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Low levels of population structure among geographically distant populations of *Pteropus vampyrus* (Chiroptera: Pteropodidae)

Susan M. Tsang[1, 2, 3, 5], Sigit Wiantoro[4], Maria Josefa Veluz[3], Nancy B. Simmons[2], and David J. Lohman[1, 3]

[1]*Department of Biology, City College and the Graduate Center, The City University of New York, 365 Fifth Avenue, New York, NY, 10016, USA*
[2]*Department of Mammalogy, American Museum of Natural History, Central Park West and 79th Street, New York, NY, 10024, USA*
[3]*Zoology Division, National Museum of the Philippines, Padres Burgos Ave, Ermita, Manila, 1000 Metro Manila, Philippines*
[4]*Museum Zoologicum Bogoriense, Indonesian Institute of Sciences, Jl. Raya Jakarta-Bogor, Km. 46, Cibinong, 16911, Indonesia*
[5]*Corresponding author: E-mail: stsang@amnh.org*

Pteropus vampyrus, the largest bat in the world, has a broad geographic range covering much of Southeast Asia. The wide distribution of *P. vampyrus* and its ability to cross oceanic expanses makes management of this threatened species an international concern. *Pteropus vampyrus* is an essential seed disperser and pollinator of rain forest trees, many of which are ecologically and economically important. Understanding population dynamics of *P. vampyrus* is thus critical to addressing conservation issues and global health concerns. We used phylogenetic inference and population genetic indices to infer past gene flow between populations of *P. vampyrus* throughout most of the species' range. Population genetic parameters indicate low levels of nucleotide variability with high haplotype diversity across its range, implying a demographic scenario of recent population expansion after a bottleneck. Subspecies were not found to be monophyletic from the genetic data, which may reflect some level of genetic variation on even shallower time scales. The low level of population genetic structure throughout the species range is not necessarily surprising given its high vagility and seasonal migratory behavior. However, it cannot be entirely excluded that these results may reflect historical connectivity or lineage sorting issues rather than more recent persistent gene flow. These findings highlight the need for international cooperation and monitoring to ensure persistence of populations and to create a species management plan that can protect the species throughout its range. Increased genetic sampling is needed to ascertain *P. vampyrus*' commonly used dispersal routes and to assess the possibility of asymmetric gene flow among populations.

Key words: Southeast Asia, population genetics, Indonesia, flying fox, Philippines, *Pteropus vampyrus*

Introduction

Pteropus vampyrus (Linnaeus, 1758), also known as the large flying fox, has the largest wingspan of any bat species in the world (Andersen, 1912; Corbet and Hill, 1992). It is native to the Philippines, western Indonesia, and peninsular Southeast Asia where it is typically found in coastal areas living in large colonies comprising thousands of individuals (Goodwin, 1979; Corbet and Hill, 1992; Jones and Kunz, 2000). *Pteropus vampyrus* is an important seed disperser and pollinator of eco-logically and economically important plants, such as figs (*Ficus* spp.) and durian (*Durio zibethinus*) (Fujita and Tuttle, 1991; Jones and Kunz, 2000; Stier

and Mildenstein, 2005). The efficacy of flying foxes as seed dispersers requires large, healthy popula-tions (McConkey and Drake, 2006), which are in-creasingly rare as in the face of habitat conversion and intensive hunting (Mickleburgh *et al*., 1992; Mohd-Azlan *et al*., 2001; Struebig *et al*., 2007).

Pteropus vampyrus is arguably one of the most well-known *Pteropus* species in Southeast Asia. Re-searchers have investigated many aspects of *P. vam-pyrus* biology, including ecology (Mohd-Azlan *et al*., 2001; Stier, 2003; Gumal, 2004; Mildenstein *et al*., 2005; Stier and Mildenstein, 2005) and physiology (Reeder *et al*., 2006*a*, 2006*b*; Riskin *et al*., 2010). Conservation research on *P. vampyrus* has gained traction in recent years as the effects of hunting have

been recognized (Struebig *et al.*, 2007; Harrison *et al.*, 2011; Croes, 2012; Heng, 2012). Additionally, there have been more studies in the past decade focused on screening *P. vampyrus* for zoonotic pathogens and parasites including Nipah virus, Hendra virus, and *Hepatocystis* species (Yob *et al.*, 2001; Sendow *et al.*, 2006; Olival *et al.*, 2007; Wang *et al.*, 2008; Epstein *et al.*, 2009; Rahman *et al.*, 2010, 2013; Sohayati *et al.*, 2011; Breed *et al.*, 2013).

Pteropus vampyrus has been an exemplar in studies of evolutionary relationships among genera in the family Pteropodidae, as well as phylogenetic analyses of the genus *Pteropus* (Giannini *et al.*, 2008; Almeida *et al.*, 2014; S.M.T., S.W., M.J.V., N. Sugita, N.B.S., and D.J.L., unpublished data). However, population dynamics within this or any other *Pteropus* species have never been investigated across their range. A previous study of *P. vampyrus* population structure was limited in its taxonomic and genetic sampling, but suggested little genetic differentiation among Sundaic populations (Olival, 2008). A clear understanding of this species' genetic diversity is important for conservation management, managing of gene flow in rainforest tree species, and modeling disease ecology of zoonotic pathogens. Unfortunately, population genetic data on this or any other *Pteropus* species are too sparse to even speculate on these questions. We aim to remedy this lacuna on the population dynamics and connectivity of *P. vampyrus* so that conservation management needs can be met and questions related to gene flow and potential routes of pathogen dispersal can be addressed. This study will provide a framework for future research, including targeted satellite telemetry projects to acquire observational evidence of the dispersal patterns we infer. We hypothesize that gene flow between *P. vampyrus* populations may be adequately described by an isolation-by-distance model of migration, with populations becoming increasingly genetically differentiated as geographic distance between them increases. Lack of genetic differentiation between Sundaic populations (Olival, 2008) suggests that some degree of gene flow exists among populations, while past research on Malaysian *P. vampyrus* suggests that dispersal is localized (Epstein *et al.*, 2009). Infrequent dispersal between distant populations would result in population structure that increases with geographic distance. Conversely, frequent gene flow among *P. vampyrus* populations would reduce the signal of an isolation-by-distance model and result in little differentiation across the range of the species.

