1   **Population genetic structure in the insular Ryukyu flying fox, *Pteropus dasymallus***

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3   Shiang-Fan Chen[1], Chung-Hao Juan[1], Stephen Rossiter[2], Teruo Kinjo[3], Dai Fukui[4], Kuniko
4   Kawai[5], Susan M. Tsang[6,7], Maria Josefa Veluz[7], Hiroko Sakurai[8], Nian-Hong Jang-Liaw[9],
5   Keiko Osawa[10], Wen-Ya Ko[11], Masako Izawa[12]

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7   [1] Center for General Education, National Taipei University, Taipei, Taiwan
8   [2] School of Biological and Chemical Sciences, Queen Mary, University of London, London,
9   UK
10   [3] Okinawa Zoo & Museum, Okinawa, Japan
11   [4] University of Tokyo Hokkaido Forest, University of Tokyo, Hokkaido, Japan
12   [5] Department of Biology, Tokai University, Hokkaido, Japan
13   [6] Department of Mammalogy, American Museum of Natural History, USA
14   [7] Mammalogy Section, National Museum of Natural History, Manila, Philippines
15   [8] Hirakawa Zoological Park, Kagoshima, Japan
16   [9] Conservation and Research Center, Taipei Zoo, Taiwan
17   [10] Freelance writer, Saitama, Japan
18   [11] Department of Life Sciences and Institute of Genome Sciences, National Yang-Ming
19   University, Taipei, Taiwan
20   [12] Biology Program, Faculty of Science, University of the Ryukyus, Okinawa, Japan

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22   **Correspondence:**
23   Shiang-Fan Chen: Center for General Education, National Taipei University, Taipei, Taiwan.
24   Email: schen@mail.ntpu.edu.tw
25   Wen-Ya Ko: Department of Life Sciences and Institute of Genome Sciences, National Yang-
26   Ming University, Taipei, Taiwan. Email: wenko@ym.edu.tw
27   Masako Izawa: Biology Program, Faculty of Science, University of the Ryukyus, Okinawa,
28   Japan. Email: izawa@sci.u-ryukyu.ac.jp

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31 **Abstract**
32 Small isolated populations are vulnerable to both stochastic events and the negative
33 consequences of genetic drift. For threatened species, the genetic management of such
34 populations has therefore become a crucial aspect of conservation. Flying foxes (*Pteropus* spp,
35 Chiroptera) are keystone species with essential roles in pollination and seed dispersal in
36 tropical and subtropical ecosystems. Yet many flying fox species are also of conservation
37 concern, having experienced dramatic population declines driven by habitat loss and hunting.
38 The Ryukyu flying fox (*Pteropus dasymallus*) ranges from Japan and Taiwan to the northern
39 Philippines, and has undergone precipitous population crashes on several islands in recent
40 decades. To assess population genetic structure and diversity in *P. dasymallus*, and its likely
41 causes, we analyzed mitochondrial and microsatellite DNA. Both markers showed significant
42 genetic differentiation among most island populations with patterns of isolation-by-distance.
43 However, while mitochondrial haplotypes showed some mixing across the region, likely
44 reflecting historical colonization and/or dispersal events, microsatellites markers showed clear
45 subdivisions corresponding to the position of deep ocean trenches. The current distribution of
46 *P. dasymallus* and its subspecific diversity therefore appears to have arisen through vicariance
47 coupled with a long history of restricted gene flow across oceanic barriers. We conclude that
48 isolated island subgroups should be managed separately, with efforts directed at reducing
49 further declines.
50
51 Keywords: genetic differentiation, island biogeography, oceanic dispersal, Pteropodidae,
52 Ryukyu Islands, vicariance

Introduction

Small and isolated populations are vulnerable to stochastic events and the effects of genetic drift, potentially leading to the loss of diversity, reduced reproductive fitness, and increased risks of extinction (Ellstrand & Elam 1993; Frankham 2010; Jordan et al. 2016). The genetic management of such populations is therefore considered an essential aspect of conservation, particularly for endangered and threatened species. With detailed genetic information, we can effectively monitor the loss of genetic diversity and precisely estimate population parameters such as population size fluctuation, admixture, and gene flow, all of which contribute to our understanding of long-term species survival (Allendorf et al. 2010; Shafer et al. 2015). Genetic and nongenetic (e.g., behavioral, ecological, demographic, and environmental) considerations can therefore be integrated to enhance the efficiency of conservation programmes and further formulate appropriate management strategies (Hoban et al. 2013; Polechová & Barton 2015; Frankham et al. 2017).

Old World fruit bats (Chiroptera: Pteropodidae) are keystone species that play essential roles in pollination and seed dispersal in tropical and subtropical ecosystems (Cox et al. 1991; Fujita & Tuttle 1991). Aside from promoting long-distance seed dispersal needed for forest restoration (Nyhagen et al. 2005; Shilton & Whittaker 2009), these species also pollinate a number of economically important plants, including durian (Aziz et al. 2017). Yet despite acting as major providers of ecosystem services, Old World fruit bats face a range of threats. Over recent decades, the combined impacts of habitat loss, forest degradation, and hunting for bushmeat have all led to severe and rapid population declines (Mickleburgh et al. 2002), with wide-ranging negative ecological and other impacts (Cox & Elmqvist 2000; McConkey & Drake 2006; Florens et al. 2017). Currently, of the nearly 200 recognised species, over a half are of conservation concern, including around 30 *Pteropus* species (IUCN 2019).

Larger-bodied Old World fruit bats are generally considered to be strong and capable fliers with extensive home-ranges. For example, genetic analyses of *P. scapulatus* and *Eidolon helvum* have suggested gene flow can occur over thousands of kilometres, across mainland Australia and Africa, respectively (Sinclair et al. 1996; Peel et al. 2013). Although long distance movements in large Old World fruit bats might result from natal dispersal events, or from storms and typhoons, their capacity for long distance day-to-day movements, even among islands, is likely to be an adaptive trait for tracking ephemeral food resources. For example, long-distance inter-island movements have been recorded and/or inferred indirectly from genetic data in several *Pteropus* species, e.g., *P. dasymallus inopinatus*, *P. medius*, *P. niger*, *P. tonganus*, and *P. vampyrus* (McConkey & Drake 2007; Nakamoto et al. 2011a; Larsen et al. 2014; Tsang et al. 2018; Olival et al. 2019). In these taxa, populations on adjacent islands can appear as single panmictic units. However, in other flying fox species, e.g., *P. livingstonii*, *P. mariannus*, and *P. samoensis*, genetic structure can be present among island groups, indicative of restricted gene flow (Brown et al. 2011; Russell et al. 2016; Ibouroi et al. 2018). These contrasting scenarios require different conservation management approaches; in the former case, island groups might be managed as a single entity, while in the latter case, islands populations might be better treated as distinct evolutionarily significant units (ESU), and thus managed separately (Epstein et al. 2009; Oleksy et al. 2019).

