species tree only, see Fig. 2.2 for more

details on nodal support from other methods. External morphological measurements used for species identification from newly captured specimens are reported in Table 2.5.

The personatus *species group, a Wallacean clade*

Two medium-sized *Pteropus* form an early-diverging clade: *P. personatus* and *P. lombocensis*. The nuclear tree indicates that the two species are deeply divergent sister taxa (BPP = 0.78). *P. lombocensis* individuals from different islands in the Lesser Sundas form mildly substructured monophyletic populations according to the Bayesian tree with all individuals (BPP = 1). The mitochondrial data place *P. lombocensis* in a different position. While incomplete lineage sorting is usually weaker in mitochondrial DNA than nuclear DNA (Rosenberg, 2002), the PhyloNet analysis supported a model indicating incomplete lineage sorting, not hybridization. The distribution of *P. lombocensis* overlaps with both *P. vampyrus* and *P. alecto*, though its dissimilarity in both size (smaller) and behavior (not found in gregarious colonies) from either one of these species makes hybridization unlikely, but not impossible.

Table 2.5. Measurements of external characteristics for specimens used in this study. Weight is in grams, all other measurements are in millimeters. Blank cells are missing measurements if field conditions prevented taking them. Field numbers are used since museum catalog numbers are not yet available.

ID	Species	Age/Sex	WT	FA	HB	TAIL	EAR	TIB	HF
SS002	*Pteropus alecto*	A ♂	590	165	230	-	31.09	77.57	13.46
SS004	*Pteropus alecto*	A ♀	580	165	240	-	33.99	75.95	48.07
SS025	*Macroglossus minimus*	A ♂	12	39	64	-	15.08	15.90	11.13
SS026	*Rousettus celebensis*	A ♂	66	76	106	25.2	18.12	34.28	19.65
SS027	*Chironax melanocephalus*	A ♂	15	47	72	-	12.26	16.33	10.58
SS028	*Rousettus linduensis*	A ♂	81	77	120	31.2	19.11	35.09	20.06
SS029	*Macroglossus minimus*	A ♂	14	41	64	-	15.48	15.96	10.91
SS030	*Acerodon celebensis*	A ♂	400	143	225	-	33.34	58.90	43.49
SS031	*Acerodon celebensis*	A ♀	400	133	205	-	30.95	58.15	37.84
SS032	*Acerodon celebensis*	A ♀	370	137	200	-	30.2	57.86	39.30
SS033	*Pteropus alecto*	A ♀	610	174	250	-	29.93	78.37	48.28
SS034	*Pteropus alecto*	A ♂	580	161	235	-	32.58	77.28	48.23

SS035	*Pteropus alecto*	♀	300	138	195	-	29.08	61.60	43.03
SS036	*Pteropus alecto*	♂	310	138	208	-	30.01	63.93	48.98
SS037	*Pteropus alecto*	A ♂	390	147	210	-	28.44	69.10	50.16
SS038	*Pteropus alecto*	♂	310	142	200	-	28.35	61.41	47.10
SS039	*Pteropus alecto*	♀	340	142	180	-	26.03	61.71	41.70
SS040	*Pteropus alecto*	A ♀	490	160	222	-	27.59	69.54	45.80
SS041	*Pteropus alecto*	A ♀	505	160	235	-	30.91	70.02	44.60
SS049	*Pteropus alecto*	A ♀	380	155	225	-	29.8	71.51	46.51
SS050	*Pteropus alecto*	A ♀	450	172	215	-	30.56	78.29	48.62
SS051	*Pteropus hypomelanus*	A ♂	210	123	190	-	25.18	56.08	37.98
SS052	*Pteropus hypomelanus*	A ♀	300	135	190	-	26.61	59.94	40.03
SS053	*Pteropus hypomelanus*	A ♂	310	138	200	-	27.15	61.93	43.27
SS054	*Pteropus alecto*	A ♂	610	175	265	-	33.11	79.26	52.07
SS057	*Pteropus alecto*	A ♀	500	156	230	-	28.36	73.03	46.51
SS064	*Nyctimene cephalotes*	A ♂	36	67	99	25.6	15.36	24.18	14.12
SS065	*Nyctimene cephalotes*	A ♂	40	65	108	22.6	15.15	23.13	14.78
SS066	*Syconycteris australis*	A ♀	19	47	74	-	14.51	16.29	11.57
SS067	*Syconycteris australis*	A ♀	22	48	80	-	16.26	18.18	12.58
SS068	*Dobsonia viridis*	A ♂	160	109	155	23.0	24.05	48.23	24.38
SS069	*Macroglossus minimus*	A ♀	12.5	40	66	-	12.18	17.02	9.92
SS070	*Dobsonia viridis*	A ♂	180	110	145	27.0	25.48	51.04	24.72
SW001	*Pteropus vampyrus*	A ♂	1340	251	315	-	39.41	101.73	61.68
SW002	*Pteropus vampyrus*	A ♂	1343	250	350	-	41.01	108.92	63.03
SW003	*Pteropus vampyrus*	Juv ♂	300	116	160	-	32.25	54.10	52.50
SW006	*Pteropus vampyrus*	A ♂	1380	215	278	-	39.39	100.02	66.72
SW007	*Pteropus vampyrus*	A ♀		225					
SW008	*Pteropus vampyrus*	A ♂		220					
SW009	*Pteropus vampyrus*	A ♀		195					
SW010	*Pteropus vampyrus*	A ♂		210					
SW011	*Pteropus vampyrus*	A ♀		215					
SW013	*Pteropus hypomelanus*	A ♂	550	165	240	-	29.40	77.90	50.36
SW014	*Pteropus hypomelanus*	A ♂	500	165	240	-	29.03	84.54	50.48
SW077	*Pteropus vampyrus*	A ♂	700	188	245	-	41.22	96.42	68.11
SW078	*Pteropus vampyrus*	A ♂	1300	210	273	-	36.78	104.00	63.55
SW105	*Pteropus chrysoproctus*	♀	290	138	190	-	27.08	62.39	48.73
SW106	*Pteropus chrysoproctus*	A ♀	780	180	265	-	30.81	79.91	52.00
SW107	*Pteropus chrysoproctus*	♀	380	150	210	-	32.09	66.14	49.26
SW108	*Pteropus chrysoproctus*	♂	420	144	220	-	30.91	65.72	52.66
SW120	*Pteropus temminckii*	A ♀	200	106	155	-	22.71	47.10	32.38
SW121	*Pteropus melanopogon*	Juv ♂	150	91	146	-	23.71	38.31	45.33
SW123	*Pteropus temminckii*	A ♂	150	97	160	-	23.92	45.59	30.09
SW124	*Pteropus temminckii*	A ♀	160	102	140	-	22.88	44.86	31.51

SW125	*Rousettus amplexicaudatus*	SA ♂	48	77	112	24.2	20.18	33.19	
SW126	*Pteropus ocularis*	♂	400	144	195	-	26.29	60.89	44.38
SW127	*Pteropus vampyrus*	A ♀	630	182	240	-	43.41	87.78	51.91
SW128	*Pteropus vampyrus*	A ♂	950	186	275	-	40.37	90.23	56.68
SW131	*Pteropus vampyrus*	A ♀	880	198	290	-	42.26	97.37	56.32
SW132	*Pteropus vampyrus*	A ♂	680	195	265	-	42.04	98.04	59.17
SW133	*Pteropus vampyrus*	A ♂	560	172	225	-	40.25	84.36	57.50
SW134	*Pteropus hypomelanus*	A ♂	940	185	280	-	39.42	90.96	56.94
SW140	*Pteropus vampyrus*	A ♀	600	165	230	-	37.76	81.75	56.39
SW143	*Pteropus lombocensis*	A ♂	215	101	150	-	25.66	45.22	35.65
SW144	*Pteropus lombocensis*	A ♀	360	122	180	-	26.47	52.04	35.71
SW145	*Pteropus lombocensis*	A ♀	360	122	178	-	25.79	56.54	37.04
SW146	*Acerodon* sp. Lombok	A ♂	520	147	230	-	35.35	64.80	50.28
SMT207	*Acerodon jubatus*	Juv ♀	145	109	149	-	23.30	31.40	11.50
SMT214	*Pteropus pumilus*	SA ♂	110	113	152	-	16.27	35.49	10.46
JBS111	*Acerodon jubatus*	A ♂	1160	210					
MJV418	*Acerodon jubatus*	A ♀	1060	202					
MJV419	*Pteropus* cf. *hypomelanus*	A ♂	580	170					
MJV420	*Pteropus vampyrus*	A ♂	860	192					
MJV435	*Pteropus vampyrus*	A ♀	960	189					
MJV436	*Pteropus vampyrus*	A ♀	1000	192					
MJV451	*Pteropus dasymallus*	SA ♀	-	125					
MJV458	*Pteropus dasymallus*	SA ♂	210	120					
MJV504	*Pteropus* cf. *vampyrus*	A ♂	856	193					
MJV505	*Pteropus* cf. *vampyrus*	A ♀	456	172					

Two of the *P. personatus* specimens (T24 and T26) were sister to one another (BPP = 1) and formed a basal clade with *P. lombocensis* within *Pteropus*. However, a third individual identified as *P. personatus* (T41) was found to be sister to one of the outgroup taxa, *Nyctimene cephalotes* (BPP = 1) (*e.g.*, it is sister to the genus, not to that species explicitly). All of these individuals were captured on Ternate, North Maluku, Indonesia and identified as *P. personatus* initially by S. Wiantoro. Polyphyly of *P. personatus* would explain why previous research has suggested *P. personatus* may not be a *Pteropus* species (Almeida, 2014)—it may be that the original description of *P. personatus* is a true *Pteropus*, but a similar, sympatric non-*Pteropus* species is also recorded from here and often confused as *P. personatus*. By providing evidence

that *P. personatus* is a true *Pteropus*, assignment of the name to the species group is appropriate as it is the oldest taxonomic name.

The pelagicus *species group, an early-diverging South Pacific clade*

A strongly supported basal radiation in the South Pacific is reconstructed in the species tree comprising (*P. molossinus* (*P. gilliardorum*, *P. woodfordi*) (BPP = 1). Following the data available from Almeida et al. (2014), *P. macrotis*, *P. pelagicus*, *P. tokudae*, and *P. mahaganus* all also belong in this clade, though *P. tokudae* and *P. pelagicus* are not strongly supported. *Pteropus pelagicus* is the oldest name. This radiation shares morphological and dietary similarities (narrower skulls and smaller teeth often found in nectar and pollen feeding species) (Flannery, 1995; Buden et al., 2013), rather distinct compared to most other *Pteropus* which subsist primarily on fruit. Data from a greater number of specimens would be necessary for determining the relationships of each of these lineages in a biogeographic context.

The scapulatus *species group, a unique Australian lineage*

Pteropus scapulatus is the only Australian lineage found in an early diverging position in the *Pteropus* tree (BPP = 0.61), though it is weakly supported. *Pteropus scapulatus* is morphologically distinct from other Australian *Pteropus*. These bats have narrower skulls and are much smaller than other Australian *Pteropus*, which fits with their predominantly nectar-based diet (Churchill, 2008). No previous research suggests that *P. scapulatus* hybridize with the other three common Australian species (discussed in greater detail below), despite being sympatric (Sinclair et al., 1996; Vardon & Tidemann, 1999). However, the PhyloNet analysis indicates there may be incomplete lineages sorting between *P. scapulatus*, *P. lombocensis*, and *P. rayneri*. These three species are not sympatric, but they do share a nectarivorous diet and *P. lombocensis* and *P. rayneri* are distributed in areas adjacent to the range of *P. scapulatus*. More

fine-scale data and specimens of *P. rayneri* will be needed to resolve the evolutionary dynamics and taxonomic position of *P. scapulatus*.

The vampyrus *species group, a "widespread" species clade*

The *vampyrus* species group forms a clade of species that are generally large-bodied, fly long distances, and form large colonial aggregates (Corbet & Hill, 1992; Jones & Kunz, 2000; Francis, 2008). A polytomy of ((*P. vampyrus, P. lylei*), (*P. giganteus*[1], *P. rufus*), (*P. pumilus* (*P. dasymallus*, *P. pselaphon*)) is sister to *P. niger*. The position of *P. niger*, which is poorly supported (BPP = 0.52), may be a result of a great deal of missing nuclear data due to the poor quality of the sample. The final species tree in Fig. 2.2 is presented without *P. niger*, since the missing data significantly affected posterior probabilities in the *vampyrus* species group. Taking into account only strongly supported relationships, there are two clades in the *vampyrus* species group, one that includes all species in the group on and west of Sundaland and a second clade that diverged eastward, including the Philippines and Micronesia.

The species tree analysis also corroborates the position of *P. pselaphon* and *P. dasymallus* in the *vampyrus* species group using the full nuclear data. However, in Almeida et al. (2014), *P. pselaphon* was sister to all other *vampyrus* species group members, which may have been an artifact in their study of using only fragmentary mitochondrial data totaling 381bp, which I resolve here by using a fuller dataset and species tree methods. Instead, *P. pselaphon* was found to be sister to *P. dasymallus*, with the relationship (*P. pumilus* (*P. dasymallus*, *P. pselaphon*). It should be noted that our specimens of *P. dasymallus* were from the Batañes, a

[1] This study maintains the name *P. giganteus* instead of *P. medius* as suggested by Mlikovsky (2012), as *P. giganteus* is the more commonly recognized name and nomenclatural stability is needed for preserving conservation gains in the countries of origin for this species (India, Pakistan, Bangladesh, Bhutan, Nepal, Myanmar, Sri Lanka, and Maldives), as changing names may addle application of environmental laws.

Philippine archipelago north of Luzon, not Japan, so it is not simply a case of the Japanese species forming their own radiation. The biogeography of this clade will be revisited in Chapter 3, as they form a monophyletic clade in the northeastern edge of the genus' distribution.

The two large-bodied Moluccan species, *P. melanopogon* and *P. chrysoproctus*, are sister species. They form a Moluccan radiation with *P.* cf. *caniceps* (see *griseus* group for discussion of M64 specimen). Both of these species previously were classified by Andersen (1912) in separate species groups: *P. melanopogon* was classified under the *livingstonii* species group and *P. chrysoproctus* was classified under the *chrysoproctus* species group. *Pteropus aruensis* and *P. keyensis* likely belong to this radiation as well, as they are part of the *P. melanopogon* species complex. Previously recognized similarities between *P. chrysoproctus* and *P. argentatus* would also result in classification of *P. argentatus* under this radiation.

The species tree analysis suggest that the traditionally recognized *vampyrus* species group is sister to a clade of (*P. poliocephalus* (*P. melanopogon*, *P. chrysoproctus*)) based on nuclear data alone. The phylogenetic position of *P. poliocephalus* was drastically different in the full dataset though—it nested with the *pelagicus* species group when mitochondrial data were included. Our increased sampling of nuclear data in the species tree does not support this basal position of *P. poliocephalus*—even from individual nuclear genes. *Pteropus poliocephalus* either clustered with the large Moluccan *Pteropus* species or all other *vampyrus* species. Almeida et al. (2014) found a similarly discordant position for *P. poliocephalus* based on mitochondrial as compared to nuclear data and suggested that this is due to hybridization rather than lineage sorting. *Pteropus poliocephalus* is known to hybridize with the *P. alecto* (Webb & Tidemann, 1995). Putative hybridization between *P. poliocephalus* and the sympatric and synchronously breeding *P. conspicillatus* has also been suggested through observed mating, though the viability

of hybrids is unknown (Parsons et al., 2010). Both *P. alecto* and *P. conspicillatus* are part of the *griseus* species group and are not closely related to *P. poliocephalus*.

Despite not having many of the species available for inclusion in this dataset, previously published data from O'Brien et al. (2009) suggest that *P. seychellensis*, *P. aldabrensis*, and *P. rodricensis* will be included in this species group. Almeida et al. (2014) suggested that a *P. livingstonii* + *P. voeltzkowi* clade forms its own species group. Given what is known about the natural history of other members of the *vampyrus* species group, minor genetic differences are not enough to warrant a separate species group, following the earlier criteria set in this study for common morphological, ecological, and behavioral traits being used for species group classifications. Its life history and morphological traits are similar to many of the other *vampyrus* species group members (large-bodied, colonial, frugivores, robust skulls) (Andersen, 1912; Gerlach, 2004), and this Indian Ocean radiation is similar to the Moluccan radiation above in that it is distinct from the main Sundaic radiation, but is still a part of the *vampyrus* species group. Presumably, the extinct Mauritian *P. subniger* would also have belonged to the *vampyrus* species group, though its teeth are smaller and narrower (Andersen, 1912), suggesting a nectarivorous diet. The unusual cranial morphology (skull shape, dentition, ear shape, and size) of *P. subniger* and *P. rodricensis* suggest a sister-species relationship as compared to other African *vampyrus* species group members. Andersen (1912) lists *P. rodricensis* in the same species group as *P. molossinus* and remarks on the similarity of the delicate dentition of *P. subniger* to that of the nectarivorous *P. molossinus*, but did not recognize the similarities between *P. rodricensis* and *P. subniger*. The precise relationship of each of the Indian Ocean radiations within the *vampyrus* species group are unknown until the inclusion of more Indian Ocean *Pteropus* is possible.

Inclusion of *P. subniger* would necessitate the use of high-throughput sequencing methods, as the species is only represented by nineteenth-century specimens in a few European collections.

The *vampyrus* species group is both the most species rich and geographically diverse clade in *Pteropus*. Where *Pteropus* are distributed, there is at least one member of the *vampyrus* species group, with most regions supporting a small radiation. Given the high dispersal capability inherent in this species group, its presence in many of the islands in the Paleotropics is predicted. However, few natural history data are available for these species, so few inferences about speciation mechanisms can be made. More ecological information about diet selection, roosting habitat, or mating behaviors may help to understand these divergences in the future.

The temminckii *species group, a unique lineage*

Pteropus temminckii is sister to the *vampyrus* species group radiation (BPP = 0.71). The nuclear and mitochondrial signals were discordant for this species. The nuclear loci generally agreeed with one another on the position of temminckii as sister to the vampyrus species group. However, the mitochondrial data indicated a significantly different phylogenetic position—either sister to other Moluccan *Pteropus* (*cyt-b*) or nested within the *hypomelanus* species group (D-loop). The morphological features of *P. temminckii* are significantly different from those of the *vampyrus* species group—they are smaller, the skull is proportionately shorter and less robust, and the fur is dense and colored uniformly tan-blond across the body (Andersen, 1912; Corbet & Hill, 1992; Flannery, 1995). *Pteropus temminckii* generally occurrs as solitary roosters in a variety of forest habitats, and has never been encountered as colonial aggregates (*pers. obs.*) as one would with most species in the *vampyrus* group.

The griseus *species group, an island species group*

At the base of the *griseus* species group is *P. ocularis*, a rare Central Moluccan species. Andersen (1912) had previously categorized *P. ocularis* within a *conspicillatus* species group as a smaller cousin, as the light rings around its eyes superficially resembled the rings around the eyes of *P. conspicillatus*. However, the *P. conspicillatus* specimen analyzed in this study was strongly supported to be sister to *P. neohibernicus* (BPP = 0.91) in the species tree, though additional individuals would allow for more rigorous tests of introgression. A closer relationship between *P. ocularis* and *P. conspicillatus* is still a possibility though since the PhyloNet analysis weakly supported the possibility of hybridization between *P. alecto* and other members of the *griseus* species group (*P. griseus* and *P. conspicillatus*). Previous research has shown that *P. conspicillatus* and *P. alecto* can produce viable hybrid offspring (Fox, 2006). If the individual used in this study was the descendent of a lineage with a history of introgression with *P. alecto*, then the position of *P. conspicillatus* will likely shift in the tree.

There was also clear evidence of introgression at some level between the Lesser Sundaic and Australian populations of *P. alecto* and other members of the *griseus* species group from *P. alecto morio* and *P. alecto gouldi* populations in the Lesser Sundas and Australia, respectively (on the species tree as *P. alecto gouldi*). The Sulawesi *P. alecto alecto* all form their own monophyletic clade sister to all other *griseus* group species. A more comprehensive look at *P. alecto* population genetics, along with potential lineage sorting or introgression issues in this clade, will be discussed in Chapter 5. Given that the Sulawesi populations represent the nominotypical form and are distinctly separate (*e.g.*, not hybridizing) from other *Pteropus* species, its phylogenetic position in the species tree should be where *P. alecto* is considered to be located within the genus. There is also a monophyletic clade of *P. tonganus*, though a clear divide between the populations on Samoa and Vanuatu exists according to the MrBayes trees.

Further study of the biogeographic dynamics between the Samoa, Fiji, and Vanuatu populations would be possible within *P. tonganus* had specimens from Fiji been available.

In the species tree, *P. pelewensis* and *P. yapensis* were sister taxa, but with a weak posterior probability (BPP = 0.51). BPP species delimitation could not verify these two should be treated as separate species and primarily mitochondrial differences underlie their bifurcation. Both of these species were previously considered subspecies of *P. mariannus*, and were recognized as full species in the most recent edition of *Mammal Species of the World* (Simmons, 2005) following Flannery (1995), which lacked an explanation for this designation. However, the two taxa are incredibly similar, and pelage coloration does not vary greatly between the two species (both have brown undersides, with yellowish necks and mantles). Only the average size of the species differs: mean forearm length in male *P. pelewensis* is 114.5 mm whereas *P. yapensis* is 130 mm (Flannery, 1995) and condylobasal length averages 53.4 mm and 57.3 mm, respectively (Almeida et al., 2014). It is possible that these two species diverged so recently that few markers are reciprocally monophyletic (Knowles & Maddison, 2002). In another recent study of populations of the closely related *P. mariannus* from Palau, Guam, Rota, and the Mariana Islands (Brown et al., 2011) suggests that gene flow between islands exists, which is also possibly in the case of *P. pelewensis* and *P. yapensis*. These two species should be treated as conspecific under the name *P. pelewensis*, as suggested by Almeida et al. (2014), pending additional population studies. Inclusion of samples of *P. mariannus* and *P. ualanus* would be needed for a more complete understanding of Micronesian flying foxes and biogeography.

The *P. hypomelanus* species complex was represented by 4 of 17 recognized subspecies (sensu Corbet and Hill 1992), along with a few other newly discovered populations: *P. h. hypomelanus* (from the type locality, Ternate), *P. h. macassaricus* (from Sangir), *P. h.*

cagayanus (from the Visayas), an unnamed *P. hypomelanus* population from Pulau Panjang (north of West Java), and an unnamed *P. hypomelanus* population from the island of Madura (north of East Java). While a majority of all subspecies fell into a clade with *P. alecto*, *P. griseus*, *P. tonganus*, *P. pelewensis*, and *P. neohibernicus* (all members of the *griseus* species group, sensu Almeida et al. 2014), one of the individuals representing the nominotypical form (P. h. hypomelanus) from Ternate (M64) was found to be nested within the *vampyrus* species clade instead (originally labeled as "*P.* cf. *hypomelanus*"). The second individual from Ternate (M50), originally labeled "*Pteropus* sp. Ternate," clustered with all other *P. hypomelanus* individuals. Sigit Wiantoro originally captured these two individuals (M50 and M64) in 2011 on the same expedition. The confusion in species identity suggests that some of the "*P. h. hypomelanus"* in North Maluku may be a cryptic species or actually represent the sympatric *P. caniceps*. Given the position of M50 on the species tree, this specimen is considered here as a representative individual of the true *P. h. hypomelanus* (BPP = 1). The M64 specimen, which appeared as sister to the Moluccan radiation in the *vampyrus* species group, is likely an individual of the sympatric *P. caniceps*, a North Moluccan species that is almost indistinguishable from larger forms of *P. hypomelanus* externally (Flannery, 1995), and not a new cryptic species. There are slight morphological variations in the skull and teeth noted by Andersen (1912), such as larger orbits and broader palatal ridge and rostrum, that suggest that *P. caniceps* belonged to the *vampyrus* species group, but no molecular data were previously available given the dearth of specimens from Maluku. Additional cranial measurements will be needed from this specimen to corroborate this assertion, but M64 is considered here to represent *P.* cf. *caniceps*.

Other members of the *griseus* group are nested among the *P. hypomelanus* subspecies as (*P. griseus* (*P. admiralitatum* (*P. pelewensis* (*P. tonganus* (*P. conspicillatus*, *P.*

neohibernicus))))). The position of the various subspecies of *P. hypomelanus* was well-supported by the species tree (BPP > 0.95), but poorly supported by the other analyses. Verification using BPP 2.2 species delimitation methods provided support for splitting *P. hypomelanus* subspecies into individual species. A revision of the *P. hypomelanus* species complex is needed using high-throughput sequencing data, as these species are rather closely related and may have diverged only within the last million years. It is unclear if and how either of the Javan subspecies is related to the various subspecies named from the Riau Islands, and will require inclusion of collection skins in future studies, as some of these populations are exceedingly rare or extinct in the wild. *P. h. cagayanus* was sister to the subspecies from Sangir (an archipelago north of Sulawesi), *P. h. macassaricus*. As this represents the northeastern extent of the range of *P. hypomelanus*, a stepping stone model of speciation would predict that these would form a singular clade representing constant gene flow from Wallacea to the Philippines, which is what the phylogeny suggests. Including *P. h. tomesi* could more rigorously test this hypothesis, a subspecies not represented in this dataset, from Mindanao, in the southern half of the Philippines.

The samoensis *species group, a second Pacific radiation*

A second radiation of Pacific species includes *P. anetianus* (Vanuatu), *P. vetulus* (New Caledonia), *P. capistratus* (Bismarck Archipelago), *P. rayneri* (Solomon Islands and Bougainville), and *P. samoensis* (American Samoa, Fiji, Samoa). The molecular data from Almeida et al. (2014) strongly support the inclusion of three Solomon Island endemics *P. rennelli*, *P. nitendiensis*, and *P. cognatus* in this species group as well. *Pteropus fundatus* (Vanuatu) and *P. tuberculatus* (Solomon Islands) were also nested weakly within this group (Almeida et al., 2014). There are morphological similarities that suggest the inclusion of the extinct species *P. brunneus* (Percy Island, Australia), *P. pilosus* (Palau), *P. coxi* (Samoa), and *P.*

allenorum (Samoa) in this species group (Helgen et al., 2009; Almeida et al., 2014), but the multiple colonization events of the Pacific islands already apparent from distantly related *griseus* group members means that this classification should be approached with caution. The species tree and subsequent validation approaches do not support treating *P. capistratus ennisae* as its own species as suggested by Almeida et al. (2014). The species tree had low posterior support (BPP = 0.56) at that node and BPP 2.2 could not verify that they were independently evolving lineages. The distinct *P. vetulus*, which has converged in some superficial ways with *Pteralopex* monkey-faced bats (Flannery, 1995), was recovered within this clade (BPP = 0.81). Since so many of the species in this clade are missing (8 of 13 missing) from the species-tree analysis, testing for biogeographic models of diversification among the Pacific Islands would not be possible at . Additionally, there is evidence of low posterior probabilities due to incomplete lineage sorting, likely due to shallow internodes with a large ancestral population.

New species

A pale pteropodid with a distinct, brown facial mask (similar to the mask of *P. capistratus*) was captured on Lombok (SW146, MZB 36977)—the first masked pteropodid ever found on Lombok—and is unquestionably a new species. It shares a great deal of morphological similarity with the enigmatic North Moluccan *P. personatus* but is unequivocally not sister to it, as it is nested well within the genus *Acerodon*. Using external characters, we identified it as belonging to Pteropodini (sensu Bergmans 1997) and cranial morphology indicates it is not a *Pteropus*. The squared-shaped M1 suggests that it is an *Acerodon* (as compared to a longer M1 that would be expected in *Pteropus*). The species tree clearly indicates that the individual falls outside of crown *Pteropus* and is nested within *Acerodon* outgroup species. It does not conform to the species description for *A. mackloti* (tan in color, forearm range of 139 to 145 mm,

Andersen, 1912), the Lesser Sundaic endemic, though further comparison to a series will be needed to confirm measurement comparisons. A formal species diagnosis for this specimen is in progress, and it is temporarily represented on this tree as "*Acerodon* sp. nov. Lombok" (Fig. 2.2).

The specimen T41 from Ternate, North Maluku, Indonesia was initially identified as *P. personatus* due to its external similarities (pale, with a brown mask), and superficially similar cranial features. The overall size, especially the diagnostic forearm size, of this pteropodid is slightly smaller than that of *P. personatus* (89.8 mm, compared to average 93.25 mm in *P. personatus*), a significant difference in mid-sized pteropodids. The cranial morphology must be re-examined before a formal species diagnosis is conducted for this specimen. The molecular data places this species outside of *Pteropus*, though more outgroup taxa are needed to verify its precise relationship to other pteropodids. It may be similar to other masked pteropodid species with narrow skulls, such as those belonging to the genus *Styoctenium*. It remains represented in the tree as "T41."

Relative divergence rates

Table 2.6. Bayes factors for models with *k* shifts relative to the null model of 0 shifts from BAMM analysis. Bolded is the best overall model as compared to the null model, a single rate shift.

Shifts	Bayes factor
0	1.0
1	**401.03233**
2	377.61007
3	247.58536
4	128.57026
5	65.66056

Multiple independent BAMM runs were conducted, and they converged on similar posterior distributions. Model comparisons using Bayes factor evidence is strongly in favor of a rate shift as compared to the null model of no rate shifts (Table 2.6). The BAMM analysis suggests that a single rate shift is the most credible scenario within the genus *Pteropus*. Not all *Pteropus* had an elevated rate of diversification though. An increase in diversification rate at the node joining

the *temminckii*, *vampyrus*, *samoensis*, and *griseus* species group radiations was the most credible rate shift configuration (Fig. 2.4a Rank 1, posterior = 0.43). The mean time-averaged clade-specific rate at this node was much higher (11.09159) than the background rate (3.689555). Notably, the second most likely model was also a single rate shift that included all of the above taxa and *P. scapulatus* (Fig. 2.4b, Rank 2, posterior = 0.29).

Gigantism in Pteropus

My species tree reveals that gigantism evolved either twice (independently in each of the *vampyrus* and *griseus* species groups) or once at the node joining the *griseus*, *temminckii*, *vampyrus*, and *samoensis* species groups (with a subsequent loss or relaxation of effect on body size in the *temminckii* and *samoensis* species groups). Selective pressures for gigantism changing over time would be assumed, as there are a few members of the *vampyrus* and *griseus* species groups that are medium-sized. Based on the species tree, the most parsimonious model would assume that gigantism evolved independently twice. When taken into consideration with home range size, it may be that there is little selective pressure for *temminckii* and *samoensis* species group members to evolve gigantism because their home ranges are restricted to rather small islands. Some of the largest members of the *vampyrus* and *griseus* species groups fly some of the greatest distances to forage when necessary (Palmer & Woinarski, 1999; Palmer et al., 2000; Epstein et al., 2009). These larger home range sizes may not be necessary on smaller islands, since there are fewer competitors for the same resources (Wilson & Graham, 1992).