MATERIALS AND METHODS

We sampled fresh tissues from adult individuals in populations throughout Indonesia and the Philippines (Appendix). We took 4 mm^2 wing biopsy punches from a majority of the bats represented, and opportunistically collected liver samples from specimens vouchered for the reference collections of the Museum Zoologicum Bogoriense or National Museum of the Philippines, following standard protocols (Corthals *et al.*, 2015). Bat capture, handling, and sampling 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 granted from the Ministry of Foreign Research and Technology and the Ministry of Forestry and Environment in Indonesia, and the Department of Environment and Natural Resources and the Biodiversity Management Bureau in the Philippines. Canopy mist nets were set up 20 to 30 m above the ground by having a local tree climber tie the nets to a pole extending above the highest trees in flyways identified during prior reconnaissance. A single rope went through both sides of the mist net loops to create a double pulley system to allow for efficient lowering of the net. We used either 6 m, 9 m, or 12 m nets 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 animal. Bats were placed into individual cloth holding bags that were misted with water to keep them cool until the bats could be processed. Additional tissue samples were obtained from wild-caught bats from Lubee Bat Conservancy (Gainesville, Florida), Lee Kong Chian Museum of Natural History (formerly Raffles Museum of Biodiversity Research, Singapore), and the Royal Ontario Museum (Toronto, Canada). By combining tissue samples from bats we collected with material obtained from other institutions, we were able to sample 39 individuals representing colonies across the species' range (Fig. 1): Philippines ($n = 11$, combined three sites: Negros Occidental, Leyte, Palawan), Borneo ($n = 2$), Sumatra ($n = 7$), Java ($n = 8$), Bali ($n = 5$), Flores/Sumbawa (eastern Lesser Sundas, $n = 3$), and peninsular Southeast Asia ($n = 3$). We assumed that each colony was a separate population that did not interbreed with the others, as inferred by recognized subspecies differences or by large geographic distances (≥ 500 km) separating the colonies. Each of the six recognized *P. vampyrus* subspecies (Table 1 and Fig. 1 — Corbet and Hill, 1992; Koopman, 1993, 1994; Simmons, 2005) was represented in at least one of the populations, with the exception of unsampled *P. v. edulis*, which is restricted to Timor, for a total of 39 individuals. Samples of *Acerodon celebensis*, *Pteropus hypomelanus*, and *P. alecto* were included as outgroup taxa to root the tree. We generated all sequence data used in our analyses.

Tissue samples were extracted using a Qiagen DNEasy Blood and Tissue Kit. Two mitochondrial loci, five nuclear exons, and three nuclear introns were amplified: cyt-*b* (Kocher *et al.*, 1989), D-loop (Brown *et al.*, 2011), ATP7A, BDNF, PLCB4 (Eick *et al.*, 2005), RAG-1, RAG-2 (Giannini *et al.*, 2008), COPS7A-4 (Igea *et al.*, 2010), FGB-7 (Nesi *et al.*, 2011), and STAT5A (Piaggio and Perkins, 2005) (Table 2). Thermal cycle profiles were as follows: 35 cycles of initial denaturation at 95º C for 1 min, annealing at 52º C for 30 s, extension at 72º C for 2 min; then a final extension at 72º C for 3 min. Successfully amplified PCR products were cleaned using ExoSAP or a vacuum manifold. Products were run on an Applied Biosystems 3730xl automated sequencer using ABI Big Dye version 3.1. Genes were aligned using Geneious 5.4.3
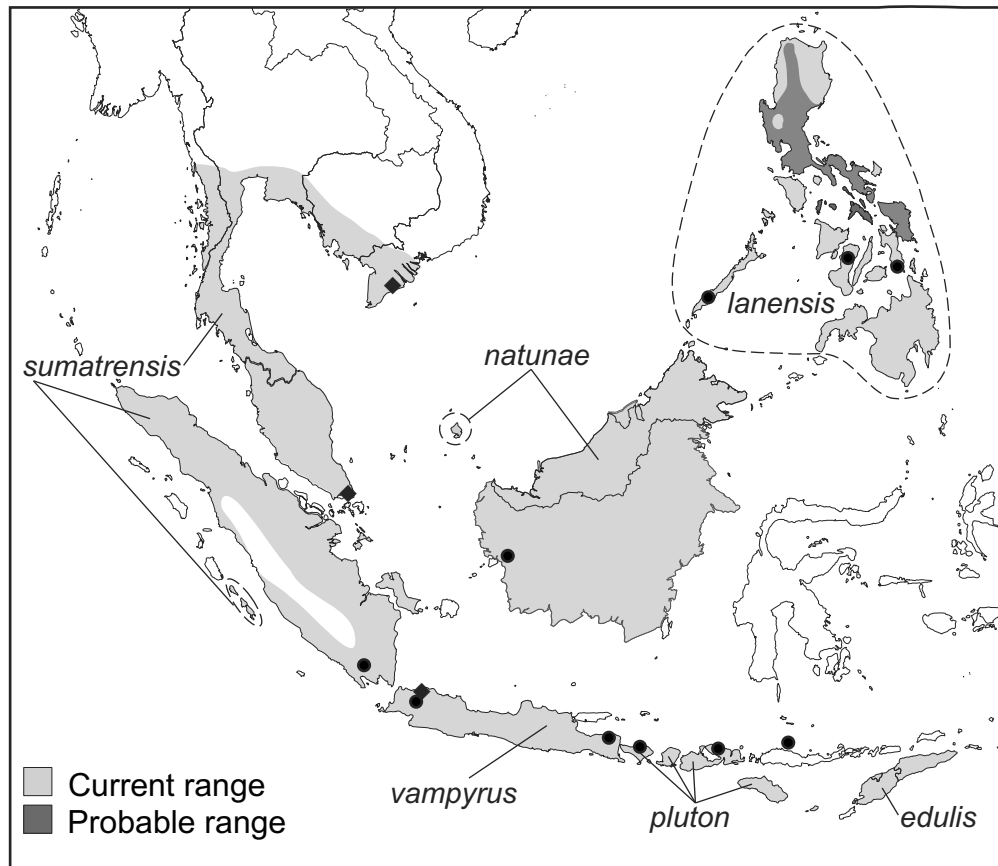
FIG. 1. Range map of *P. vampyrus* subspecies following to the IUCN species listing (Bates, 2008). Sampling localities for fresh tissue are marked with black circles, museum loans with black diamonds. Some specimen loans were from the same locality as the wild-caught specimens, and are marked with only the black circle for clarity