The Ryukyu flying fox (*Pteropus dasymallus*) is distributed from the Ryukyu Archipelago of Japan through Taiwan to the northern Philippines (Kinjo & Nakamoto 2009; Figure 1). Five subspecies are recognized, with populations from Ryukyu Archipelago classified into four subspecies based on their respective island group ranges (Daito flying fox, *P. d. daitoensis*; Erabu flying fox, *P. d. dasymallus*; Orii's flying fox, *P. d. inopinatus*; and Yaeyama flying fox, *P. d. yayeyamae*). The fifth subspecies, from Taiwan, is recognized as the Formosan flying fox

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101 (*P. d. formosus*) (Yoshiyuki 1989; Mickleburgh et al. 1992), while a population in the
102 Philippines has been discovered more recently and has yet to be named formally as a subspecies
103 (Heaney et al. 1998). This latter population occurs north of Luzon on two oceanic island groups,
104 the Batanes and Babuyan Islands, and its unclear subspecific status in part reflects the logistical
105 difficulty in traveling to these islands.

106 *P. dasymallus* is currently categorized as Vulnerable by the IUCN Red List (IUCN 2019),
107 however, the local conservation status differs among the five subspecies (Vincenot et al. 2017).
108 In particular, the Daito, Erabu, and Formosan flying foxes are all characterized by small
109 populations numbering approximately 100-300 individuals (Saitoh et al. 2015). All three are
110 also protected by national laws, with the Daito and Erabu flying foxes designated as 'Natural
111 Monuments' of Japan, and the former also designated as a 'National Endangered Species'.
112 Similarly, the Formosan flying fox is also afforded protection, designated as an 'Endangered
113 Species' in Taiwan. In contrast, the two subspecies Orii's and Yaeyama flying foxes, and the
114 Philippine population, appear more common and are not classified as locally threatened
115 (Heaney et al. 1998; Nakamoto et al. 2011b; Saitoh et al. 2015).

116 The Daito flying fox is geographically restricted to only two small islands of the Daito Islands
117 (Minamidaito-jima and Kitadaito-jima, Figure 1), where most natural habitat has been
118 converted into farmland, and where typhoons are the major current threat (Saitoh et al. 2015).
119 The Erabu flying fox is found on the Ōsumi Islands and Tokara Islands, representing the
120 northern limit of this species (Yoshiyuki 1989). In Taiwan, the Formosan flying fox was once
121 abundant in its original main habitat on Lyudao (Green Island) 30.6 km off the southeastern
122 coast of Taiwan; however, this island population experienced dramatic hunting and habitat loss
123 in the 1970s and 1980s (Lin & Pei 1999), leading to its near extinction, with only four
124 individuals have been recorded in recent years (Chen 2009). In 2004, an additional small
125 population of the Formosan flying fox was recorded for the first time on Gueishan Island
126 (Turtle Island), 9.7 km off the northeastern coast of Taiwan, and some individuals have
127 occasionally been found on the main island of Taiwan since 2006 (Wu 2010). At the present
128 time, nothing is known about the origin of the flying foxes on Gueishan Island and Taiwan's
129 main island.

130 To date, the phylogenetic relationships and population divergence among the subspecies of *P.*
131 *dasymallus* remains obscure but is likely to reflect the geography of the Ryukyu Archipelago.
132 This island chain comprises around 150 islands, extending for 1200 km between the main
133 islands of Japan to Taiwan. Although several adjacent Ryukyu islands were connected by a
134 land bridge in the Last Glacial Maximum (LGM) (Ota 1998), other islands remained isolated
135 from each other. In fact, genetic studies of several taxonomic groups have revealed divergence
136 between populations from the Northern, Central, and Southern Ryukyus (Ota 1998, 2000),
137 areas which remained separated in the LGM by two deep tectonic straits, the Tokara Gap
138 (Tokara Strait) and the Kerama Gap (Miyako Strait) (Figure 1). The Tokara Gap lies between
139 Akuseki-jima and Kodakara-jima in the Tokara Islands, while the Kerama Gap, the widest
140 strait in the Ryukyu Islands, lies between Okinawa-jima and Miyako-jima (Nakamura et al.
141 2013). Therefore, both of these gaps are likely to have formed geographical barriers, so
142 promoting genetic drift and divergence among the Northern, Central and/or Southern Ryukyu
143 populations (Toda et al. 1997; Lin et al. 2002). Of these, Taiwan is geographically closest to
144 the Southern Ryukyus (110 km), and both species and genetic diversity have been found to be
145 more similar among Taiwan and the Southern Ryukyus than between these islands and other
146 regions (>270 km) (Ota 2000; Tominaga et al. 2015). To the south, the two oceanic island
147 groups of the Batanes and the Babuyan Islands were formed about three million years ago (late
148 Pliocene), with continued uplift and volcanism into the Pleistocene. The Batanes is closer to
149 the southern tip of Taiwan (150 km, Bashi Channel) than they are to Luzon (200 km) (Bellwood

4

150 & Dizon 2013). However, the genetic structure of mammals on the island chain has seldom
151 been addressed (Yoshikawa et al. 2016).

152 Here we examined genetic diversity and population genetic structure in *P. dasymallus* using
153 mitochondrial DNA (mtDNA) and microsatellite markers. We aimed to (1) assess the genetic
154 diversity of *P. dasymallus* across the different island groups, (2) examine whether genetic
155 differentiation exists among these groups in line with their subspecies designations, (3)
156 examine the pattern of genetic structure, and (4) examine the relationships between both the
157 newly-recorded Formosan flying fox individuals (from Gueishan Island and Taiwan's main
158 island) and the little-studied Philippine samples, and the other subspecies. Our hypotheses were
159 as follows: First, we hypothesized that the Daito, Erabu, and Formosan flying fox subspecies
160 will show lower genetic diversity than the other subspecies due to their relatively small
161 population sizes. Second, we hypothesized that genetic differentiation exists in *P. dasymallus*
162 and corresponds to the respective subspecies identities. Third, like in many other species in this
163 region, this differentiation can be also accounted for by regional deep sea trenches: the Tokara
164 and Kerama Gaps in the Ryukyu Archipelago, and the Bashi Channel between Taiwan and the
165 northern Philippines. Finally, we hypothesized that the Formosan flying fox individuals, newly
166 recorded on Gueishan Island and Taiwan's main island, will share a similar genetic structure
167 with bats from the nearest Yaeyama Islands, suggesting a colonization event. An understanding
168 of the genetic structure and degree of gene flow among different island groups of *P. dasymallus*,
169 along with insights into whether these patterns are ancient or new, can help to inform
170 conservation management decisions, including whether or not to treat small populations
171 separately, or whether to translocate isolated vagrants.

