1 A phylogenetic test of sympatric speciation in the Hydrobatinae (Aves: Procellariiformes)

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Abstract

Phylogenetic relationships among species can provide insight into how new species arise. For example, careful consideration of both the phylogenetic and geographic distributions of species in a group can reveal the geographic models of speciation within the group. One such model, sympatric speciation, may be more common than previously thought. The Hydrobatinae (Aves: Procellariiformes) is a diverse subfamily of Northern Hemisphere storm-petrels for which the taxonomy is unclear. Previous studies showed that *Hydrobates* (formally *Oceanodroma*) *castro* breeding in the Azores during the cool season is sister species to *H. monteiroi*, a hot season breeder at the same locations, which suggests sympatric speciation by allochrony. To test whether other species within the subfamily arose via sympatric speciation by allochrony, we sequenced the cytochrome *b* gene and five nuclear introns to estimate a phylogenetic tree using multispecies coalescent methods, and to test whether species breeding in the same geographic area are monophyletic. We found that speciation within the Hydrobatinae appears to have followed several geographic modes of divergence. Sympatric seasonal species in Japan likely did not arise through sympatric speciation, but allochrony may have played a role in the divergence of *H. matsudairae*, a cool season breeder, and *H. monorhis*, a hot season breeder. No other potential cases of sympatric speciation were discovered within the subfamily. Despite breeding in the same geographic area, hydrobatine storm-petrels breeding in Baja California (*H. microsoma* and *H. melania*) are each sister to a species breeding off the coast of Peru (*H. tethys* and *H. markhami*, respectively). In fact, antitropical sister species appear to have diverged at multiple times, suggesting allochronic divergence might be common. In addition, allopatry has likely played a role in divergence of *H. furcata*, a north Pacific breeder, and *H. pelagius*, a north Atlantic breeder. This study demonstrates that a variety of mechanisms of divergence have

played a role in generating the diversity of the Hydrobatinae and supports the current taxonomy of the subfamily.

1. Introduction

Understanding the phylogenetic relationships among species can give insight into how lineages diverged and how new species arise. The process of speciation can be organized based on the geographic overlap of incipient species during divergence. These models of speciation range from complete geographic isolation of incipient species (allopatric speciation) to complete geographic overlap of incipient species (sympatric speciation) with various degrees of geographic overlap in between (peripatric and parapatric speciation; Coyne and Orr, 2004; Fitzpatrick et al., 2009). Although the importance of sympatric speciation as a mode of species generation has been controversial, recent evidence suggests that it may be more common than previously thought. For example, sympatric speciation appears to have been involved in the origin of cichlid species (e.g. *Pundamilia nyererei* and *P. pundamilia*; Seehausen and van Alphen, 1999), palm trees (*Howea belmoreana* and *H. forsteriana*; Gavrillets and Vose 2007), subterranean blind mole rats (*Spalax galili*; Hadid et al., 2013), and apple maggots (*Rhagoletis pomonella*; Smith, 1988). Sympatric speciation by ecological segregation has also arguably occurred in birds, including *Nesospiza* buntings in the Tristan de Cunha archipelago (Ryan et al., 2007) and Galápagos warbler finches *Certhidea olivacea* and *C. fusca* (Tonniss et al., 2005). Sympatric speciation can also occur by allochrony, where individuals that breed at different times are less likely to encounter each other leading to reduced gene flow (Hendry and Day, 2005). For example, in sympatric periodic cicadas (*Magicicada* species), one population emerges on a 13-year cycle whereas another population emerges on a 17-year cycle, resulting in

assortative mating and population divergence (Sota et al., 2013). Allochronic speciation can also occur as a result of populations breeding in different seasons, as in summer and winter populations of the pine processionary moth (*Thaumetopoea pityocampa*; Santos et al., 2011).

The Hydrobatinae (Aves: Procellariiformes; Genus: *Hydrobates*, Carboneras and Bonan, 2014; formerly genera *Oceanodroma*, *Halocyptena*) is a diverse subfamily of mostly Northern Hemisphere seabirds that includes several examples of genetic divergence of sympatric allochronic populations (Table 1). Both the band-rumped storm-petrel *Hydrobates castro* spp. (Monteiro and Furness 1998; Snow and Snow, 1966) and Leach's storm-petrel (*H. leucorhoa*; Power and Ainley, 1986) have populations that nest in different seasons (hot and cool) on the same island, often using the same burrows. Results from past and ongoing population genetic studies suggest that these seasonal populations originated sympatrically by allochrony (Friesen et al., 2007a; M.F. Silva et al. 2016; Taylor et al., unpubl.). Among other results, this led to distinction of the hot-season breeders from the Azores archipelago as a cryptic species, Monteiro's storm-petrel (*H. monteiroi*; Bolton et al., 2008; Smith et al., 2007). Similarly, previous and on-going studies have found genetic differences between sympatric hot-season and cool-season populations of *H. castro* in the Galápagos (Smith and Friesen, 2007), *H. jabejabe* in Cape Verde (Deane, 2011; Friesen et al., 2007a), and *H. leucorhoa* in Guadalupe (*H. l. cheimomnestes* and *H. l. socorroensis*; Friesen et al., unpubl.).