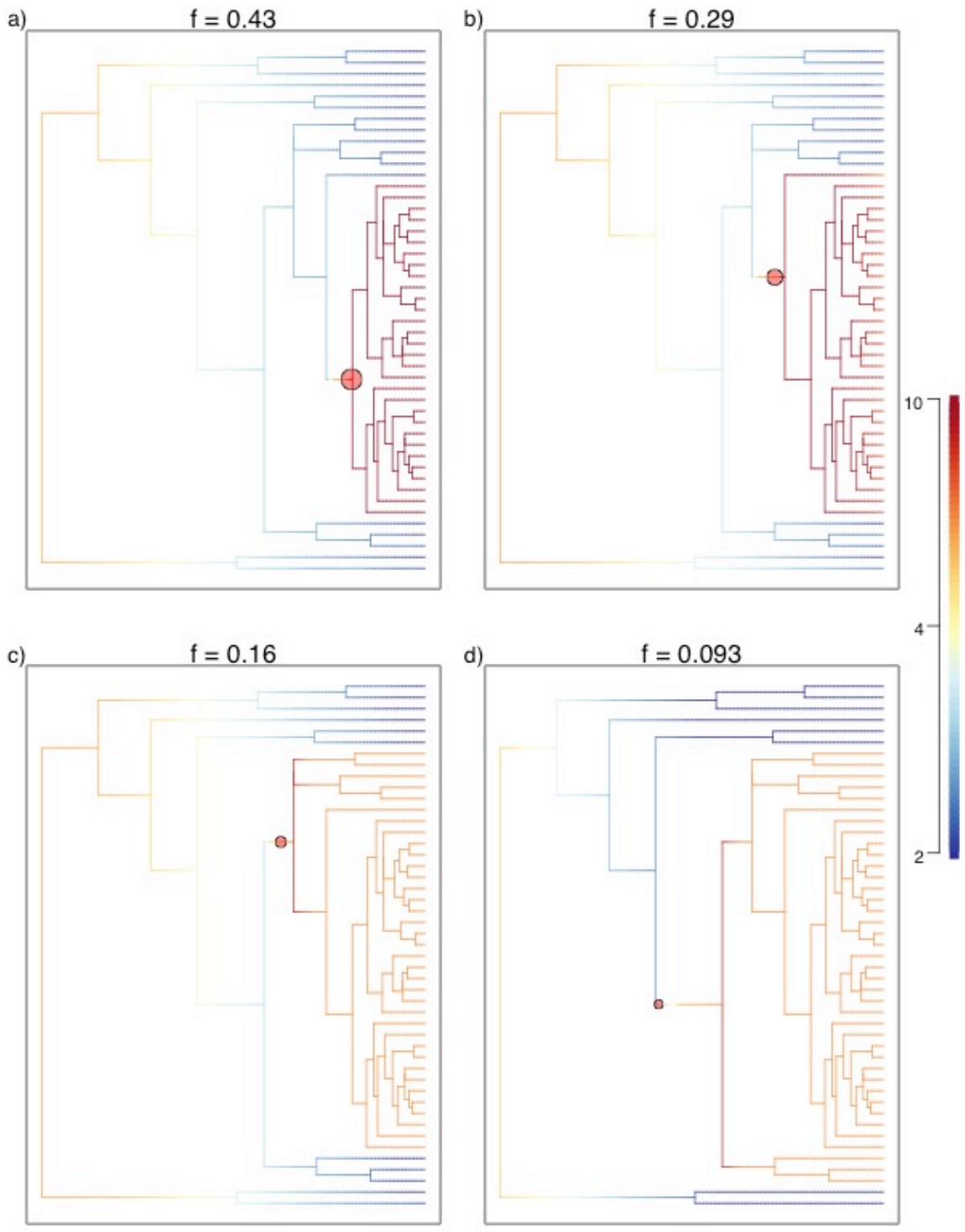


Figure 2.4. Most credible rate shift scenario results from BAMM analyses. Scenarios are in descending order from most credible on, with posterior values above each plot. Red circles indicate where rate shifts are located. Red indicates rate acceleration and blue indicates rate deceleration. The legend on the right is a histogram of the frequency of rates.

Of note though is that the ancestral node of these four species groups is also the most credible node where a diversification rate increase occurred according to the BAMM analysis. There are likely other ecological or geographical forces affecting body size, but it is possible that body size could have potentially acted as a key innovation allowing for greater dispersal capability and leading to increased ability for *Pteropus* species to colonize remote islands. For instance, the dominance of wind-dispersed dipterocarps in Southeast Asia (Fleming et al., 1987) and masting in forests (Appanah, 1993) means that pteropodids may need to disperse over greater distances to forage than their Neotropical counterparts. A masting event can lead to *Pteropus* following resource availability over great distances to a new area, leading to initial isolation. The bats subsequently do not disperse long distances as the need to do so is reduced, and only forage locally, eventually leading to speciation. More data would be needed to test if dispersal capability linked to body size changes is the reason for this rate acceleration in *Pteropus*, or if there are other factors tied to this diversification increase. General conceptual hypotheses for gigantism also suggest that extreme isolation, small island areas, thermoregulatory needs, and ecological release may result in gigantism (Lomolino et al., 2012), though how these factors affect *Pteropus* directly have yet to be tested due to lack of natural history data for many of the largest species.

Implications for Conservation and Disease Ecology Studies

The taxonomic findings reported here are especially important for global challenges in conservation and disease ecology. The nuclear data generated here may be of great importance to conservation biologists who want to identify sympatric *Pteropus* species that are morphologically similar but may have potentially hybridized recently(*e.g.*, *P. lylei* and *P. vampyrus* in zoos). Knowing that the *Pteropus* molecular clock ticks particularly slowly is

important to conservation, as bottlenecks in genetic variability may be a result of deeper historical (*e.g.* evolutionary) factors and not necessarily crashes caused by recent habitat loss or hunting. To detect changes in population size due to anthropogenic factors, more rigorous models may be necessary, which may necessitate the collection of a larger number of loci and individuals. Alternatively, inclusion of samples from historical specimens for comparisons of haplotypes and genetic diversity of historical populations to modern populations using coalescent models may allow for more precise identification of population bottlenecks. These analyses coupled with data on land use changes may provide a more accurate picture of how *Pteropus* populations are affected by habitat conversion.

These results also provide stronger empirical evidence for previous assumptions about the genus. Taxa that are highly dispersive and have directed flight (*e.g.*, destination not controlled by wind currents only), were generally assumed to have low genetic divergence, even on the spatial scales necessary for dispersal to remote islands (Gillespie et al., 2012). It was generally assumed that widespread *Pteropus* species are panmictic due to their high dispersal capability (Sinclair et al., 1996; Webb & Tidemann, 1996; Olival, 2008; Olival et al., 2013). However, panmixia has only been empirically shown once with limited taxon and gene sampling (Olival, 2008). In other cases, a lack of structure was found across multiple populations though within a single biogeographic area, a species may be panmictic (*e.g. P. conspicillatus*, Fox et al., 2009). For disease ecologists, panmixia may mean constant gene flow increases the potential for pathogens to spread between non-adjacent countries. For conservation biologists, panmixia means that loss of other populations may affect the level of genetic diversity within the species. It is entirely likely that some populations have constant gene flow between them despite geographic distance whereas others do not, but that it depends heavily on the landscape. In the

lycaenid butterfly *Lampides boeticus*, population structure was present throughout archipelagic areas, but was entirely lacking (*e,g,*, panmictic) from peninsular Southeast Asia to east Africa (Lohman et al., 2008). Based on studies of other volant taxa, we would expect a similar pattern in *Pteropus*.

According to disease ecology theory, the genetic similarity detected among *vampyrus* species group members would lead to predictions of increased likelihood of pathogen spillover (*e.g.*, host switching) from the original host. This prediction would mean that all *Pteropus* species of the *vampyrus* species group may be acting as a single pool of pathogens, and host species boundaries are porous enough to allow pathogen transmission. In this case, retroviruses would be shared among *vampyrus* species group members with higher likelihood than would be predicted from probabilistic pathogen transmission models. Understanding how host genetic diversity can affect pathogen spread is especially important in this group, as member species are commonly found from Sundaland and the Philippines to Madagascar in large colonies that often come into contact with dense human populations. Additionally, since most *Pteropus* species are closely related, viral diversity may be promoted (Huang et al., 2015). In a recent global analysis of carnivores, parasite diversity was negatively correlated with evolutionary distinctiveness (defined by phylogenetic terminal branch length) (Huang et al., 2015). Parasite abundance is also negatively correlated to taxonomic distance in small mammals (Krasnov et al., 2004). These studies suggest that the close phylogenetic relationship of *Pteropus* would promote high levels of viral diversity and abundance.

While *P. alecto* does not belong to the *vampyrus* species group, it has also received a great deal of attention from the biomedical community due to outbreaks of the Hendra virus (Paramyxoviridae) in Australia. The precise relationship of pathogens hosted by *P. alecto* to

pathogens hosted by *vampyrus* species group members remains unclear, but the low level of genetic diversity in the genus may allow for spillover events to occur despite *P. alecto* being distantly related to *vampyrus* species group members. This could be tested in a real scenario, as hybridization between Australian *Pteropus* is common. Spillover between *P. alecto* and the closely related, sympatric *P. conspicillatus* may be more common, and pathogens are predicted to be more similar between these two species. Spillover between *P. alecto* and the distantly related, sympatric, *P. poliocephalus* would accelerate pathogen evolution according to disease ecology theory, as pathogens would have to evolve more mechanisms for adapting to new host (Hatcher & Dunn 2011).

The single rate acceleration found in *Pteropus* may also have affected pathogen diversification. Presumably, associated pathogens would have also diverged quickly and there may be a radiation of closely related pathogens at that same node. Previous research on parasites in carnivores (Huang et al., 2015) and primates (Nunn et al., 2004) also found greater parasite diversity in rapidly evolving clades. If that is the case, there may be a large pool of pathogens that could easily spread between hosts who are very closely related and genetically similar. This would mean that the disease profile for *Pteropus* species in large radiations, such as the *vampyrus* species group, may have a higher level of pathogen diversity as compared to other *Pteropus* species groups.

Conclusion

Efforts towards determining species-level relationships between *Pteropus* have always suffered from a paucity of samples from Wallacea, and the inclusion here of multiple nuclear loci for many species brings additional clarity to the evolutionary history of this genus. The inclusion of many previously unsampled species in the tree reduced the long branches in the phylogeny.

This study also highlights the importance of nuclear data for global comparisons in this clade, as mitochondrial-nuclear gene tree discordance suggests that hybridization or incomplete lineage sorting results in misleading inferences from mitochondrial data. Divergent mitochondrial signals can be a result of hybridization or lineage sorting, and bottlenecked island populations with small effective population sizes may further enhance the effects of genetic drift (Knowles & Richards, 2005). Mitochondrial data may still be informative for intraspecific studies or studies of sister species, but is clearly insufficient for understanding such a complex genus-level evolutionary history as that of *Pteropus*.

Despite the inclusion of multiple nuclear loci and the use of species tree methods, there remain some poorly supported nodes in this phylogeny, and more loci are likely needed to fully resolve some internal nodes within the genus *Pteropus*. Rapid radiation causing short intermodal periods may have contributed to low support at internal nodes prior to the burst of recent speciation in the *temminckii*, *vampyrus*, *samoensis*, and *griseus* species groups. Further resolution of *Pteropus* could perhaps be achieved using next-generation sequencing techniques, which would provide additional data and would also allow the inclusion of recently extinct or rare and remote taxa unlikely to be sampled again. Genomic data from next generation sequencing would allow for the sampling of several magnitudes more loci for understanding not only species level relationships, but also intraspecific relationships.

CHAPTER 3

Biogeography of the genus *Pteropus* in the Indo-Australian Archipelago

Abstract

Pteropus is a genus of highly mobile bats native to the island landscapes of the Indo-Australian Archipelago (IAA). Islands provide opportunities for isolation from sister populations, promoting speciation. Most of the landmasses in the IAA are oceanic in origin, and the ability of organisms to disperse to these islands can vary. For volant taxa in the IAA, dispersal and founder-event speciation should therefore be the dominant biogeographic forces instead of vicariance. To empirically test this hypothesis, *Pteropus* serves as a suitable model system since it has multiple widespread and endemic species on every landmass in the IAA. I implemented the DEC and DEC+J model in BioGeoBEARS and the BBM model in RASP 3.0 to determine what biogeographic forces shaped the genus *Pteropus*. Wallacea was found to be the center of origin of the genus, with dispersal as the most common scenario through which lineages diverged. Founder-event speciation was similarly found to be the mechanism for expansion of *Pteropus* species into Micronesia and islands in the western Indian Ocean. The rate of dispersal for *Pteropus* is a magnitude higher than most other volant taxa, such as flycatchers and *Papilio* butterflies, again highlighting the importance of dispersal in the genus.

Introduction

The genus *Pteropus* (Chiroptera: Pteropodidae) presents a unique opportunity to model the biogeography of a species-rich taxon distributed throughout Southeast Asia, which includes the Indo-Australian Archipelago (IAA), one of the most geologically complex tropical areas in the world (Lohman et al., 2012). One or more of the approximately 65 species in Pteropus are found on every major landmass in the IAA, with some taxa being endemic while others are

widespread (Simmons, 2005). This diversity and distribution pattern makes it possible to answer questions about the influence of geography on population biology and dispersal dynamics within the genus. *Pteropus* diversity is concentrated in Indonesia and the South Pacific, with additional radiations on islands in the western Indian Ocean and Micronesia (Corbet & Hill, 1992). This pattern of distribution is similar to some bird (Moyle et al., 2012; Harris et al., 2014) and insect species (D.J. Lohman, unpublished data), but has never been empirically studied before using bat taxa.

Unlike other regions, the IAA presents unique challenges to accepted null models in biogeography. Many of the landmasses in the IAA are comprised of young crustal terranes, ancient continental rafts, or some combination of the two (Hall, 2002). Examples of these include the Philippines, Sulawesi, and New Guinea (Hall, 2002; Lohman et al., 2012; Stelbrink et al., 2012). Biotic studies in the IAA are few, especially in Wallacea, an area extending east of Wallace's Line to the Sahul Shelf west of Lydekker's Line. The major islands found in this region are Sulawesi, the Moluccan Islands, and the Lesser Sundaic Islands. Most *Pteropus* species native to this area are only known from studies of museum collections (Andersen, 1912; Bergmans, 2001; Helgen et al., 2009); few recent expeditions in the last twenty years have investigated bat biodiversity in the area (Kitchener & Maryanto, 1995). This is an unfortunate circumstance, as the level of species diversity and population structure in *Pteropus* species suggests that there are many unresolved taxonomic issues remaining (Chapter 2). Based on the species diversity of *Pteropus* in the region, Wallacea is potentially important to understanding the ancestral site of origin and mechanism of diversification of *Pteropus*,. Previous ancestral area reconstructions of the genus relied heavily on taxonomic sampling from South Pacific species, and reconstructed ancestral ranges may thus have been at least in part a result of sampling

artifacts (Almeida et al., 2011). Mechanisms of diversification cannot be explored without a clear understanding of its biogeographic history.

Speciation requires separation of daughter lineages, and islands in the IAA would seem to provide myriad opportunities for speciation. In previous studies of Southeast Asian terrestrial biota, island size is often predicted to be positively correlated with species diversity, resulting in Borneo being predicted as the place of origin for many taxa (de Bruyn et al., 2014). This is not necessarily true of *Pteropus* species. Compared to the diversity on much smaller islands in Wallacea and the South Pacific, Borneo is relatively species poor, with only two species (Mickleburgh et al., 1992). Seram has four *Pteropus* species despite being 2% the size of Borneo (Corbet & Hill, 1992; Mickleburgh et al., 1992). The Samoan Islands also had four *Pteropus* species in the recent past (two are now extinct), despite being 0.5% the size of Borneo (Helgen, 2009). The number of *Pteropus* species per island does not appear to be proportional to an island's size, with the exception of among the Pacific Islands (Fig. 3.1). Smaller islands are often disproportionately species rich, perhaps because most *Pteropus* prefer coastal areas, which abound on small islands. This pattern fits with predictions of the "small island effect" where species richness is independent of island area when island size is too small (*e.g.* lower than the variable evolutionary threshold, Lomolino & Weiser, 2001).

Previous molecular studies of *Pteropus* included a geographically biased subset of taxonomic diversity and sampled too few species for confident ancestral area reconstruction (Fox & Waycott, 2007; Olival, 2008; O'Brien et al., 2009; Brown et al., 2011; Larsen et al., 2014). There were no representative taxa from an important biogeographic area (Wallacea) in previous phylogenetic studies of the genus (Giannini et al., 2008), or only fragmentary mitochondrial data from a handful of taxa (Almeida et al., 2014). As previously discussed in Chapter 2, I have

overcome this issue with more thorough taxonomic sampling and increased geographic sampling throughout Southeast Asia. This allows for the inference of the evolution of species' ranges through time as a fully resolved tree is necessary for most ancestral area reconstruction methods.

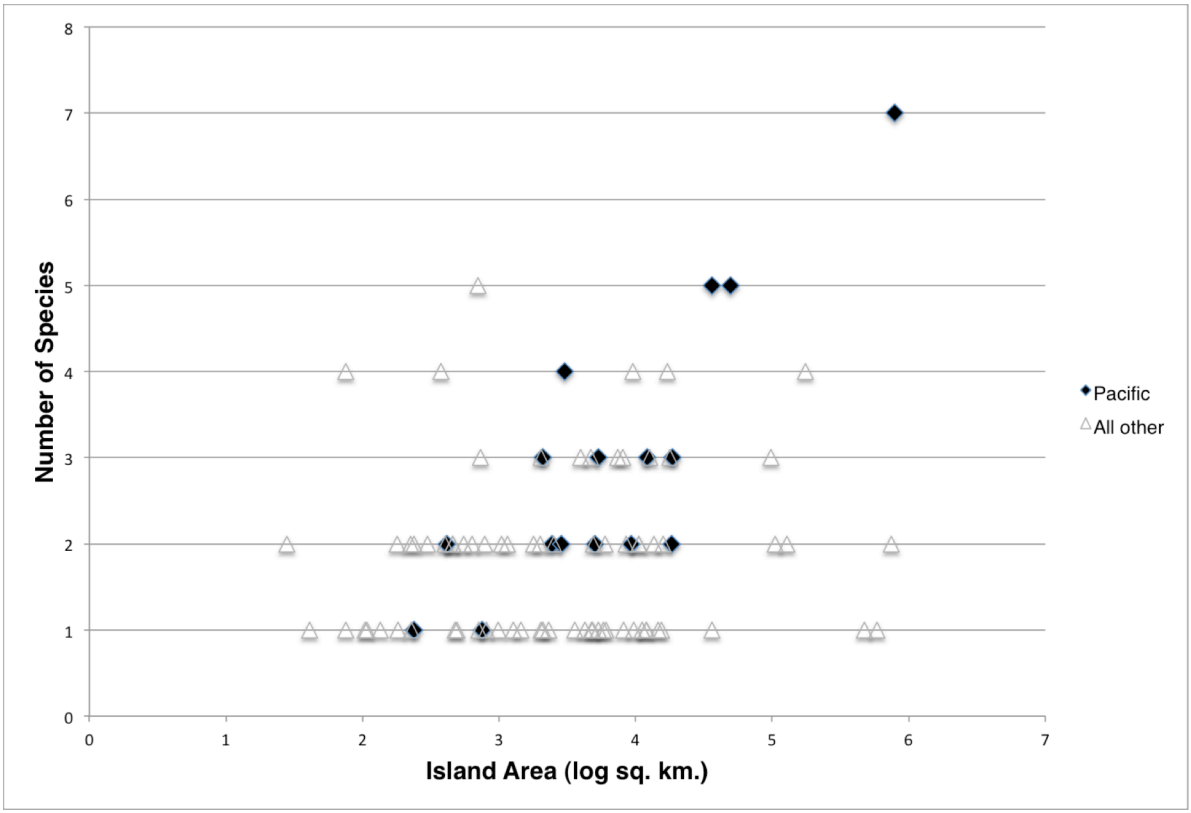


Figure 3.1. Log island area in relation to number of *Pteropus* species. Only major island and archipelagoes were included (see Appendix 3.1). Number of *Pteropus* species derived from Mickelburgh et al. (1992), Corbet and Hill (1992), Simmons (2005), and Helgen (2009). All models showed a significant relationship (overall model: adjusted $R^2 = 0.07$, $p < 0.05$), although only in the South Pacific did island area explain a substantial portion of the variance in species richness (Pacific Islands only: adjusted $R^2 = 0.69$, $p < 0.05$; all other categories combined: adjusted $R^2 = 0.06$, $p < 0.05$). The highest number of species is New Guinea, which is not only large but in close proximity to the Wallacean origin of *Pteropus*, both factors which may have contributed to its high species richness.

Departures from the Null Hypothesis of Vicariance in Biogeographic Inference

When inferring biogeographic scenarios to explain the distribution of extant species, vicariance is typically viewed as far more likely than dispersal (Morrone, 2009; Ronquist &

Sanmartín, 2011). However, the cyclic fission and fusion of landmasses in the IAA complicates its geologic history (Lohman et al., 2012). The presence of many taxa on oceanic islands that have never had a mainland connection, and the plausibility and observation of overwater dispersal by volant animals, suggests that dispersal is an important factor explaining species distributions in the IAA (Kodandaramaiah, 2009; Shilton & Whittaker, 2009). Vicariance as the null model may be especially uninformative for *Pteropus* since at least some species are undeterred by large oceanic expanses (*e.g.*, Roberts et al., 2012). With volant species, the increased complexity of high dispersal capability presents challenges to modeling population and species dynamics. *Pteropus* can fly more than 50 km a night to forage (Jones & Kunz, 2000; McConkey & Drake, 2006), and long distance dispersal seems common.

Recent historical biogeographic inferences of ancestral areas have been based on the DEC (dispersal-extinction-cladogenesis) model, and inferences have been implemented in Lagrange (Ree & Smith, 2008) or DIVA (Ronquist, 1997). However, the assumptions in DIVA are often times too simple or inflexible (Kodandaramaiah, 2010) to characterize the multiple mechanisms affecting *Pteropus* populations and species. DEC models improve upon biogeographic inference by increasing the scenarios that can be evaluated, but still exclude some scenarios that are likely in a highly vagile group such as *Pteropus*: 1) daughter species cannot both inherit a widespread ancestral range, 2) vicariant ranges are not allowed to be split evenly (*e.g.*, ABCD cannot become AB and CD daughter ranges), and 3) long-distance dispersal is not modeled (Matzke, 2014). *Pteropus* includes species that are both widespread (*e.g. P. vampyrus*) and restricted (*e.g. P. chrysoproctus*) in range, and previous biogeographic research suggests that rely a great deal on long-distance migration to reach remote areas (O'Brien et al., 2009); the inclusion of founder-event speciation would likely more accurately infer ancestral area estimates.

Additionally, the role of local extinction may be stronger than in other terrestrial vertebrates that occupy continental areas since *Pteropus* species are often restricted to small islands. Moreover, the true ranges of *Pteropus* species may be even further restricted to specific habitats, such as mangroves (*e.g.* Aul et al., 2014; Bates et al., 2014), resulting in predictions of higher extinction risk given a small species range size. Rejection of vicariance as the null model and the increased significance of both dispersal and extinction make reconstruction methods reliant on vicariance models inappropriate for biogeographic analyses in *Pteropus*.

Sympatric Diversity

Biogeographic inference will provide insight into the mechanisms for the distributions observed in island systems. Many examples of multiple sympatric congeners co-occurring in small island ecosystems exist. However, it is unclear whether these sympatric species are a result of multiple colonization events (*e.g.*, dispersal) or *in situ* radiations after a single colonization event. Determining which mechanism explains the diversity of sympatric species would allow for further understanding of drivers of speciation in these organisms and ecosystems. Both mechanisms leave distinct molecular signatures, and they can be tested using the resolved phylogeny. A single colonization event and subsequent *in situ* radiation would result in sympatric species being sister to one another on the phylogeny. Alternatively, multiple colonization events as a result of dispersal will result in sympatric species not being sister taxa on the phylogeny.

Methods

The BEAST species tree described in Chapter 2 was used for biogeographic analyses. The zoogeographic areas were defined as: Wallacea (A), New Guinea + Australia + South Pacific (B), Sundaland + South Asia (C), the Philippines (D), Micronesia (E), and western Indian

Ocean (F) (Fig 3.2). These designations were based on current understanding of geological history per Hall (2002). Impossible area combinations were excluded from the analysis to more realistically model how biogeographic areas are distributed in space (*e.g.*, Wallacea and Africa is not a possible combination). The maximum number of areas was set to four, matching the maximum number of areas occupied by any taxa in the analyses. Area assignments for each species was based on known distribution data according to Simmons (2005). Ancestral area estimation was implemented using BioGeoBEARS (Matzke, 2013), which accounts for founder-event speciation (the +J model) and compares multiple methods of biogeographic inference (DEC, DIVALIKE, and BAYAREALIKE) in a single R package, for a total of six models. BioGeoBEARS allows for tests of biogeographic scenarios under each of the different model types common to biogeographic inference using a single analytic framework. BioGeoBEARS' implementation of DIVALIKE and BAYAREALIKE models are modified to fit within a likelihood framework and are simply for exploration of whether or not results would vary greatly between methods. I will therefore focus on the presentation of results from the DEC and DEC+J models, as those were the intended models implemented by BioGeoBEARS. The *d* (dispersal) and *e* (extinction) parameters were allowed to vary freely. Results from the DEC and DEC+J analyses implemented in BioGeoBEARS were compared to those of DEC implemented in the Python script for Lagrange (Ree & Smith, 2008). Results from the BAYAREALIKE and BAYAREALIKE+J analyses implemented in BioGeoBEARS were compared to those of BBM (Bayesian binary MCMC) analyses implemented in RASP 3.0 (Reconstruct Ancestral State in Phylogenies; Yu et al., 2014). RASP ancestral area reconstruction was simulated using two independent runs with 10 chains each for 1 million generations and sampled every 100 generations at a temperature of 0.1 under the F81+Γ model to allow for estimated rate

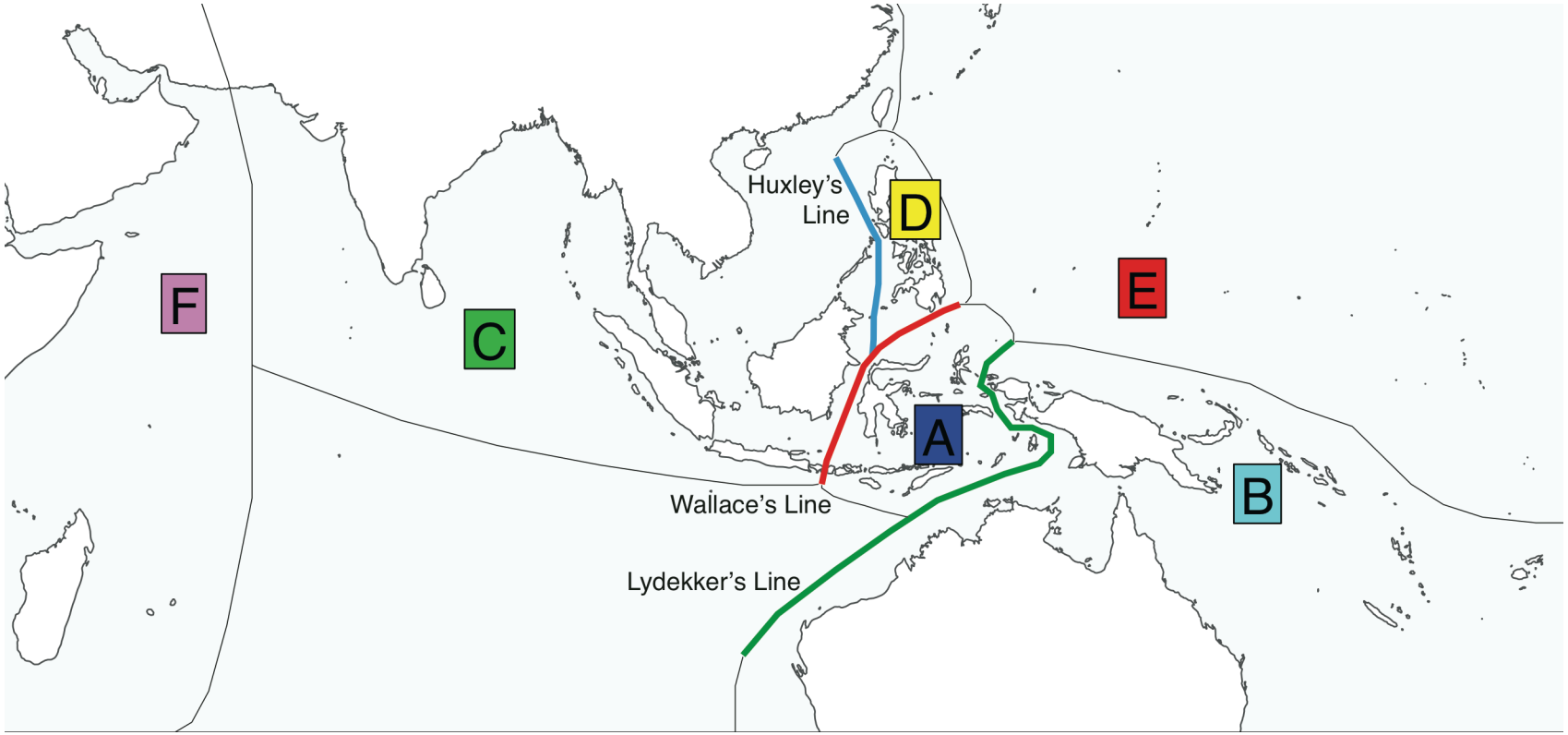


Figure 3.2. Map of biogeographic areas used in this study, labeled as follows: Wallacea (A), New Guinea + Australia + South Pacific (B), Sundaland + South Asia (C), the Philippines (D), Micronesia (E), and Africa (F). The Japanese islands of Ryukyu and Bonin were included as part of Micronesia due to their proximity and shared geologic history. Wallace's Line (red) and Lydekker's Line (green) are indicated on the map to separate the Sunda Shelf, Wallacea, and the Sahul Shelf. Huxley's Line (blue) indicates the split between Palawan and the rest of the Philippines.

frequencies and rate variation between sites. In addition to the anagenetic models tested by RASP allows for a scenario where widespread sympatry occurred. This scenario is possible within *Pteropus* given the number of widespread species, and inclusion of RASP would all for these comparisons to be made. The *Pteropus* and *Acerodon* species tree was attached u supertree methods to the pteropodid tree of Almeida et al. (2011) to ensure that the smaller outgroups from this study did not bias the ancestral area estimates. This larger set of outgr did not change estimates for *Pteropus*, and have been omitted for clarity. For the purposes biogeographic analyses, "widespread" here is defined as any distribution that is three of m the biogeographic areas.