172

173 **Methods**

174 **<u>Sampling</u>**

175 We obtained samples of *P. dasymallus* opportunistically over a period of 10 years (2009-2019)
176 from wild-caught individuals and carcasses found in the wild, as well as rescued and/or captive
177 individuals. All samples originated from eight different Taiwanese and Ryukyu islands, and
178 were classified into the five subspecies based on their geographical source following
179 Mickleburgh et al. (1992). A total of 77 samples were analyzed for this study after removing
180 duplicate samples and putative parents or offspring of other individuals, as outlined below.
181 Sample sizes per subspecies were 36 Formosan, 10 Yaeyama, 22 Orii's, 1 Erabu, and 8 Daito.
182 Formosan flying fox samples were further divided into three groups denoted as TW1 (Gueishan
183 Island), TW2 (Lyudao), and TW3 (Taiwan's main island) according to the islands from which
184 they originated. Yaeyama flying fox samples originated from Iriomote-jima and Ishigaki-jima,
185 Orii's flying fox samples from Okinawa-jima, Erabu flying fox samples from Kuchinoerabu-
186 jima, and Daito flying fox samples from Minamidaito-jima (Figure 1, Appendix 1).

187 Samples ranged from wing membrane biopsies, blood and frozen muscle tissue to fecal samples.
188 Wing membrane samples were collected with a 3-mm biopsy punch and placed in 99.5%
189 ethanol, Allprotect Tissue Reagent (Qiagen), or silica beads until extraction. For the blood
190 samples, a volume of 0.5 cc was taken by a professional veterinarian and preserved in
191 ethylenediaminetetraacetic acid (EDTA) anticoagulant. Frozen muscle tissue obtained from
192 specimens, and fresh feces, were stored in 99.5% ethanol or RNAlater RNA Stabilization
193 Reagent (Qiagen).

194 **<u>DNA extraction and amplification</u>**

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195 To extract genomic DNA from wing membrane, blood, and frozen muscle samples, we used
196 DNeasy Blood and Tissue Kits (Qiagen). For fecal samples, we used the QIAamp Investigator
197 Kit or QIAamp Fast DNA Stool Mini Kit (Qiagen).

198 We amplified a section of the mtDNA control region using the primers BovL 14987 (5'-CGC-
199 ATA-TGC-AAT-CCT-ACG-A-3') and BovR 15967 (5'-GCG-GGT-TGC-TGG-TTT-CAC-3'),
200 which we designed for this study. Polymerase chain reaction (PCR) was carried out in a total
201 volume of 15 μl, containing 20-100 ng of template DNA, 0.25μl of 10 μM of each primer, and
202 7.5 ul of Quick Taq HS DyeMix (TOYOBO). Amplification was performed with the following
203 profile: 2 min at 94ºC followed by 30 cycles of 30 s at 94ºC, 30s at annealing temperature
204 (55°C), 50 s at 68ºC, and a final extension of 10 min at 68ºC. PCR products were run on an
205 ABI 3730XL DNA Analyzer (Applied Biosystems). The chromatograms were edited and
206 aligned in the program of SeqMan and MegAlign (DNASTAR). We also obtained three
207 published *P. dasymallus* partial control region sequences from GenBank and Dryad, for one
208 Yaeyama flying fox from Irabu-jima (accession NC_002612.1) (Nikaido et al. 2000), and two
209 individuals collected from the Batanes Islands, with one from Batan Island and the other from
210 Sabtang Island (accessions MJV458 and MJV451, respectively), representing the Philippine
211 population (Tsang et al. 2019).

212 For microsatellite DNA analysis, 108 species-specific markers were generated by Genetic
213 Identification Services (CA, USA). To quantify polymorphism and characteristics of these loci,
214 we used a subset of samples. In total, 26 loci were polymorphic and used for subsequent
215 genotyping 76 samples (Supporting Information S1). For this, PCRs were carried out in a total
216 volume of 10 μl, containing approximately 10-50 ng of template DNA, 0.5 μl of 10 μM of each
217 primer, and 5 μl of Quick Taq HS DyeMix. Amplification was performed with the following
218 profile: 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at annealing temperature
219 (54°C), 1 min at 68°C, and a final extension of 10 min at 68°C. PCR products were also run
220 on an ABI 3730XL DNA Analyzer, and allele scoring was performed using the software
221 GeneMarker 4.2 (SoftGenetics). Identity and parentage analyses were performed using Cervus
222 3.0.7 (Kalinowski et al. 2007) to identify duplicate samples and parentage. Samples with
223 exactly matching genotypes across all loci were determined as duplicates, and removed.
224 Parentage was determined based on no allele mismatches. Only one sample from each duplicate
225 or parent-offspring pair was included for further analyses.

226 **MtDNA analysis**

227 Based on mtDNA data, we estimated the number of haplotypes, haplotype diversity (h),
228 nucleotide diversity (π), and average number of pairwise differences for each subspecies. We
229 determined the extent of genetic differentiation by applying an analysis of molecular variance
230 (AMOVA) (Excoffier et al. 1992). Only populations with sample sizes greater than one were
231 included. The total variance was partitioned into variance components attributable to within
232 and among subspecies. To measure the degree of genetic differentiation among subspecies, the
233 derived index of the total population was estimated. The significance of the differentiation was
234 tested by performing 20,000 random permutations. These analyses were performed using
235 Arlequin 3.5.2.2 (Excoffier & Lischer 2010).

236 We also estimated pairwise differentiation among subspecies, and examined isolation by
237 distance for genetic distances, estimated by Φ_{ST}, among islands. For geographical distances
238 (km) among pairwise islands, linear Euclidean distances between the centers of pairwise
239 sampling islands were computed based on latitudinal and longitudinal coordinates. A total of
240 eleven islands were included in the analysis of isolation by distance (Gueishan Island, Lyudao,
241 the main island of Taiwan, Iriomote-jima, Ishigaki-jima, Irabu-jima, Okinawa-jima,
242 Kuchinoerabu-jima, Minamidaito-jima, Batan Island, and Sabtang Island). The significance

6

243 level was assessed using a Mantel test with 20,000 permutations in Genepop (web version)
244 (Raymond & Rousset 1995; Rousset 2008).

245 To further visualize genetic structure with respect to subspecies, we generated a haplotype
246 network. An unrooted maximum likelihood tree was generated in MEGA X (Kumar et al. 2018)
247 and converted into a haplotype network using Haplotype Viewer (Center for Integrative
248 Bioinformatics Vienna).

249 **Microsatellite DNA analysis**

250 Deviation from Hardy-Weinberg equilibrium (HWE) at each microsatellite locus and
251 subspecies, and linkage disequilibrium for each pair of loci, were tested using the Markov chain
252 method (10,000 dememorization steps, 1,000 batches and 10,000 iterations per batch). We
253 assessed statistical significance using Bonferroni correction for multiple comparisons. For each
254 microsatellite locus, we recorded the number of alleles (N_A), observed heterozygosity (H_O),
255 and expected heterozygosity (H_E). For each subspecies, we derived diversity indexes, including
256 the mean number of alleles (N_a), allelic richness corrected for unequal sample size (A_C), and
257 the mean H_O and H_E. The average pairwise relatedness (RI) of each subspecies was calculated
258 to infer relationships between individuals (Ritland 1996).