The Hydrobatinae also includes other potential cases of sympatric speciation among the 14 extant and one recently extinct species. Storm-petrels are pelagic, but mostly breed on isolated islands and exhibit high natal philopatry (Brooke, 2004; Warham, 1990). Breeding at their natal site can limit gene flow, likely promoting genetic differentiation between populations (Power and Ainley, 1986). Most species breed in the Northern Atlantic and Pacific oceans, while

a few breed in the Southeastern Pacific (Fig. 1). *H. leucorhoa* and *H. castro* are widely distributed in both the Atlantic and Pacific (temperate, and tropical/subtropical, respectively; Brooke, 2004; Warham, 1990) with several defined subspecies and geographic variants (Table 1). Within the *H. castro* complex, in addition to recent recognition of *H. monteiroi* as a good species, *H. jabejabe* (previously classified under *H. castro*) breeding on Cape Verde has been identified as a full species (Sangster et al., 2012; Taylor et al., unpubl.). Within *H. leucorhoa*, four subspecies have been recognized: *H. l. socorroensis*, hot season breeder on Guadalupe I.; *H. l. cheimomnestes*, cool season breeder on Guadalupe I.; *H. l. chapmani*, hot season breeder on islands off Baja California; and *H. l. leucorhoa*, which breeds throughout the North Atlantic and North Pacific oceans (Power and Ainley, 1986). In the North Pacific, the fork-tailed storm-petrel (*H. furcata*) breeds around the Aleutian Islands and the Pacific coast of Canada (Brooke, 2004; Warham, 1990). In the North Atlantic, one subspecies of European storm-petrel (*H. pelagicus pelagicus*) breeds in the Atlantic and the other subspecies (*H. p. melitensis*) breeds in the Mediterranean (Cagnon et al., 2004; Sangster et al., 2012).

Storm-petrels appear to have a few major hotspots of diversity (Fig. 1). For instance, many storm-petrel species breed in the waters around Baja California, Mexico including the least storm-petrel (*H. microsoma*), black storm-petrel (*H. melania*), ashy storm-petrel (*H. homochroa*), three subspecies of *H. leucorhoa*, and the recently extinct *H. macrodactyla* (Brooke, 2004; Warham, 1990). A diversity hotspot also occurs off the coast of Peru, where Markham's storm-petrel (*H. markhami*), and one wedge-rumped storm-petrel subspecies (*H. tethys kelsalli*) breed (Brooke, 2004; Warham, 1990). The other wedge-rumped storm-petrel subspecies (*H. tethys tethys*) breeds around the Galápagos (Brooke, 2004; Warham, 1990). The breeding range of Hornby's storm-petrel (*H. hornbyi*) is unknown as nests have not been found;

however observations of it in the waters around Chile and Peru, and observations of fledglings inland, suggest that it nests in this area (Brooke, 1999).

Another hotspot of diversity occurs in the Northwestern Pacific around Japan where Matsudaira's storm-petrel (*H. matsudairae*), Swinhoe's storm-petrel (*H. monorhis*), Tristram's storm-petrel (*H. tristrami*), *H. leucorhoa*, and *H. castro* breed (Brooke, 2004; Warham, 1990). Similar to *H. castro* on the Azores archipelago, these species breed in different seasons in the same geographic area (Brooke, 2004). *H. tristrami* breeds in the cool season from October to June on northwestern Hawaiian Is. and on islands in the Ogasawara Archipelago of Japan (McClelland et al., 2008). *H. matsudairae* also breeds in the cool season but has a breeding range restricted to Iwo Island (Chiba et al., 2007). Historically, *H. matsudairae* and *H. tristrami* bred sympatrically on Iwo I., but their current breeding sites do not overlap (Chiba et al., 2007, Ornithological Society of Japan, 2012). *H. monorhis* breeds in the hot season from June to October (Brooke, 2004) on islands off of Japan, Korea, far east Russia, and China, with its largest breeding colony on Kutsujima I. in the Sea of Japan (Ornithological Society of Japan, 2012; Sato et al., 2010). Currently, *H. tristrami* and *H. monorhis* breed sympatrically at two breeding sites (Hachijo-Kojima I. and Kozushima I.) but in different seasons (McClelland et al., 2008; Ornithological Society of Japan, 2012; Sato et al., 2010). The difference in breeding times between these species in the same geographic area suggests they may have arisen through sympatric speciation by allochrony.

Despite these interesting geographic distributions, the phylogenetic relationships among the species are not well characterized, with only a few partial phylogenies (e.g. Friesen et al., 2007; M.C. Silva et al., 2016; Nunn and Stanley, 1998; Penhallurick and Wink, 2004). For the present study, we used gene trees and a multispecies coalescent model (*BEAST; Degnan and

Rosenberg, 2009; Heled and Drummond, 2010) based on one mitochondrial segment and five nuclear introns to estimate phylogenetic relationships among all extant species and subspecies (Table 1). Specifically, given previous evidence for sympatric speciation by allochrony (above), we used a phylogenetic approach to test whether the sympatric species with non-overlapping breeding seasons in Japan (*H. tristrami* in the cool season and *H. monorhis* in the hot season) may have arisen through sympatric speciation by allochrony. In addition, we tested whether species breeding in the same geographical area (e.g. Peru, Baja California area) are monophyletic as predicted under sympatric speciation.