and MAFFT 7.0 (Katoh and Standley, 2013). To infer population history, a phylogeny of all *P. vampyrus* individuals were reconstructed based on a gene tree using a partitioned Bayesian analysis of all 10 loci implemented in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Gene substitution models were estimated using jModelTest2 (Darriba *et al.*, 2012) (Table 2). The analysis was simulated for 10 million generations, with a sampling frequency of 1,000 generations with 25% burn-in. We evaluated models of demographic history by first mapping the geographic provenance of each specimen onto the MrBayes consensus tree. The null hypothesis of no migration between populations predicts that individuals from the same population would form a monophyletic clade. If there was prior gene flow between populations, individuals would not necessarily be most closely related to others in the same geographically defined population. An isolation-by-distance model predicts that populations that are geographically adjacent would be more closely related to one another than to those that are distant (e.g., the amount of gene flow is negatively related to geographical distance). However, long-distance dispersal can facilitate gene flow between geographically distant individuals, resulting in colonies comprised of individuals that are not necessarily each other's closest relative.

Five commonly used population genetic diversity indices were calculated for each genetic marker using DnaSP (Rozas *et al.*, 2003). 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 (Nei, 1987). The Watterson parameter theta (θ) estimates 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) aids in calculation of the mutation rate and Tajima's D. Under an infinite alleles model, S is equivalent to the total number of mutations. Tajima's D compares patterns of genetic variation to a neutral model which allows for interpretation of biological scenarios such as selection or population size changes (Tajima, 1989). 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).

RESULTS

All nuclear and mitochondrial genes were amplified successfully and uploaded to GenBank (Accession Nos. MG920856–MG921147 — see Appendix).

TABLE 1. List of recognized subspecies of *P. vampyrus*. Names follow Simmons (2005), with information from Corbet and Hill (1992) and Koopman (1993, 1994) considered as well

Subspecies	Type locality	Range	Synonyms
edulis	Timor (Indonesia)	Timor	*funereus*
lanensis	Mindanao (Philippines)	Philippines	
natunae	Panjang, Natuna (Indonesia)	Natuna Islands, Borneo	
pluton	Bali (Indonesia)	Lesser Sundas	*kopangi*
sumatrensis	Sumatra (Indonesia)	Sumatra, Peninsular Southeast Asia	*malaccensis*
vampyrus	Java (Indonesia)	Java	*celaeno, caninus, javanicus, kalou, kelaarti, nudus, phaiops, pteronotus*

The poorly resolved intraspecific phylogenetic consensus tree inferred by MrBayes for all genes suggests low levels of population structure among most *P. vampyrus* populations (Fig. 2A, individual gene trees in Supplementary Fig. S1). No single subspecies was recovered as a monophyletic group. The mitochondrial tree (Fig. 2B) generally agreed with the nuclear tree in that there were no subspecies recovered as a monophyletic clade. Having another individual from the same colony as the closest relative on the tree was only estimated in a handful of instances (e.g*.,* the Southern Leyte colony or the Flores colony). The single individual sampled from peninsular Southeast Asia was more closely related to specimens from Sumatra and West Java, as predicted by an isolation-by-distance (IBD) model. Peninsular Malaysia is approximately 1,000 km from southern Vietnam (even farther if bats avoid flying over water), whereas peninsular Malaysia is only ca. 700 km from sites where we sampled in South Sumatra and West Java. Satellite telemetry has recorded *P. vampyru*s flying from peninsular Malaysia to Sumatra over the Strait of Malacca (Epstein *et al*., 2009), but direct radio telemetric observations of

long distance dispersal have not been made outside of peninsular Malaysia. The IBD model of population structure on the mainland of Southeast Asia could be further tested by including populations from Thailand, Cambodia, and southern Myanmar. However, many populations, particularly those on islands of the Indo-Australian Archipelago, include individuals more closely related to bats on another island than to others from the same colony or population: closest relatives could not be predicted by an isolation-by-distance model. For example, some Philippine individuals were found to be sister to Sumatran, Javan, or Lesser Sundaic individuals.