Results and Discussion

Models utilizing the founder-event speciation parameter consistently outperformed without it (DEC –lnL = -109.5, DEC+J –lnL = -93.95, p = 2.40E-08) (Table 3.1). Likeliho scores and ancestral area estimation under each of the six models did not vary greatly (Tab 3.2), and I will focus on reporting the results under the DEC+J model. Ancestral area estim from DEC+J implemented in BioGeoBEARS (Fig. 3.3), DEC in Lagrange (Fig. 3.4), and I in RASP (Fig. 3.5) were generally similar to each other, though nodes at the root of *Pterop* estimated under RASP estimated a dispersal event from Wallacea unequivocally instead of likelihoods of dispersal from Wallacea or Wallacea + the South Pacific. Extinction (e) was small, non-zero number, and none of the methods found that scenario to be likely. Dispersa the most commonly estimated scenario, though founder-event speciation was also recovere nodes related to range expansion into Africa and Micronesia.

Table 3.1. Model performance comparisons implemented in BioGeoBEARS of each of the biogeographic models with and without the founder-event speciation parameter (+J). Models with +J significantly outperformed null models in every type of ancestral area estimation.

Null Model	Alt. Model	LnL(null)	LnL(alt)	*p* value	AIC
DEC	DEC+J	-109.5	-93.95	2.40E-08	223.1
DIVALIKE	DIVALIKE+J	-116.8	-99.11	2.80E-09	237.5
BAYAREALIKE	BAYAREALIKE+J	-148.7	-101.6	2.80E-22	301.5

Table 3.2. Ancestral area estimations under each of the models implemented by BioGeoBEARS. Dispersal (*d*), extinction (*e*), and jump (*j*) parameter estimates were also reported. Bolded is the best model according to likelihood scores, the DEC+J model.

Models	Likelihood	*d*	*e*	*j*
DEC	-109.52691	0.2910336	1.00E-12	0
DEC+J	**-93.95459**	**0.1429571**	**1.00E-12**	**0.04955305**
DIVALIKE	-116.75608	0.4510047	1.00E-12	0
DIVALIKE+J	-99.10639	0.2110676	1.00E-12	0.0475716
BAYAREALIKE	-148.72571	0.4777523	2.65E+00	0
BAYAREALIKE+J	-101.62144	0.1836749	1.00E-07	0.05277385

The most significant difference between the DEC+J and BBM models was ancestral area estimation at the node joining the *samoensis* species group to the *temminckii* + *vampyrus* species groups. Under DEC+J, ancestral areas at those nodes are estimated to be the result of narrow vicariance or sympatric speciation in a subset of areas, resulting in a widespread ancestral area (ABCD). Under BBM in RASP, ancestral areas are estimated to be the result of separate dispersal events to the South Pacific (B) and Wallacea (A) with subsequent radiations in each of the respective species groups. The BAYAREALIKE+J ancestral area estimations from BioGeoBEARS agree with the RASP results. These different ancestral area estimates may be due to the failure of RASP to estimate narrow vicariance and sympatry in a subset of areas, which are potential scenarios when considering the biogeographic histories of a mixture of widespread and restricted species such as those in *Pteropus*. It is possible that this ancestral area estimate may

also have been a result of the simplified designations of some areas in Wallacea and the South Pacific necessary to reduce the number of areas in the analysis.

The *griseus* species group, which consists of primarily insular taxa, had a Wallacean ancestral area. The *vampyrus* species group ancestral area estimate was widespread. The *samoensis* species group, which is sister to the *vampyrus* + *temminckii* species groups was estimated to have shared that widespread ancestor, but subsequently speciated in the South Pacific. The *samoensis* species group is the only instance of a large clade (*e.g.*, more than three species) in a single biogeographic area. Most other sympatric species occurrences were a result of multiple colonization events of the same area. With the Samoan species (*P. tonganus* and *P. samoensis*), these multiple colonization events were to multiple small, remote islands, such as Fiji, Tonga, Samoa, and American Samoa. Micronesia was colonized twice from different routes—colonization in the *vampyrus* species was through the Philippines, but colonization in the *griseus* species group was through the South Pacific. Australia was also colonized multiple times, through either a combined ancestral area of Wallacea plus the South Pacific, or from each individual area.

One of the only other examples of a radiation that was apparently the result of *in situ* speciation within a single archipelago is that of the Moluccan *Pteropus* species: *P. caniceps*, *P. melanopogon*, and *P. chrysoproctus*. The Moluccan Islands and North Moluccan Islands all existed in some form as oceanic terranes by the early Miocene (20 mya), but geological activity did not stop until the late Pliocene (3 mya), resulting in their present-day configuration (Hall, 2002). Colonization of the Moluccas by this radiation would be close to the estimated time scale for when this group would have diverged, and it is possible that they were able to radiate as some of the first colonizers to these oceanic islands.

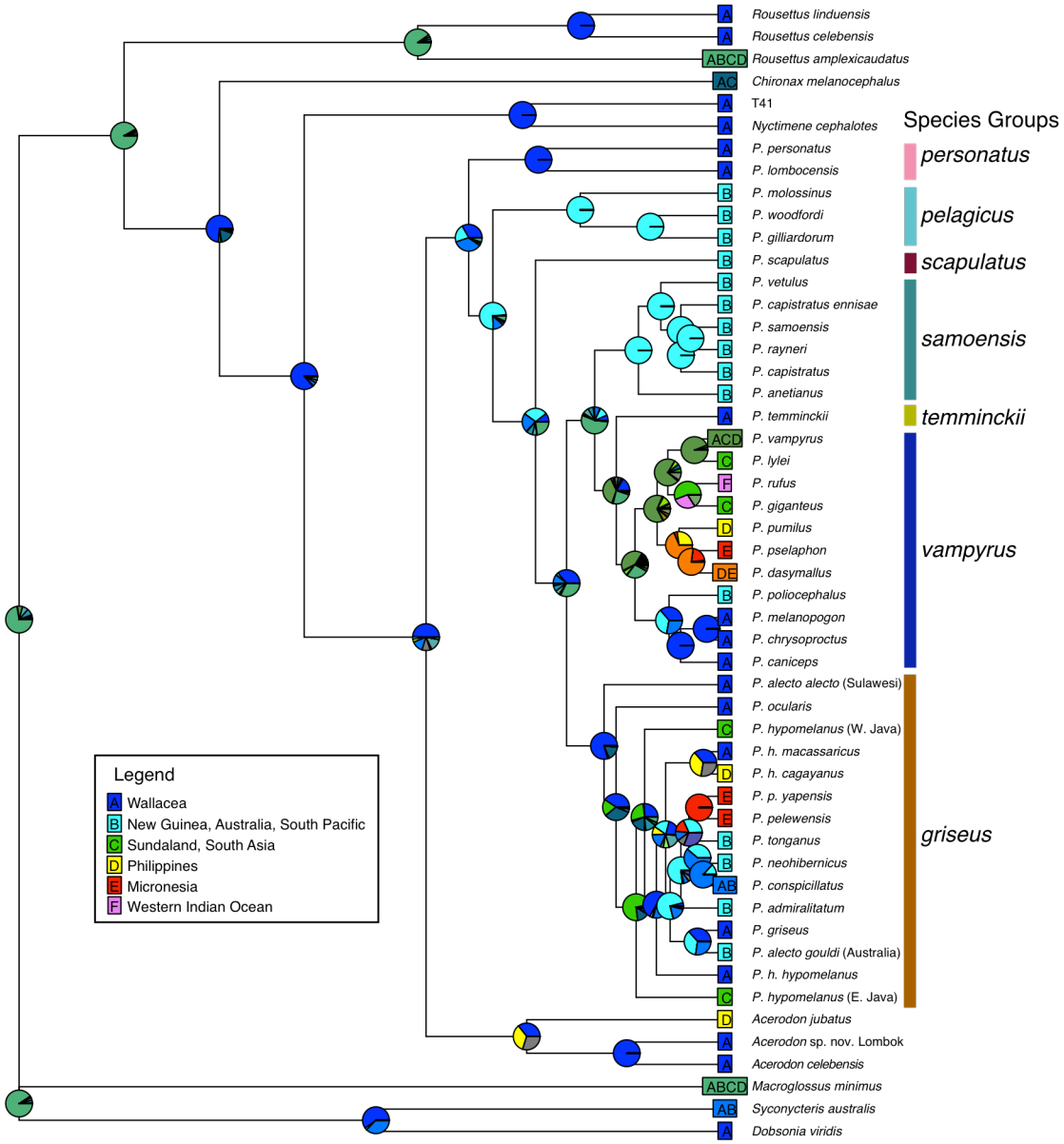


Figure 3.3. DEC+J results from BioGeoBEARS analysis. Ancestral area estimations at nodes represent areas before instantaneous speciation event. "Corner" estimates (states immediately after speciation) have been omitted for clarity, as they generally agree with descendent areas. Species groups are listed on the right. Multiple areas are represented by combining colors of component areas.

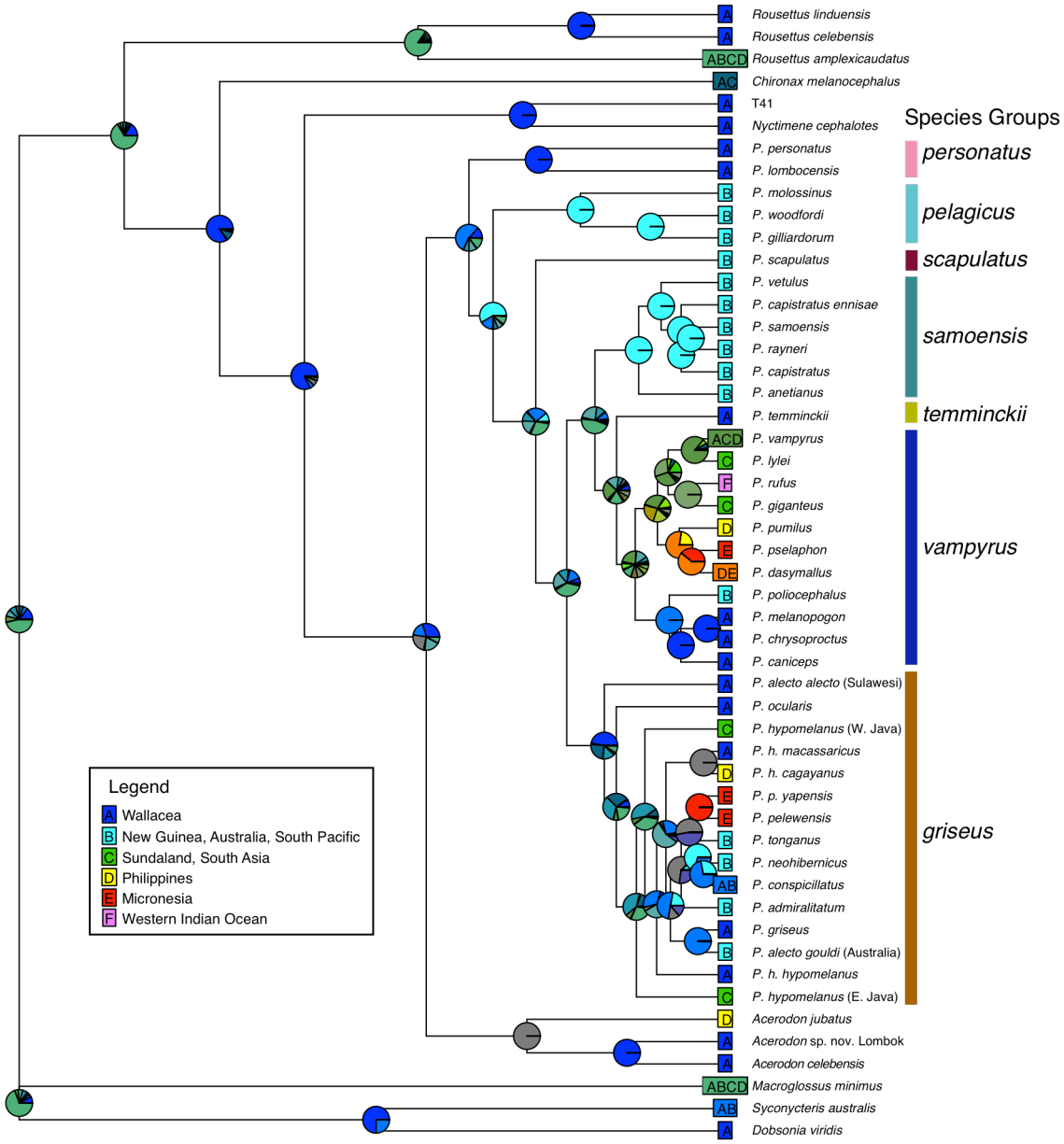


Figure 3.4. DEC results from Lagrange analysis. Results were plotted using R scripts available from the BioGeoBEARS package. Ancestral range estimations generally agreed with those from DEC+J. At several nodes (in grey), the DEC model could not estimate ancestral ranges.

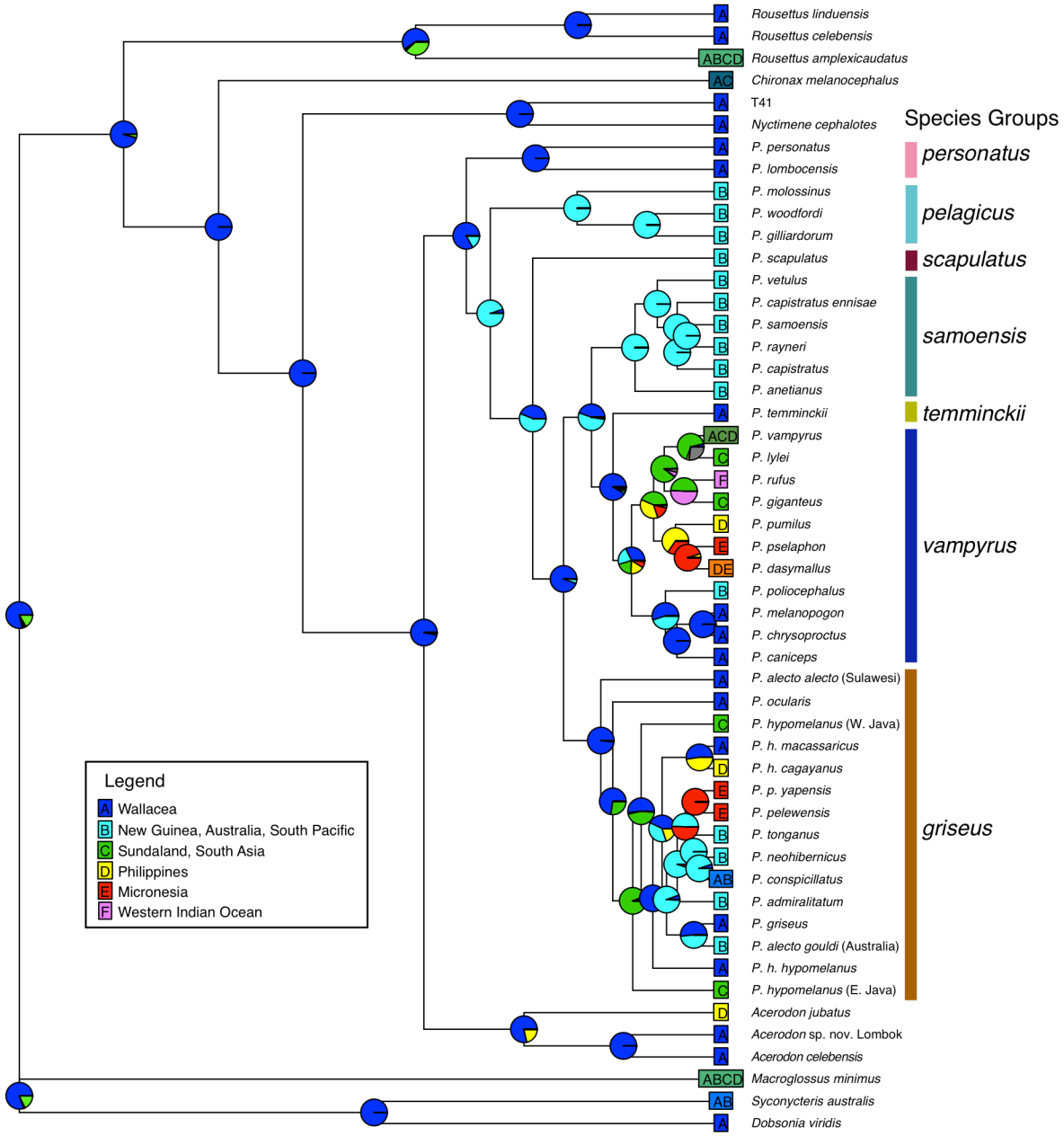


Figure 3.5. BBM results from RASP 3.0. Results from RASP were recoded and plotted using R scripts from BioGeoBEARS. The most significant difference from ancestral area estimations in the DEC+J model is the different mechanisms at the node joining the *samoensis* species group to the *temminckii* + *vampyrus* species groups. Under BBM, the node is estimated to result in two dispersal events into two different biogeographic areas (South Pacific and Wallacea, respectively).

The *P. pumilus* (*P. pselaphon*, *P. dasymallus*) clade in the Philippines and Micronesia is apparently the result of dispersal from the rest of the *vampyrus* species group's widespread ancestral area. Based on the estimated ancestral ranges before and after each node, this radiation is likely the result of sympatric speciation in a subset of areas and subsequent dispersal, as vicariance would not be possible. The Izu-Bonin-Mariana Arc is estimated to have formed through transformation of an oceanic fault into a subduction zone with subsequent volcanic activity during the Eocene (Stern & Bloomer, 1992; Ishizuka et al., 2006). *Pteropus pselaphon* is an endemic Bonin Island species, and these islands have never been connected to any continental crust, making vicariance impossible.

Founder-event speciation was clearly a common mechanism for long-distance dispersal and expansion of the clade into Africa and Micronesia. Inferred founder events may become more common if each of the major South Pacific archipelagoes was treated as a separate area. Combining all South Pacific islands into a single area was necessary for this study, which covered such a wide geographic range, but a focused study on the *samoensis* species group and its radiation in the South Pacific would be important to understanding the role of founder-event speciation in remote island systems.

Wallacea has played a significant role in the evolution of *Pteropus*, a clear and novel result that has emerged from comparisons developed for the first time here. As a clade that thrives on islands, the archipelagoes of Indonesia and the South Pacific offer great opportunity for isolation, and thus speciation. *Pteropus* cannot be defined as being predominantly part of the Asian or the Australian biotas; instead it appears to have a distinct Wallacean origin with daughter lineages extending both to the east and the west. High dispersal ability has resulted in multiple crossings of both Wallace's Line in the west and Lydekker's Line in the east. To better

understand fine-scale biogeographic histories of individual *Pteropus* species, future analyses may need to split areas in Southeast Asia and the South Pacific even further, according to geological histories. This would require a computationally more intensive method for incorporating a large number of areas, such as that of BayArea (Landis et al., 2013). Data from a few missing taxa must first be obtained before BayArea can be implemented, but it is a promising method for unraveling biogeographic relationships and mechanisms in such a complex region.

Islands, especially oceanic islands such as the majority of those in Wallacea and the South Pacific, apparently acquire biotic diversity primarily through dispersal (and subsequent evolution). When comparing dispersal in *Pteropus* to other terrestrial taxa in the few published studies also implementing BioGeoBEARS, the *d* parameter representing dispersal rate (rate of range addition along a branch) is at least a magnitude higher in *Pteropus* than in non-volant organisms (Pyron, 2014) and plants (Matzke, 2014). Not all volant taxa rely on dispersal instead of vicariance, as dispersal may be related to other life history traits or the geographical location. For instance, the Neotropical nymphalid butterfly genus *Calisto* owes its current distributions to ta combination of vicariant events, with a few instances of long distance dispersal when founder-event speciation is taken into account (Matos-Maraví et al., 2014). Dispersal parameter estimates in this case are two magnitudes lower than those recovered in *Pteropus* in this study. Future comparisons to dispersal parameters in other volant taxa found in the IAA that have been posited to be good dispersers are needed. Having these comparisons would be important to understanding how communities are assembled in remote island ecosystems, as these taxa also often carry seeds and affect primary productivity on islands directly.

Repeated colonization events from a single core area are commonly estimated as the scenario for other volant taxa that share the same distribution as *Pteropus* (Moyle et al., 2012, D. J. Lohman, unpublished data). Fruit doves (genus *Ptilinopus*), which share a similar ecological niche with *Pteropus*, are generally thought to have originated in New Guinea with separate radiations into Asia and Melanesia (Cibois et al., 2014). However, this inference may be due to limitations from the Lagrange DEC model—there are two instances of possible founder-effect speciation from New Guinea to Asia and Micronesia. Repeated colonization events may be more common than previously thought in volant taxa with high dispersal capability and mechanisms that have resulted in present distributions may need to be reconsidered.

The breadth of the distribution and exceptional species richness of *Pteropus* is not matched by any other pteropodid genus. However, there are other pteropodid genera with more than ten species, such as *Nyctimene* and *Dobsonia*. These genera have radiated in the South Pacific and Wallacea as well, and species are characterized as being good dispersers able to survive in severely resource-limited areas or disturbed areas (Flannery, 1995). The small gape size of *Nyctimene* is thought to be important to seed dispersal of early successional plants, as many of these plant species often produce small fruits with many small seeds (Muscarella & Fleming, 2007). It is possible that *Nyctimene* are not as geographically widespread as *Pteropus* simply because they are much smaller and cannot fly as great distances (*Nyctimene* species forearm size range from 50 to 80 mm, *Pteropus* species forearm size range from 90 to 220 mm). *Dobsonia* species, which are much closer in size to *Pteropus* (forearm size ranges of 95 to 160 mm), may be limited by their dependence on caves as day roosts or on availability of foliage roosts (Flannery, 1995; K. Helgen, *pers. comm.*). Comparison of biogeographic histories in these other pteropodid genera is of great interest, though not currently possible given the lack of

resolved phylogenies for *Nyctimene* and *Dobsonia*. Biogeographic comparisons would also necessitate splitting Wallacea and the South Pacific into smaller fragments, as mentioned before, as *Nyctimene* and *Dobsonia* species primarily occur in those areas. This offers a unique opportunity to study multiple pteropodid species with overlapping ranges to determine if there may be simultaneous patterns of divergences across pteropodid lineages when new islands arise.

Size does not guarantee that large pteropodid species will be long-distance dispersers. The monkey-faced bats (*Pteralopex* spp. and the closely allied monotypic *Mirimiri acrodonta*, previously classified as *Pteralopex acrodonta*) are larger-bodied than most *Pteropus* species (forearm size range 111 to 160 mm), but these bat species are restricted to the Solomon Islands (and *Mirimiri* in Fiji) (Helgen, 2005). *Pteralopex* is a forest-dependent clade that is incredibly sensitive to human disturbance and habitat conversion (Flannery, 1995). With such a narrow niche compared to *Pteropus*, this would likely preclude *Pteralopex* from being able to colonize areas unless a forest is established on an island already. Additionally, the wings of *M. acrodonta* have been noted to be suited for slow, maneuverable flight, likely for avoiding obstacles in dense primary forests (Flannery, 1995). This may affect their ability to disperse long distances, and may explain why monkey-faced bats are restricted to small islands.

Conclusion

The importance of dispersal to the biogeographic history of *Pteropus* is distinctly tied to its ability to both colonize and speciate. The degree and potential for colonization of islands or subsequent *in situ* speciation are generally dependent on island area, age, and distance from mainland (Lomolino, 2000; Lomolino & Weiser, 2001), but the data on *Pteropus* diverge from the usual model predictions. As a relatively young clade, *Pteropus* apparently have used their high dispersal capability to reach young, remote, oceanic islands with few competitors. The data

presented here have shown dispersal to be a powerful mechanism that should be considered of greater importance than vicariance, and should be considered when studying the biogeography of volant taxa in the IAA.

CHAPTER 4

High levels of inferred gene flow among geographically distant populations of *Pteropus vampyrus* across their range in Southeast Asia

Abstract

Understanding the population structure of the Large Flying Fox (*Pteropus vampyrus*) is critical to addressing regional challenges faced by conservation biologists and disease ecologists. The distribution of *P. vampyrus* is one of the widest of all *Pteropus* species in Southeast Asia, and its ability to cross large oceanic expanses makes management of this threatened species an international issue. *Pteropus vampyrus* is an important seed disperser and pollinator in forest ecosystems, but is also a natural reservoir host for emerging infectious pathogens. I used phylogenetic inference and population genetic indices to infer gene flow between populations and modeled past migration events and frequencies using MIGRATE. Population genetic parameters indicate low levels of nucleotide variability with high haplotype diversity, likely implying a demographic scenario of recent population expansion after a bottleneck. Both the phylogeny and the MIGRATE inferences indicate that *P. vampyrus* from geographically distant populations frequently intermix. These findings indicate that *P. vampyrus* acts as a near panmictic population across its broad range. For conservation, this may mean that protection of the species requires international cooperation and monitoring to ensure metapopulations persist. For disease ecology, this suggests that *P. vampyrus* is likely capable of pathogen transmission across international boundaries. The low genetic variability and large size of the host gene pool also may lead to more aggressive pathogens evolving in the host system. Increased genetic sampling is needed to more accurately determine commonly used dispersal routes or asymmetric gene flow between populations. Furthermore, protection of the species and its habitats is

important, as environmental stress will likely lead to increasing frequency of emergence of infectious pathogens.

Introduction

Pteropus vampyrus (the Large Flying Fox or Malayan Flying Fox) is the largest bat species in the world (Andersen, 1912; Corbet & Hill, 1992). It is native to the Philippines, western Indonesia, and peninsular Southeast Asia, often occurring in large, colonial aggregations of thousands of individuals in coastal areas (Goodwin, 1979; Corbet & Hill, 1992; Jones & Kunz, 2000). *Pteropus vampyrus* is an important seed disperser and pollinator of ecologically and economically important plants, such as figs (*Ficus* spp.) and durian (*Durio zibethinus*) (Fujita & Tuttle, 1991; Jones & Kunz, 2000; Stier & Mildenstein, 2005). In general, the efficacy of flying foxes as seed dispersers depends on maintaining large, healthy populations (McConkey & Drake, 2006), which is becoming difficult as their populations are dwindling rapidly in the face of habitat conversion and intensive hunting (Mickleburgh et al., 1992; Mohd-Azlan et al., 2001; Struebig et al., 2007). The species is listed under CITES Appendix II and by IUCN as Near Threatened, but few national laws exist in Southeast Asia to enforce protection. Given the current, significant declines across its range due to overhunting, the species may soon be categorized as Vulnerable (Bates et al., 2014).

There is little or no local momentum for regional protection of *P. vampyrus* in Southeast Asia, where local residents lack incentive for biodiversity conservation (*e.g.*, Harada, 2003) and access to environmental education (Sulistyawati et al., 2006). There is minimal to no enforcement of quotas or hunting bans and seizure activities are rarely initiated by local enforcement agencies (Nijman, 2005; Shepherd & Njiman, 2008). In the entirety of the range of *P. vampyrus*, hunting bans only exist in 3 of 16 Malaysian states and federal territories (Heng,

2012). Incidental protection due to the proximity of a colony to religious sites or government grounds exists in Thailand (Bumrungsri, *pers. comm.*), Cambodia (Ravon et al., 2014), Vietnam (L.Q. Dang, *pers. comm.*), the Philippines (*pers. obs.*), Bali (*pers. obs.*), and Myanmar (*pers. obs.*), but none of these sites have legal protection, resulting in continued hunting or persecution of flying foxes. In Indonesia, flying foxes are not listed as a protected species (Maryanto et al., 2008). In Borneo, excessive hunting of *P. vampyrus* threatens the continued persistence of populations (Struebig et al., 2007; Harrison et al., 2011). In Malaysia, population modeling suggests that current levels of hunting are unsustainable (Epstein et al., 2009). Populations in Sumatra and Java are occasionally hunted for medicinal use (Croes, 2012), though hunters have expressed the greater difficulty in locating populations in recent years (*pers. obs.*). Steep declines in *P. vampyrus* populations across its range can result in dire consequences for forest regeneration, as much of their preferred diet consists of early successional plant species (Stier & Mildenstein, 2005).

The burgeoning interest in *P. vampyrus* as a natural reservoir host for emerging infectious pathogens has resulted in a plethora of recent species-specific studies on viral detection and disease ecology, especially with the increasing availability of molecular detection tools (Yob et al., 2001; Sendow et al., 2006; Olival et al., 2007; Wang et al., 2008; Rahman et al., 2010, 2013; Sohayati et al., 2011; Breed et al., 2013). These viral studies and reports have quickly outpaced those in other fields such as ecology (Mohd-Azlan et al., 2001; Stier, 2003; Gumal, 2004; Mildenstein et al., 2005; Stier & Mildenstein, 2005), conservation (Struebig et al., 2007; Harrison et al., 2011; Croes, 2012; Heng, 2012), and physiology (Reeder et al., 2006a, 2006b; Riskin et al., 2010). Studies of evolutionary relationships have included *P. vampyrus*, but have never delved deeply into the subspecies and population dynamics (Giannini et al., 2008; Almeida

et al., 2014). However, understanding population genetic diversity and frequency of gene flow between populations can have a direct effect on studies of viral transmission (*e.g.* predicting source populations of pathogens). The aim of this chapter is to remedy this lacuna so that conservation management needs can be met and questions related to population connectivity can be addressed. I hypothesize that *P. vampyrus* are panmictic across its range. Alternatively, individuals in populations that are geographically closer are more closely related. This is the first study sampling populations of *P. vampyrus* across its broad distribution and aims to provide indirect evidence regarding population connectivity by using genetic data to model migration. Having some foundational framework can in the future allow for the creation of targeted satellite telemetry projects for each region to get more specific, direct evidence of gene flow.