2. Materials and methods

2.1 Sample collection and DNA extraction

Tissue, feather or blood samples were obtained for representatives of all extant species of the storm-petrel subfamily Hydrobatinae (Table 1). At least two individuals per species were included in our analysis. Whenever possible, two individuals from each named subspecies or previously identified genetically differentiated conspecific population were also included (e.g. Cagnon et al., 2004; Smith et al., 2007). We also attempted to extract DNA from museum specimens of *H. macrodactyla*; however, these resulted in very poor quality DNA and so this species was not included in the study. Previous studies showed that Hydrobatinae is a monophyletic group, sister to the Oceanitinae (Burleigh et al., 2015; Nunn and Stanley, 1998; Warham, 1990); one Wilson's storm-petrel (*Oceanites oceanicus*) was therefore used as an outgroup.

DNA was extracted using either a PureLink Genomic DNA Mini-kit (Life Technologies, Burlington, ON), a DNEasy Blood & Tissue Kit (Qiagen, Toronto, ON), or a standard phenol-

chloroform procedure (Sambrook et al., 1989) depending on the quality of the tissue. For museum samples that were collected before 1971 (Table 1), the DNEasy animal tissue protocol was used with the following modifications (Ibarguchi et al., 2006; Ibarguchi, 2011): (i) toepads were soaked in phosphate buffered saline for 39 - 44 hours before beginning the DNA extraction, (ii) the digestion time was increased to 55 – 68 hours, (iii) an extra 20 mg/mL of proteinase-K was added every seven hours to aid tissue digestion, and (iv) the elution step was performed with 50 µL of elution buffer and incubated at room temperature for 15 minutes before centrifugation. All toepad extractions were performed alongside negative controls, and subsequent polymerase chain reactions (PCRs) included the negative extractions as a contamination control.

2.2 Laboratory methods: cytochrome *b*

The mitochondrial cytochrome *b* (MT-CYB) gene was amplified from all samples using a suite of published and newly designed primers (for primer sequences and sources, See Appendix, Table A.1). The primer pair L14863/b6 was used for most samples using standard PCR protocols following Patterson et al. (2011). Annealing temperature varied between 47°C to 55°C, depending on the species. For museum specimens with poor DNA quality, internal primers were designed to amplify small overlapping fragments (~150-200 bp) that covered the length of MT-CYB (Appendix Table A.1). PCR products were sequenced in both directions using a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA) at the Génome Québec Innovation Centre (McGill University, Montreal, QC). Sequences were verified from chromatograms and aligned using CLUSTALW (Larkin et al., 2007) as implemented in BIOEDIT (Version 7.0.9.0; Hall, 1999).

2.3 Laboratory methods: nuclear introns

186 Previous studies have shown that resolution of phylogenetic relationships depends more on the
187 number of independent loci than the number of individuals sequenced (Heled and Drummond
188 2010). Five nuclear introns were amplified for one randomly selected individual of each species:
189 1) fibrinogen beta chain (Human Genome Organisation nomenclature: FGB); 2) lipoprotein
190 lipase (LPL); 3) ornithine decarboxylase (ODC1); 4) triosephosphate isomerase (TPI1); and 5)
191 enolase 1 (ENO1) (for primer sequences and references see Appendix Table A.1). At the time of
192 this study, the very closely related *H. jabejabe* and *H. castro* were considered conspecific
193 (Friesen et al., 2007) and so are represented by one *H. jabejabe* individual from Cape Verde. In
194 addition, the relationships within *H. castro* and *H. monteiroi* have been investigated elsewhere
195 using similar techniques (M.F. Silva et al. 2016), whereas our study attempts to clarify the
196 divergence of the newly described *H. jabejabe* and the *H. castro/H. monteiroi*. Internal primers
197 were used to amplify degraded samples (Appendix Table A.1). PCRs were conducted using
198 standard protocols with annealing temperatures ranging from 50 – 60°C. Sequences were verified
199 from chromatograms and heterozygous sites were identified based on the presence of two peaks
200 of approximately equal size. PHASE (Version 2.1; Stephens and Donnelly, 2003) was used to
201 determine haplotypes for heterozygotes with more than one polymorphic site. Haplotypes that
202 could not be resolved statistically with posterior probability greater than 0.90 were coded using
203 IUPAC ambiguity codes. Intron sequences were aligned using CLUSTALW, and the four gamete
204 test (Hudson and Kaplan, 1985) implemented in DNASP (Version 5.10.00; Librado and Rozas,
205 2009) was used to test for intralocus recombination. When recombination was detected, the
206 longest non-recombining block of sequence was retained for all subsequent analyses. To assess
207 how much the perceived recombination in our data affected downstream multispecies coalescent

analyses (e.g. Edwards et al., 2016; Springer and Gatesy, 2015), full intron sequences were also used as input in separate phylogenetic analyses.