Genetic diversity indices for *P. vampyrus* were low for each marker, except for the hypervariable mitochondrial D-loop (Table 2). Populations of *P. vampyrus* appear to be almost panmictic in all markers. Most F_{ST} values were lower than 0.1, and Tajima's D was not significant for most of the genes, indicating lack of deviation from the null model of evolution. For genes where results were significant, all of the values of Tajima's D were negative, suggesting a population expansion after a recent bottleneck.

TABLE 2. Genetic substitution models and genetic diversity indices for *P. vampyrus* in this study. Genetic substitution models were estimated using jModelTest2. π — nucleotide diversity, *h* — haplotype diversity, θ — Watterson estimator, S — segregating sites, F_{ST} — fixation index

Gene	Model	π	*h*	θ	S	Tajima's D	D significance	F_{ST}
mitochondrial								
cyt-*b*	TrN	0.00651	0.992	0.01430	55	-2.0168	< 0.05	0.00469
D-loop	HKY+G	0.27709	0.998	0.19520	307	1.6310	ns	0.01092
nuclear								
RAG-1	HKY+G	0.00245	0.690	0.00733	17	-2.2505	< 0.01	0.14924
RAG-2	HKY	0.00325	0.892	0.00560	15	-1.3738	ns	0.08514
STAT5A	TVM	0.00512	0.776	0.01263	18	-2.0607	< 0.05	0.02837
PLCB4	HKY	0.00173	0.316	0.00558	6	-1.8999	< 0.05	0
BDNF	TVM	0.00136	0.280	0.00355	5	-1.6553	ns	0.06474
FGB7	TrN	0.00499	0.917	0.01053	23	-1.7910	ns	0.04418
COPS7A4	HKY+G	0.00310	0.830	0.00417	9	-0.8306	ns	0.08428
ATP7A	K81+G	0.00124	0.492	0.00320	7	-1.7662	ns	0.08619

Discussion

The results suggest potentially moderate to high levels of gene flow in the populations of *P. vampyrus*. The potential for gene flow between sites may reflect known aspects of the species natural history as highly vagile animals with large foraging ranges (Epstein *et al*., 2009) and seasonal migration (e.g., Soegiharto, 2009). The lack of genetic diversity in the nuclear introns used is unusual compared to previous studies that have utilized the same genes in bats — for instance, FGB-7 was previously used to study the phylogeography of three African pteropodids and had much higher levels of genetic variability (Nesi *et al*., 2011). COP7A-4 was designed by Igea *et al*. (2010) specifically to target highly variable introns, and was recommended from a recent study of rhinolophid bats for studies of recently diverged taxa (Dool *et al*., 2016). Additionally, similar population patterns have been found in another island flying fox species, *P. niger* (Larsen *et al*., 2014). While this study cannot completely exclude the possibility of incomplete lineage sorting and historical connectivity leading to similar tree topologies, the current patchy resource landscape may be a contributing factor to migratory behavior. Deforestation leading to loss of local food sources for *P. vampyrus*, coupled with their ability to disperse, is likely leading to more connectivity; and therefore, more gene flow. Additionally, optimal habitats for roosting are decreasing rapidly, and would lead to more contact between what may have originally been two separate populations.

Variation in fruit availability across the Southeast Asian landscape (Cannon *et al*., 2007) may explain some of the high degree of connectivity between the populations sampled, particularly as forested areas are now highly fragmented. Many forests in Southeast Asia are dominated by trees in the Dipterocarpaceae, which typically reproduce synchronously en masse ('mast fruiting') at non-regular intervals and induce trees in other families in the same area to do the same (Sakai, 2002). Many animals in the region are capable of long distance dispersal to take advantage of the surfeit of temporally and spatially patchy resources afforded by the mast flowering (e.g., *Apis dorsata*, the Asian giant honeybee — Itioka *et al*., 2001) and fruiting (e.g., *Sus barbatus*, Bornean bearded pig — Curran and Leighton, 2000). Since *P. vampyrus* is a generalist frugivore (Stier and Mildenstein, 2005) and is capable of dispersing over large areas (Epstein *et al*., 2009), colonies often move to locales with abundant

food instead of staying at a single roost throughout the year. For instance, the resident colony in the Bogor Botanical Garden in West Java, Indonesia, leaves from June to October, which is the peak of the dry season on Java (S.M.T., personal observation). Presumably the same individuals return after each hiatus, though the reason for leaving — perhaps foraging or mating — remains unclear. Individuals sampled from the sprawling Indonesian archipelago were sometimes most closely related to Philippine individuals or specimens from mainland Southeast Asia — not necessarily other specimens from the Indonesian archipelago.