259 Like mtDNA, we also conducted genetic structure analyses, including AMOVA, pairwise
260 differentiation, estimated by F_{ST}, and isolation by distance, for microsatellite data. Eight islands
261 were included here (Irabu-jima, Batan Island, and Sabtang Island were excluded, where no
262 microsatellite data was available). These analyses were performed in GenAlEx 6.51 (Peakall
263 & Smouse 2006, 2012) or Genepop (web version).

264 To examine relationships among populations based on multilocus microsatellite genotype data,
265 we inferred the number of genetically distinct clusters using the Bayesian clustering approach
266 implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003). An admixture
267 ancestry model with correlated allele frequencies was used with a burnin period of 100,000
268 iterations followed by 1,000,000 Markov chain Monte Carlo (MCMC) repetitions. The number
269 of ancestral populations (K) was set to 1 to 10. Ten independent runs for each K to confirm
270 consistency across runs were performed with prior information on population origins. The best
271 number of K was determined based on the mean likelihood ($L(K)$) and variance for each K
272 value and the *ad hoc* statistic ΔK with the Evanno method using the program Structure
273 Harvester (Evanno et al. 2005; Earl & vonHoldt 2012). The output data were generated and
274 visualized with Clumpak 1.1 (Kopelman et al. 2015).

275

276 **Results**

277 **MtDNA analysis**

278 Analyses of partial mtDNA control region sequences revealed 33 haplotypes with 22
279 parsimony informative sites from a total of 80 *P. dasymallus* samples encompassing the five
280 recognized subspecies and the Philippines population. Haplotype diversity (h), nucleotide
281 diversity (π), and pairwise difference averaged over all samples were 0.948, 0.012, and 3.556,
282 respectively. A summary of the genetic diversity is presented in Table 1. The Philippine
283 population showed the highest diversity. On the other hand, the Daito flying fox consistently
284 showed the lowest diversity. We excluded the Erabu flying fox individuals from the
285 subspecies-level analyses given that only one sample is available.

286 The AMOVA revealed genetic differentiation among the five analyzed populations (the
287 Formosan, Yaeyama, Orii's, and Daito flying foxes and Philippine population with sample
288 sizes greater than one). The Φ_{ST} value was 0.140, which was significantly different from zero

289 (P < 0.001, Table 2). This indicated that approximately 14.0% of the total mtDNA genetic
290 variation was accounted for by the differences among subspecies. The magnitude of the
291 pairwise differentiation varied markedly with the lowest value shown in the pair of the
292 Formosan and Yaeyama flying foxes. The Daito flying fox and Philippine population presented
293 relatively high values of differentiation with other counterparts (Table 3).

294 We found a significant positive correlation between pairwise genetic and geographical
295 distances based on Mantel tests (r^2 = 0.115, P < 0.05, Figure 2), indicating that genetic
296 differentiation in *P. dasymallus* across the Ryukyu, Taiwanese and Philippine islands fits an
297 isolation-by-distance model. Pairwise genetic distances also showed that the TW1 Formosan
298 flying fox has a close relationship with Yaeyama populations from Iriomote-jima and Ishigaki-
299 jima (0.082and 0.016, respectively), with pairwise distances that are lower than those between
300 TW1 and both the TW2 and TW3 populations from the same subspecies (0.252 and 0.143,
301 respectively).

302 The haplotype network indicated that one of the Philippine samples (MJV451) from Sabtang
303 Island showed a relatively deeper genetic divergence with respect to the other samples. On
304 the other hand, the haplotypes presented by Japanese or Taiwanese samples were genetically
305 close to each other (Figure 3). Of these, two haplotypes were the most common, shared by
306 11and 10 individuals mainly found in Okinawa-jima (Orii's flying fox) and Gueishan Island
307 (TW1 Formosan flying fox), respectively.

Microsatellite analysis

309 Genotype analysis based on 26 polymorphic microsatellite loci from 76 *P. dasymallus* samples
310 of five subspecies revealed a moderate degree of polymorphism across subspecies. The number
311 of alleles at each locus was 5.27 ± 2.29, ranging from 2 to 10 across all the samples. The mean
312 H_O and H_E values were 0.536 and 0.544, respectively. The highest diversity was recorded in
313 the Formosan or Yaeyama flying fox. In contrast, and in line with the mtDNA data, the Daito
314 flying fox harboured the lowest diversity (Table 1). The F_{IS} values were all not significant,
315 implying no major deviations from HWE. Exact tests showed that four locus-population
316 combinations deviated from HWE; however, there was no consistent pattern according to either
317 subspecies or locus. No loci pair was detected in linkage disequilibrium. Finally, average
318 pairwise relatedness was significant in three analyzed population except the Yaeyama. Daito
319 flying fox showed a particularly high value of 0.147.

320 The AMOVA based on microsatellite data showed significant genetic differentiation among
321 subspecies. The F_{ST} value was 0.069 (P < 0.001, Table 2). The Formosan and Yaeyama flying
322 foxes were the only pair without significant differentiation. A pattern of isolation by distance
323 was also shown here (r^2 = 0.298, P < 0.01, Figure 2).

324 An analysis of genetic structure using STRUCTURE revealed clear substructure among
325 geographical locations and subspecies identities (Figure 4). The most likely number of genetic
326 clusters was four (K= 4) as inferred using the Evanno method based on the highest ΔK and
327 mean likelihood value without an increase in variance. All or nearly all of the Daito and Orii's
328 flying fox samples, respectively, were assigned unambiguously to their own clusters, with the
329 exception of one individual of Orii's flying fox. A number of individuals also showed evidence
330 of partial inferred ancestry. Formosan flying foxes from TW2 (from Lyudao) and TW3 (from
331 Taiwan's main island), and Erabu flying foxes, were assigned to a different genetic cluster. On
332 the other hand, Formosan flying foxes from TW1 (from Gueishan Island) and Yaeyama flying
333 foxes showed a greater admixture across different genetic clusters with full or partial
334 membership. For K = 3, the Erabu and Orii's flying foxes were grouped together. The

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335   Formosan and Yaeyama flying foxes showed an admixture of different genetic clusters. For *K*
336   = 5, the Erabu flying fox also showed an admixture of membership.

337

Discussion

339   We examined genetic diversity and structure among the five recognized subspecies of *P.*
340   *dasymallus* from the Ryukyu Archipelago of Japan to Taiwan, and also included published data
341   from two individuals sampled from a poorly known population from Batanes, Philippines. Our
342   analyses based on mtDNA control region sequences and 26 microsatellite markers revealed
343   significant genetic differentiation among island groups, broadly supporting the subspecies
344   identities based on geographical locations (Figure 4).