2.4 Phylogenetic analysis: MT-CYB gene tree estimation

As a first estimate of hydrobatine phylogeny, a gene tree was estimated for MT-CYB for all samples. Individual sequences were collapsed into unique haplotypes, and MRBAYES (Version 3.1.2; Ronquist and Huelsenbeck, 2003) was used to estimate a gene tree. The best-fit nucleotide substitution model, as determined using the Akaike Information Criterion (AIC) in JMODELTEST (Version 0.1.1; Guindon and Gascuel, 2003; Posada, 2008; Appendix Table A.1) was applied to the data, and the data set was partitioned by codon position (Saccone et al., 1990). The model was run for 10 million generations sampling every 100 trees with a burn-in of 2500. Parameters of the nucleotide substitution model were allowed to vary. Convergence was assessed by examining the traces of parameters and by monitoring the average standard deviation of split frequencies between two runs. This analysis was repeated three times to ensure convergence on the same topology. All trees were visualized using FIGTREE (Version 1.3.1; <http://tree.bio.ed.ac.uk/software/figtree/>).

2.5 Phylogenetic analysis: Nuclear gene tree estimates

Nuclear intron sequences were reduced to haplotypes in TCS (version 1.21; Clement et al., 2000), and gene trees were estimated independently for each intron. For *H. matsudairae*, introns *ENO1*, *ODC1* and *TPII* could not be amplified and thus this species was not included in these gene tree analyses. Gene trees were estimated in MRBAYES using the nucleotide substitution model of best-fit, as determined in JMODELTEST (Appendix Table A.1). Each nuclear locus also

included sequence from the flanking exon(s); intron and exon sequences were included in different partitions for each analysis. Insertion-deletion (indel) polymorphisms were also included in a separate partition, with each indel coded as a binary character (presence versus absence). All other settings of MRBAYES were as given for the MT-CYB analysis.

2.6 Phylogenetic analysis: *BEAST tree estimates

For the *BEAST analysis, two alleles from each species for each gene were included. Missing data were included for species instead of excluding taxa as BEAST analyses have shown to be robust to missing data (see Zheng and Wiens, 2015). The Hasegawa-Kishino-Yano substitution model with a proportion of invariant sites and gamma distribution rate variation (HKY+I+G; Hasegawa et al., 1985) was used for all loci and each codon position for MT-CYB was given its own partition. Each locus had its own nucleotide substitution model and a strict molecular clock model with its own rate. A Yule speciation tree prior was used, as this prior has been noted to be suitable for species-level phylogenies (Drummond et al., 2006). *BEAST was run with a Markov chain Monte Carlo (MCMC) length of 1.0×10^8 generations, with trees sampled every 2000 generations (for a total of 50,000 sampled trees). Of these, the first 10% were discarded as burnin for a total of 45,000 retained trees. To assess convergence, the *BEAST analysis was run three times and, once convergence of the MCMC had been established (by monitoring effective sample size (ESS) values and comparing the three runs to one another in TRACER; version 1.6; Rambaut et al., 2014), the resultant log and tree files were combined using LOGCOMBINER (Version 1.7.2; Drummond and Rambaut, 2007). The final result was a sample of 135,000 trees (3 x 45,000), which was used for all subsequent calculations.

2.7 Dating of major clades

We also used *BEAST to obtain rough estimates of the divergence times of major clades that were supported with high posterior probability in the MT-CYB tree (see Results). In the *BEAST analysis, the MT-CYB substitution rate was fixed and the rates for all other loci were estimated relative to MT-CYB. The result of this configuration is that divergence dates are based on the information contained in all loci, but calibrated using established MT-CYB substitution rates. The MT-CYB rate was calibrated at 2% sequence divergence per million years (Lovette, 2004; Weir and Schluter, 2008). However, some studies have shown that 2% is not an accurate estimate of sequence divergence and suggest a slower divergence rate for mitochondrial genes (e.g. for the *Pterodroma*/*Diomedea* split; cytochrome *b* 0.422%/my, Pereira and Baker, 2006; complete mitochondrial genome 0.32 – 0.36%/my, Pacheco et al., 2011). Nunn and Stanley (1998) found that MT-CYB evolution was slower in procellariiforms than in other birds (see also Lovette, 2004) and correlated with body size. They estimated a divergence rate of 0.92%/my for the members of the Oceanitinae, which are similar in body size to species in the Hydrobatinae. Therefore, we also estimated dates with a divergence rate of 0.92%/my.

3. Results

3.1 Sequence variation

Sequences of the entire MT-CYB gene, 1143 bp including 396 variable sites, were obtained from 46 individuals (GENBANK: KU217327-KU217371). Sequence ambiguity was found at only one site and may be due to true mitochondrial (mtDNA) heteroplasmy or a sequence artifact, and was coded as missing data. Several lines of evidence indicated that mtDNA was amplified, as opposed to nuclear homologs of mtDNA (numts; following Friesen and Anderson, 1997),

including that sequences of some species were either identical to or very similar to sequences on GENBANK from Nunn and Stanley (1998), who used several criteria for determining amplification of true mtDNA. Although the shorter length of our PCR amplifications from museum specimens makes the co-amplification of numts more likely (Ibarguchi et al., 2006; Sorenson and Quinn, 1998), the following evidence suggests that the amplifications were true mtDNA: (1) sequences from the museum samples were consistent with sequences from non-museum samples of the same species, differing by 0-3 bp, (2) sequences of individual museum samples of the same species were consistent with each other, (3) there was no evidence of co-amplification as seen by double peaks on chromatograms, and (4) overlapping sections of the small internal fragments matched exactly.