This high rate of inferred dispersal has important consequences for the species' ecology and conservation, and suggests that transnational management plans are essential. International strategies for protecting *P. vampyrus* do not exist, nor do initiatives to monitor populations that cross national boundaries (e.g., the population studied by Epstein *et al*. (2009), which occurs from peninsular Malaysia to Sumatra). Legislation similar to the Convention on the Conservation of Migratory Species of Wild Animals (CMS: http://www.cms.int) or the Agreement on the Conservation of Bats in Europe (EUROBATS) can act as a model for how *Pteropus* may be protected regionally. The provisions in these agreements require that signatory parties agree to restrict the capture of bats, except for scientific research, species recovery programs, traditional subsistence needs, or extraordinary circumstances. Raising awareness of conservation issues related to bats, protection of roost and foraging sites, and promotion of research that can benefit conservation management are just some of the activities covered by these legal agreements. Using these agreements as models may help build future conservation policies that can help protect migratory bat species such as *P. vampyrus*. For instance, the Philippines is the only Southeast Asian country that is a signatory party of CMS but it is possible to create agreements under CMS that include non-signatory countries.

Clinal variation in *P. vampyrus* body size has been noted in the literature, though pelage coloration has not been linked to particular subspecies (Andersen, 1912; Corbet and Hill, 1992). Our analyses suggest that no subspecies are monophyletic; genome-scale data could establish or refute the monophyly of subspecies with more evidence. The authors have previously collected extensive morphologic data from all the subspecies of *P. vampyrus* that suggest there are significant differences between subspecies that uphold the validity of these

S. M. Tsang, S. Wiantoro, M. J. S. Veluz, N. B. Simmons, and D. J. Lohman

FIG. 2. Intraspecific phylogenetic tree of *P. vampyrus* based on A) all 10 loci and B) mitochondrial loci only, analyzed with MrBayes 3.2 simulated for 10 million generations, with a sampling frequency of 1,000 and 25% burn-in. Posterior probabilities are indicated above each node. The topology of the mitochondrial tree does not differ greatly from the nuclear tree, and both suggest near panmixia throughout the species range

B

ROM-110949 *P. v. sumatrensis* Vietnam
0.9425 LBC-904503 *P. v. vampyrus* West Java
SW010 *P. v. pluton* Bali
0.9998 LBC-930092 *P. v. sumatrensis* Sumatra
0.6035 MJV504 *P. v. lanensis* Palawan
0.9265 SMT211 *P. v. lanensis* Negros Occidental
SMT212 *P. v. lanensis* Negros Occidental
0.8059 LBC-930088 *P. v. sumatrensis* Sumatra
0.8601 MJV420 *P. v. lanensis* Southern Leyte
0.9915 SW127 *P. v. natunae* West Kalimantan
SW009 *P. v. pluton* Bali
0.7073 LBC-930089 *P. v. sumatrensis* Sumatra
MJV505 *P. v. lanensis* Palawan
0.5832 SW131 *P. v. sumatrensis* South Sumatra
0.9885 SW011 *P. v. pluton* Bali
SW077 *P. v. vampyrus* East Java
0.693 LBC-930091 *P. v. sumatrensis* Sumatra
SW006 *P. v. vampyrus* West Java
LBC-930093 *P. v. sumatrensis* Sumatra
0.8686 MJV419 *P. v. lanensis* Southern Leyte
0.727 MJV435 *P. v. lanensis* Southern Leyte
SW002 *P. v. vampyrus* West Java
MJV436 *P. v. lanensis* Southern Leyte
0.7758 0.9278 SW133 *P. v. pluton* Flores
0.6279 SW134 *P. v. pluton* Flores
0.7756 SW128 *P. v. natunae* West Kalimantan
ROM-110948 *P. v. sumatrensis* Vietnam
SW001 *P. v. vampyrus* West Java
SW008 *P. v. pluton* Bali
SW078 *P. v. vampyrus* East Java
SW132 *P. v. sumatrensis* South Sumatra
SW140 *P. v. pluton* Sumbawa
WRS-G1339 *P. v. sumatrensis* Singapore F1
SW003 *P. v. vampyrus* West Java
0.8869 SMT213 *P. v. lanensis* Negros Occidental
0.7192 SW007 *P. v. pluton* Bali
0.5923 LBC-904502 *P. v. vampyrus* West Java
0.5231 SMT208 *P. v. lanensis* Negros Occidental
SMT210 *P. v. pluton* Bali