345   Differences in the patterns of differentiation recovered by the two types of markers provide
346   insights into the history of connectivity of these island populations. Notably, while we detected
347   no deep divergence among any of the individuals from the five subspecies, the haplotypes
348   showed evidence of only weak sorting with respect to island (Figure 3). Overall this pattern
349   points to gene flow in the past, either through recurrent gene flow or colonization and
350   admixture, alongside evidence of isolation and genetic drift in some cases, likely reflecting
351   small population sizes. The central position of orange haplotype, which was most abundant in
352   the Orii's flying fox, suggests that this taxon might have served as a source of other populations
353   across the Ryukyu and Taiwanese islands. In this scenario, the other subspecies populations
354   were founded by colonization events, eastward to the Daito Islands and westward to Taiwan.
355   While we were only able to examine two sequences from the Philippine population, these
356   showed evidence of high levels of divergence with respect to each other, with the bat sampled
357   from Sabtang Island also showing clear separation from all other samples. Further study,
358   including sampling of bats from the Babuyan Islands, is needed to assess the likely causes of
359   this apparent deep structure.

360   Our microsatellite analyses revealed a cline in genetic diversity from the highest in the
361   Formosan and Yaeyama flying foxes, to the lowest in the Daito flying fox (Table 1). Of all the
362   taxa, the Daito flying fox was seen to form a separate cluster with no admixture across different
363   values of K. The genetic distinctiveness of the Daito flying fox can be explained by the
364   comparatively large geographical distance between the remote easternmost Daito Islands and
365   the other Ryukyu islands (approximately 360 km east off of Okinawa-jima) coupled with the
366   absence of islands that could serve as stepping-stones for dispersers. The Daito Islands are
367   uplifted coral islands that lie on the Philippine Sea Plate and are thought to have emerged
368   approximately 1.2 to 1.6 million years ago in the mid-Pleistocene (Shiroma et al. 2015; Knez
369   et al. 2017). Consequently, unlike the other continental islands, the oceanic Daito Islands have
370   never been connected to a land mass by a land bridge during a glacial period. Instead, the
371   Ryukyu Trench (Figure 1) - a deep, broad water body that separates the Daito Islands from the
372   Eurasia Plate - has served as a significant geographical barrier to gene flow. We conclude that
373   the low genetic diversity and high differentiation from other subspecies suggest the Daito
374   subspecies arose from a historical event involving long-distance oceanic dispersal and has since
375   experienced geographical and reproductive isolation. Similar differentiation and restricted
376   gene flow between the Daito Islands and other Ryukyu islands lying on the Eurasia Plate has
377   been reported for the elegant scops owl (*Otus elegans*) (Hsu 2005).

378   In addition to the Daito flying fox, we also found differentiation among other populations
379   across the Ryukyu Archipelago based on multilocus genotypes, notably between the Erabu,
380   Orii's, and the Yaeyama flying foxes. In the case of Erabu, the inclusion of just one sample
381   strongly limits our interpretations about this population. In contrast, the results for Orii's and

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382   Yaeyama were more surprising, especially in light of the mtDNA data. Such subdivisions based
383   on ncDNA are strongly concordant with the position of deep-sea channels, including the
384   Tokara and Kerama Gaps, that separate the Northern, Central and Southern Ryukyus. Thus
385   differentiation among these island groups, appears to have been driven by their long-term
386   isolation from each other, a consequence of the fact that they were not connected by land-
387   bridges during the Last Glacial Maximum.

388   In spite of the strong genetic subdivisions detected, our results also showed a significant
389   positive correlation between genetic and geographical distance that is consistent with a pattern
390   of isolation by distance across Taiwan and the Ryukyu Islands. Isolation by distance is typically
391   considered to be a consequence of migration-drift equilibrium, whereby recurrent gene flow
392   follows a stepping-stone pattern, and is thus more likely to occur among neighboring
393   populations (Kimura & Weiss 1964). Nevertheless, trends of isolation by distance can also be
394   generated through a colonization process, in which the contribution of genetic drift outweighs
395   that of gene flow. Finally, drift and thus isolation by distance might also be more easily detected
396   at larger spatial scales due to the higher probability that barriers will occur over greater
397   distances (see Bossart & Prowell 1998). Indeed, in our study system, it is notable that island
398   groups characterised by stronger genetic differentiation were also more likely to occur on
399   opposite sides of deep-sea trenches (e.g., Hutchison & Templeton 1999).

400   Previous studies have reported gene flow among flying fox populations over hundreds to
401   thousands of kilometres, although these have tended to focus on movements over land or along
402   coast lines. In our study, similar genetic profiles of populations from Taiwan and Yaeyama
403   support genetic mixing via movements across water, coupled with the formation of a land-
404   bridge in the LGM. Indeed, these two subspecies are geographically closest and the least
405   genetically structured. On the other hand, strong differentiation among island populations of
406   flying foxes separated by 200-300 km, such as between Orii's and Yaeyama, suggests that this
407   distance represents an upper limit for recurrent gene flow in these bats. Nonetheless, despite
408   overall clear genetic differentiation based on microsatellites, our structure-based clustering
409   analyses did reveal a small number of putative migrants. In particular, two individuals recorded
410   in Taiwan (TW1) and one in Yaeyama, appear to be individuals of the Orii's flying fox.

411   A surprising result of this study was the high recorded genetic diversity in the Formosan flying
412   fox from Gueishan Island (TW1). The inhabitants living on Gueishan Island before 1977, when
413   the island was designated as a military control area, claimed that no flying fox had been seen
414   on the island (Wu 2010), and thus this population is considered to be newly established via
415   oceanic dispersal. Although this population appears to show a closer relationship with the
416   Yaeyama flying fox from Iriomote-jima than with the other populations on Taiwan (TW2 and
417   TW3), its high diversity likely stems from genetic admixture involving several different genetic
418   clusters (e.g., Comas et al. 2004). Indeed, our results indicate that the TW1 population likely
419   has multiple ancestral origins with putative founders from Yaeyama, TW2 (from Lyudao),
420   TW3 (from the main island of Taiwan) and/or the Philippine population.

421   A combination of one or more explanations could account for the genetic diversity found on
422   Gueishan Island. First, flying foxes might have arrived on Gueishan Island as a result of strong
423   winds associated with seasonal typhoons or the winter northeast monsoon. Second, individual
424   bats may have actively dispersed in search of resources. A third scenario is that active dispersal
425   was driven by population expansion of the Yaeyama flying fox population. Yonaguni-jima of
426   Japan, the westernmost margin of the distribution of the Yaeyama flying fox (Kinjo &
427   Nakamoto 2009), is 107 km from Gueishan Island. According to Nakamoto et al. (2011a),
428   Yaeyama flying foxes have been presumed to be dispersing eastward across the sea to a new
429   insular habitat approximately 50 km away (from Tarama-jima to Miyako-jima). The flying fox

430 population on Yonaguni-jima or the neighboring islands may also expand westward to
431 Gueishan Island with the help of winds, forming a widely distributed and diverse population.