The nuclear intron sequences averaged 362 bp long (range 113-607 bp) with 16-35 variable sites (Appendix Table A.1; GENBANK: KU217372- KU217444). The four gamete test detected possible recombination events in parts of the nuclear sequences for all loci, thus the largest sequence blocks with no evidence of recombination for each locus were used for further analyses (average 214 bp, range 112-358 bp; Appendix Table A.1). Individuals with one polymorphic site were represented with two alleles. Of the individuals that had sequence polymorphism at two or more sites (n=14), PHASE was able to separate the alleles in all individuals with confidence except for alleles for FGB in *H. tristrami*, *H. matsudairae*, *H. tethys* and *H. hornbyi*, and ODC1 in *H. monorhis*, *H. monteiroi*, *H. microsoma* and *H. hornbyi*. In these cases the ambiguities were coded as missing data for phylogenetic analyses. The estimated nucleotide substitution models varied among introns, and were specified accordingly in subsequent analyses (Appendix Table A.1).

3.2 Phylogenetic analysis using gene trees

Sequences from individuals of the same species always grouped together in the MT-CYB gene tree but sequences of conspecific individuals did not always group by sampling site (Fig. 2). Local races of *H. castro* all grouped together in one paraphyletic clade with *H. monteiroi*, with the hot season breeders from Azores (*H. monteiroi*) being sister to the cool season *H. castro* individuals from Azores. *H. leucorhoa* was monophyletic, with *H. l. chapmani* from Baja California basal to the Atlantic and Pacific individuals (Fig. 2). However, one *H. l. socorroensis* individual, a hot season breeder from Guadalupe, did not group with the other individuals from Guadalupe (Fig. 2), possibly as a result of recent ancestry and incomplete lineage sorting (R. Taylor et al., unpubl. data).

Overall, four major clades received high support within the Hydrobatinae in the MT-CYB tree: 1) *H. castro*, *H. monteiroi*, and *H. jabejabe*, 2) *H. melania*, *H. markhami*, *H. microsoma*, and *H. tethys*, 3) *H. tristrami*, *H. monorhis*, *H. matsudairae*, *H. homochroa*, *H. hornbyi* and *H. leucorhoa*, and 4) *H. furcata* and *H. pelagicus* (Fig. 2). Although relationships among these clades were unclear, the relationships among species within these clades received strong support (Fig. 2). No single intron gene tree was fully resolved; however, many of the major clades found in the MT-CYB tree were also supported by individual nuclear gene trees suggesting that all gene trees provided support for the multispecies coalescent tree (Appendix Fig. A1. i-v). Gene trees estimated using full intron sequences did not differ in overall topology from trees estimated using the non-recombining segments, but the support for some of the nodes tended to be weaker (see Appendix Fig. A1. i-v).

3.3 Phylogenetic analysis using multispecies coalescent methods

The four major clades found in the MT-CYB tree as well as phylogenetic relationships within each of these clades received high posterior probabilities in the *BEAST tree analysis. Three interesting relationships are apparent within the *BEAST tree (Fig. 3): 1) *H. monorhis*, a hot season breeder in Japan received high support as sister to *H. matsudairae*, a cool season breeder in Japan (clade F); 2) There was strong support for two pairs of species in the eastern Pacific, each with one species breeding north of the equator around Baja whereas the other species breeds south of the equator around Peru (*H. microsoma* and *H. tethys*, clade E; *H. melania* and *H. markhami*, clade H); and 3) *H. furcata* (which breeds in the north Pacific) and *H. pelagicus* (north Atlantic) received strong support as sister species (clade B). Topology of the *BEAST tree estimated using the non-recombining segments of the introns or using the full intron sequences did not differ significantly in topology, but offered weaker support for some nodes (Appendix Fig. A2)

3.4 Divergence time estimates

As a 0.92% divergence rate is more realistic for hydrobatine storm-petrels (see Section 2.7), we have chosen to discuss the divergence dates estimated using this rate. However, divergence dates calculated with 2% sequence divergence rates are presented in Table 2 and are roughly half of the dates estimated with 0.92% sequence divergence. Results suggest that the Hydrobatinae originated 16.44 (11.28-22.22) mya assuming a divergence rate of 0.92%/my, with most lineages originating more than 4 mya (Table 2). Recent speciation events were the cool- (*H. castro*) and hot-season (*H. monteiroi*) breeders from Azores, and the divergence of *H. melania* and *H. markhami* across the equator in the eastern Pacific.