1

1

LBC-904529 *Pteropus hypomelanus*
0.9982 SW012 *Pteropus hypomelanus*
0.9987 SW013 *Pteropus hypomelanus*
SW014 *Pteropus hypomelanus*
SMT207
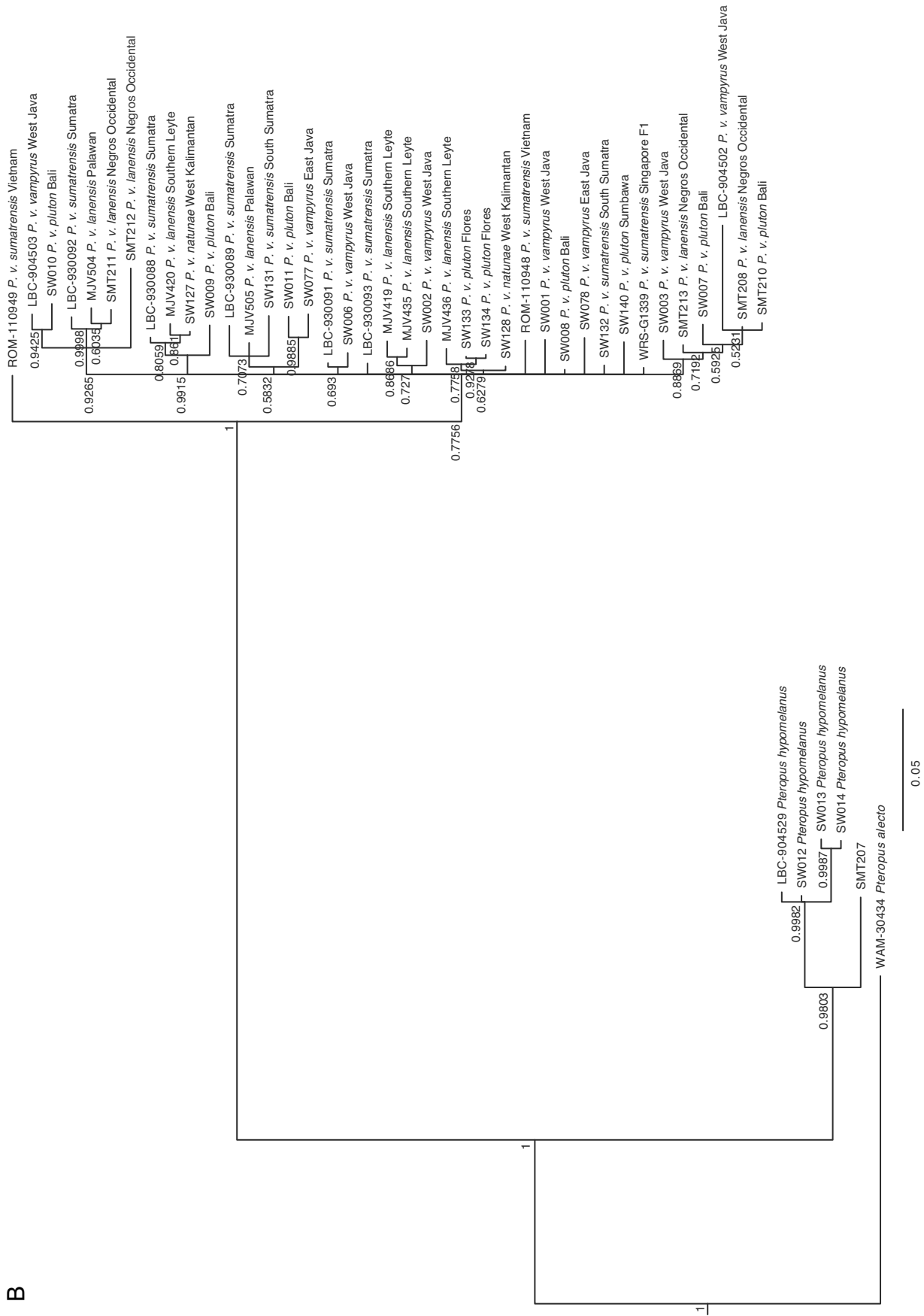0.9803 WAM-30434 *Pteropus alecto*

1

0.05

FIG. 2. Continued

names (S.W. and S.M.T., unpublished data). These morphologic subspecies boundaries are incongruous with our genetic results that do not find subspecies to be monophyletic. This suggests that there may indeed be some genetic variation, but on comparatively shallow time scales. However, these data included primarily historical specimens, and do not account for recent changes in distribution due to threats posed by overhunting and habitat loss. To determine whether there has been a more recent shift in the validity of the subspecies, measurements from more recent populations of poorly known subspecies (e.g., *P. v. edulis*) are needed. The low level of genetic variability of *P. vampyrus* throughout its species range suggests little possibility for local adaptation, and may be related to the ability of *P. vampyrus* to occupy such a variety of different habitats. Genomic tests for recent adaptations may indicate whether there are population-level differences due to localized environmental differences (e.g., diet and different microbiomes) or anthropogenic exposure (e.g., urbanization has led to selection for heavy metal tolerance in mouse populations — Harris *et al.*, 2013).

Colony size is an ecologically important trait in pteropodid bats, one that has implications for population structure and genetic diversity patterns, since it affects their migration patterns, roosting ecology, and mating behavior (Wilson and Graham, 1992; Brooke *et al.*, 2000; Jones *et al.*, 2003; Fahr *et al.*, 2015). In *Pteropus giganteus*, a large and closely-related habitat generalist, colonial species, habitat fragmentation due to land use change has resulted in smaller colonies that are more numerous because fewer trees are available for roosting at any one site (Hahn *et al.*, 2014). Fragmentation of large colonies is also likely occurring in *P. vampyrus* (S.M.T., personal observation). In the past, large (> 2,000 individuals) colonies were found in the Philippines and throughout Indonesia, but colonies of that size are now limited to a small number of areas with little or no human disturbance and to large, undisturbed forests — particularly pristine mangrove forests. The largest *P. vampyrus* colony in the world (ca. 20,000 individuals) is in Subic Bay in the Philippines, which is on the site of a former American naval base with a large dipterocarp forest and a buffer zone surrounding it (Mildenstein *et al.*, 2005). Another large colony (> 5,000 individuals) found through this study was on a remote island surrounded by mangrove forest in a marine protected area in Flores, Indonesia (S.M.T., personal observation). In both of these cases, regular disturbance

at the roosting site is not possible given the restricted accessibility to local people. In highly disturbed islands, such as Java, Sumatra, West Kalimantan, and Bali, colonies of *P. vampyrus* may number fewer than 1,000 individuals (S.M.T., personal observation). Further work is needed to determine the impact of smaller colony size on the genetic stability and persistence of the species, but little is currently known about yearly roosting ecology patterns of *P. vampyrus* colonies in most parts of their range.

