Molecular analysis of a storm petrel specimen from the Marquesas Islands, with comments on specimens of *Fregetta lineata* and *F. guttata*

by Alice Cibois, Jean-Claude Thibault, Mary LeCroy & Vincent Bretagnolle

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Summary.—An old museum specimen of a storm petrel from the Marquesas Islands (French Polynesia) was sampled genetically. This specimen has been alternatively attributed to Black-bellied Storm Petrel *Fregetta tropica*, or described as a new taxon. Its plumage also recalls the recently rediscovered New Zealand Storm Petrel *F. maoriana*. However, molecular phylogenetic analysis revealed that this specimen is closely related to some individuals of White-bellied Storm Petrel *F. grallaria*, which species is apparently non-monophyletic.

Storm petrels are small seabirds divided into two families, the Oceanitidae (Austral storm petrels) and Hydrobatidae (Northern storm petrels), which are apparently not sister taxa (Hackett *et al*. 2008). Austral storm-petrels have short, round wings, usually square-ended tails and long legs. They typically forage while gliding slowly with their legs dangling on the surface of the ocean. Systematics within the family are complex with five genera and eight species recognised (Dickinson & Remsen 2013), including recently rediscovered species (e.g. New Zealand Storm Petrel *Fregetta maoriana*; Stephenson *et al*. 2008), new species (e.g. Pincoya Storm Petrel *Oceanites pincoyae*; Harrison *et al*. 2013) and potentially undescribed forms (Shirihai *et al*. 2015), while previously established taxonomy is strongly debated (e.g. species delimitation of Black-bellied Storm Petrel *Fregetta tropica* and White-bellied Storm Petrel *F. grallaria*, which might breed sympatrically on Gough Island, South Atlantic: Brooke 2004, Flood & Fisher 2011, Howell 2012).

New Zealand Storm Petrel belongs to the 'streaked' white-bellied forms of storm petrels, the so-called '*pealea*' phenomenon described *c*.60 years ago (genera *Fregetta*, *Nesofregetta* and *Garrodia*: Murphy & Snyder 1952). Five particular '*pealea*' specimens held in various museums have confused the systematics of South Pacific storm petrels for decades (*cf*. Stephenson *et al*. 2008 for a complete review), being assigned to three different genera over the years, *Fregetta*, *Thalassidroma* or *Pealea* (taxon *lineata*). The situation has been much clarified with the rediscovery of *Fregetta maoriana*, to which three of the five specimens refer, including the type of *F. maoriana* (Robertson *et al*. 2011). The other two specimens, according to Murphy & Snyder (1952), are a streaked bird presumed to be *F. grallaria*, collected off the Marquesas (see below) and another streaked specimen, which is considered to be a *F. tropica*, collected at Upolu, Samoa, in 1839 by Peale (see Discussion).

Taxonomic placement of *F. maoriana* was ambiguous based on plumage comparison, so a genetic analysis was conducted to assign this taxon to genus (Robertson *et al*. 2011). Here, we address the identity of the Marquesan specimen using a similar approach. This bird was collected at sea off Ua Pou (Marquesas Islands) on 15 September 1922 by R. H. Beck during the Whitney South Sea Expedition (WSSE). A female, it was registered in the American Museum of Natural History, New York (AMNH 194110) under the species name *Fregetta lineata* (Peale, 1848), now a junior synonym of *Fregetta tropica* (Gould, 1844). According to Murphy (1924), who cited the WSSE logbook: 'the bird was feeding in a streak of oily water in company with large numbers of *Bulweria* [petrels] and *Fregetta grallaria*'.

Figure 1. Specimen AMNH 194110, (a) ventral view and (b) side view of head (Matthew Shanley / © AMNH), and (c) ventral view of specimen USNM 15713 (Vincent Bretagnolle)

Mathews (1933) re-examined the specimen and assigned the bird to the new taxon, *Fregetta guttata* (not *Fregettornis guttata*; Murphy & Snyder 1952), based on the presence of dark belly streaking (Fig. 1a, b), which was thought to be absent in White-bellied Storm Petrel at that time. Murphy & Snyder (1952), however, found this plumage difference insufficient for recognition of a new taxon and assigned this specimen instead to *F. grallaria*. Overall, Murphy & Snyder (1952) and Jouanin & Mougin (1979) considered all of the '*lineata*' specimens to be aberrant plumages of distinct species of storm petrels. However, we found the assignment of AMNH 194110 to *F. grallaria* to be questionable, first because this species has not otherwise been recorded in the Marquesas (thus questioning Beck's identification in the field), apart from subfossil bones attributed to *Fregetta* sp. (Steadman 2006), and second because the specimen's odd plumage recalls the streaked belly of *F. maoriana*. Clearly, the identity of this specimen, based on its provenance and plumage, is uncertain. Here we address this issue using phylogenetic analysis of mitochondrial (cytochrome-*b*) DNA of this specimen and other storm petrels in the Oceanitidae and Hydrobatidae.

Material and Methods

A small fragment of toe pad was sampled from AMNH 194110. It was washed with sterile water before extraction, and total genomic DNA was extracted from small pieces

TABLE 1
Primers used in this study.