4. Discussion

According to Coyne and Orr (2004), four criteria must be met for sympatric speciation to be inferred: the species must (1) have sympatric distributions, (2) be mostly reproductively isolated, (3) exhibit a sister relationship, and (4) have had little chance of an allopatric phase in their history. In the present study, the MT-CYB tree provided high support for the previously found sister relationship between cool-season (*H. castro*) and hot-season (*H. monteiroi*) band-rumped storm-petrels from Azores (M.F. Silva et al. 2016), supporting the hypothesis that *H. monteiroi* arose through sympatric speciation by allochrony (Friesen et al., 2007a). The MT-CYB tree also indicated that seasonal populations of *H. leucorhoa* on Guadalupe I. (*H. l. cheimomnestes*, cold season breeders, are sister to *H. l. socorroensis*, hot season breeders) may have arisen through allochronic speciation but these relationships are being investigated further with larger sample sizes (Taylor et al., unpubl.). In the *BEAST tree, the divergence of *H. jabejabe* from the *H. castro*/*H. monteiroi* clade of 4.21 mya is consistent with M.F. Silva et al. (2016) estimates of divergences within the *H. castro*/*H. monteiroi* clade occurring later than 200,000 years ago.

However, no other potential cases of sympatric speciation were found. In Japan, *H. tristrami* (cool season breeder) used to breed in sympatry with *H. matsudairae* during the cool-season and currently breeds in sympatry with *H. monorhis* at two breeding sites but in the opposite season (Chiba et al., 2007; Ornithological Society of Japan, 2012; McClelland et al. 2008; Sato et al., 2010). However, *H. tristrami* is more closely related to other species breeding in the Pacific Ocean (*H. leucorhoa*, *H. hornbyi* and *H. homochroa*, Fig. 3) than to species breeding in Japan. Interestingly, *H. monorhis*, a hot-season breeder in Japan, is sister to *H. matsudairae*, a cool-season breeder, diverging 6.63 (3.87-9.32) mya, but these species do not breed on the same islands (Chiba et al., 2007; Ornithological Society of Japan, 2012; Sato et al.,

2010). Thus, although species in Japan may not be a classic case of sympatric speciation, allochrony may have been involved in the development of reproductive isolation between *H. monorhis* and *H. matsudairae*.

The environmental heterogeneity within the eastern Pacific allows for many species of storm-petrels to co-occur (Spear and Ainley, 2007). However, within the Hydrobatinae, species breeding in the same geographic area are not always monophyletic as would be expected under sympatric speciation. Antitropical distributions of sister species seem to have arisen in the eastern Pacific several times, where one species that breeds north of the equator around Baja California is sister to a species breeding south of the equator off the coast of Peru: *H. melania* and *H. markhami*; and *H. microsoma* and *H. tethys* (Fig. 3). This has also been described in invertebrates, vertebrates and plant taxa, especially in the marine realm (Burridge, 2002; Lindberg, 1991). Possible mechanisms of divergence include 1) colonization by a species from one side of the tropics to the other (speciation by dispersal), or 2) extinction of tropical populations of a widespread species, isolating the northern and southern populations through vicariance (Burridge, 2002 and references therein; Lindberg, 1991). During the Pliocene (5.3 - 2.58 mya), both the emergence of the Isthmus of Panama and the formation of the Humboldt Current off the coast of Peru altered the eastern Pacific Ocean currents (Lindberg, 1991), possibly facilitating divergence of *H. microsoma* and *H. tethys* approximately 7.57 (4.74-10.79) mya. Similarly, periods of global cooling altered the sea level during the Pleistocene (2.58 - 0.01 mya), which likely induced changes in near-shore upwelling intensity and location along the eastern Pacific (Lindberg, 1991). This may have allowed easier dispersal of the common ancestor of *H. melania* and *H. markhami* across the tropics approximately 0.16 (0.00 – 0.56) mya. Similar coastal environments (Californian and Peru-Chilean biogeographic provinces;

392 Briggs and Bowen, 2013) may have allowed colonizing individuals to adapt to the new
393 environment and/or genetic drift may have caused divergence during a time when conditions in
394 the tropical eastern Pacific were less conducive to dispersal. For example, antitropical sister
395 species of Pacific fish have been found to arise multiple times during recent glaciation events,
396 likely as a result of decreased gene flow across the equator (Burridge, 2002). Changes in ocean
397 productivity as a result of oceanographic alterations during glaciation events may have
398 influenced foraging behaviour and/or increased competition for suitable nest sites, leading to
399 divergence of storm-petrels (Spear and Ainley, 2007).

400 The strong support for monophyly of *H. pelagicus* (eastern Atlantic) and *H. furcata*
401 (Pacific) may suggest allopatric speciation within this subfamily. Although seabirds are strong
402 fliers and can travel long distances, land acts as a barrier to gene flow for most seabirds,
403 probably because they cannot feed inland and some cannot take flight from land (Friesen et al.,
404 2007b; Friesen, 2015). Friesen (2015) found that gene flow was restricted between populations
405 that were separated by large land or ice masses including the continent of North America, the
406 Isthmus of Panama and/or Arctic sea ice in all of 16 seabird species that have been studied.
407 Results of the present study suggest that *H. furcata* and *H. pelagicus* diverged 10.31 (6.99-14.26)
408 mya. Both the Bering Strait (e.g. for common murre, *Uria aalge*, Morris-Pocock et al., 2008)
409 and the Isthmus of Panama (e.g. for *Calonectris* shearwaters, Gómez-Díaz et al., 2006) have
410 been shown to be drivers of seabird diversification. The Bering Strait flooded approximately 5.3
411 mya and now provides a Trans-Arctic Interchange (Gladenkov et al., 2002) where dispersal most
412 commonly occurs from the North Pacific Ocean into the North Atlantic Ocean (e.g. molluscs;
413 Briggs and Bowen, 2013; Vermeij, 1991). However, sea ice and inhospitable ecological
414 conditions may currently restrict gene flow through the Arctic in seabirds (e.g. Morris-Pocock et