The low level of population structure may mean that connectivity between populations of *P. vampyrus* is moderate to high, which has direct consequences for understanding how bat-borne pathogens may have evolved and may evolve in the future. Understanding population genetic diversity and frequency of gene flow among host populations can have a direct effect on studies of pathogen transmission (e.g., predicting source populations — Woolhouse *et al.*, 2005; Epstein and Field, 2015) and other aspects of pathogen biology (e.g., infection cycles, rates of transmission, and degree of host damage — Lion and Boots, 2010; Morand and Krasnov, 2010; Carlsson-Granér and Thrall, 2015). Some studies have challenged these predictions — either by showing no increase in pathogen aggressiveness in a contiguous host population (Tack *et al.*, 2014) or that increased host population connectivity may also result in higher degrees of host resistance to pathogens, meaning that diseases are more likely to appear in isolated populations instead (Carlsson-Granér and Thrall, 2002; Jousimo *et al.*, 2014). The role that host biology, particularly population connectivity plays in maintaining pathogen persistence and prevalence, is then an important factor to consider in studying transmission dynamics and essential to informing strategies for combating the spread of zoonotic pathogens in the event of a pandemic. As anthropogenic pressure increasingly stresses *P. vampyrus* populations, the potential for pathogen transmission and outbreaks increase (Daszak *et al.*, 2001; Dobson and Foufopoulos, 2001; Plowright *et al.*, 2008, 2015). Biodiversity loss has been linked to the increasing pathogen emergence (Daszak *et al.*, 2001; Patz *et al.*, 2004; Pongsiri *et al.*, 2009), and safeguarding flying fox populations and natural spaces may decrease the likelihood of transmission.

Steep declines in *P. vampyrus* populations across the range of this species might also result in dire consequences for forest regeneration, as the fruits of early successional plant species constitute the bulk of their preferred diet (Stier and Mildenstein, 2005).

Excessive hunting of *P. vampyrus* in Borneo threatens the continued persistence of populations (Struebig *et al.*, 2007; Harrison *et al.*, 2011), and population modeling in Malaysia suggests that current levels of hunting are unsustainable (Epstein *et al.*, 2009). Flying foxes in Sumatra and Java are occasionally hunted for medicinal purposes (Croes, 2012), though hunters have found it increasingly difficult to locate populations in recent years (S.M.T., personal observation). Persistent hunting of *P. vampyrus* throughout its range leading to population crashes should be explored further by including sampling of historical specimens in future studies.

Pteropus vampyrus 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 ongoing, significant declines across its range due to overhunting, the species may soon be categorized as Vulnerable (Bates *et al.*, 2008). There is little or no local incentive for regional protection of *P. vampyrus* in Southeast Asia, and local residents throughout most of this region 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 on bats, and seizure activities are rarely initiated by local enforcement agencies (Nijman, 2005; Shepherd and Njiman, 2008). Despite their CITES status, flying foxes in Indonesia are not listed as a protected species (Maryanto *et al.*, 2008). Although the species is found broadly across Malaysia, hunting bans exist in only three of 16 Malaysian states and federal territories (Heng, 2012), and in Thailand and Cambodia (Epstein *et al.*, 2009). Incidental protection due to the proximity of *Pteropus* colonies to religious sites or government grounds occurs in Thailand (S. Bumrungsri, personal communication), Cambodia (Ravon *et al.*, 2014), Vietnam (L. Q. Dang, personal communication), and the Philippines, Bali, and Myanmar (S.M.T., personal observation), but none of these sites have legal protection to deter hunting or persecution of flying foxes. The health of *P. vampyrus* populations should be considered both a conservation and a public health issue to the Association of Southeast Asian Nations (ASEAN) member nations, with the exception of Lao PDR, where *P. vampyrus* does not occur. Southeast Asia is one of the most densely populated areas in the world (United Nations Department of Economic and Social Affairs Population Division, 2015) and these issues should be addressed as a precautionary measure, not a reactionary one.

SUPPLEMENTARY INFORMATION

Contents: Supplementary Fig. S1. Intraspecific phylogenetic tree of *P. vampyrus* based on each molecular marker analyzed separately with MrBayes 3.2 simulated for 10 million generations, with a sampling frequency of 1,000 and 25% burn-in. Posterior probabilities are indicated above each node. Supplementary Information is available exclusively on BioOne.

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APPENDIX   ⇨

Sample information for specimens of *P. vampyrus* used in this study, including subspecies identifications, collection localities, sample numbers, and GenBank accession info for genetic data derived from each sample. LBC = Lubee Bat Conservancy, FL, USA; RMBR = Raffles Museum of Biodiversity Research/Lee Kong Chiang Museum of Natural History, Singapore; ROM = Royal Ontario Museum, Canada. Due to the potential direct threats to colonies, latitude and longitude data for wild-caught specimens were omitted, but available via correspondence with the authors