432

Conclusions

434 Our findings from mitochondrial and nuclear markers support the current division of
435 subspecies of *P. dasymallus* from the Ryukyu Archipelago and Taiwan. Genetic subdivisions
436 among some island groups appear to reflect a lack of long-distance movements across water,
437 coupled with the presence of deep-sea channels that prevented the formation of land-bridges
438 during the LGM. We also find evidence that the recent colonization of Taiwan has involved
439 founders from several distinct clusters. Taken together, we conclude that highly isolated and
440 genetically distinct populations, such as Daito, should be treated as separate management units.
441 On the other hand, bats from adjacent islands that show strong evidence of recent and frequent
442 gene flow can be managed as a single population. The comparatively higher level of divergence
443 between the Philippine sample from Sabtang Island and all the other sampled bats highlights
444 the importance of future work to establish the status of this population. More generally, our
445 results indicate that the evolutionary and ecological forces shaping the pattern of the genetic
446 structure in *P. dasymallus* are dynamic and ongoing. As a taxon that ranges from the temperate
447 northern Ryukyu Archipelago and subtropical Taiwan to the tropical northern Philippine
448 islands, this species may serve as an excellent model for studying the processes driving island
449 biogeography. Future studies that combine new sequencing technologies with more extensive
450 sampling of the Philippine populations are expected to improve the current understanding of
451 the phylogeography of *P. dasymallus* among islands.

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Table 1. Genetic diversity of *Pteropus dasymallus* subspecies estimated with mitochondrial DNA control region and 26 microsatellite loci. N: sample size, H: number of haplotype observed, *h*: haplotype diversity, π: nucleotide diversity, *k*: average number of nucleotide differences, N_a: mean number of alleles per locus, A_C: allelic richness, H_O: observed heterozygosity, H_E: expected heterozygosity, RI: relatedness, F_{IS}: inbreeding coefficient. The Erabu flying fox was excluded for subspecies-level analyses given that only one sample is available. Abbreviation: FF, flying fox.

Population	mtDNA					Microsatellite DNA						
	N	H	*h*	π	*k*	N	N_a	A_C	H_O	H_E	RI	F_{IS}
Formosan FF	36	16	0.889	0.012	3.557	36	4.885	3.599	0.574	0.601	0.004*	0.060
Yaeyama FF	11	10	0.982	0.011	3.200	10	3.731	3.480	0.596	0.584	0.005	0.031
Orii's FF	22	9	0.844	0.008	2.377	22	3.846	3.351	0.540	0.575	0.029*	0.083
Erabu FF	1	1	-	-	-	1	-	-	-	-	-	-
Daito FF	8	3	0.679	0.007	2.214	7	2.885	2.885	0.434	0.418	0.147*	0.040
Philippines	2	2	1.000	0.043	13.000	-	-	-	-	-	-	-

16

672 Table 2. Analysis of molecular variance (AMOVA) for *Pteropus dasymallus*. Five populations,
673 including the Formosan, Yaeyama, Orii's, and Daito flying foxes and Philippine population,
674 with sample sizes greater than one are included in the mtDNA analysis. The first four are
675 included in the microsatellite analysis.
676

Source of	mtDNA		Microsatellite DNA	
	variation	P value	variation (%)	P value
Among	13.97	< 0.001*	6.91	< 0.001*
Within subspecies	86.03		93.09	

677 *: statistically significant
678
679

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680 Table 3. Pairwise genetic differentiation between *Pteropus dasymallus* subspecies or
681 population. Φ_{ST}, above diagonal based on mtDNA data; F_{ST}, below diagonal based on
682 microsatellite data. The Erabu flying fox is excluded. Statistical significance is also provided.
683 Abbreviation: FF, flying fox.
684

| Population	Formosan FF	Yaeyama FF	Orii's FF	Daito FF	Philippines
Formosan FF		0.000[NS]	0.096*	0.166*	0.407*
Yaeyama FF	0.011[NS]		0.079*	0.158*	0.361*
Orii's FF	0.057*	0.036*		0.247*	0.519*
Daito FF	0.132*	0.139*	0.159*		0.439*