al., 2008). For example, black guillemots (*Cepphus grylle*) are thought to have originated when ancestral guillemots dispersed from the North Pacific to the North Atlantic through the Bering Strait and were subsequently isolated due to the harsh conditions of the Arctic (Kidd and Friesen, 1998). The Central American Seaway could also have been a route of dispersal between the Atlantic and Pacific Oceans until approximately 3 mya when the Isthmus of Panama emerged, potentially isolating populations (Coates and Obando, 1996). For example, the elevated terrain of the Isthmus of Panama is thought to act as a physical barrier to dispersal in *Calonectris* shearwaters (Gómez-Díaz et al., 2006) and in the masked booby (*Sula dactylatra*, Steeves et al., 2005).

Our results suggest that the hydrobatine storm-petrels are a monophyletic group, and also agree with recent taxonomy changes (subsuming *Oceanodroma* into genus *Hydrobates*, Carboneras and Bonan, 2014). Phylogenetic relationships that were strongly supported in this study are consistent with previous partial phylogenies (e.g. M.C. Silva et al., 2016; Nunn and Stanley, 1998; Penhallurick and Wink, 2004). Large-scale phylogenetic studies of relationships within Aves either have very little representation of hydrobatine species (e.g. Burleigh et al., 2015; Claramunt and Cracraft, 2015; Pacheco et al., 2011) and/or estimate divergence rates based on different loci (e.g. Jetz et al., 2012). No other study has put a time estimate on the divergence of Hydrobatinae and Oceanitinae but have estimated older divergences. Divergence dates estimated using previously published rates for the divergence of albatrosses and petrels would result in older divergence dates than reported in this study (e.g. Jetz et al., 2012).

In summary, the diversification of hydrobatine storm-petrel species appears to have been influenced by a variety of mechanisms. Although the sympatric species breeding in Japan (*H. tristrami* and *H. monorhis*) are not sister species, *H. monorhis* and *H. matsudairae* (which breed

in different seasons) are sisters, suggesting that allochrony may have played a role in divergence. Several species of storm-petrels breeding in the eastern Pacific have antitropical distributions, suggesting isolation by inhospitable habitat has led to speciation multiple times within the subfamily. In addition, allopatry has likely played a role within the subfamily with the divergence of the sister species *H. pelagicus* and *H. furcata* inhabiting the Atlantic and Pacific oceans, respectively. Future population genetic studies between species pairs would provide a better understanding of the mechanisms of speciation occurring within the Hydrobatinae. With the exceptions of the *H. castro* species complex and *H. leucorhoa*, sympatric speciation does not appear to be prevalent within northern hemisphere storm-petrels but allochrony may play a role. Understanding both geographical and ecological characteristics of a species can lead to a better appreciation of how new lineages arise.

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730 estimation with BEAST? *Mol. Phylogent. Evol.* 85, 41-49.

731 Table 1. Sources and number of samples included in the study. Samples are archived at Queen's University unless otherwise indicated.

Species	Subspecies	Collection Location	Breeding season	Tissue Source (N, date of collection for museum tissue)	Reference for ESU/geographic variant
<i>Oceanities oceanicus</i>	<i>oceanicus</i>	Livingston I., Antarctica		Blood (1)	
<i>Hydrobates castro</i>	-	Galápagos Is.		Blood (2)	Smith et al., 2007
	-	Japan		Blood (2)	
	-	Azores	cool	Blood (2)	
<i>H. monteiroi</i>	-	Azores	hot	Blood (2)	Bolton et al., 2008
<i>H. jabejabe</i>	-	Raso I., Cape Verde		Blood (2)	Sangster et al., 2012
<i>H. pelagicus</i>	<i>pelagicus</i>	Iceland	May - June	Blood (2)	Cagnon et al., 2004
	<i>melitensis</i>	Balearic Is, Mediterranean		Blood (2)	
<i>H. furcata</i>	<i>furcata</i>	Commander Is, Aleutian Is.	April - June	Blood (2)	
	<i>plumbea</i> *	Southeastern Alaska		Toepad (1, 1954) ^a	
<i>H. leucorhoa</i>	<i>leucorhoa</i>	Newfoundland, Canada		Blood (2)	Friesen et al., unpubl.
	<i>leucorhoa</i>	British Columbia, Canada		Blood (2)	
	<i>socorroensis</i>	Guadalupe I.	hot	Soft tissue (2)	
	<i>heimomnestes</i>	Guadalupe I.	cool	Soft tissue (2)	
	<i>chapmani</i>	Baja California		Toepad (2, 1971)	
<i>H. microsoma</i>	-	Baja California	June - July	Blood (2)	
<i>H. homochroa</i> *	-	Baja California	April - July	Blood (1), toepad (1, 1936) ^b	
<i>H. melania</i>	-	Baja California	May - June	Blood (2)	