APPENDIX

Subspecies	Specimen ID	Locality	cyt-*b*	D-loop	ATP7A	BDNF	FGB7	PLCB4	RAG-1	RAG-2	STAT5A
P. v. vampyrus	LBC-904502	West Java		MG921055	MG920997	MG920909	MG920955	MG921121	MG920867	MG921025	MG920928
P. v. vampyrus	LBC-904503	West Java	MG921085	MG921056	MG921004	MG920898	MG920957	MG921122	MG920871	MG921019	
P. v. sumatrensis	LBC-930088	Sumatra	MG921102	MG921057	MG920999	MG920901	MG920977	MG921119	MG920862	MG921024	MG920943
P. v. sumatrensis	LBC-930089	Sumatra	MG921086	MG921058	MG920998	MG920902	MG920972	MG921125	MG920880	MG921023	MG920945
P. v. sumatrensis	LBC-930091	Sumatra			MG921000	MG920888	MG920971	MG921120	MG920863	MG921043	
P. v. sumatrensis	LBC-930092	Sumatra		MG921059	MG921002	MG920913	MG920978	MG921117	MG920870	MG921016	
P. v. sumatrensis	LBC-930093	Sumatra	MG921081		MG921003		MG920954	MG921124	MG920869	MG921046	
P. v. vampyrus	MZB-36229/SW001	West Java	MG921091	MG921065	MG920986		MG920956	MG921113	MG920861	MG921027	MG920942
P. v. vampyrus	MZB-36230/SW002	West Java	MG921084	MG921066	MG920985		MG920963	MG921118	MG920860	MG921033	
P. v. vampyrus	MZB-36231/SW003	West Java	MG921099	MG921067		MG920904			MG920868	MG921031	MG920924
P. v. vampyrus	MZB-36248/SW006	West Java	MG921097	MG921068	MG921001	MG920890	MG920964	MG921130	MG920887	MG921026	
P. v. pluton	SW007	Bali	MG921103			MG920912		MG921131	MG920858	MG921029	MG920941
P. v. pluton	SW008	Bali	MG921105		MG920993		MG920958	MG921132	MG920865	MG921015	MG920920
P. v. pluton	SW009	Bali	MG921109		MG920994	MG920889	MG920969	MG921133		MG921040	
P. v. pluton	SW010	Bali				MG920905	MG920952	MG921143			MG920922
P. v. pluton	SW011	Bali	MG921108	MG921069	MG921008	MG920914	MG920981	MG921140	MG920859	MG921034	MG920923
P. v. vampyrus	MZB-36947/SW077	East Java	MG921100	MG921070	MG921009	MG920916	MG920951	MG921142	MG920856	MG921041	MG920930
P. v. vampyrus	MZB-36948/SW078	East Java	MG921098	MG921071	MG920992	MG920900	MG920983	MG921138	MG920864	MG921048	
P. v. natunae	MZB-36930/SW127	West Kalimantan	MG921088	MG921072	MG921013	MG920903	MG920974	MG921116	MG920884	MG921047	MG920935
P. v. natunae	MZB-36931/SW128	West Kalimantan	MG921082	MG921073	MG921011	MG920893	MG920973	MG921112	MG920881	MG921021	MG920937
P. v. sumatrensis	MZB-36933/SW131	South Sumatra	MG921080	MG921074	MG921005	MG920899	MG920962	MG921114	MG920872	MG921022	MG920944
P. v. sumatrensis	SW132	South Sumatra	MG921079	MG921075	MG920990	MG920895	MG920976	MG921115	MG920885	MG921018	MG920936
P. v. pluton	MZB-36937/SW133	Flores	MG921087	MG921076	MG921010		MG920965	MG921129			MG920946
P. v. pluton	MZB-36938/SW134	Flores		MG921077			MG920968	MG921127			MG920929
P. v. pluton	MZB-36934/SW140	Sumbawa	MG921083	MG921078	MG920984	MG920892	MG920953	MG921128	MG920879	MG921017	MG920947
P. v. sumatrensis	ROM-110949	Vietnam	MG921094	MG921061	MG920995	MG920908		MG921144		MG921020	MG920931
P. v. sumatrensis	ROM-110948	Vietnam	EF584230	MG921060		MG920918		MG921126			MG920933
P. v. lanensis	PNM-7427/MJV419	Southern Leyte, Philippines			MG921012	MG920919	MG920961	MG921147	MG920866	MG921035	MG920938
P. v. lanensis	PNM-7428/MJV420	Southern Leyte, Philippines	MG921092	MG921050			MG920960	MG921146		MG921030	MG920927
P. v. lanensis	PNM-6911/MJV435	Southern Leyte, Philippines	MG921093	MG921051	MG920988	MG920907	MG920959	MG921111	MG920886	MG921044	
P. v. lanensis	PNM-7429/MJV436	Southern Leyte, Philippines	MG921089	MG921052	MG920989	MG920911	MG920967	MG921110	MG920877	MG921045	MG920925
P. v. lanensis	PNM-7430/MJV504	Palawan, Philippines	MG921096	MG921053	MG920987	MG920891	MG920970	MG921137	MG920875	MG921028	MG920934
P. v. lanensis	PNM-7431/MJV505	Palawan, Philippines	MG921090	MG921054		MG920897	MG920966	MG921145	MG920873	MG921036	MG920921
P. v. lanensis	SMT208	Negros Occidental, Philippines	MG921101		MG921006	MG920906	MG920950	MG921135	MG920857	MG921037	MG920926
P. v. lanensis	SMT210	Negros Occidental, Philippines	MG921104			MG920910	MG920949	MG921134	MG920883		
P. v. lanensis	SMT211	Negros Occidental, Philippines	MG921106	MG921062	MG921014	MG920896	MG920980	MG921136	MG920878	MG921039	MG920940
P. v. lanensis	SMT212	Negros Occidental, Philippines		MG921063						MG921042	
P. v. lanensis	SMT213	Negros Occidental, Philippines	MG921107	MG921064	MG921007	MG920917	MG920979	MG921141	MG920874	MG921032	MG920932
P. v. sumatrensis	WRS-G1339	Malaysia (Singapore Zoo F1)	MG921095	MG921049	MG920996	MG920894	MG920982	MG921123	MG920876	MG921038	MG920939
P. hypomelanus	SW012	Madura			MG920991	MG920915	MG920975	MG921139	MG920882		MG920948