Methods

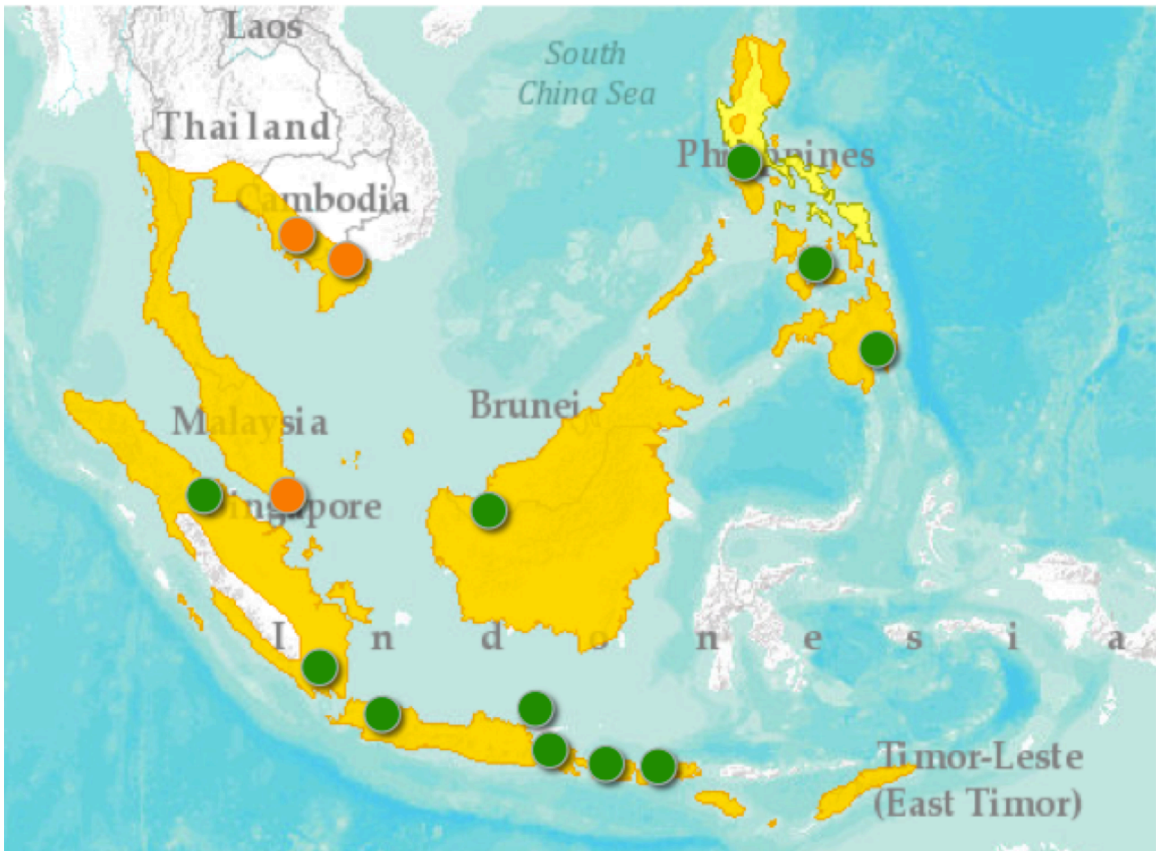


Figure 4.1 Map of specimen localities overlain on distribution map of *P. vampyrus* from IUCN Red List (Bates et al., 2014). Green dots represent freshly collected tissue, orange dots represent museum loans.

Acquisition of specimens and genetic data was described in Chapter 2. These specimens represent populations from across the range of *P. vampyrus* (Fig. 4.1)—from Negros Occidental (Philippines), West Kalimantan (Borneo), Sumatra, Java, Bali, Flores, and peninsular Southeast Asia. I assumed a maximum of seven possible populations given the geographical breadth of my sampling—the most geographically comprehensive study to date. Five population genetic indices commonly used by conservation geneticists were calculated for each gene using DnaSP (Rozas et al., 2003) to provide a general understanding of genetic diversity. Nucleotide diversity (π) is the average number of nucleotide differences per site between two randomly chosen sequences. Haplotype diversity (*h*) is a measure of the uniqueness of a haplotype within a population and calculated as the probability that two randomly selected haplotypes are not the same. Both are important for understanding genetic variability. The Watterson estimator (θ) allows for an estimation of the mutation rate since θ is four times the effective population size (N_e) times the mutation rate (μ) ($\theta = 4N_e\mu$). The number of segregating sites (S) is important as well for the estimation of the mutation rate and calculation of Tajima's D. Under the infinite alleles model, S is the same as the total number of mutations. Tajima's D is a statistical test to determine if genes are behaving under the neutral model or not, which allows for interpretation of biological scenarios such as selection or population size changes. Populations were also compared using the allelic fixation index, F_{ST} (Nei, 1973). Other measures of genetic variation between populations have been recently presented as an alternative to F_{ST} (*e.g.*, G'_{ST}, D), but given the low variability among *Pteropus*, F_{ST} remains the best measure of population differentiation (Whitlock, 2011). DnaSP also calculates pairwise effective migration rates (Nm) from F_{ST}, where N is the effective population size and m is the migration rate.

To make inferences of population history, phylogenies of all *P. vampyrus* individuals were reconstructed based on a gene tree using a partitioned Bayesian analysis of all 11 loci implemented in MrBayes 3.2 (Ronquist & Huelsenbeck, 2003). The analysis was simulated for 10 million generations, with a sampling frequency of 1000 and 25% burn-in. Models for all loci used were the same as those reported in Chapter 2 (see Table 2.3). A total of 40 *P. vampyrus* individuals representing the 7 populations were included in the ingroup, with a single *Acerodon celebensis*, *P. hypomelanus*, and *P. alecto* used as outgroup taxa to root the tree. The null hypothesis of no migration between populations would predict that individuals from the same population would form a monophyletic clade. However, if there was migration, individuals would not necessarily be most closely related to others in the same putative population. If migration occurred, isolation by distance would predict that populations that are geographically adjacent would be more closely related to one another than those that are geographically more distant (*e.g.*, gene flow results in a shared molecular signature). However, long-distance dispersal facilitates gene flow between geographically distant individuals, resulting in individuals from different populations being more closely related, regardless of population identity. A clustering analysis was conducted in Structure (Pritchard et al., 2000) and the most probable number of populations (k) was chosen via the methods described by Evanno et al. (2005).

To estimate past migration rates and frequency of migration events, a Bayesian inference of all parameters was implemented in MIGRATE (Beerli & Palczewski, 2010) using a Bayesian framework. Given the low rate of variation of sampled nuclear loci, migration analyses were conducted using only mitochondrial genes and two nuclear genes with higher nucleotide variability, STAT5A and FGB7. Mutation rates were calculated within the program as a relative rate among loci given the sequence data from a Watterson's estimator for each locus. Per-locus

test runs using a constant mutation rate were also conducted for comparative purposes and for refining priors for the multilocus analyses. F_{ST} values were used to estimate starting parameters and ten replicate runs were conducted in a single analysis. Skyline plots generated by MIGRATE visualized migration rate changes through time between population pairs.

Results

Table 4.1 Genetic diversity indices for a) *Pteropus vampyrus* and b) the genus *Pteropus*. Both are presented here to allow for a comparison in an evolutionary context. Nucleotide diversity is π, haplotype diversity is *h*, the Watterson estimator is θ, and segregating sites is S. Comparisons of gene flow using the allelic fixation index F_{ST} and Nm are included here for *P. vampyrus*. It may seem that *P. vampyrus* has an especially low level of nucleotide diversity, but when compared to the genus level genetic variability, it is only a magnitude lower.

a) *Pteropus vampyrus* (n = 40)

Gene	**π**	***h***	**θ**	**S**	**Tajima's D**	**D sig.**	**F_{ST}**	**Nm**
mitochondrial								
cyt-b	0.00651	0.992	0.0143	55	-2.01679	*, P < 0.05	0.00469	53
D-loop	0.27709	0.998	0.1952	307	1.631	NS	0.01092	22.64
nuclear								
RAG-1	0.00245	0.690	0.00733	17	-2.25052	**, P < 0.01	0.14924	1.43
RAG-2	0.00325	0.892	0.0056	15	-1.37383	NS	0.08514	2.69
STAT5A	0.00512	0.776	0.01263	18	-2.06068	*, P < 0.05	0.02837	8.56
PLCB4	0.00173	0.316	0.00558	6	-1.89994	*, P < 0.05	0	3.68
BDNF	0.00136	0.280	0.00355	5	-1.65533	NS	0.064742	3.46
FGB7	0.00499	0.917	0.01053	23	-1.79096	NS	0.04418	5.41
PSMB8	0.00466	0.858	0.00461	10	0.03106	NS	0	1.37
COPS7A4	0.0031	0.830	0.00417	9	-0.83058	NS	0.08428	2.71
ATP7A	0.00124	0.492	0.0032	7	-1.76615	NS	0.08619	2.65

b) Genus *Pteropus* (n = 188)

Gene	**π**	***h***	**θ**	**S**	**Tajima's D**	**D sig.**
mitochondrial						
cyt-b	0.09148	0.9955	0.1317	584	-1.00276	NS
D-loop	0.41537	0.9973	0.15751	327	5.33022	***, P < 0.001
nuclear						
RAG-1	0.00849	0.843	0.02592	61	-2.07616	*, P < 0.05
RAG-2	0.01227	0.964	0.03022	106	-1.89316	*, P < 0.05
STAT5A	0.02984	0.908	0.07243	86	-1.87146	*, P < 0.05
PLCB4	0.01393	0.723	0.03781	44	-1.87218	*, P < 0.05
BDNF	0.00353	0.678	0.0174	32	-2.33763	**, P < 0.01
FGB7	0.01608	0.872	0.05551	84	-2.21939	**, P < 0.01
PSMB8	0.01441	0.015	0.04203	76	-2.12799	*, P < 0.05
COPS7A4	0.01873	0.956	0.04667	79	-1.9333	*, P < 0.05
ATP7A	0.0074	0.854	0.02337	68	-2.11243	*, P < 0.05

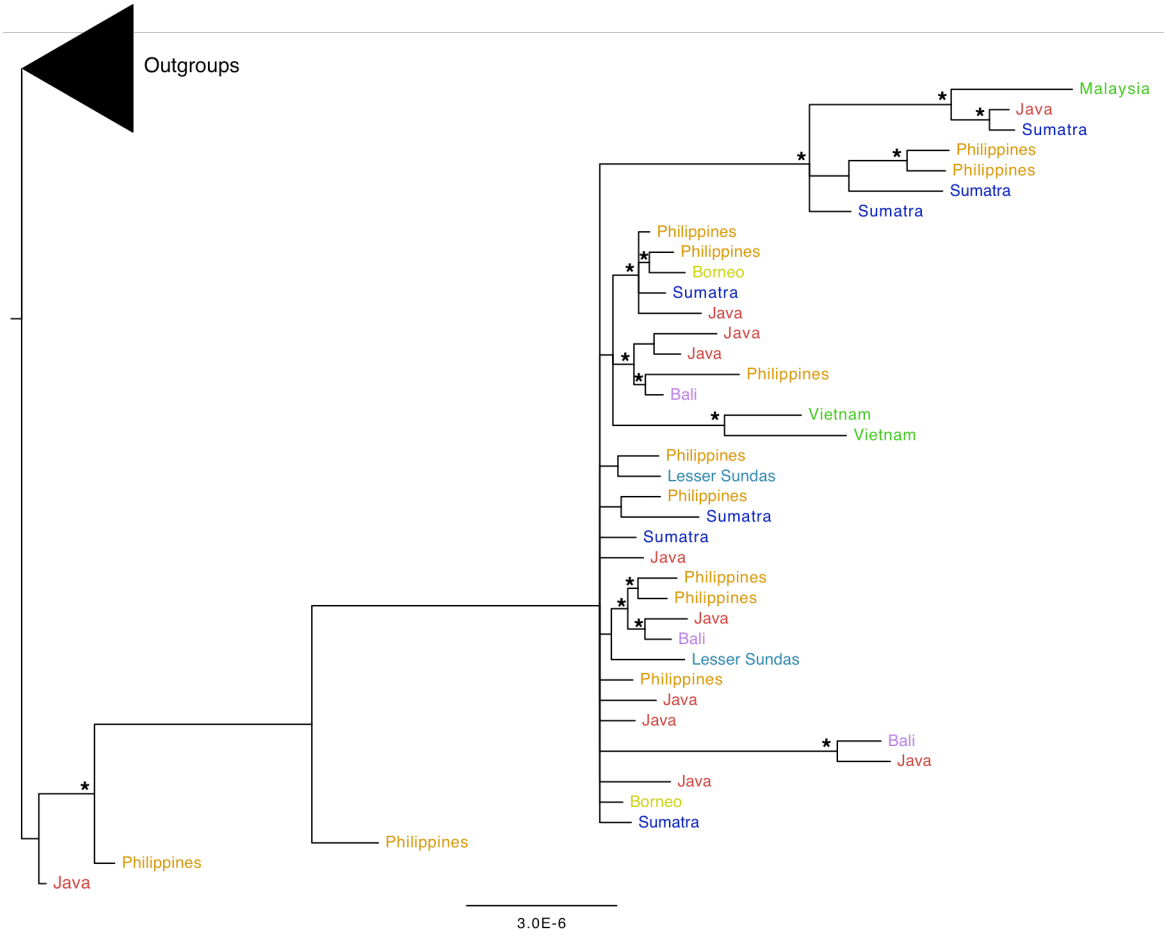


Figure 4.2 Phylogenetic relationships of all *P. vampyrus* sampled. The outgroups are collapsed together as a black triangle. Starred nodes indicate posterior probabilities over 0.9. Specimens are color-coded to their putative populations based on their sampling locality. Most individuals were most closely related to individuals from a different population.

The variability of all *P. vampyrus* genetic diversity indices was low for each gene, except for the hypervariable mitochondrial D-loop (Table 4.1a), which agrees with the low level of genetic diversity found for the genus *Pteropus* in Chapter 2. To put this in perspective, *P. vampyrus* has a similar range for π as mitochondrial nucleotide diversity in Asian elephants, another slowly evolving mammal, which has values of π ranging from 0.00195 to 0.01643 between populations, and an overall π of 0.0176 in Asia. Populations of *P. vampyrus* were almost panmictic in all of the genes, with F_{ST} values lower than 0.1 in most genes. Tajima's D

was not significant for most of the genes in *P. vampyrus*. However, Tajima's D was significant and negative for a majority of the genes at the genus level for *Pteropus*. This suggests a population expansion after a recent bottleneck, and may partly explain the depressed levels of genetic diversity in *Pteropus*. The high degree of haplotype diversity despite low nucleotide diversity also supports a demographic scenario of population expansion after a recent bottleneck. The only gene that exhibited a significant, large positive value for Tajima's D was D-loop, which suggests a sudden population contraction instead.

The phylogenetic analyses suggested *P. vampyrus* has high levels of gene flow between populations (Fig. 4.2). The Structure results also indicated that *P. vampyrus* essentially are acting as a single population ($k = 1$, lnL=-1731.3). The MIGRATE analyses also suggested that these migration events have occurred at high frequencies in the recent past with no significant asymmetry in any particular direction (Fig. 4.3 for an example, all remaining plots in Appendix 4.1). However, individuals from the continental areas clustered differently from those on islands. In the island populations, individuals were not most closely related to others from the same colony or population. Populations also did not follow predictions from isolation by distance, with some Philippine individuals being found to be sister to Sumatran, Javan, or Lesser Sundaic individuals (Fig. 4.2). However, on the continent, specimens from Vietnam were more closely related to one another. The peninsular Malaysian specimen was more closely related to two individuals from Sumatra and West Java, which would be predicted by an isolation by distance model. Vietnam and Malaysia were treated as two separate populations in the final MIGRATE analysis as a result of this finding. Peninsular Malaysia to southern Vietnam is approximately 1000 km (though longer if avoiding flight over water), whereas peninsular Malaysia to south Sumatra and West Java is approximately 700 km. Bats have been recorded flying from

peninsular Malaysia to Sumatra over the Strait of Malacca using satellite telemetry (Epstein et al., 2009), but direct observations of long distance dispersal have not been recorded outside of peninsular Malaysia. An isolation by distance model on the mainland of Southeast Asia could be further tested by including populations from Thailand, Cambodia, and southern Myanmar and a larger sample of marker loci.

Discussion

Pteropus vampyrus is essentially panmictic across its range, confirming my hypothesis. Given that *P. vampyrus* has a generalist diet and is capable of dispersing over large areas, it is not surprising that populations are interconnected as a result of traveling increased distances, perhaps to facilitate foraging in an increasingly fragmented habitat. This might first be interpreted as good news for conservation biologists, as protection of the species in some parts of its range may be adequate for protection of the genetic diversity of the species as a whole. However, this highlights the need for transnational strategies for conservation, as individuals cross country boundaries often and threats in one part of its range may severely affect population persistence. Additionally, this study did not test for genomic adaptations in each population to the local environment. Clinal variation in size in *P. vampyrus* has been recorded in older studies, though pelage coloration was not tied specifically to subspecies names (Andersen, 1912; Corbet & Hill, 1992). Since *P. vampyrus* is able to occupy such a wide range of habitats, it is unlikely that they are locally adapted. To detect localized adaptations, next generation sequencing data will be needed, as tests for recent selective sweeps may indicate whether there are population-level differences due to localized environments or anthropogenic exposure (*e.g.*, mining in Borneo may have similar effects on bat populations as urbanization has on mouse populations,

such as in Harris et al. (2013), by changing allele frequencies of loci related to adapting to heavy metal exposure).

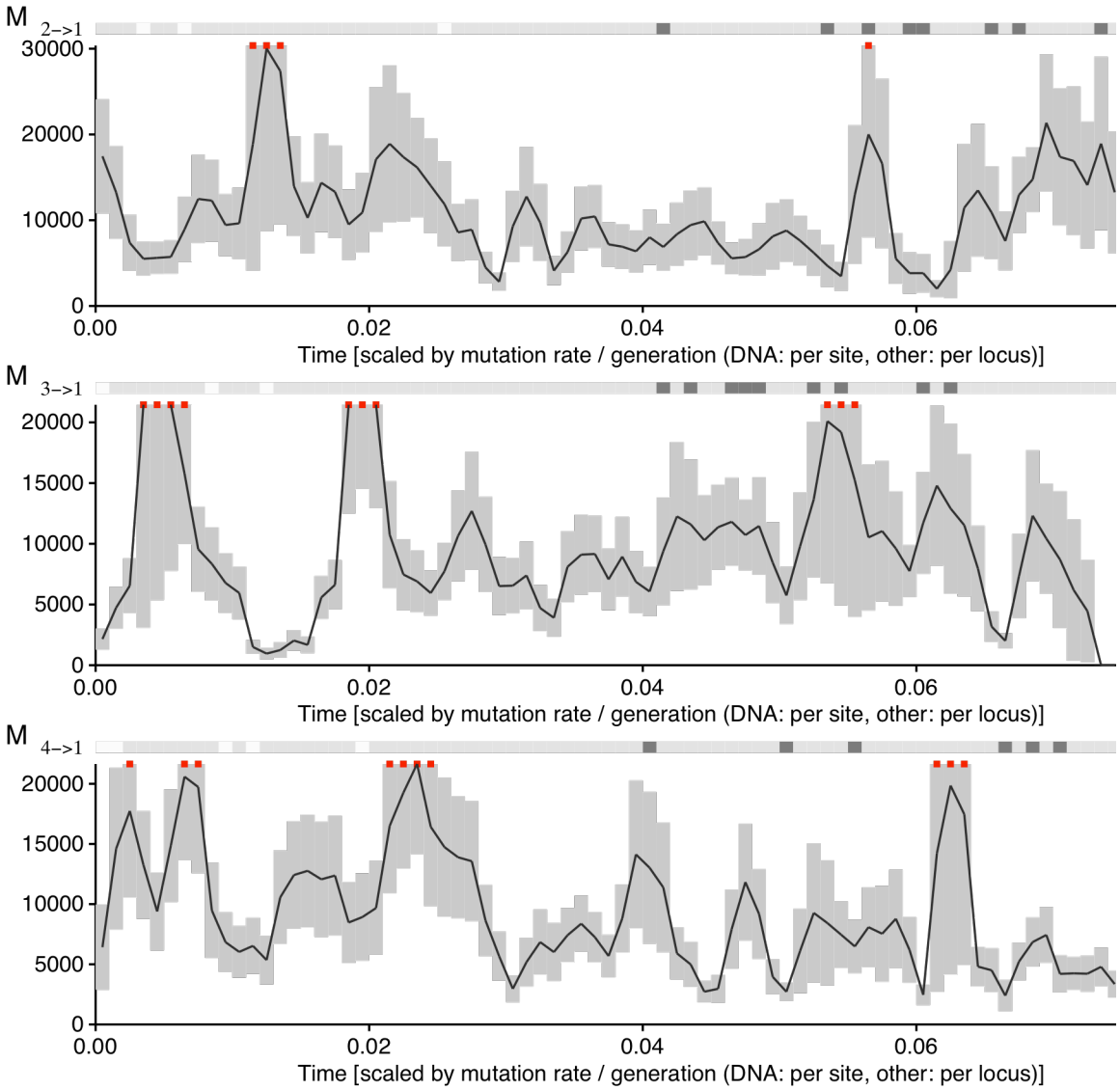


Figure 4.3. Example of a skyline plot generated by analyses in MIGRATE for migration rate through time between populations. Populations noted here in the subscripts for M are: 1) Sumatra, 2) Java, 3) Borneo, and 4) Bali. Time is scaled by coalescent units on the X-axis and M is the migration rate on the Y-axis. Red dots mean that either upper quantile or main values was higher than the Y-axis. See Appendix 4.1 for all additional MIGRATE plots.

The high level of connectivity among *P. vampyrus* populations may also be a methodological artifact given the genes used in the study, but that is unlikely. The *vampyrus* species group radiated approximately 2 mya, near the beginning of the Pleistocene, with *P. vampyrus* diverging from its sister species, *P. lylei*, within the last million years (Chapter 2). Since *Pteropus* exhibit such low levels of genetic variability, the recent population divergence in *P. vampyrus* may not have been enough time for lineage sorting issues to be resolved. Gene tree results (Chapter 2) have also suggested mitochondrial introgression between *P. vampyrus*, *P. alecto*, and *P. lombocensis* in the Lesser Sundas, though tests for hybridization provided only weak support.

In the closely-related large, generalist colonial species *P. giganteus*, habitat fragmentation due to land use change has been found to be an issue, apparently resulting in a larger number of smaller colonies because fewer trees were available for roosting (Hahn et al., 2014). A similar effect is likely in *P. vampyrus*, as large populations were only observed in some areas of the Philippines and the Lesser Sundas where there are large undisturbed forests and mangroves (*pers. obs.*). In highly disturbed areas, such as Java, Sumatra, West Kalimantan, and Bali, colonies of *P. vampyrus* were smaller than 1000 individuals (*pers. obs.*). Further research is necessary to determine the impact of smaller colony size on the genetic stability and persistence of the species, but currently little is known about yearly roosting patterns of *P. vampyrus* in Indonesia and metapopulation connectivity among roosting colonies to assess this.

Connectivity between *P. vampyrus* populations, to the point where populations are nearly panmictic, has direct consequences for our understanding of how viral systems evolve. Disease ecology theory predicts that connected populations will produce more aggressive pathogens with higher resistance than will occur in isolated populations with localized dispersal (Morand &

Krasnov, 2010; Carlsson-Graner & Thrall, 2015). This is a direct result of the host population essentially serving as a single population. Localized extirpation of a pathogen may be temporary; the pathogen may be harbored in one of many host populations and be transmitted again. This prediction of host population connectivity to pathogen aggressiveness (*e.g.* how infective a pathogen is) can be directly tested through isolating and characterizing pathogens in *P. vampyrus* and comparing them to other *vampyrus* species group members that have more isolated populations exhibiting lower population connectivity (*e.g. P. melanopogon* or *P. chrysoproctus*, which also do not overlap in range with *P. vampyrus*). However, connectivity may also mean that hosts can share pathogen resistance that may have developed in a single population within a metapopulation, allowing for equilibrium to exist between hosts and pathogens (Carlsson-Graner & Thrall, 2015). The high level of panmixia found in *P. vampyrus* is in contrast to that of *P. alecto*, the other well-studied flying fox in terms of disease ecology, which exhibits some level of population substructure across its range (discussed in further in Chapter 5), meaning that pathogen aggressiveness and resistance may also contrast with what is found in pathogens from *P. vampyrus*. Increased genetic sampling (*e.g.*, more loci) would provide more data for modeling dispersal routes across a metapopulation to better model potential routes of transmission for pathogens in both species.

Conclusion

Pteropus vampyrus exhibit high degrees of gene flow between geographically distant populations throughout their range in Southeast Asia. Knowledge of host population structure is essential to the formation of strategies for combating the spread of zoonotic pathogens in the event of a pandemic. The analyses demonstrate that a concerted effort across many international borders is needed, as *P. vampyrus* readily cross into Malaysia, Indonesia, Singapore, Vietnam,

the Philippines, and presumably Thailand, Cambodia, and Brunei as well. Biodiversity loss has been linked to the increasing global disease incidences (Daszak et al., 2001; Patz et al., 2004; Pongsiri et al., 2009), and safeguarding flying fox populations and natural spaces can decrease the likelihood for transmission and pathogen prevalence. As increasing environmental pressure stresses *P. vampyrus* populations more, the potential for pathogen spread and outbreaks increases (Daszak et al., 2001; Dobson & Foufopoulos, 2001). That means the health of *P. vampyus* populations should be both a conservation and public health issue to every member nation of ASEAN (with the exception of Lao PDR, where *P. vampyrus* do not occur). Southeast Asia is one of the most densely populated areas in the world (United Nations Population Division, 2013) and these issues must be addressed as a precautionary measure, not a reactionary one.

CHAPTER 5

Population structure of *Pteropus alecto* in Indonesia and Australia and its implications for disease ecology

Abstract

Pteropus alecto is a colonial species of flying fox native to Indonesia, Australia, and Papua New Guinea. However, most research effort has focused on Australian populations and little is known about the species in the Indonesian portion of its range. The relationship of Indonesian populations of *P. alecto* to Australian populations is important for both conservation management decisions and studies of pathogen transmission. I sampled 24 individuals from 4 putative populations of *P. alecto* from North Sulawesi, Central Sulawesi, the Lesser Sundas, and Australia. Inference from phylogenetic reconstruction of species relationships and Structure analyses indicated that colonies from Sulawesi have been acting as a single population, but there is some degree of population structure between Sulawesi, the Lesser Sundas, and Australia. The Lesser Sundaic population is more closely related to the eastern Australian population despite being geographically closer to Sulawesi. The high genetic diversity of populations from both Sulawesi and Australia suggest a potential for higher diversity of pathogens in those populations. The separation of Sulawesi from other *P. alecto* populations highlights the need for protection of Sulawesi populations threatened by bushmeat hunting, as they represent a distinct genetic lineage not found in any other part of the species' range.

Introduction

Pteropus alecto (Temminck, 1837) is a colonial, tropical flying fox species ranging from the Lesser Sundas and Sulawesi in Indonesia to southern Papua New Guinea and the northern and eastern coasts of Australia (Hutson et al., 2014). It is the most abundant flying fox species in

Australia (Halpin et al., 2011), though extreme heat events there have recently led to massive die-offs (Welbergen et al., 2008, 2014) and it still faces intensive levels of hunting in Sulawesi (Lee et al., 2005; Sheherazade & Tsang, 2015). It is one of the only species of *Pteropus* that has been extensively studied from an ecological (Palmer et al., 2000; Markus & Hall, 2004), behavioral (Vardon & Tidemann, 1998, 1999; Vardon et al., 2001; Markus, 2002; Phillips et al., 2007), and medical perspective (Field, 2004; van den Hurk et al., 2009; Halpin et al., 2011). It is the only *Pteropus* species with a high coverage genome (Zhang et al., 2013), with some studies already studying receptor and interferon expression as it relates to potential infection (Cowled et al., 2011; Cowled, Baker, Zhou, Tachedjian, & Wang, 2012; Janardhana et al., 2012; Zhou, Cowled, Wang, & Baker, 2013). Most of this work was conducted solely on Australian populations, and very little is known about *P. alecto* populations from other parts of their range.

Much of the genomic and medical research regarding *P. alecto* has been largely driven by the discovery of Hendra virus in 1994 and a subsequent unprecedented series of outbreaks of Hendra virus in northeastern Australia in 2011 (Smith et al., 2011b). Hendra virus belongs to the genus *Henipavirus* (family Paramyxoviridae) and it is widely recognized as an emerging (*e.g.*, increasing in incidence in the past twenty years) zoonotic pathogen that causes highly fatal encephalitis (Drexler et al., 2012). *Henipavirus* has a wide range of hosts, a unique feature for a paramyxovirus (Wang et al., 2008), and a contributing factor to its interest to the medical research community. Medical interest in paramyxoviruses aligns with a more general recognition of pteropodid bats as natural reservoir hosts for many emerging infectious pathogens (Calisher et al., 2006). However, most of this work is still focused on viral discovery; relatively little research has been conducted from the perspective of host ecology and host population dynamics. Host population dynamics, such as degree of host population connectivity, can be used as proxy

measures for viral dispersal routes. Given that there is still no standardized direct method for tracking flying foxes (Smith et al., 2011a), population connectivity studies of dispersal will need to rely on indirect measures from population genetics.

Novel viral emergence has been tied to anthropogenic environmental changes (Daszak et al., 2001; Chapman et al., 2005). Persistent human encroachment on natural spaces will continue to increase the potential for pathogens to be transmitted from bat hosts to other animals that they would not have any contact with naturally. For instance, it is unknown whether the pathogens already found in Australian populations of *P. alecto* are capable of infecting livestock, or whether these pathogens are also found in Indonesian or Papua New Guinea populations of *P. alecto*. Infectious pathogens pose the greatest threat in North Sulawesi, where intensive consumption of *P. alecto* and another flying fox, *Acerodon celebensis*, increases contact between bats, intermediary hosts, and humans. Bushmeat hunting is one of the biggest threats to large-bodied bats (Mickleburgh et al., 2009), but little is known about how increasing hunting pressure on *P. alecto* populations (Lee et al., 2005; Sheherazade & Tsang, 2015) is affecting viral density and abundance in host species. The degree of connectivity between bushmeat markets on different islands increases the potential for pathogens to be spread by humans or intermediary hosts, greatly increasing the potential geographic scope of pathogen transmission. For instance, bats are brought live to Beserhati in North Sulawesi only killed once they are at the market, exacerbating issues of interspecific contact.