685 [NS]: nonsignificant
686 *: significant at $P < 0.05$
687
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690 Figures
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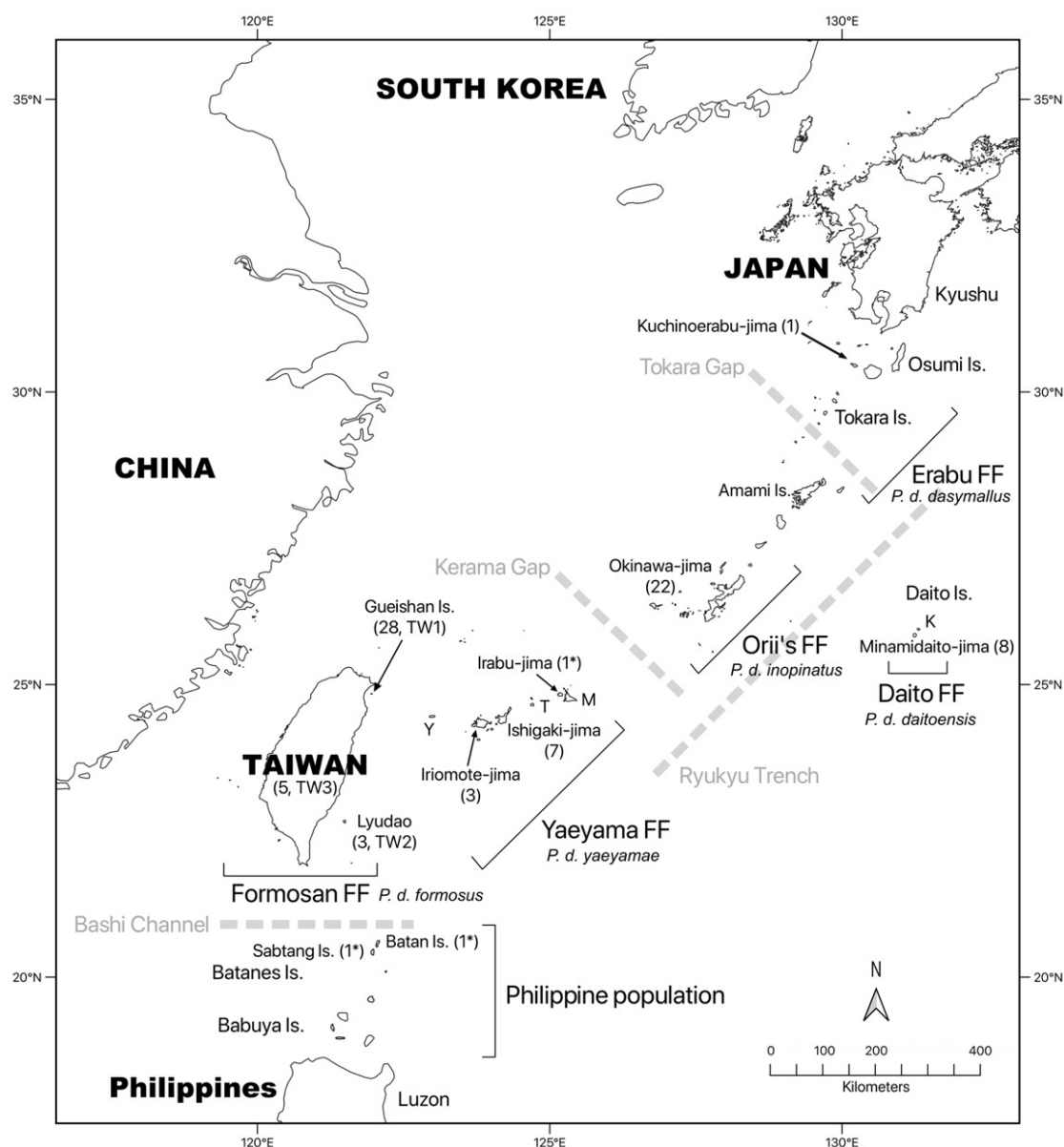
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694 Figure 1. Map of the Ryukyu Archipelago, Taiwan, and northern islands of the Philippines
695 showing the distribution of *Pteropus dasymallus*. The locations where the samples used in
696 this study originated is also shown with corresponding sample size presented in brackets. An
697 asterisk indicates DNA sequences acquired from Genbank or Dryad. Abbreviation: FF, flying
698 fox; Y, Yonaguni-jima; T, Tarama-jima; M, Miyako-jima; K, Kitadaito-jima.
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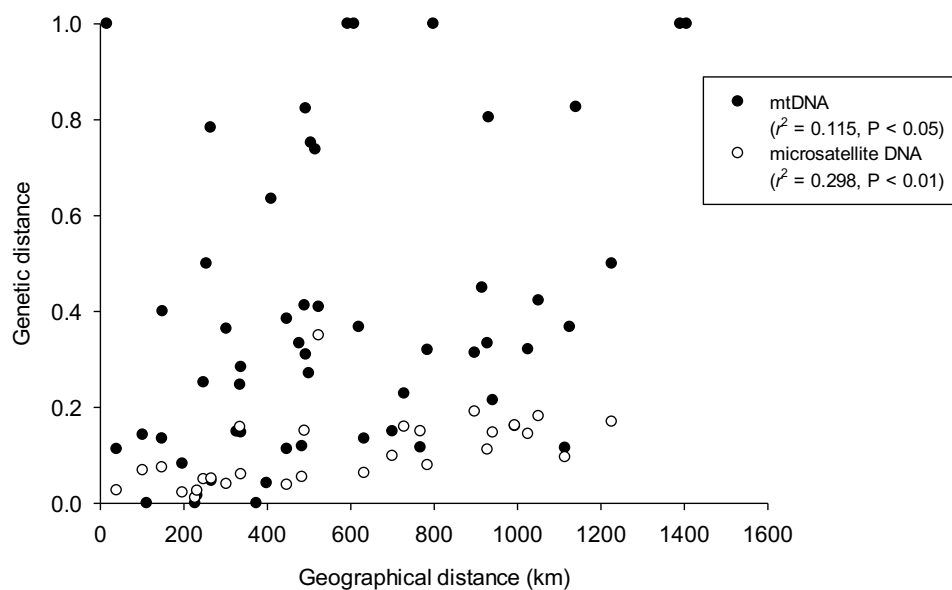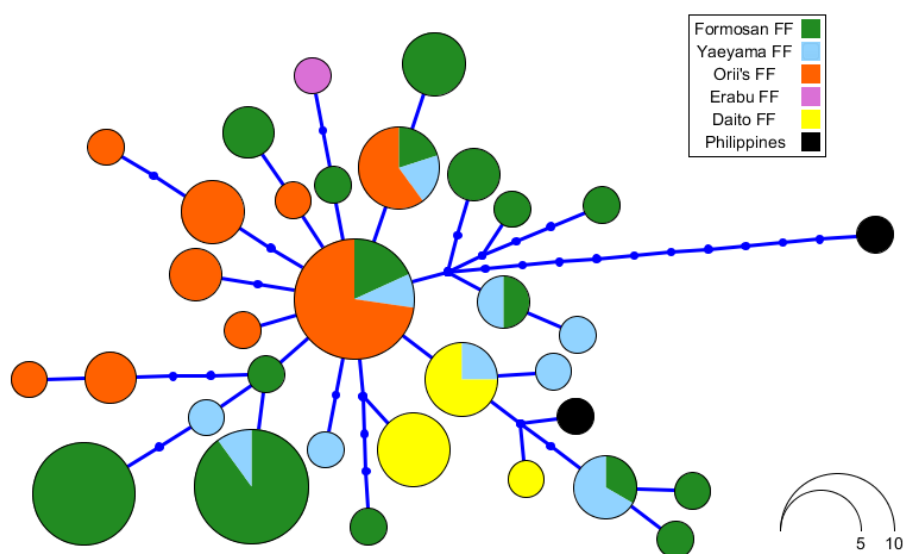

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722
723   Figure 2. Relationships between genetic and geographical distances in *Pteropus dasymallus*
724   in pairwise comparisons among islands.

725

726
727 Figure 3. Haplotype network for *Pteropus dasymallus*. Each color represents a subspecies or
728 population. The size of each circle is proportional to haplotype frequency. Each line segment
729 and small dot represent a single mutational step and an inferred intermediate haplotype,
730 respectively.
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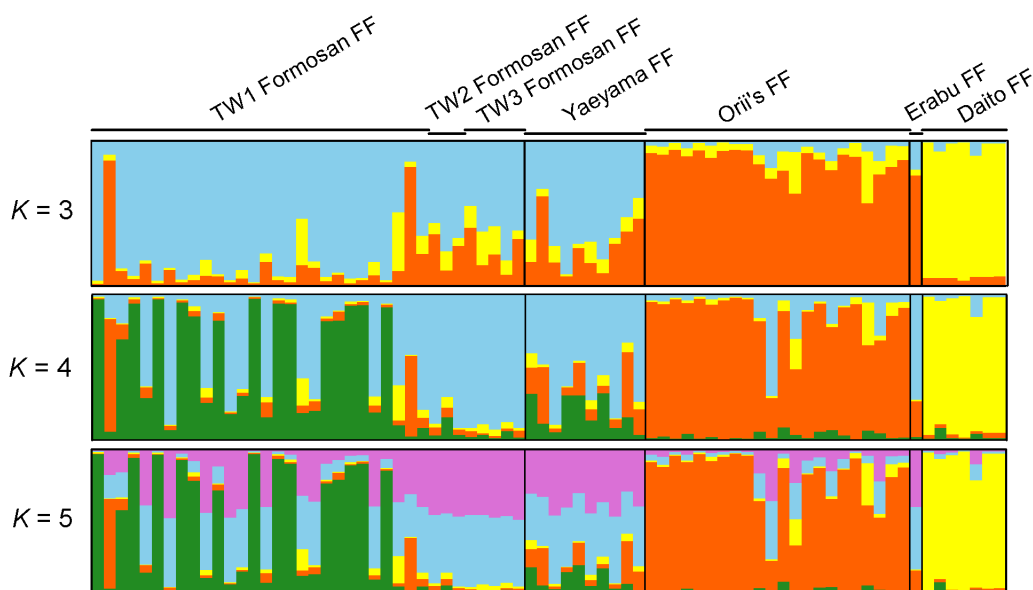
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737   Figure 4. Genetic structure of 76 *Pteropus dasymallus* individuals from five subspecies based
738   on a Bayesian STRUCTURE analysis. Each vertical bar represents one individual. Each color
739   represents a genetic cluster. The length of each color in a certain vertical bar is proportional
740   to the probability of assigning the individual to the corresponding cluster. The value of *K*
741   indicates the possible number of clusters. The most likely number of clusters with the highest
742   value of Δ*K* is four. The vertical black lines separate subspecies in different island groups.
743   The Y-axis represents proportion, with a range between 0 and 1. Abbreviation: FF, flying
744   fox.
745
746
747