<i>H. markhami</i> *	-	Peru	June - August	Soft tissue (1) ^c
<i>H. tethys</i>	<i>tethys</i>	Galápagos Is.	May - June	Soft tissue (2) ^e
	<i>kelsalli</i>	Peru		Soft tissue (1) ^c
<i>H. hornbyi</i> *	-	Peru, Chile	hot	Feather (1), toepad (1, 1949) ^b
<i>H. matsudairae</i> *	-	Bonin Is, Japan	October - June	Toepad (2, 1923, 1949) ^{b,d}
<i>H. monorhis</i>	-	Japan	June - October	Blood (2)
<i>H. tristrami</i>	-	Hawaii, North Pacific	October - June	Soft tissue (2) ^f

732 *degraded museum sample, sequenced in smaller fragments using internal primers

733 ^aRoyal Ontario Museum (ROM), Toronto, Ontario (ROM#133859)

734 ^bOrnithology Department, Museum of Comparative Zoology (MCZ), Cambridge, MA (MCZ281258, MCZ289163, MCZ276792)

735 ^cAcademy of Natural Sciences (ANSP), Philadelphia, PA (Tissue #17751, ANS185037)

736 ^dAmerican Museum of Natural History (AMNH), New York, NY (AMNH#269829, AMNH#784874)

737 ^eNational Museum of Natural History (NMNH), Washington, DC (USNM#B06760, USNM#B06761)

738 ^fUniversity of Washington Burke Museum, Seattle, WA (UWBM#89754, UWBM#55740).

Table 2. Time to most recent common ancestor as estimated in *BEAST for clades with high posterior probabilities in the MT-CYB and *BEAST tree. Divergence rates were chosen as a higher (2%; Lovette 2004; Weir and Schluter 2008) and lower limit (0.92%; Nunn and Stanley, 1998) and dates are presented in million years with 95% highest posterior density estimates. Clades refer to Fig. 3.

Clade	Species included	Time to most recent common ancestor in million years (95% highest posterior density)	
		2% divergence rate	0.92% divergence rate
A	Ingroup –hydrobatine storm-petrels	7.56 (5.19 – 10.22)	16.44 (11.28 – 22.22)
B	<i>H. tristrami</i> , <i>H. leucorhoa</i> , <i>H. hornbyi</i> , <i>H. homochroa</i> , <i>H. monorhis</i> , <i>H. matsudairae</i>	5.10 (3.32 – 7.08)	11.08 (7.21 – 15.40)
C	<i>H. furcata</i> , <i>H. pelagicus</i>	4.74 (3.22 – 6.56)	10.31 (6.99 – 14.26)
D	<i>H. tethys</i> , <i>H. microsoma</i> , <i>H. melania</i> , <i>H. markhami</i>	4.48 (2.89 – 6.34)	9.73 (6.28 – 13.78)
E	<i>H. microsoma</i> , <i>H. tethys</i>	3.48 (2.18 – 4.96)	7.57 (4.74 – 10.79)
F	<i>H. matsudairae</i> , <i>H. monorhis</i>	2.93 (1.78 – 4.28)	6.63 (3.87 – 9.32)
G	<i>H. jabejabe</i> , <i>H. monteiroi</i>	1.94 (1.07 – 2.93)	4.21 (2.33 – 6.37)
H	<i>H. melania</i> , <i>H. markhami</i>	0.07 (0.00 – 0.26)	0.16 (0.00 – 0.56)

Figure captions:

Fig. 1. Geographic distribution of hotspots of storm-petrel diversity depicted with approximate breeding distribution of some hydrobatine storm-petrels. Geographic locations mentioned in the text are labelled in grey. *H. castro* and *H. leucorhoa* are global breeders breeding in the Atlantic and Pacific Oceans (not shown).

Fig 2. Cytochrome-*b* gene tree depicting the phylogenetic relationship between species, subspecies and local races of hydrobatine storm-petrels. Posterior probabilities above 0.8 are indicated on the tree and a “*” indicates a posterior probability of 1.0. Bolded numbers represent clades mentioned in the text, and coloured branches represent mentioned relationships. All species and subspecies breed in the local hot season except for the branches coloured in blue, indicating cool season breeders. Green branches represent species breeding north of the equator off the coast of Baja California whereas orange branches represent species breeding off the coast of Peru.

Fig 3. Hydrobatine multispecies coalescent tree estimated in *BEAST. Posterior probabilities above 0.8 are indicated on the tree and a “*” indicates a posterior probability of 1.0. Divergence dates are labelled in million years ago (mya) for nodes with high posterior probabilities. Branches are coloured according to relationships measured in the text. Blue branches represent cool season breeders while red branches represent hot season breeders in the same area. All other species breed in the local hot season. Green branches represent species breeding north of the

765 equator off the coast of Baja California whereas orange branches represent species breeding off
766 the coast of Peru.

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Figure 1

Figure 2

Figure 3