In Chapter 5, phylogenetic and population genetic inferences of *P. alecto* metapopulation history will determine connectivity between populations in Indonesia and Australia. The population dynamics of *P. alecto* are important from both a conservation and a disease perspective, especially given the different challenges each population faces. *Pteropus alecto*

populations are known to change roosting sites according to seasonal changes that reflect shifts in both food availability and the bat's reproductive cycle (Vardon et al., 2001). This suggests frequent migration between roost sites, but it remains unclear whether migration over water is common, or whether bats only migrate to other sites on the same land mass. Knowledge of population connectivity will better inform studies of pathogen evolution and transmission across international borders.

Methods

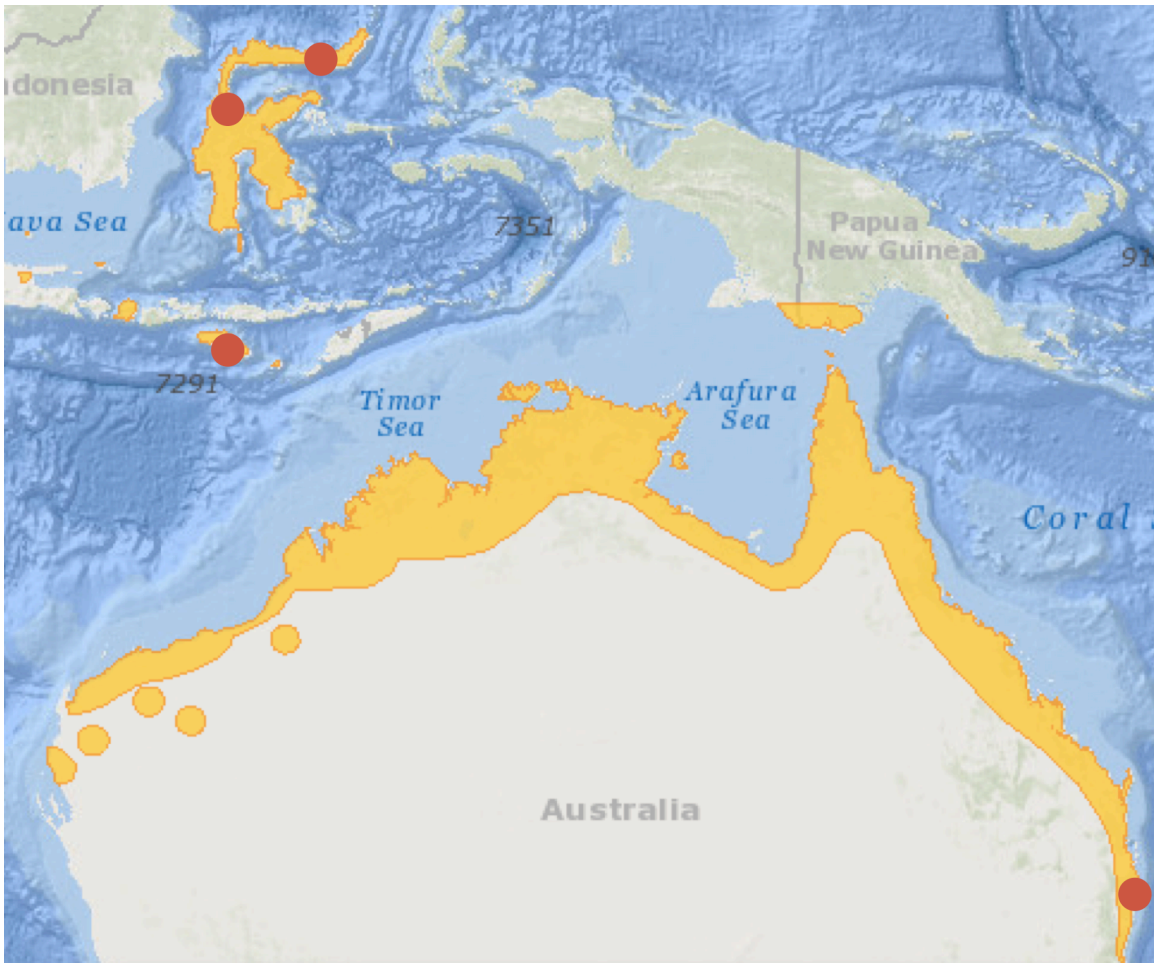


Figure 5.1. Map of sampling localities overlain on IUCN Red List range map for *P. alecto*. Red dots indicate where four populations were located.

Samples were collected from four localities for *P. alecto* (Fig. 5.1), totaling 24 individuals. Indonesian *P. alecto* were collected from both northern and central Sulawesi populations. Tissue loans from the Western Australian Museum and Australian Museum represented populations from the Lesser Sundas and eastern Australia, respectively. Methods of DNA sequencing and model selection were as described in Chapter 2. Four outgroup taxa were used to root the tree: *Pteropus vampyrus*, *P. admiralitatum*, *Acerodon celebensis*, and *Rousettus celebensis*. A phylogenetic tree was inferred using all available data and implemented in MrBayes 3.2 (Ronquist & Huelsenbeck, 2003), run for 10 million generations sampled every 1,000 generations and discarding the first 25% of sampled trees as burn-in. Haplotype networks were reconstructed as minimum spanning trees for each gene using genetic distances generated from MEGA6 (Tamura et al., 2013) and visualized using HapStar (Teacher & Griffiths, 2011). A clustering analysis was conducted in Structure (Pritchard et al., 2000) and the most probable number of populations (k) was chosen via the methods described by Evanno et al. (2005). Structure results indicated that $k = 3$ is the most probable scenario (Ln = -363.7). Therefore the Sulawesi localities were treated as a single population in additional analyses. The same population genetic indices were calculated as those from Chapter 4 for *P. vampyrus*.

The species tree from Chapter 2 suggested that populations of *P. alecto* in Australia are potentially interbreeding with other sympatric *Pteropus* species. Mitochondrial and nuclear gene tree discordance suggest that there are incomplete lineage sorting in nuclear genes, and potential hybridization in both mitochondrial genes. Based on the mitochondrial gene trees, the Australian *P. alecto* are hybridizing with *P. conspicillatus*, whereas the Lesser Sundaic *P. alecto* are hybridizing with *P. griseus* (Chapter 2). Previous research using both backcrossing observations and genetic data suggested the possibility of hybridization between *P. alecto* and both *P.*

poliocephalus (Webb & Tidemann, 1995) and *P. conspicillatus* (Fox, 2006). To test this hypothesis, I implemented a maximum likelihood comparison as in Lohse & Frantz (2014) to compare *P. alecto* populations (separated into Sulawesi and Australian populations) to *P. conspicillatus*. The analysis was not possible with *P. poliocephalus* due to the poor quality of the data (*i.e.*, too much missing data). This method required breaking down comparisons to triplet populations and small genomic blocks of 100bp each in order to increase efficiency in parameter estimation. Calculations were solved using notebooks for Mathematica 8 written by Lohse & Frantz (2014).

Results

Evidence of gene flow between the North and Central Sulawesi populations was detected in the phylogenetic analyses, as individuals from either population formed mixed clades (Fig. 5.2). Nucleotide and haplotype diversity (Table 5.1a) were similar to that of *P. vampyrus* presented in Chapter 4. Tajima's D were mostly not significant, meaning that genes were behaving neutrally. However, the allelic fixation index F_{ST} was higher for each locus in *P. alecto*, indicating a higher degree of population substructure than in *P. vampyrus*. As a result, the pairwise effective migration rate (Nm) was lower than in *P. vampyrus*. Both the phylogenetic tree and the haplotype networks for each gene support the conclusion that there is a minor degree of substructure, with the break being between Sulawesi and the Lesser Sundas-Australia. When considered by population, Sulawesi and Australia were both far more diverse than the Lesser Sundas (Table 5.1b).

The haplotype networks show a clear break between the Sulawesi population and the Lesser Sundaic-Australian populations, for instance, in both mitochondrial D-loop (Fig. 5.3a) and nuclear STAT5A (Fig. 5.3b) signal (all other haplotype networks in Appendix 5.1). F_{ST}

values between the Australian and Lesser Sundaic populations were smaller than between the Sulawesi and Lesser Sundaic populations, despite being geographically closer (Table 5.2). In the Sulawesi population, the star topology of many of the genes suggests a rapid expansion from a small founder population. This scenario would fit with what is known of *Pteropus* evolution and biogeographic history—that species may often be the result of dispersal or founder-effect speciation to islands (from biogeographic analyses in Chapter 3) and that the genus is experiencing population expansion after a recent bottleneck (from Tajima's D in Chapter 4). The low nucleotide and haplotype diversity within *P. alecto* also suggests a rapid expansion from a small founder population.

The potential hybridization of *P. alecto* with other sympatric *Pteropus* suggested in Chapter 2 may be the cause of the large break between Sulawesi and other populations of *P. alecto*. However, the calculations to test for hybridization using the Lohse and Frantz (2014) method were inconclusive due to insufficient variability in the data. *Pteropus alecto* is a rather young species from Wallacea that is approximately Pliocene in age (Chapters 2 and 3). The results of Chapter 2 suggest that the species expanded into the Lesser Sundas and Australia in the Pleistocene and hybridized with sympatric *Pteropus* species. To determine what admixture scenario is most likely in such a young clade, genomic level data will be needed. No hybridization in Sulawesi *P. alecto* was detected, though this may be because there are no other *Pteropus* species common to the main island of Sulawesi. Unlike in the Lesser Sundas and Australia, Sulawesi does not have any other extant flying foxes that are of a similar size.

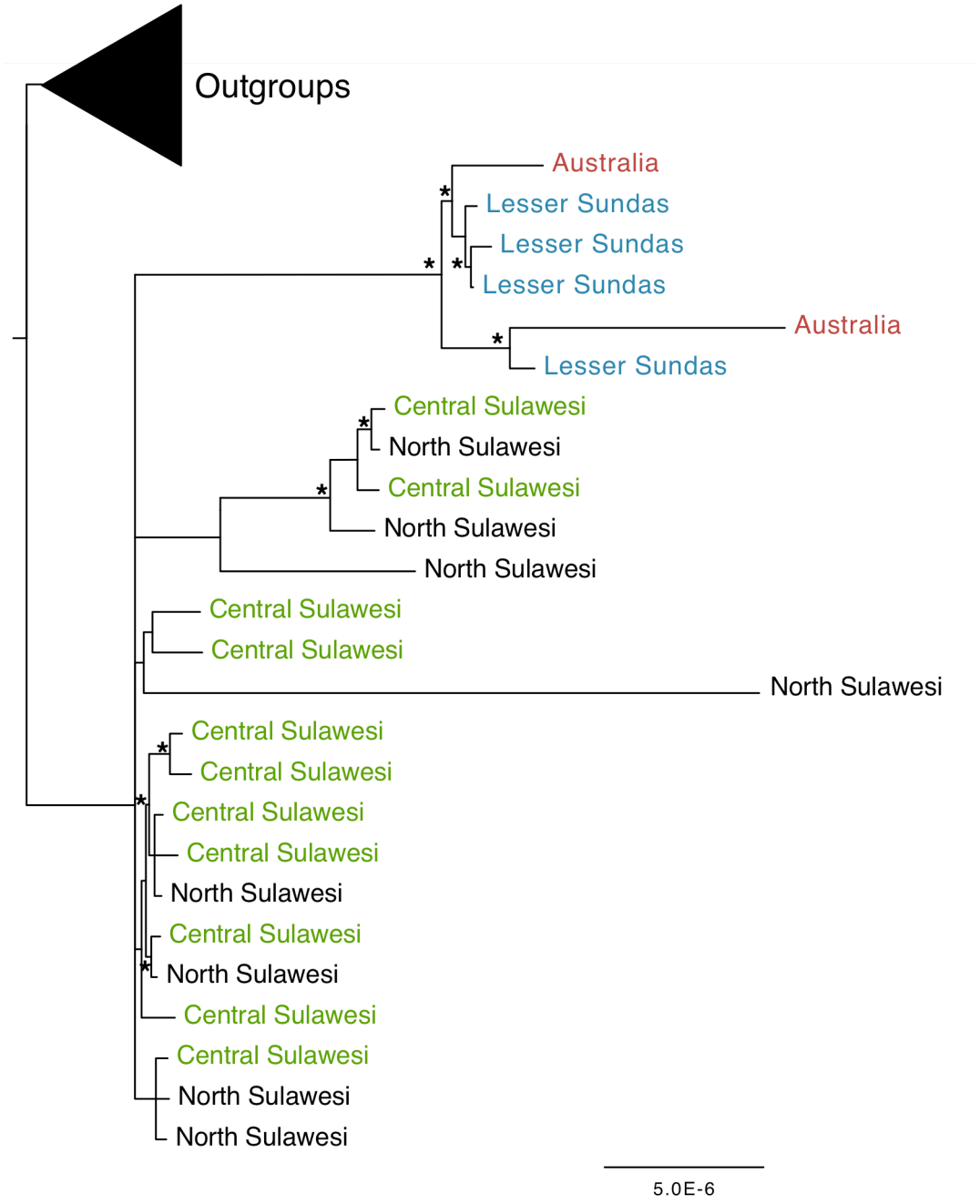


Figure 5.2. Phylogenetic tree of *Pteropus alecto* individuals inferred from MrBayes. Asterisks above nodes indicate posterior probabilities greater than 0.9. Central and North Sulawesi individuals intermix consistently, resulting in low posterior probabilities due to the lack of informative content in the data. Australian individuals were not monophyletic, both being more closely related to different Lesser Sundaic *P. alecto* than to each other.

Table 5.1. Genetic diversity indices for *Pteropus alecto.* F_{ST} values were generally higher than those for *P. vampyrus*, indicating at least some level of population substructure.

a) *Pteropus alecto*

Gene	π	*h*	θ	S	Tajima's D	D sig.	Fst	Nm
mitochondrial								
cyt-b	0.08352	0.992	0.09399	636	-2.1682	**, P < 0.01	0.08608	2.65
D-loop	0.32837	1	0.5841	308	2.50725	*, P < 0.05	0.04688	5.08
nuclear								
RAG-1	0.00261	0.279	0.00262	6	-0.46942	NS	0.11093	2
RAG-2	0.0039	0.903	0.00713	18	-1.62046	NS	0.11214	1.98
STAT5A	0.00955	0.725	0.00968	14	-1.02868	NS	0.25055	0.75
PLCB4	0.00026	0.071	0.00026	1	-1.15142	NS	0.05003	4.75
BDNF	0.00126	0.271	0.00126	5	-1.79547	NS	0.05486	4.31
FGB7	0.00354	0.818	0.00356	15	-1.85999	*, P < 0.05	0.05116	4.64
PSMB8	0.0064	0.934	0.00645	11	-0.69829	NS	0.05723	4.12
COPS7A4	0.00658	0.866	0.00758	12	-0.4627	NS	0.11432	1.94
ATP7A	0.00117	0.279	0.00117	8	-2.12302	*, P < 0.05	0.0577	4.08

b) Combined for all genes comparisons by population

	π	S
Australia	0.5099	206
Lesser Sundas	0.08705	68
Sulawesi	0.2842	281

Table 5.2. Comparison of F_{ST} values between each population pair. The Lesser Sundaic population is more similar to Australian population, despite being geographically closer to Sulawesi.

Population 1	Population 2	FST
Australia	Lesser Sundas	0.02406
Lesser Sundas	Sulawesi	0.51851
Sulawesi	Australia	0.12521

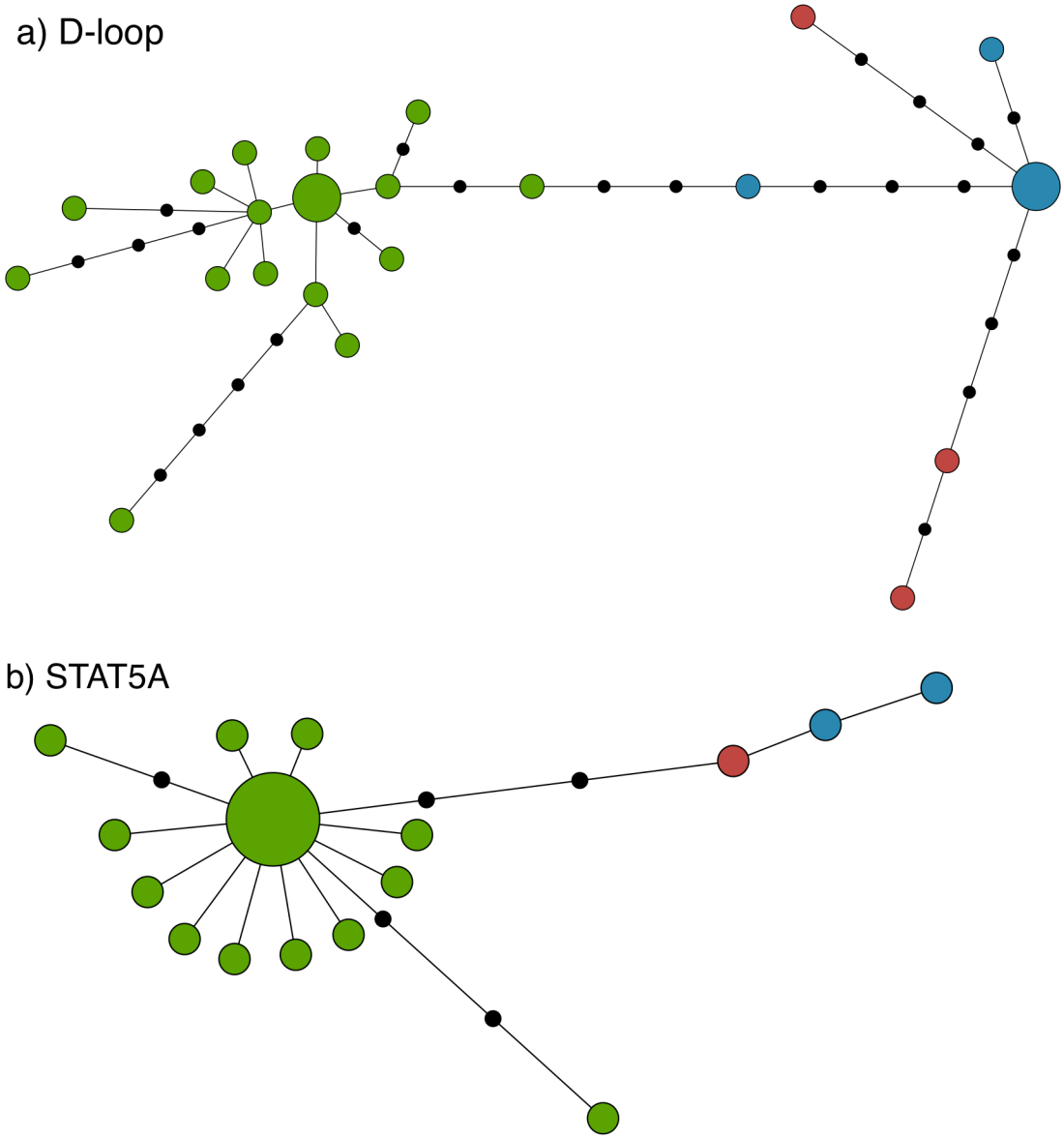


Figure 5.3. Haplotype networks of *P. alecto* for a) D-loop and b) STAT5A. Populations are Sulawesi (green), Lesser Sundas (blue), and Australia (red). Smallest circles represent a single individual and are scaled up according to number of haplotypes. Black dots indicate a single base pair change. The Sulawesi population is entirely distinct from the Lesser Sundaic and Australian populations in all loci.

Discussion

The metapopulation breaks among Sulawesi, the Lesser Sundas, and Australia agree with recognized subspecies designations in *Pteropus alecto* (Corbet & Hill, 1992), which demonstrate some degree of morphological variation. *Pteropus a. alecto* (Sulawesi) is the largest of the subspecies, *P. a. morio* (Lesser Sundas) is the smallest, and *P. a. gouldi* (Australia) has a narrower rostrum, palate, and smaller teeth (Bergmans & Rozendaal, 1988; Corbet & Hill, 1992). Variation in skull morphology of *P. a. gouldi* may result in part from hybridization with other Australian *Pteropus*, as intermediary morphological traits have been noted in previous research (Webb & Tidemann, 1995).

Recognizing distinct populations of *P. alecto* has direct consequences for conservation management decisions. *Pteropus alecto* is currently listed as Least Concern by the IUCN Red List across its range, but *P. a. alecto* faces an unsustainable level of hunting in Sulawesi that must be addressed. While *P. alecto* may be thriving in Australia, Sulawesi populations have experienced steep declines since the 1970's (Lee et al., 2005; Sheherazade & Tsang, 2015). The Sulawesi populations of *P. alecto* all act as one gene pool, and the rapid loss of colonies in the north, along with expanding bushmeat trade into other provinces in Sulawesi, cannot be ignored if populations in Sulawesi are to be preserved. The species is now locally extirpated in North Sulawesi province (Lee et al., 2005; Sheherazade & Tsang, 2015) and no legal protection exists to curtail this loss. There is also no formal protection for the small handful (< 10) of large roosts that still exist in Sulawesi for *P. alecto*. These factors would categorize *P. alecto* as Near Threatened within its Indonesian range, and reevaluation by the IUCN Red List may be necessary to ensure this issue attracts enough attention to spur conservation actions. The effect that *P. alecto* population crashes have on Sulawesi flora is unknown, as there are no studies

about specific diets or host plant associations on Sulawesi. These data are essential if new agroforestry developments are to make sustainable choices that promote native biodiversity instead of harm it.

The geographic break between *P. alecto* populations across the Java Sea is unexpected based on distance or geologic history. The distance from Sulawesi to the Lesser Sundas is approximately 800 km whereas the distance between the Lesser Sundas and eastern Australia is approximately 3000 km. Isolation by distance would predict a closer relationship between Sulawesi and Lesser Sundaic populations instead, but this is not what was observed. Plant distribution data also suggests a close connection between Java, the Lesser Sundas, and Sulawesi in the recent past (Whitten et al., 2013). Why the Java Sea acts as an effective population barrier for P. alecto whereas the Timor Sea does not, despite being about the same distance across, is not clear from the data available. The potential for isolation by distance in *P. alecto* populations from the Lesser Sundas to eastern Australia could be more accurately tested if samples from northern Australia (Northern Territory, Western Australia) were available. However, this region is not as well-represented in collections due to its relative inaccessibility as compared to colonies near populated areas of eastern Australia. Colonies of *P. alecto* from northern Australia would be geographically closer to the Lesser Sundas (700 km) than to eastern Australia (3000 km), but bats would have to cross over an oceanic expanse to migrate from the Lesser Sundas to northern Australia. This may present different challenges than overland dispersal and as such dispersal may not be as frequent. This could be tested if individuals from northern Australia were available to determine to which population they are more closely related.

Based on what is known from other mammalian parasites, the high genetic variability found in both Sulawesi and Australian populations suggests that pathogens that occur in these

populations might be more diverse and more aggressive (Nunn et al., 2004; Carlsson-Graner & Thrall, 2015; Huang et al., 2015). Why outbreaks of paramyxoviruses have been reported in Australia but not Sulawesi is unclear, but the data presented here suggest nucleotide diversity in Australia is even higher than Sulawesi, and perhaps that has led to the evolution of more aggressive parasites. Additionally, eastern Australian *Pteropus* are often found in urbanized areas and may experience a chronic elevated stress response that may deleteriously affect the bats as reservoir hosts and shift the equilibrium dynamics between host and pathogen (Bradley & Altizer, 2007; Plowright et al., 2008; Brearley et al., 2012). Higher levels of infection by Hendra virus in another Australian *Pteropus* species has previously been associated with nutritional stress, meaning habitat loss and climate change may also increase pathogen outbreaks (Plowright et al., 2008). Sulawesi *P. alecto* may not experience the level of stress seen in Australian populations, and in the past decade, rates of logging in Sulawesi have been low compared to other Indonesian islands (Margono et al., 2014). However, palm oil production is slated to increase rapidly in Sulawesi (Shean, 2009) and the effect this may have on pteropodid communities is largely unknown. The potential for an outbreak is high given the increasing degree of contact between *P. alecto*, humans, and potential intermediary hosts that are experiencing high levels of stress in bushmeat markets (Sheherazade & Tsang, 2015).

The higher degree of genetic diversity in Australian populations compared with other *P. alecto* populations may potentially be a result of putative admixture with other sympatric *Pteropus* species. If new haplotypes are often introduced into Australian *P. alecto* by hybridization events, that would increase the observed nucleotide diversity, especially in cases of hybridization with *P. poliocephalus* (Webb & Tidemann, 1995), with which is relatively distantly related. No genomic tests of hybridization have been conducted to determine how

common this is in Australia, which acts as a contact zone for three similarly-sized *Pteropus* species with some degree of niche overlap. Range expansions have been noted for *P. alecto* and *P. poliocephalus* in the past decade (Roberts et al., 2012b), and increasing frequency of shared camps may increase interspecific contact for pathogen transmission as well.

During field collection, concerted efforts were made to collect RNALater-preserved samples of the liver of *P. alecto*, especially since there are few samples available from Indonesian populations. A recent study of *P. alecto* and *Acerodon celebensis* from Sulawesi found novel rubulavirus-like and henipavirus-like pathogens (Sasaki et al., 2012), but little is known beyond these initial discoveries. Fecal and anal swabs were screened for viral isolates at CSIRO (Australia) and positives were sent to Duke-NUS (Singapore) for deep-sequencing of transcriptomes. However, these data were not available at the time of writing this dissertation and analyses of co-evolution between host and pathogen could not be conducted. Tests of convergence of host and viral phylogenies will be implemented in Jane (Conow et al., 2010) when possible. I predict that any viruses found in Sulawesi will be distinct from those in Australia, though there will likely be similar degrees of viral diversity in each population. Given that *P. alecto* often co-roost with *A. celebensis*, viruses in *P. alecto* could potentially jump between the two closely related host species. I predict that pathogens are more phylogenetically similar between these co-occurring host species than they are to pathogens found in Australian *P. alecto*.

The population structure presented here for *P. alecto* is in direct contrast to that of *P. vampyrus* from Chapter 4. *Pteropus vampyrus* has an essentially panmictic population, requiring a multinational strategy involving most of the member nations of ASEAN in order to address conservation and public health challenges. *Pteropus alecto*, on the other hand, has populations

that are rather distinct from one another genetically and face unique challenges in each part of their range. Management of *P. alecto* in the Lesser Sundas may involve transnational agreements with Australia, but more data should first be collected to determine the degree of connectivity between those populations. Pathogens would not have as geographically widespread a gene pool as they do in *P. vampyrus* but may instead be endemic to specific populations of *P. alecto* hosts and their diversity, abundance, and transmission rates may be controlled by different ecological factors. These ecological data are more readily available for Australian *P. alecto* but more concerted studies of natural history in Sulawesi and the Lesser Sundas are needed to be able to make comparisons among ecological correlates for pathogen prevalence among populations.

Conclusion

Pteropus alecto is extensively studied for biomedical reasons only in a part of its range. These types of studies should be extended to populations in Indonesia, which suffer from a paucity of data. Systematic surveys of Sulawesi and Lesser Sundaic pteropodids are not very common in the past decade, yet they may have important consequences for management of agroforestry development and maintenance of native plant diversity. The distinctiveness of the Sulawesi population points to a need for more study in order to address conservation challenges it faces. No effective conservation plans can be properly made if so little is known about what should be preserved in Sulawesi to promote species persistence.

CHAPTER 6

Concluding remarks and the future of research on the evolution of *Pteropus*

From the research conducted in this dissertation, I have produced a more comprehensive hypothesis of *Pteropus* species relationships by including previously unobtainable Southeast Asian *Pteropus* species. From this species tree, I was able to establish that most *Pteropus* lineages are the result of rapid radiations during the Pliocene. Rampant incomplete lineage sorting issues and putative hybridization detected from discordant nuclear and mitochondrial genes point to a need for genomic-scale datasets for understanding the evolutionary history of the genus. Relatively young clade age, short internodal periods, and rapid diversification makes *Pteropus* the ideal model system for answering questions related to hybridization, lineage sorting, and radiations using next-generation sequencing data. The genus originated in Wallacea and its high vagility allowed it to colonize new landmasses throughout the Indo-Australian Archipelago and South Pacific with dispersal and founder-event speciation as the mechanistic forces generating biogeographic distributions. These findings highlight the need to shift biogeographic theory away from the null model of vicariance, which is unsuitable for understanding phenomena that have driven distributions in this genus.

A basic understanding of flying fox evolution is critical to future conservation management and pandemic containment planning. In the latter chapters, case studies of two *Pteropus* species highlighted the different predictions in viral diversity and aggressiveness based on population connectivity and genetic diversity of the host system. Combined with viral data, metapopulation networks of host species can provide a clearer understanding of how pathogens may spread. Different population structures also mean conservation planning has to occur on different scales. For *P. vampyrus*, it requires the cooperation of many ASEAN member nations,

whereas for *P. alecto*, it requires action primarily on a provincial level. However, in both of these cases, the need for more natural history data on *Pteropus* species is underscored. For disease ecology, ecological factors leading to depression in host immunity leading to pathogen transmission cannot be modeled without accurate host natural history data. In terms of conservation—host plant associations, roosting ecology, and other basic life history data are unknown for most species, and understanding of species' fundamental niche and ecological role would inform and assist plans towards their protection and persistence.

This is the ideal time to research *Pteropus*. Armed with a basic understanding of the evolutionary and biogeographic history of the genus, questions regarding overlapping subspecies complexes and hybrid contact zones can be better addressed. High-throughput sequencing costs are decreasing rapidly and these methods are now a viable option for studying non-model organisms. New field studies to collect samples and natural history information and use of museum specimens can be combined with new sequencing technologies to address questions regarding community assemblages, biogeography, and host immunity. Working with *Pteropus* will undoubtedly lead to maximization of existing collections-based resources as well, since hundred year-old skins can be informative of baseline environmental conditions or tell a different story of adaptation to changing landscapes. In this dynamic and charismatic genus, these many different and relevant scientific phenomena can be highlighted, and in turn, used to educate others about the connection that biodiversity has to our own continued existence and well-being.