748 Appendix 1. Sample size and localities of origins of *Pteropus*
749 *dasymallus* used in this study.
750

Population name	# of samples	Localities of origins
P. d. formosus (Formosan flying fox)		
TW1	28	Gueishan Island
TW2	3	Lyudao
TW3	5	Taiwan's main island
P. d. yayeyamae	3	Iriomote-jima
(Yaeyama flying fox)	7	Ishigaki-jima
	1[a]	Irabu-jima
P. d. inopinatus (Orii's flying fox)	22	Okinawa-jima
P. d. dasymallus (Erabu flying fox)	1	Kuchnoerabu-jima
P. d. daitoensis (Daito flying fox)	7	Minamidaito-jima
Philippine population	1[b]	Batan Island
	1[b]	Sabtang Island
Total	80	

751 [a]: sequence acquired from Genbank with an accession numbers of
752 NC_002612.1 (Nikaido et al. 2000)
753 [b]: sequences acquired from Dryad with catalog numbers of MJV451 and
754 MJV458 (Tsang et al. 2019)
755
756

23

757 Supporting Information
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759 S1. Twenty-six polymorphic microsatellite loci for *Pteropus dasymallus* used in this study.
760   N_A: number of alleles; H_O: observed heterozygosity; H_E: expected heterozygosity.
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Locus	Forward primer (5'-3')	Repeat motif	Size range (bp)	N_A	H_O	H_E
A011	F: TCTGACTTGAGCCCTAAATGCA R: CCAACTGATATCTCTCTGGGTGGT	ATCT	177-213	10	0.774	0.668
A013	F: CATCTTAGCCAAACGCCAGC R: CCTTTCCCTTCTATTTTCCTGGA	ATCT	209-230	6	0.245	0.531
A206	F: CTAGTGTTAGAAATCTGGGCTATTAATGTATAC R: AAGAGATAATTGAAAGCAAAGAAAAAAGA	ATAG	231-247	5	0.590	0.558
A207	F: GCCATCGGAAATCTAATGTGC R: ACTGTCAAAACACTCTCCAATAAACAA	ATCT	203-215	4	0.490	0.489
A214	F: GGAAAGAGGTCCCAATGGCT R: TTTTGAATTCTGCATGAGAGATTTG	AGAT	182-202	6	0.539	0.531
A226	F: CTTTCCAAATGCCAACTGTTGA R: TCTAGAATGTGAAACATAAGCCTCTGA	AGAT	168-185	5	0.556	0.487
B004	F: CCGACACATGCCACTTGAGT R: CCCCATGTAATATGCTGCTTTTT	GTTT	115-123	3	0.322	0.284
B007	F: TCCGTTTTTTGCGTCAGACA R: GCCCTCGCTGTTCTGATATGA	GTTT	153-157	2	0.338	0.313
C017	F: TTTGTGGGTTTCCAGCTTCC R: GGCTTATCCAGAGCAACAGGTC	TATG	179-196	5	0.475	0.417
C305	F: TGGATTTTGTTAACCAATGTCACC R: GCCGTTTCCAATTTACTTCTCTCA	CATA	143-156	4	0.250	0.471
C312	F: AGAGTGCAAGACAGGCAGGG R: TGAACACAAAATGCAGTATATGGATG	ATGT	209-235	6	0.370	0.361
A313	F: CTTTGGGATTTCACGGGCTA R: AAAAGGTTGGCCATCCTGTG	AGAT	153-173	6	0.811	0.661
A213	F: GAACTGGGTCATTGGCTGGTA R: GTGGCTCCTGGGCTACATGT	ATCT	164-185	6	0.543	0.585
D018	F: CTCCTTCAGTTTAGGCTGTGCA R: TTTTGCCAGTGAGATGCCAA	AC	154-166	7	0.378	0.400
B215	F: CTTTTGTGTGAGCGCTGGTG R: ACTCAGCCCTCTGCTGTTCCT	GT/GTCT	174-182	5	0.559	0.503
B014	F: GCCTCAGTTTCCTCCTCTGTGA R: CCTCCACTTCGGTTTACCAAGA	AAAC	173-181	3	0.261	0.248
A224	F: CATGGCTCGTGCCTGTTG R: CCTCCCTCTTCTACTCTCTCTCCA	AGAT	177-197	6	0.624	0.595
C002	F: AGGGCAGTATGTCTCCTGAAGC R: TTTAATGCAATCCCTAATTGTTTTTTTT	ATGT	188-196	3	0.636	0.452
A019	F: ACATGGAAACGGAGGTTGGA R: CACACGGTCACAGAAGGCTG	GAT	211-214	2	0.263	0.323
A023	F: TCTTGGAAAAATAGCTTGTGGGAGA R: CCTGTCACACGGGAACCTAAA	ATAG	178-227	6	0.505	0.533
A014	F: TGGCAGCATTATTCACAATAGCA R: GCACGTGTAATAATTCCTTTCCTCTT	ATAG	212-236	7	0.675	0.645
A317	F: CCTCACAATCACAGGAGCCA R: GGGCTAGCAGAGAAAGGGAAC	CAGA/ AGAT	215-251	10	0.779	0.715
C220	F: CCACTTACTTCCAATTCTTACCAGC R: TGAGTATTTTACCACTGAGTGTGTTCG	CATA	221-236	4	0.325	0.338
A015	F: TTTGGAAAAACGACCCCCTT R: GCATCAAAGCATTAGGGAGGAA	AAC	265-286	4	0.233	0.236
A001	F: TCTCGGTCTGTTCCCTGAGG R: TGACTATTTAAGTCATTTGCCCATTT	AAC	299-302	2	0.406	0.352
A222	F: GGGTTGAGAGGAGGCAGTTCT R: CCAAATAGCTTTAGGAAGGTCCCT	ATCT	338-386	11	0.801	0.656

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