APPENDIX 2.1

List of specimens included in this study. Specimen codes follow museum catalog IDs or field numbers. Localities are listed according to major islands or archipelagoes.

Sample Code	Species	Island	Collection
M50	*Pteropus hypomelanus*	Halmahera	Wild
M64	*Pteropus caniceps*	Halmahera	Wild
SS002	*Pteropus alecto*	Sulawesi	Wild
SS004	*Pteropus alecto*	Sulawesi	Wild
SS007	*Pteropus alecto*	Sulawesi	Wild
SS033	*Pteropus alecto*	Sulawesi	Wild
SS034	*Pteropus alecto*	Sulawesi	Wild
SS035	*Pteropus alecto*	Sulawesi	Wild
SS036	*Pteropus alecto*	Sulawesi	Wild
SS037	*Pteropus alecto*	Sulawesi	Wild
SS038	*Pteropus alecto*	Sulawesi	Wild
SS039	*Pteropus alecto*	Sulawesi	Wild
SS040	*Pteropus alecto*	Sulawesi	Wild
SS041	*Pteropus alecto*	Sulawesi	Wild
SS049	*Pteropus alecto*	Sulawesi	Wild
SS050	*Pteropus alecto*	Sulawesi	Wild
SS051	*Pteropus hypomelanus*	Sangihe Islands	Wild
SS052	*Pteropus hypomelanus*	Sangihe Islands	Wild
SS053	*Pteropus hypomelanus*	Sangihe Islands	Wild
SS054	*Pteropus alecto*	Sulawesi	Wild
SS057	*Pteropus alecto*	Sulawesi	Wild
SS058	*Pteropus alecto*	Sulawesi	Wild
SS059	*Pteropus alecto*	Sulawesi	Wild
SS060	*Pteropus alecto*	Sulawesi	Wild
SW001	*Pteropus vampyrus*	Java	Wild
SW002	*Pteropus vampyrus*	Java	Wild
SW003	*Pteropus vampyrus*	Java	Wild
SW006	*Pteropus vampyrus*	Java	Wild
SW007	*Pteropus vampyrus*	Bali	Wild
SW008	*Pteropus vampyrus*	Bali	Wild
SW009	*Pteropus vampyrus*	Bali	Wild
SW010	*Pteropus vampyrus*	Bali	Wild
SW011	*Pteropus vampyrus*	Bali	Wild
SW012	*Pteropus hypomelanus*	Madura	Wild
SW013	*Pteropus hypomelanus*	Madura	Wild
SW014	*Pteropus hypomelanus*	Madura	Wild

SW077	*Pteropus vampyrus*	Java	Wild
SW078	*Pteropus vampyrus*	Java	Wild
SW105	*Pteropus chrysoproctus*	Seram, Maluku	Wild
SW106	*Pteropus chrysoproctus*	Seram, Maluku	Wild
SW107	*Pteropus chrysoproctus*	Seram, Maluku	Wild
SW108	*Pteropus chrysoproctus*	Seram, Maluku	Wild
SW120	*Pteropus temminckii*	Seram, Maluku	Wild
SW121	*Pteropus melanopogon*	Seram, Maluku	Wild
SW123	*Pteropus temminckii*	Seram, Maluku	Wild
SW124	*Pteropus temminckii*	Seram, Maluku	Wild
SW126	*Pteropus ocularis*	Seram, Maluku	Wild
SW127	*Pteropus vampyrus*	Kalimantan	Wild
SW128	*Pteropus vampyrus*	Kalimantan	Wild
SW131	*Pteropus vampyrus*	Sumatra	Wild
SW132	*Pteropus vampyrus*	Sumatra	Wild
SW133	*Pteropus vampyrus*	Flores, Lesser Sundas	Wild
SW134	*Pteropus hypomelanus*	Flores, Lesser Sundas	Wild
SW140	*Pteropus vampyrus*	Sumbawa, Lesser Sundas	Wild
SW143	*Pteropus lombocensis*	Lombok	Wild
SW144	*Pteropus lombocensis*	Lombok	Wild
SW145	*Pteropus lombocensis*	Lombok	Wild
T24	*Pteropus personatus*	Ternate	Wild
T26	*Pteropus personatus*	Ternate	Wild
T41	*Pteropus personatus*	Ternate	Wild
MJV419	*Pteropus hypomelanus*	Philippines	Wild
MJV420	*Pteropus vampyrus*	Philippines	Wild
MJV435	*Pteropus vampyrus*	Philippines	Wild
MJV436	*Pteropus vampyrus*	Philippines	Wild
MJV504	*Pteropus vampyrus*	Philippines	Wild
MJV505	*Pteropus vampyrus*	Philippines	Wild
MJV451	*Pteropus dasymallus*	Philippines	Wild
MJV458	*Pteropus dasymallus*	Philippines	Wild
SMT214	*Pteropus pumilus*	Philippines	Wild
SMT207	*Acerodon jubatus*	Philippines	Wild
SMT208	*Pteropus vampyrus*	Philippines	Wild
SMT209	*Pteropus hypomelanus*	Philippines	Wild
SMT210	*Pteropus vampyrus*	Philippines	Wild
SMT211	*Pteropus vampyrus*	Philippines	Wild
SMT212	*Pteropus vampyrus*	Philippines	Wild
SMT213	*Pteropus vampyrus*	Philippines	Wild
PD-3542	*Pteropus dasymallus*	Japan	Wild

PD-3543	*Pteropus dasymallus*	Japan	Wild
Pp010-Ppse	*Pteropus pselaphon*	Japan	Wild
Pp009-Ppse	*Pteropus pselaphon*	Japan	Wild
PV-904502	*Pteropus vampyrus*	Java	LBC
PV-904503	*Pteropus vampyrus*	Java	LBC
PV-930088	*Pteropus vampyrus*	Sumatra	LBC
PV-930089	*Pteropus vampyrus*	Sumatra	LBC
PV-930091	*Pteropus vampyrus*	Sumatra	LBC
PV-930092	*Pteropus vampyrus*	Sumatra	LBC
PV-930093	*Pteropus vampyrus*	Sumatra	LBC
PH-904525	*Pteropus hypomelanus*	Java	LBC
PH-904528	*Pteropus hypomelanus*	Java	LBC
PH-904529	*Pteropus hypomelanus*	Java	LBC
PH-904530	*Pteropus hypomelanus*	Java	LBC
PH-904540	*Pteropus hypomelanus*	Java	LBC
PP-929212	*Pteropus pumilus*	Philippines	LBC
PP-929213	*Pteropus pumilus*	Philippines	LBC
PA-30431	*Pteropus alecto*	Sumba, Lesser Sundas	WAM
PA-30434	*Pteropus alecto*	Sumba, Lesser Sundas	WAM
PA-30435	*Pteropus alecto*	Sumba, Lesser Sundas	WAM
PA-30436	*Pteropus alecto*	Sumba, Lesser Sundas	WAM
PL-32153	*Pteropus lombocensis*	Lomblen, Lesser Sundas	WAM
PL-32154	*Pteropus lombocensis*	Lomblen, Lesser Sundas	WAM
PL-37757	*Pteropus lombocensis*	Alor, Lesser Sundas	WAM
PL-37758	*Pteropus lombocensis*	Alor, Lesser Sundas	WAM
PG-35398	*Pteropus griseus*	Roti, Lesser Sundas	WAM
PG-35400	*Pteropus griseus*	Roti, Lesser Sundas	WAM
PG-42026	*Pteropus griseus*	Banda Islands	WAM
PG-42047	*Pteropus griseus*	Banda Islands	WAM
G1339	*Pteropus vampyrus*	Singapore	RMBR
CM-NK10524	*Pteropus giganteus*	India	CMNH
CM-NK10523	*Pteropus giganteus*	India	CMNH
ROM-110948	*Pteropus vampyrus*	Vietnam	ROM
ROM-110949	*Pteropus vampyrus*	Vietnam	ROM
ROM-44269	*Pteropus lylei*	Vietnam	ROM
ROM-44270	*Pteropus lylei*	Vietnam	ROM
FMNH-LRH4261	*Pteropus pumilus*	Philippines	FMNH
FMNH-SMG2872	*Pteropus pumilus*	Philippines	FMNH
MVZ-185262	*Pteropus mariannus yapensis*	Caroline Islands	MVZ
MVZ-140201	*Pteropus conspicillatus*	Papua New Guinea	MVZ
AMNH-124961	*Pteropus neohibernicus*	Papua New Guinea	AMNH

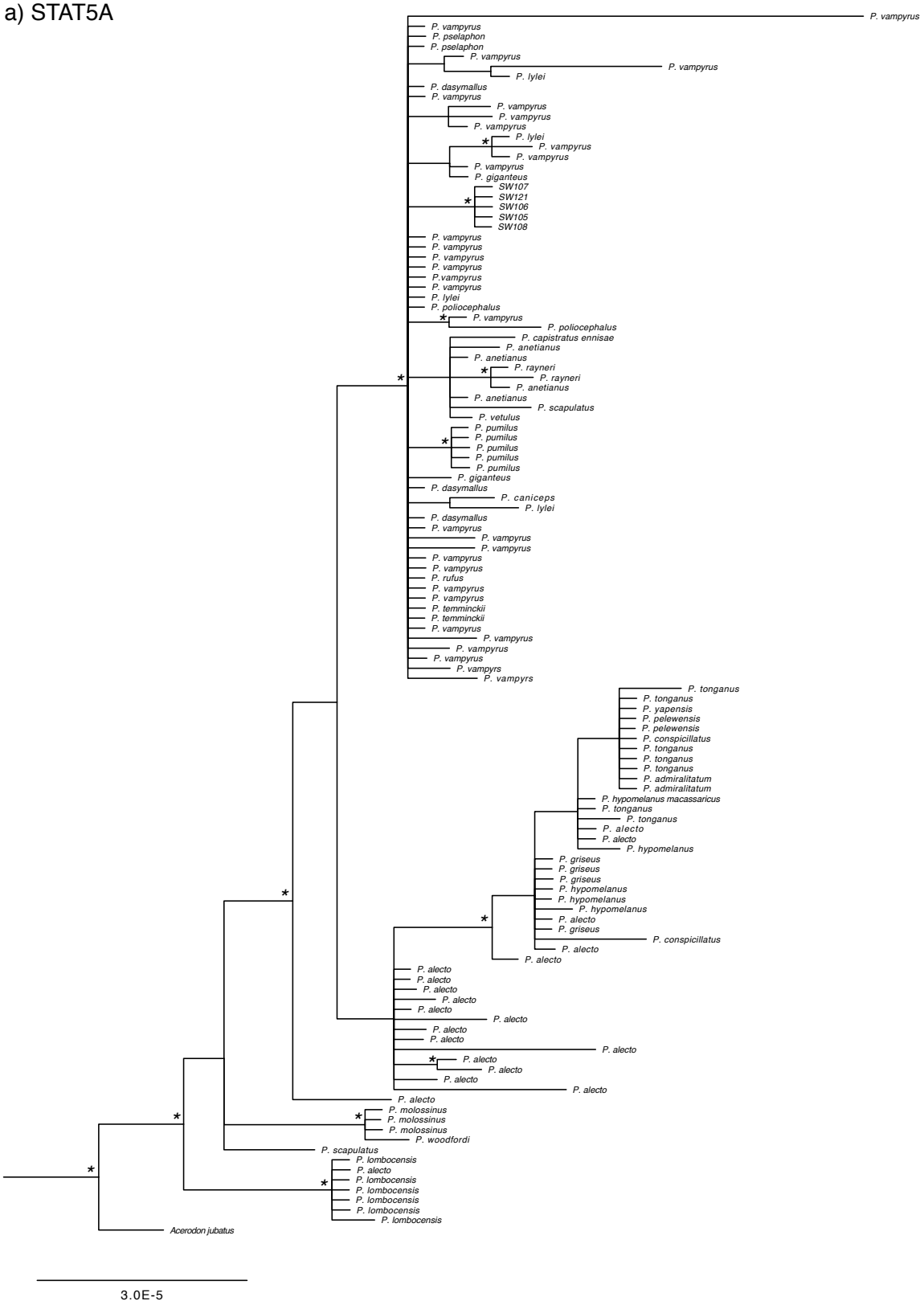
AMNH-124962	*Pteropus tonganus*	Vanuatu	AMNH
AMNH-124963	*Pteropus anetianus*	Vanuatu	AMNH
AMNH-124964	*Pteropus woodfordi*	Solomon Islands	AMNH
AMNH-124965	*Pteropus samoensis*	Samoa Islands	AMNH
AMS-M19905	*Pteropus admiralitatum*	Papua New Guinea	AMS
AMS-M20916	*Pteropus admiralitatum*	Papua New Guinea	AMS
AMS-M21582	*Pteropus conspicillatus*	Papua New Guinea	AMS
AMS-M21583	*Pteropus conspicillatus*	Papua New Guinea	AMS
AMS-M22336	*Pteropus rayneri*	Solomon Islands	AMS
AMS-M22337	*Pteropus rayneri*	Solomon Islands	AMS
AMS-M23590	*Pteropus niger*	Mauritius	AMS
AMS-M23778	*Pteropus vetulus*	New Caledonia	AMS
AMS-M24451	*Pteropus vetulus*	New Caledonia	AMS
AMS-M32409	*Pteropus alecto*	Australia	AMS
AMS-M32440	*Pteropus scapulatus*	Australia	AMS
AMS-M32441	*Pteropus scapulatus*	Australia	AMS
AMS-M32564	*Pteropus alecto*	Australia	AMS
AMS-M35495	*Pteropus poliocephalus*	Australia	AMS
AMS-M35496	*Pteropus poliocephalus*	Australia	AMS
LACM-74688	*Pteropus scapulatus*	Australia	LACM
LACM-91177	*Pteropus anetianus*	Vanuatu	LACM
LACM-91178	*Pteropus anetianus*	Vanuatu	LACM
LACM-91182	*Pteropus anetianus*	Vanuatu	LACM
LACM-91185	*Pteropus tonganus*	Vanuatu	LACM
LACM-91186	*Pteropus tonganus*	Vanuatu	LACM
UAM-104219	*Pteropus lylei*	Cambodia	UAM
UAM-104235	*Pteropus lylei*	Cambodia	UAM
UAM-104237	*Pteropus lylei*	Cambodia	UAM
UAM-104238	*Pteropus lylei*	Cambodia	UAM
UWMZ-M27499	*Pteropus rufus*	Madagascar	UWMZ
UWMZ-M27533	*Pteropus rufus*	Madagascar	UWMZ
UWMZ-M27989	*Pteropus capistratus*	Papua New Guinea	UWMZ
UWMZ-M27990	*Pteropus neohibernicus*	Papua New Guinea	UWMZ
USMN-566567	*Pteropus molossinus*	Caroline Islands	USNM
USNM-566565	*Pteropus molossinus*	Caroline Islands	USNM
USNM-566568	*Pteropus molossinus*	Caroline Islands	USNM
USNM-566587	*Pteropus pelewensis*	Palau	USNM
USNM-566588	*Pteropus pelewensis*	Palau	USNM
USNM-566597	*Pteropus tonganus*	Samoa Islands	USNM
USNM-566599	*Pteropus tonganus*	Samoa Islands	USNM
USNM-566601	*Pteropus tonganus*	Samoa Islands	USNM

USNM-566803	*Pteropus tonganus*	Samoa Islands	USNM
USNM-567239	*Pteropus pelewensis*	Palau	USNM
USNM-580018	*Pteropus capistratus ennisae*	Papua New Guinea	USNM
USNM-580021	*Pteropus gilliardorum*	Papua New Guinea	USNM
USNM-LHE1043	*Pteropus gilliardorum*	Papua New Guinea	USNM
USNM-LHE1009	*Pteropus capistratus ennisae*	Papua New Guinea	USNM
Outgroup taxa			
SS025	*Macroglossus minimus*	Sulawesi	Wild
SS026	*Rousettus celebensis*	Sulawesi	Wild
SS027	*Chironax melanocephalus*	Sulawesi	Wild
SS028	*Rousettus linduensis*	Sulawesi	Wild
SS029	*Macroglossus minimus*	Sulawesi	Wild
SS030	*Acerodon celebensis*	Sulawesi	Wild
SS031	*Acerodon celebensis*	Sulawesi	Wild
SS032	*Acerodon celebensis*	Sulawesi	Wild
SS064	*Nyctimene cephalotes*	Seram, Maluku	Wild
SS065	*Nyctimene cephalotes*	Seram, Maluku	Wild
SS066	*Syconycteris australis*	Seram, Maluku	Wild
SS067	*Syconycteris australis*	Seram, Maluku	Wild
SS068	*Dobsonia viridis*	Seram, Maluku	Wild
SS069	*Macroglossus minimus*	Seram, Maluku	Wild
SS070	*Dobsonia viridis*	Seram, Maluku	Wild
SW125	*Rousettus amplexicaudatus*	Seram, Maluku	Wild
SW146	*Acerodon* sp. Lombok	Lombok	Wild
JBS111	*Acerodon jubatus*	Philippines	Wild
MJV418	*Acerodon jubatus*	Philippines	Wild

APPENDIX 2.2

Topologies of all mitochondrial and nuclear gene trees. Asterisks indicate posterior probabilities above 0.9. Outgroups, except for *Acerodon* species, were excised for easier viewing since genetic distances were quite large in some cases. Not all individuals were sequenced at every gene. Discordance between nuclear and mitochondrial signals are in part due to incomplete lineage sorting. However, in some cases where multiple sympatric congeners occur, there was weak evidence for hybridization.

a) STAT5A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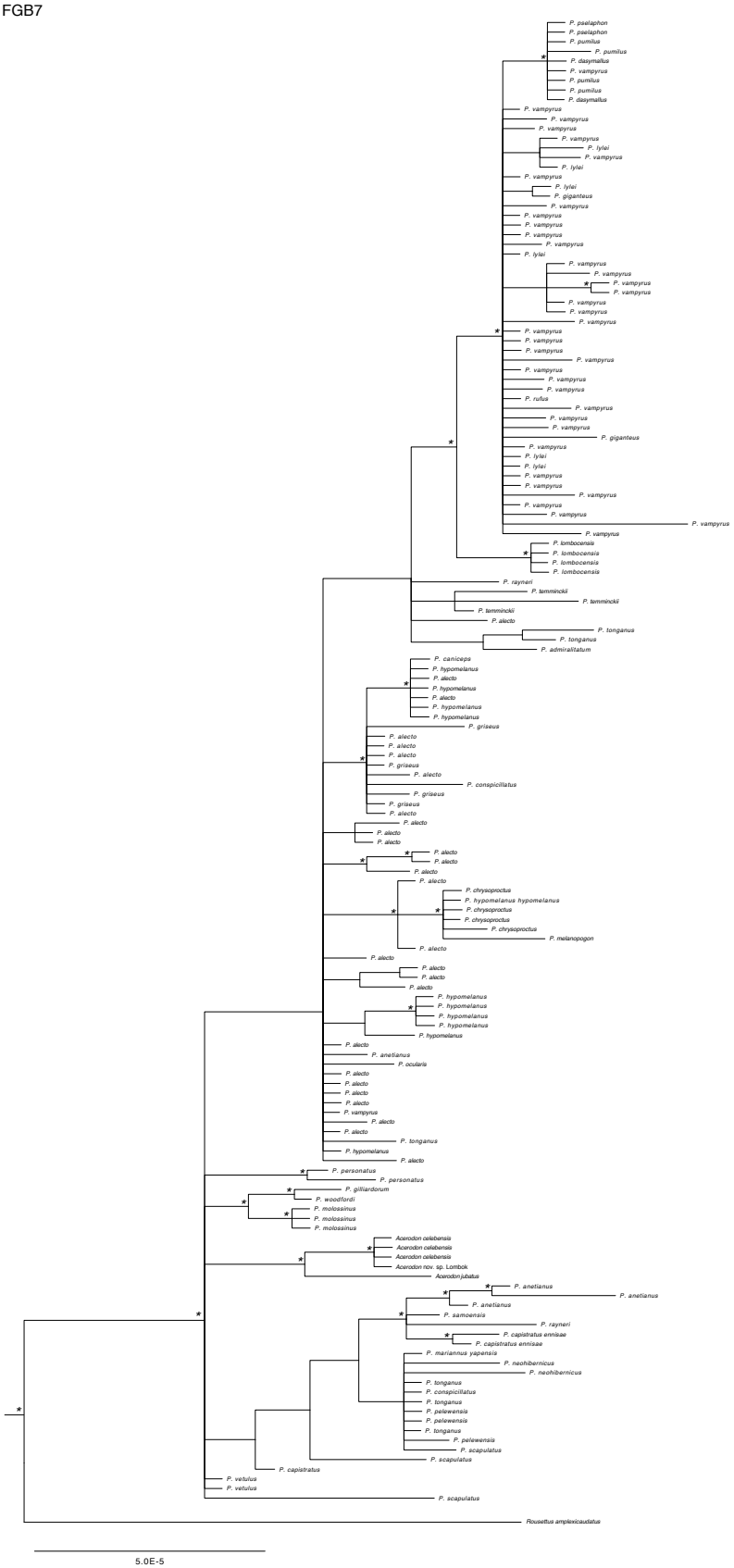
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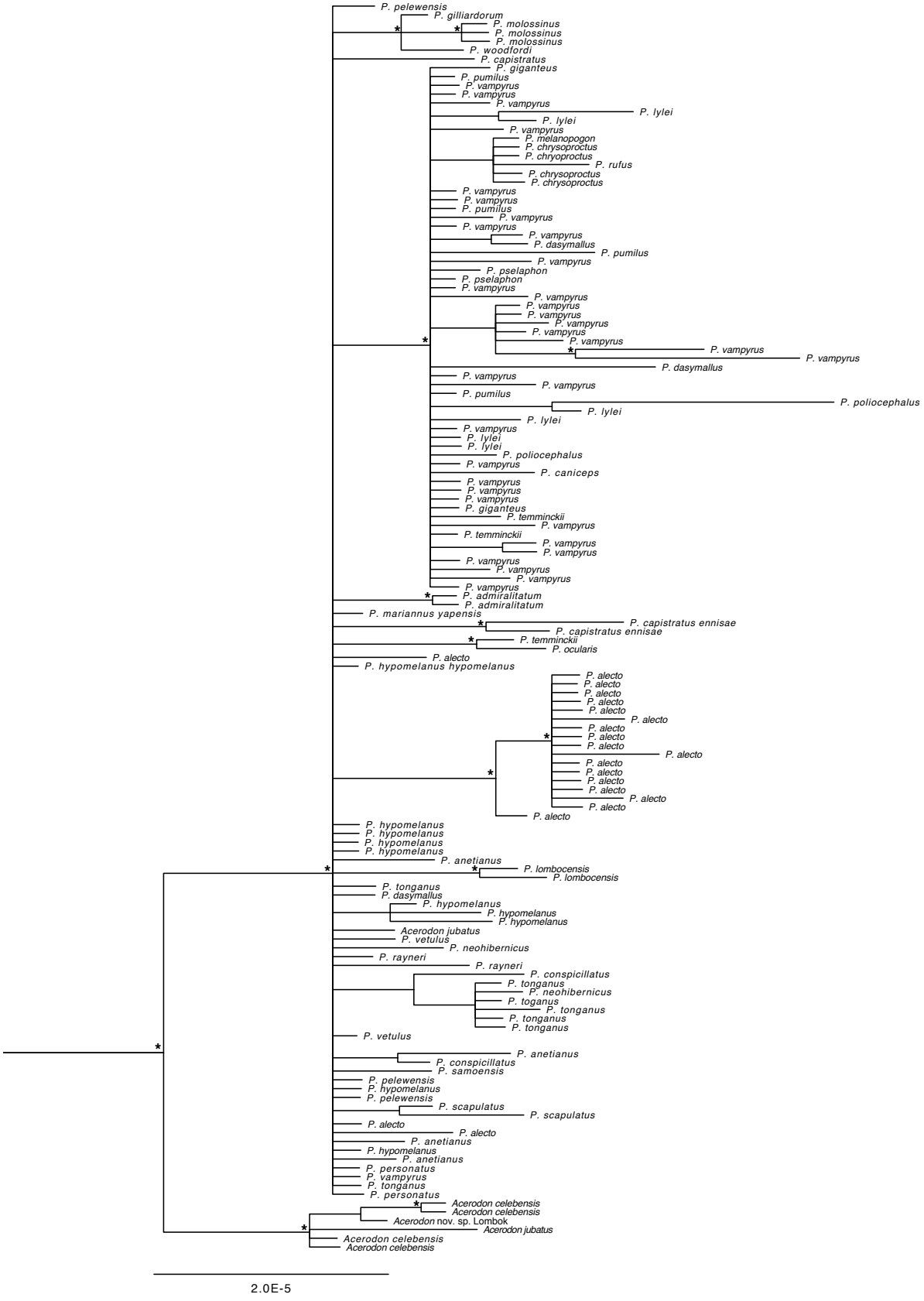
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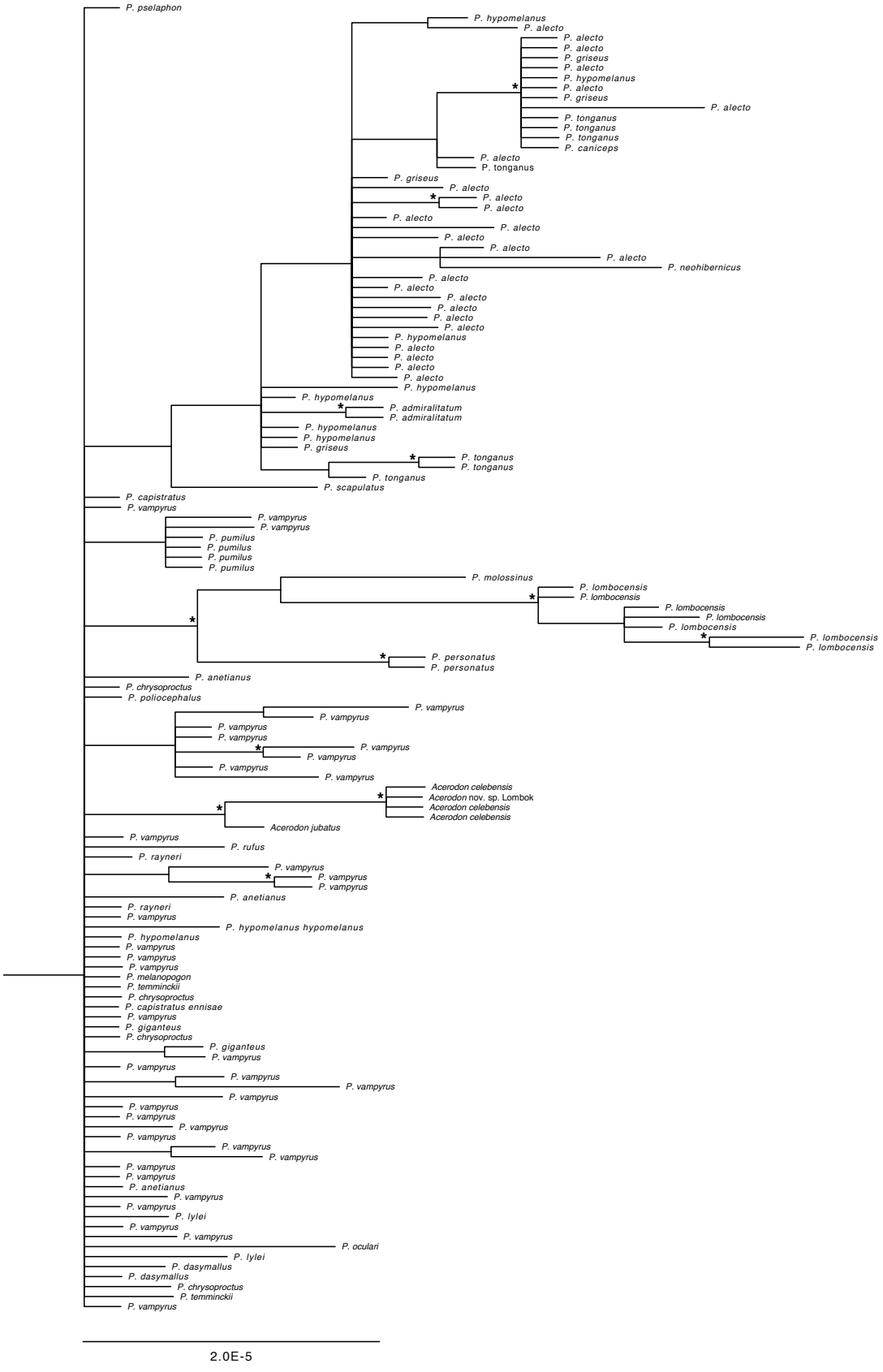
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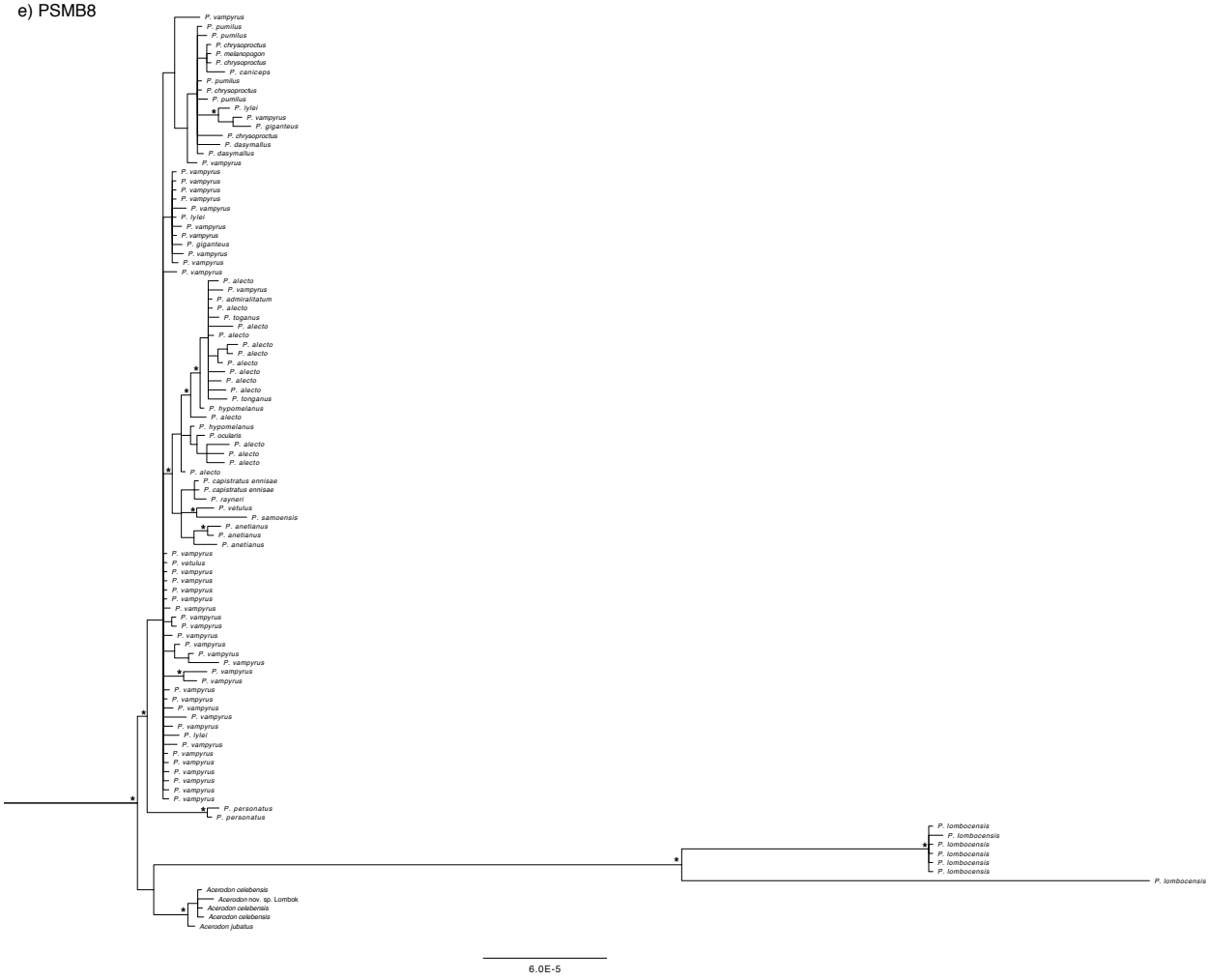
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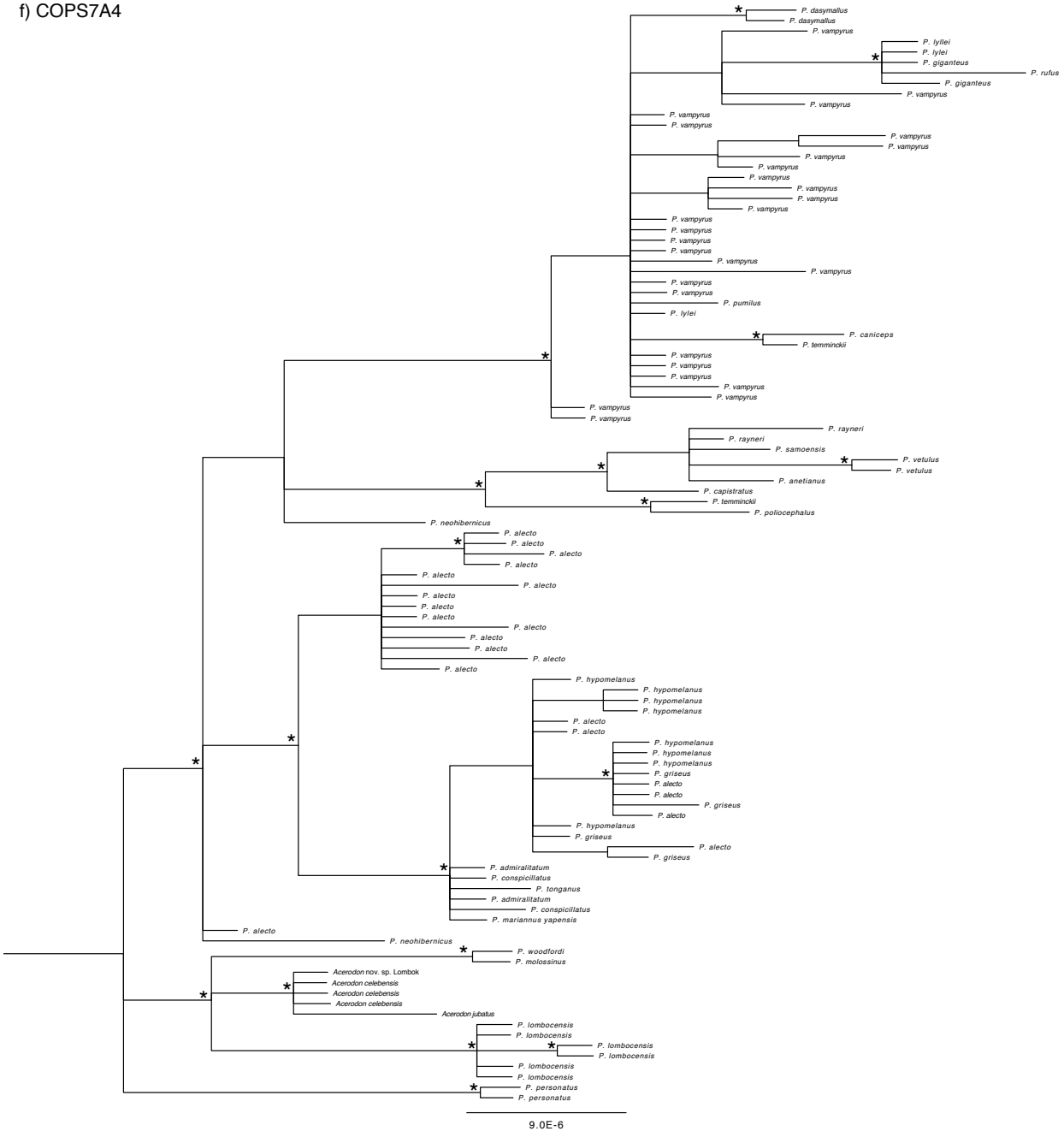
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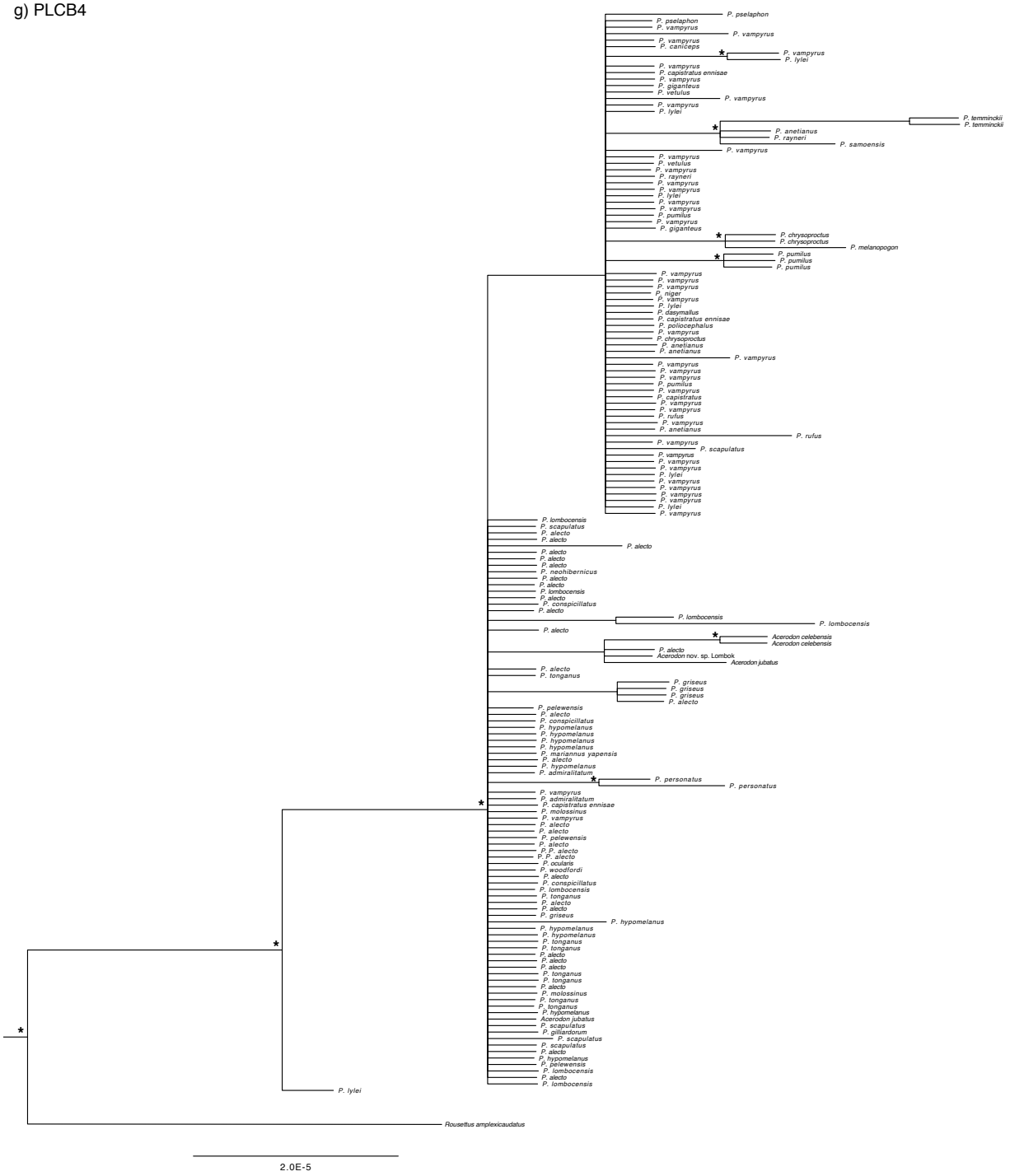
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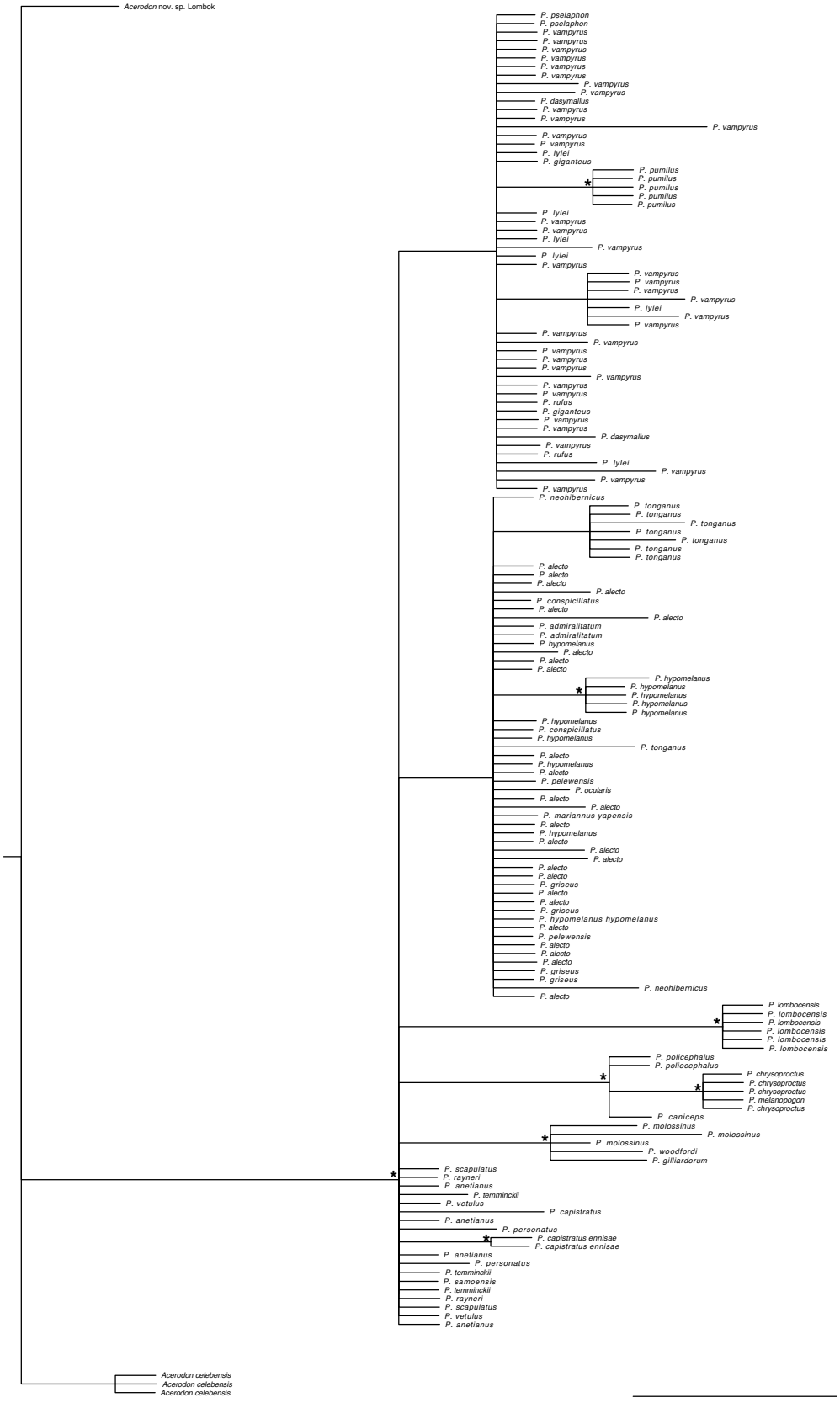
P. alecto
P. alecto
P. alecto
P. tonganus
P. tonganus
P. alecto
P. molossinus
P. tonganus
P. tonganus
P. hypomelanus
Acerodon jubatus
P. scapulatus
P. gilliardorum
P. scapulatus
P. scapulatus
P. alecto
P. hypomelanus
P. pelewensis
P. lombocensis
P. alecto
P. lombocensis
P. lylei
Rousettus amplexicaudatus
2.0E-5

h) ATP7A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P. pselaphon
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P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. dasymallus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. lylei
P. giganteus
P. pumilus
P. pumilus
P. pumilus
P. pumilus
P. pumilus
P. lylei
P. vampyrus
P. vampyrus
P. lylei
P. vampyrus
P. lylei
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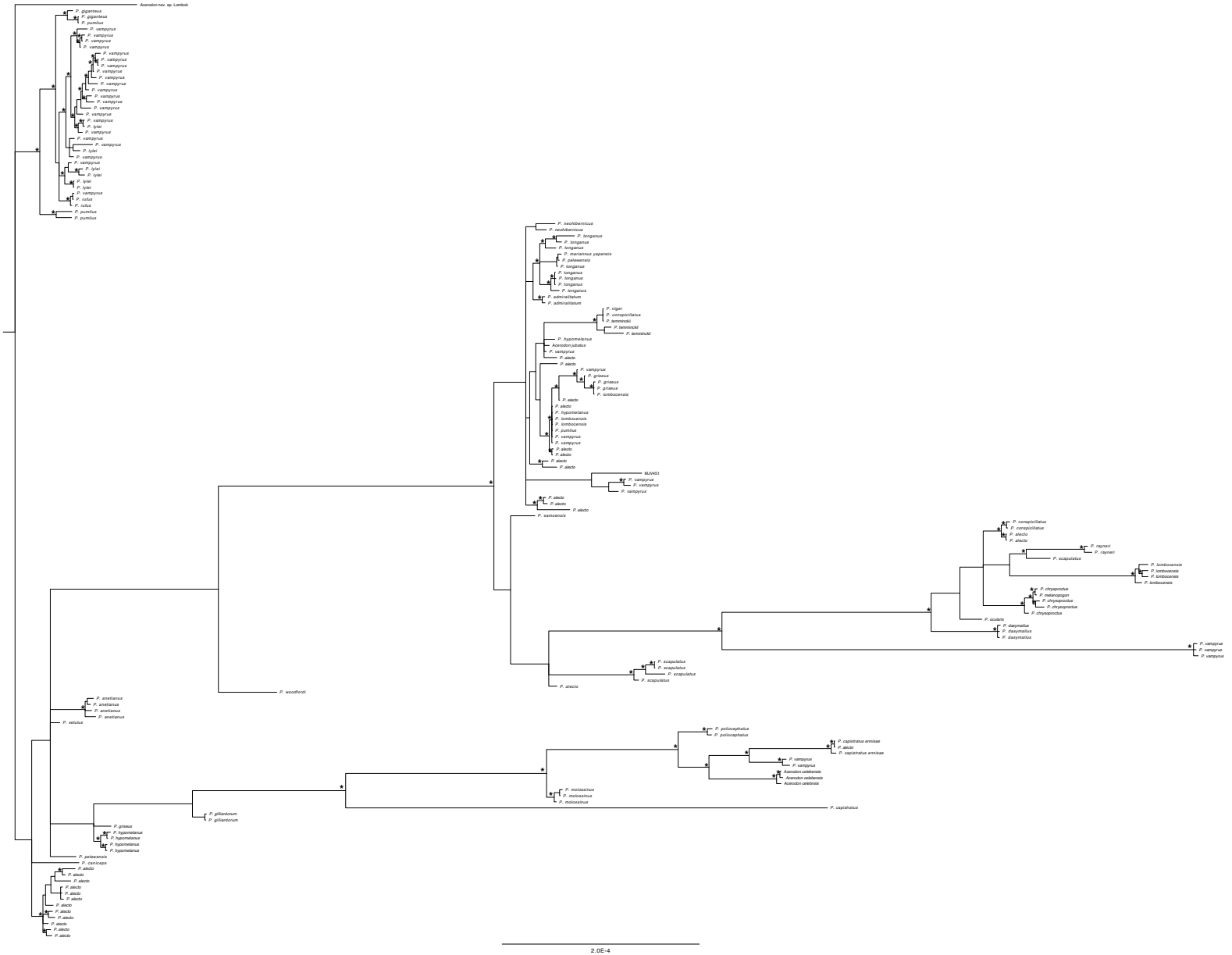
P. vampyrus
P. vampyrus
P. vampyrus
P. lylei
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. vampyrus
P. rufus
P. giganteus
P. vampyrus
P. vampyrus
P. dasymallus
P. vampyrus
P. rufus
P. lylei
P. vampyrus
P. vampyrus
P. vampyrus
P. neohibernicus
P. tonganus
P. tonganus
P. tonganus
P. tonganus
P. tonganus
P. tonganus
P. tonganus
P. alecto
P. alecto
P. alecto
P. alecto
P. conspicillatus
P. alecto
P. alecto
P. admiralitatum
P. admiralitatum
P. hypomelanus
P. alecto
P. alecto
P. alecto
P. hypomelanus
P. hypomelanus
P. hypomelanus
P. hypomelanus
P. hypomelanus
P. hypomelanus
P. conspicillatus
P. hypomelanus
P. tonganus
P. alecto
P. hypomelanus
P. alecto
P. pelewensis
P. ocularis
P. alecto
P. alecto
P. mariannus yapensis
P. alecto
P. hypomelanus
P. alecto
P. alecto
P. alecto
P. alecto
P. alecto
P. griseus
P. alecto
P. alecto
P. griseus
P. hypomelanus hypomelanus
P. alecto
P. pelewensis
P. alecto
P. alecto
P. alecto
P. griseus
P. griseus
P. neohibernicus
P. alecto
P. lombocensis
P. lombocensis
P. lombocensis
P. lombocensis
P. lombocensis
P. lombocensis
P. policephalus
P. poliocephalus
P. chrysoproctus
P. chrysoproctus
P. chrysoproctus
P. melanopogon
P. chrysoproctus
P. caniceps
P. molossinus
P. molossinus
P. molossinus
P. woodfordi
P. gilliardorum
P. scapulatus
P. rayneri
P. anetianus
P. temminckii
P. vetulus
P. capistratus
P. anetianus
P. personatus
P. capistratus ennisae
P. capistratus ennisae
P. anetianus
P. personatus
P. temminckii
P. samoensis
P. temminckii
P. rayneri
P. scapulatus
P. vetulus
P. anetianus
Acerodon celebensis
Acerodon celebensis
Acerodon celebensis
2.0E-5

i) D-loop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P. tonganus
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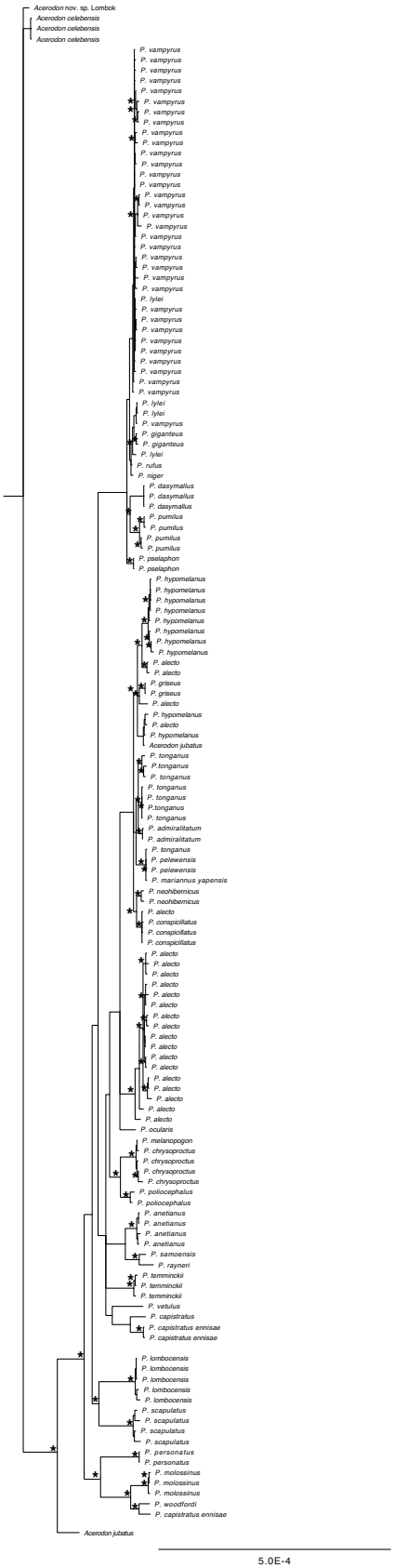
P. pelewensis
P. admiralitatum
P. niger
P. conspicillatus
P. temminckii
P. hypomelanus
Acerodon jubatus
P. vampyrus
P. alecto
P. griseus
P. lombocensis
P. pumilus
MJV451
P. scapulatus
P. capistratus
P. poliocephalus
P. molossinus
Acerodon celebensis
P. capistratus enniae
P. rayneri
P. melanopogon
P. chrysoproctus
P. ocularis
P. dasymallus
P. anellianus
P. vetulus
P. caniceps
P. rufus
P. lylei
P. pumilus
P. gigantseus
2.0E-4

j) *cyt-b*


Acerodon nov. sp. Lombok
Acerodon celebensis
Acerodon celebensis
Acerodon celebensis
P. vampyrus
P. lylei
P. rufus
P. niger
P. dasymallus
P. pumilus
P. pselaphon
P. hypomelanus
P. alecto
P. griseus
Acerodon jubatus
P. tonganus
P.tonganus
P. admiralitatum
P. pelewensis
P. mariannus yapensis
P. neohibernicus
P. conspicillatus
P. ocularis
P. melanopogon
P. chrysoproctus
P. poliocephalus
P. anetianus
P. samoensis
P. rayneri
P. temminckii
P. vetulus
P. capistratus
P. capistratus ennisae
P. lombocensis
P. scapulatus
P. personatus
P. molossinus
P. woodfordi
Acerodon jubatus
5.0E-4

APPENDIX 3.1

List of islands used for species-area comparison in Fig. 3.1. Only major islands and archipelagoes were included. Categories correspond to biogeographic areas defined in Chapter 3. Number of *Pteropus* species derived from Mickelburgh et al. (1992), Corbet and Hill (1992), Simmons (2005), and Helgen (2009).

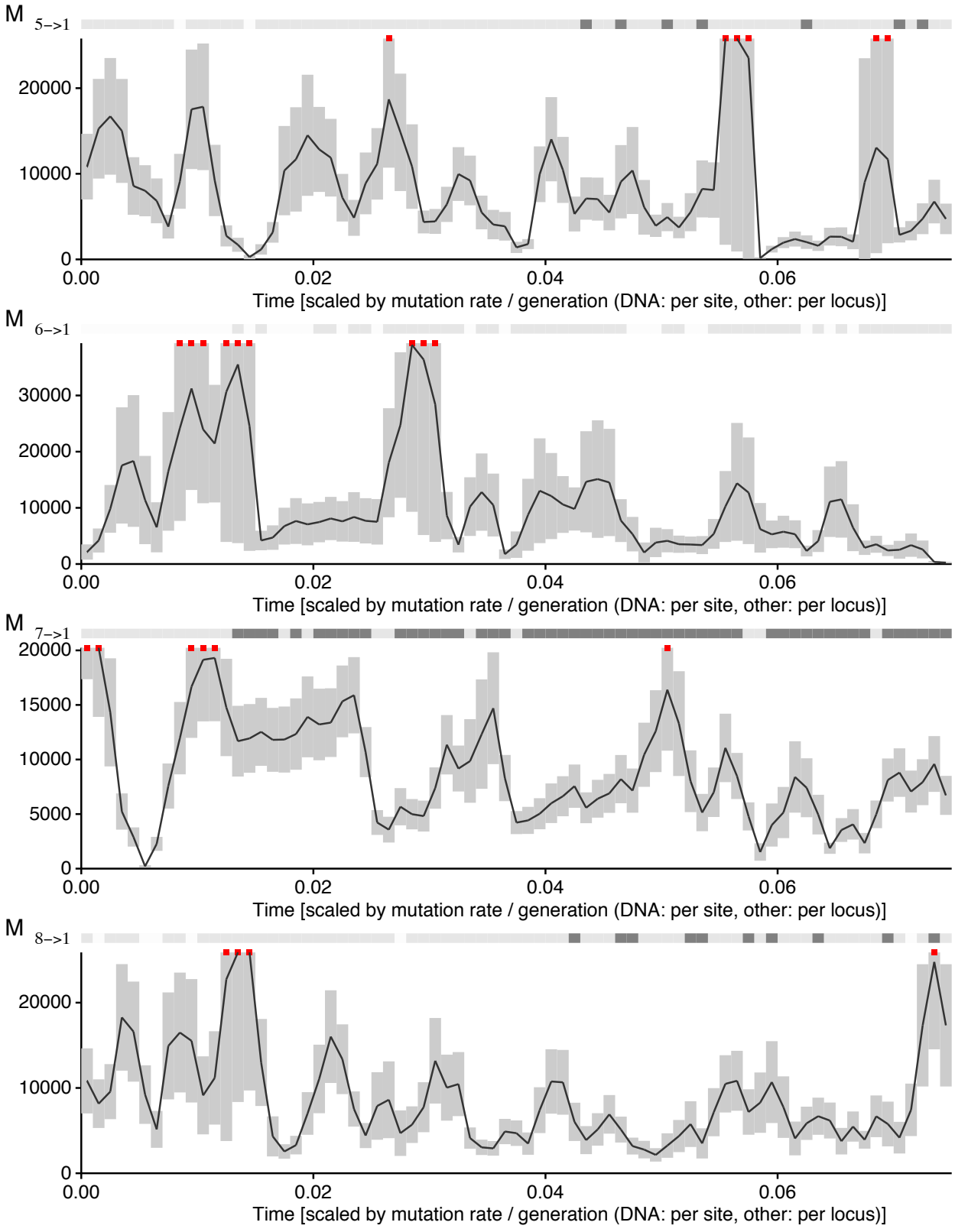
Island	Area (km^2)	log(area)	# of species	Category
Comoros	785	2.894870	2	African
Madagascar	587040	5.768668	1	African
Maldives	300	2.477121	2	African
Mauritius	2040	3.309630	3	African
Pemba	984	2.992995	1	African
Reunion	2512	3.400020	2	African
Rodrigues	108	2.033424	1	African
Seychelles	455	2.658011	2	African
Bonin	104	2.017033	1	Micronesian
Guam	549	2.739572	2	Micronesian
Mariana Islands	477	2.678518	1	Micronesian
Micronesia	702	2.846337	5	Micronesian
Palau	458	2.660865	2	Micronesian
Ryukyu Islands	1792	3.253338	2	Micronesian
Admiralty Islands	2100	3.322219	3	Pacific
Biak	2455	3.390051	2	Pacific
Bismarck Islands	49700	4.696356	5	Pacific
Bougainville	9318	3.969323	2	Pacific
Cook Islands	237	2.374748	1	Pacific
Fiji	18270	4.261739	2	Pacific
Guadalcanal	5336	3.727216	3	Pacific
New Britain	36514	4.562459	5	Pacific
New Caledonia	18575	4.268929	3	Pacific
New Georgia	5061	3.704236	2	Pacific
New Guinea	786000	5.895423	7	Pacific
New Ireland	2859	3.456214	2	Pacific
Samoan Islands	3030	3.481443	4	Pacific
Tonga	748	2.873902	1	Pacific
Trobriand Islands	415	2.618048	2	Pacific
Vanuatu	12200	4.086360	3	Pacific
Basilan	1145	3.058805	2	Philippines

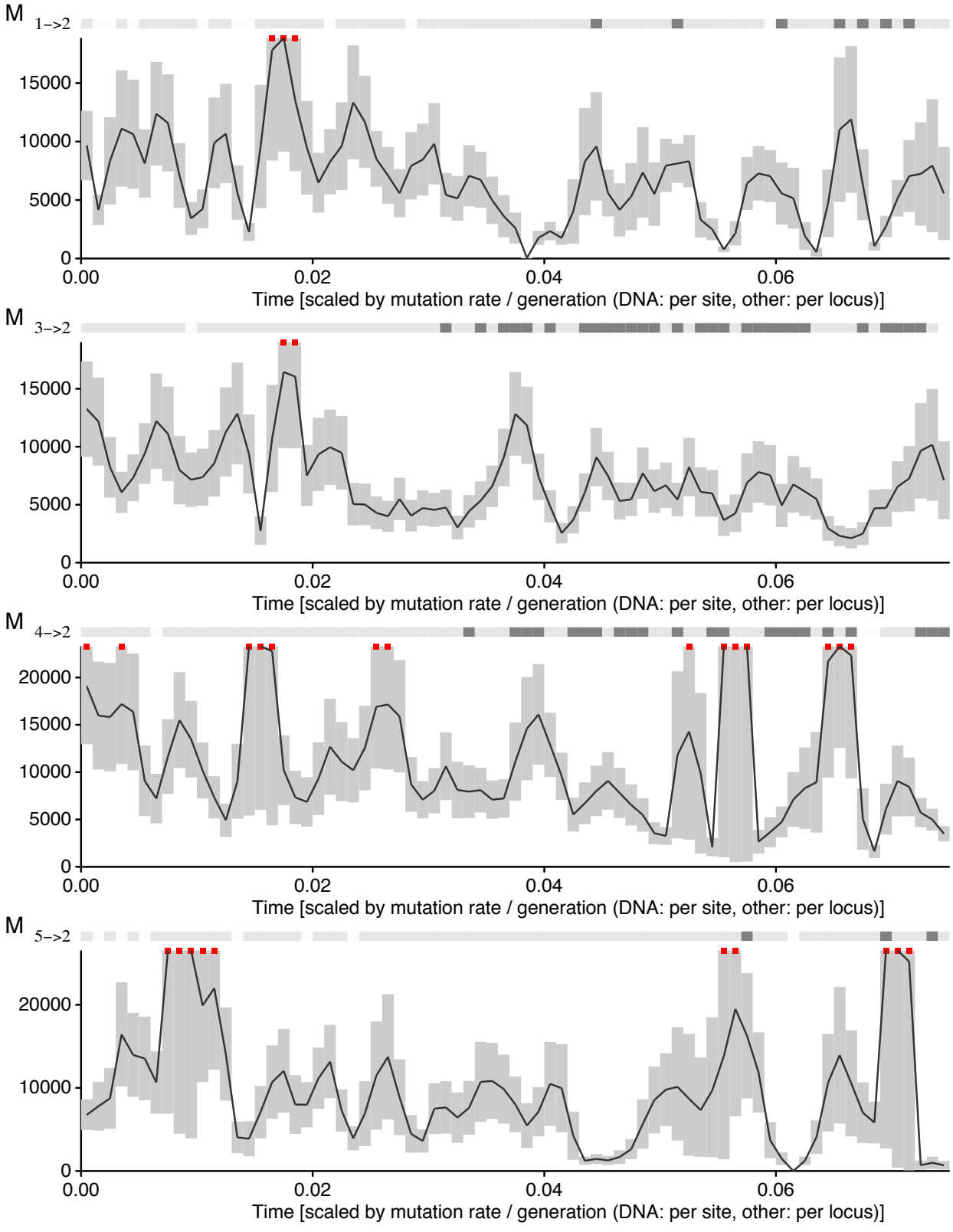
Bohol	4821	3.683137	1	Philippines
Cagayan Sulu	181	2.257679	1	Philippines
Camiguin	238	2.376577	2	Philippines
Cebu	4933	3.693111	2	Philippines
Dinagat	1036	3.015360	2	Philippines
Leyte	7368	3.867350	3	Philippines
Luzon	104688	5.019897	2	Philippines
Maripipi	28	1.447158	2	Philippines
Mindanao	97530	4.989138	3	Philippines
Mindoro	10572	4.024157	2	Philippines
Negros	12706	4.104009	3	Philippines
Palawan	14650	4.165838	1	Philippines
Panay	12011	4.079579	1	Philippines
Samar	6048	3.781612	1	Philippines
Sarangani	3601	3.556423	1	Philippines
Taiwan	36193	4.558625	1	Philippines
Anamba Islands	637	2.804139	2	Sundaic
Andaman & Nicobar Islands	8073	3.907035	3	Sundaic
Bali	5780	3.761928	1	Sundaic
Bangka	11910	4.075912	1	Sundaic
Bawean	76	1.880814	1	Sundaic
Borneo	743339	5.871187	2	Sundaic
Enganno	403	2.605305	2	Sundaic
Java	128297	5.108217	2	Sundaic
Kangean Islands	490	2.690196	1	Sundaic
Ko Samui	229	2.359835	1	Sundaic
Madura	4250	3.628389	1	Sundaic
Masalembu	41	1.612784	1	Sundaic
Mentawai Islands	6011	3.778947	2	Sundaic
Natuna	1993	3.299507	2	Sundaic
Nias	4771	3.678609	1	Sundaic
Riau Islands	8202	3.913920	1	Sundaic
Simeulue	2310	3.363612	1	Sundaic
Singapore	716	2.854913	1	Sundaic
Sumatra	473481	5.675303	1	Sundaic
Tioman	136	2.133539	1	Sundaic
Ambon	377	2.576341	4	Wallacean
Aru Islands	8563	3.932626	2	Wallacean
Bacan	734	2.865696	3	Wallacean
Banda Islands	180	2.255273	2	Wallacean

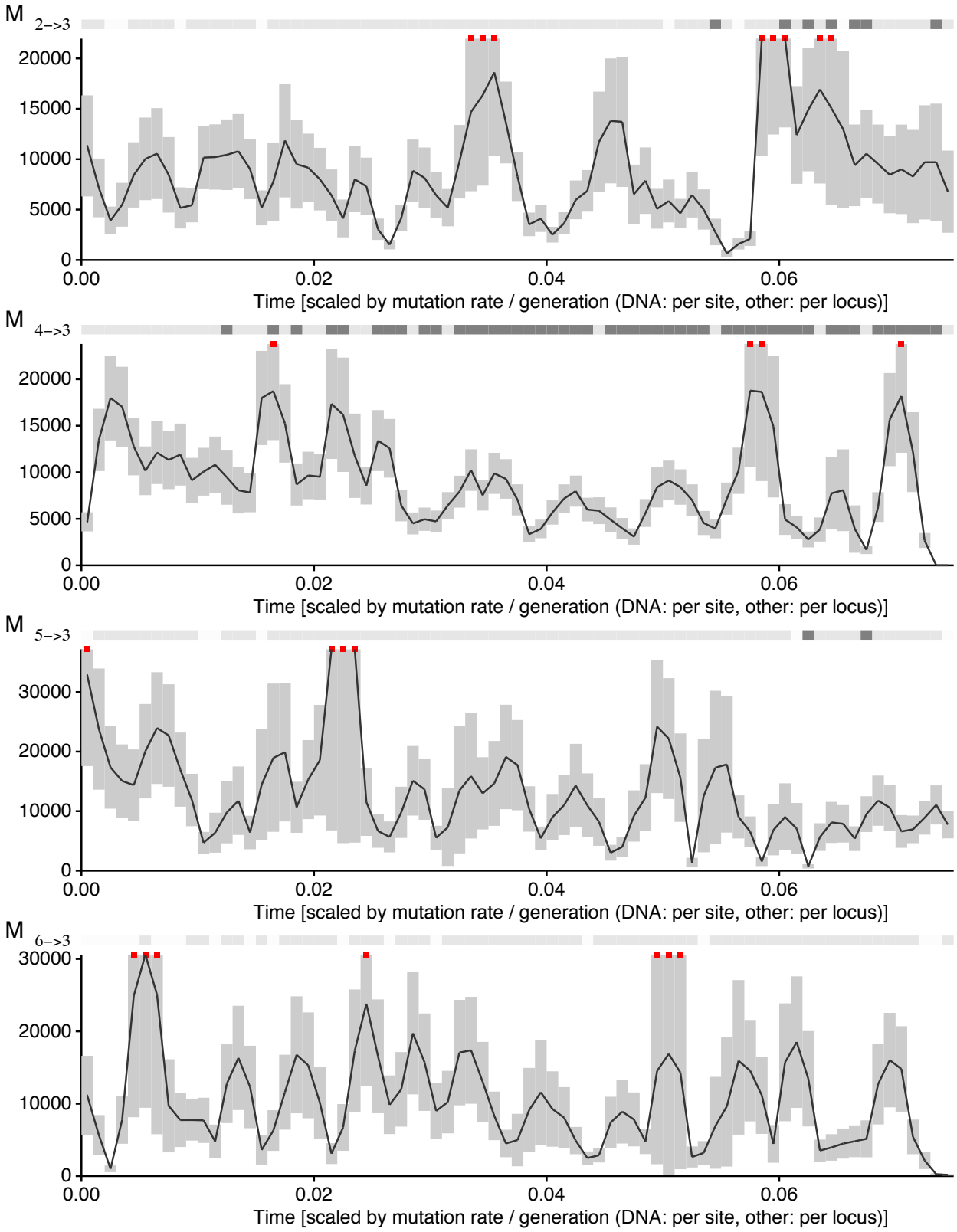
Bonerate	5307	3.724849	1	Wallacean
Buru	9505	3.977952	4	Wallacean
Flores	13540	4.131619	2	Wallacean
Gebe	224	2.350248	2	Wallacean
Halmahera	17780	4.249932	3	Wallacean
Kai Islands	1438	3.157759	1	Wallacean
Lombok	4725	3.674402	3	Wallacean
Misool	2034	3.308351	1	Wallacean
Obi	3948	3.596377	3	Wallacean
Sangihe Islands	813	2.910091	1	Wallacean
Selayar Islands	10504	4.021355	2	Wallacean
Seram	17100	4.232996	4	Wallacean
Sula Islands	9632	3.983716	1	Wallacean
Sumba	11153	4.047392	1	Wallacean
Sumbawa	15448	4.188872	1	Wallacean
Talaud Islands	1281	3.107549	1	Wallacean
Tanimbar Islands	2100	3.322219	1	Wallacean
Ternate	76	1.880814	4	Wallacean
Timor	15850	4.200029	2	Wallacean
Sulawesi	174600	5.242044	4	Wallcean

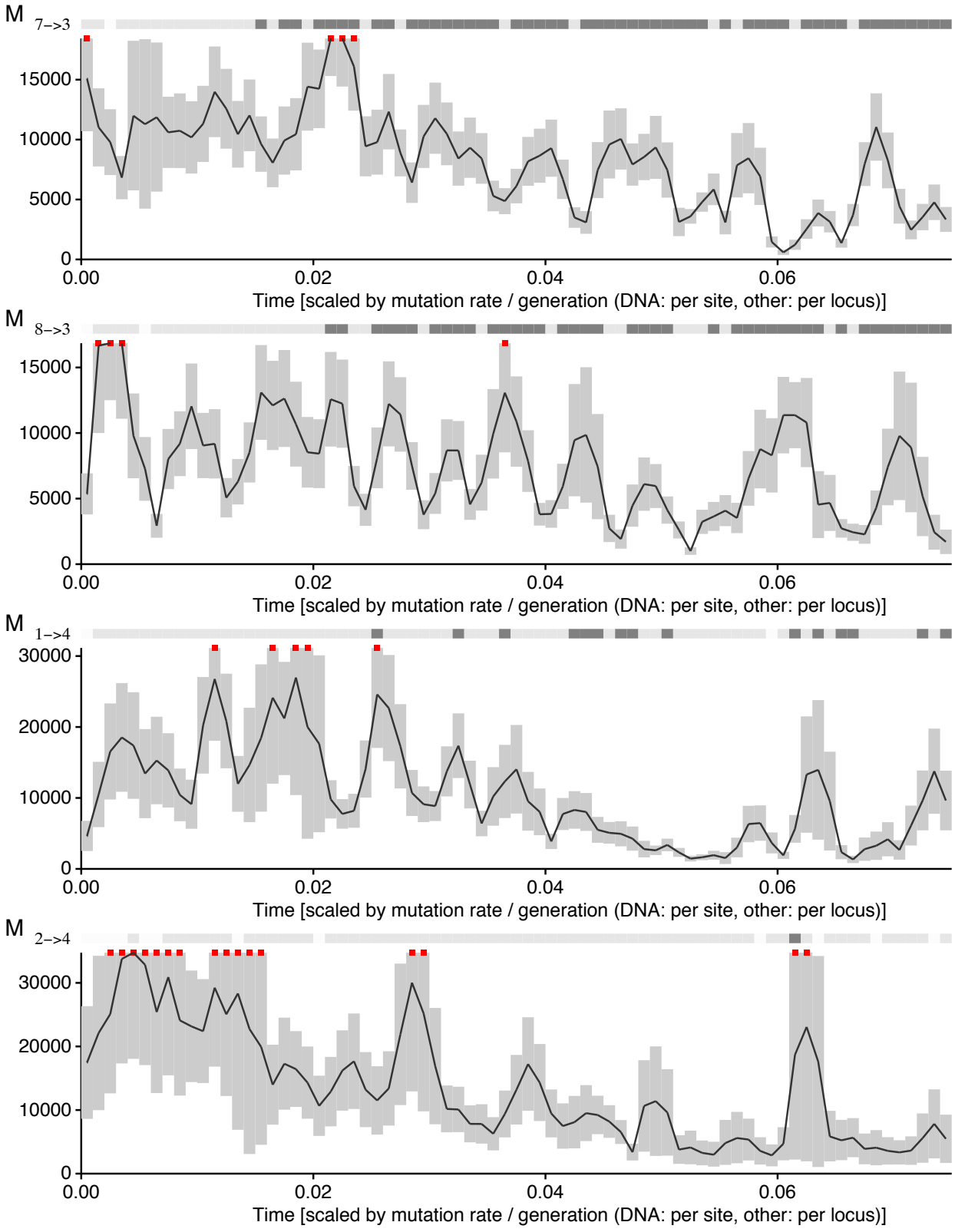
APPENDIX 4.1

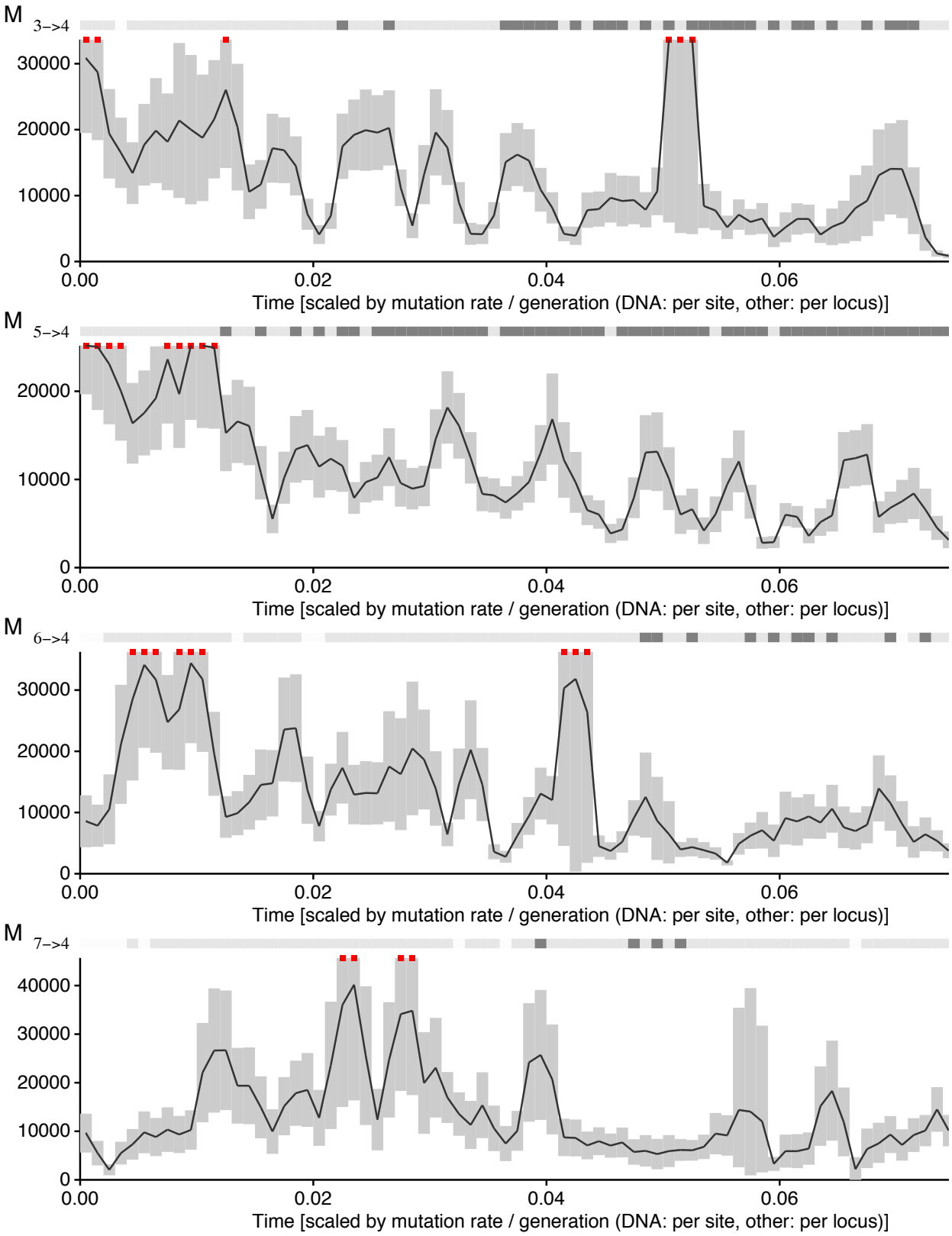
Skyline plots of migration rates through time in *P. vampyrus* populations. Populations are: 1) Sumatra, 2) Java, 3) Borneo, 4) Bali, 5) the Lesser Sundas, 6) Malaysia, 7) Vietnam, and 8) the Philippines. Time is scaled by coalescent units on the X-axis and M is the migration rate on the Y-axis. The results indicate constant migration between populations through time and no single source population. Frequency of migration also does not remain consistent through time, likely reflecting the tendency for *P. vampyrus* colonies to migrate where food resources are available (*e.g.*, during masting events).


M 5–>1
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 6–>1
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 7–>1
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 8–>1
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]


M 1–>2
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 3–>2
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 4–>2
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 5–>2
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]


M
2–>3
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M
4–>3
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M
5–>3
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M
6–>3
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]


M 7–>3
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 8–>3
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 1–>4
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 2–>4
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]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0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 5–>4
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 6–>4
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 7–>4
40000
30000
20000
10000
0
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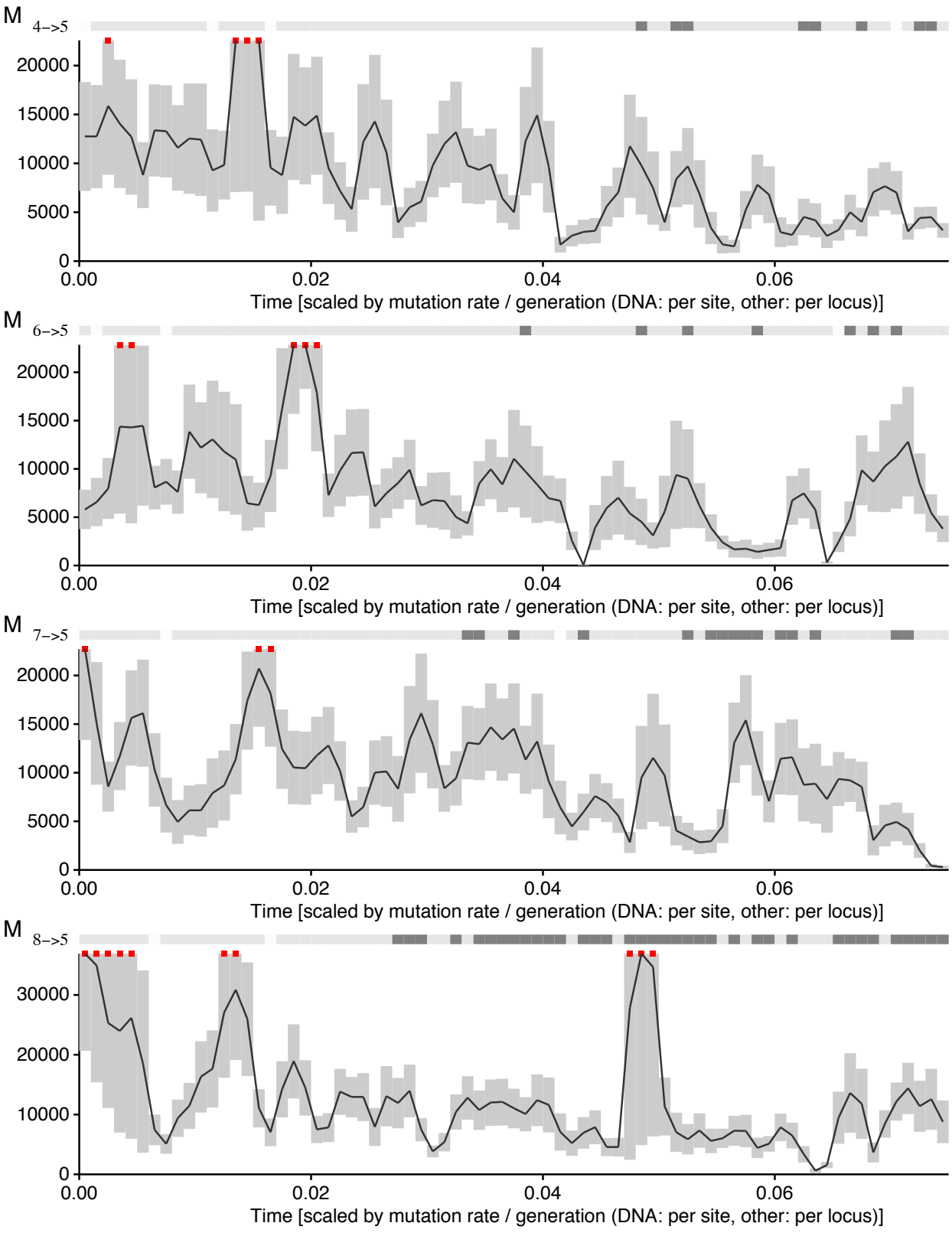
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]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15000
10000
5000
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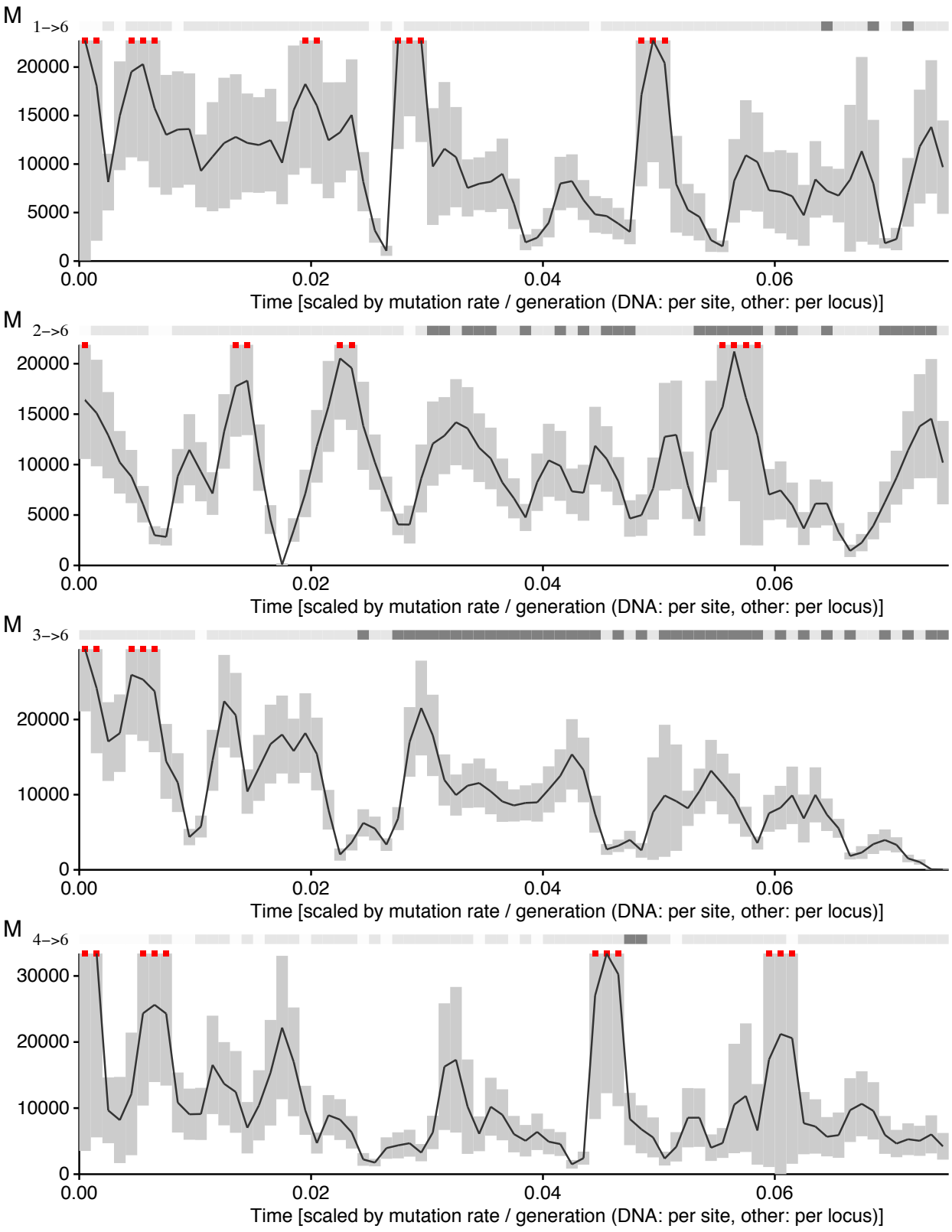
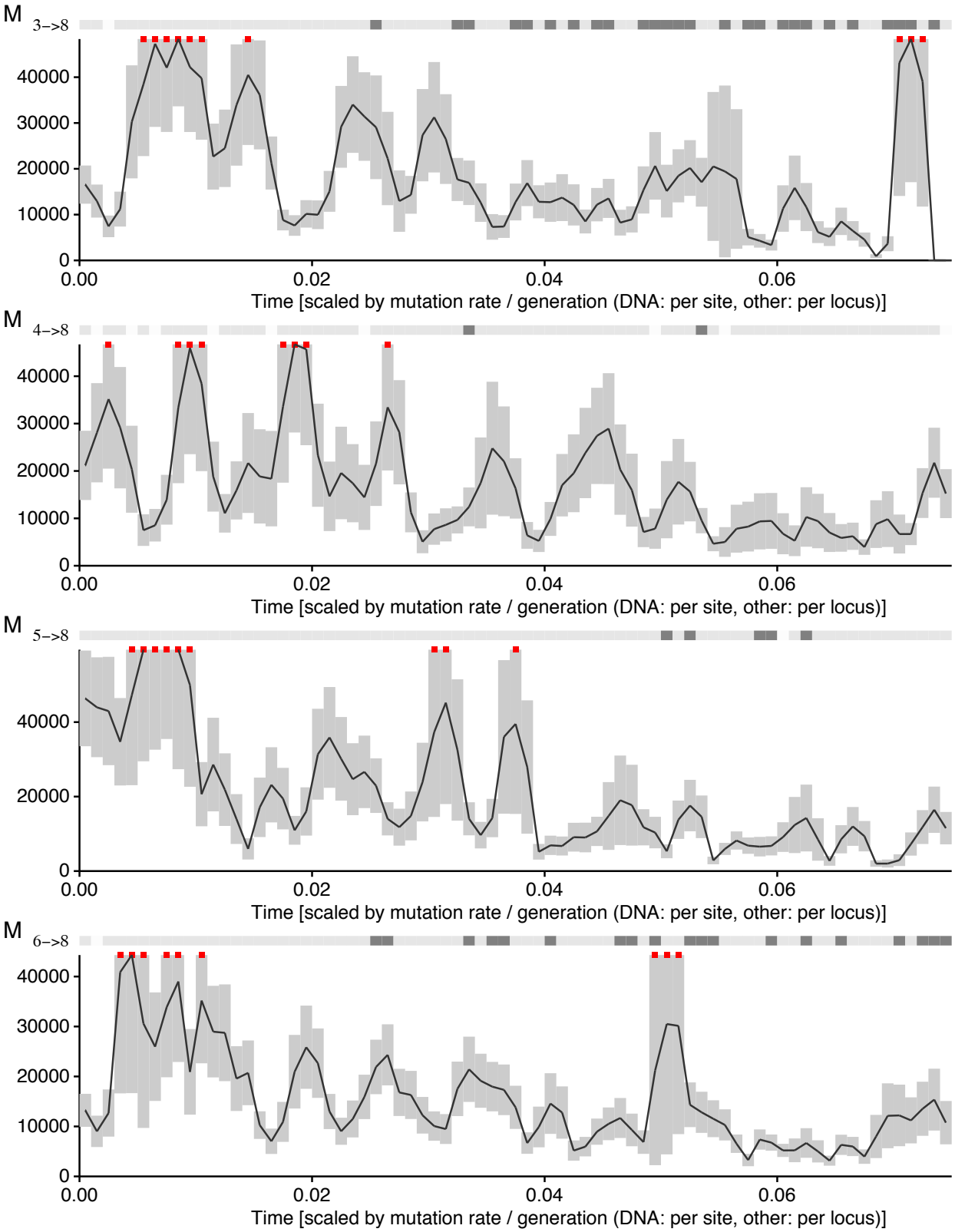
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 6–>5
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 7–>5
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 8–>5
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]


M 1–>6
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 2–>6
20000
15000
10000
5000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 3–>6
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 4–>6
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]


M 3–>8
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20000
10000
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0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 4–>8
40000
30000
20000
10000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 5–>8
40000
20000
0
0.00
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]
M 6–>8
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10000
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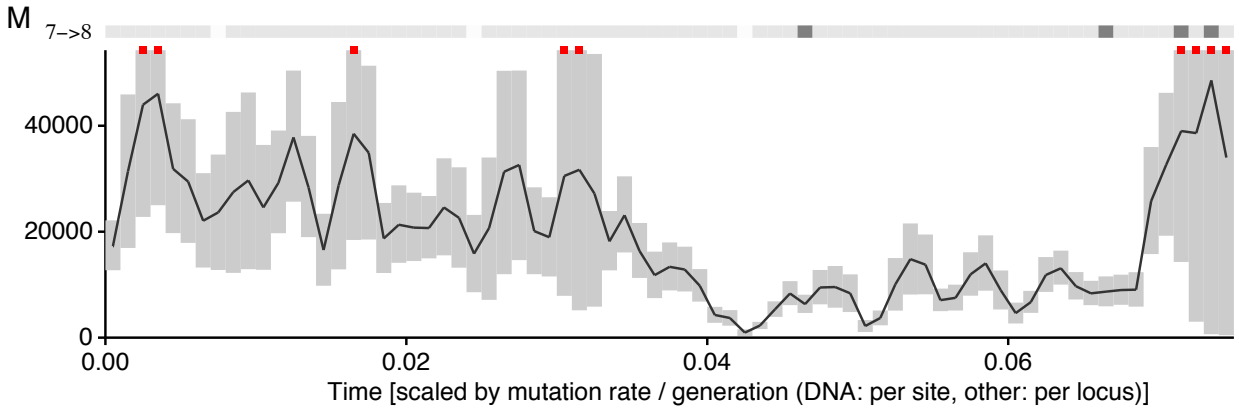
0.02
0.04
0.06
Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]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Time [scaled by mutation rate / generation (DNA: per site, other: per locus)]

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