Primer name	5′-3′ sequence	Reference
8L (L14990)	CATCCAACATCTCTGCTTGATGAAA	Modified from Kocher *et al*. (1989)
33H	TCGGCCGATGTGGAGGTAGATGC	Cibois *et al*. (2007)
L154a	CAAACGGAGCCTCATTCTT	Robertson *et al*. (2011)
B37H	CATTCTACGATGGTTTGGCC	This study
B35L	AAAGAAACCTGAAACACAGG	This study
36H	TGGGTTGTCTACTGAGAA	Cibois *et al*. (2007)
37L	GGCCAAACCTTAGTAGAATG	Cibois *et al*. (2007)
38H	GGAGTAGTATGGGTGGAATGGGA	Cibois *et al*. (2007)

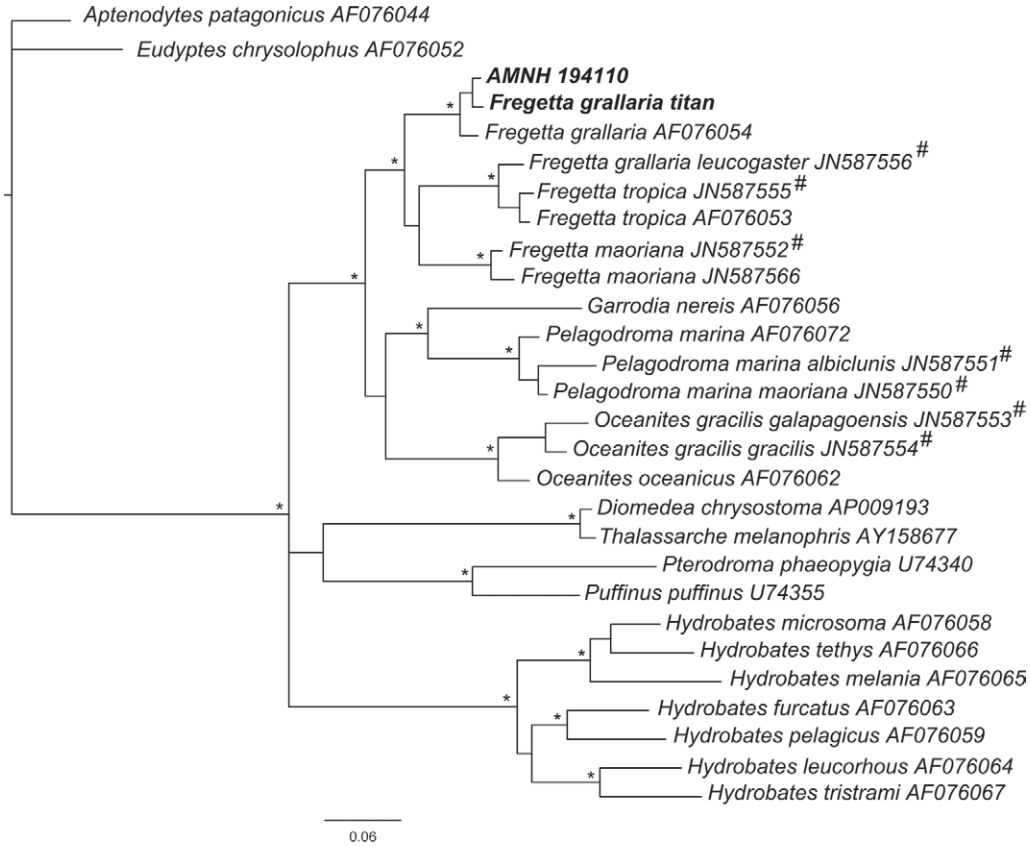

Figure 2. Phylogenetic tree estimated using Bayesian inference and cytochrome-*b* sequences. Posterior probabilities superior to 0.95 are indicated by an asterisk. GenBank numbers are indicated beside the taxon name and new sequences are in bold. # indicates short sequences (132 bp) from Robertson *et al*. (2011).

(0.5–1.0 mm^2) of skin using a commercial kit (DNeasy Tissue Kit; Qiagen, Valencia, CA). Standard extraction protocols were followed except that the time of proteinase digestion was increased from two to 12 hours, with an additional volume (20 μl) of proteinase K. All tubes and reagents were UV-treated for 30 minutes before use and extraction tubes containing no sample were used as a control for contamination. DNA extracted from museum specimens was degraded, so fragment sizes for amplification were small (*c*.200 bp). Using standard

TABLE 2
Pair-wise sequence divergence found in cytochrome-*b* sequences (% uncorrected values). The individuals studied here are indicated in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. ***Fregetta grallaria titan*** *	-													
2. *Fregetta grallaria* AF076054 *[a]	2.0													
3. *Fregetta maoriana* JN587552 [b]	6.1	6.1												
4. *Fregetta maoriana* JN587566 *[b]	6.8	7.5	0.8											
5. *Pelagodroma marina maoriana* JN587550 *[b]	7.6	9.1	7.6	6.8										
6. *Pelagodroma marina albiclunis* JN587551 *[b]	9.1	7.6	9.1	9.8	3.0									
7. *Pelagodroma marina* AF076072 *[a]	8.4	8.8	7.6	10.7	1.5	4.5								
8. *Fregetta grallaria leucogaster* JN587556 [b]	9.1	9.1	6.1	6.8	10.6	10.6	10.6							
9. *Fregetta tropica* JN587555 *[b]	9.8	9.8	5.3	6.1	9.8	11.4	9.8	2.3						
10. *Fregetta tropica* AF076053 *[a]	8.4	7.4	6.1	8.1	10.6	10.6	10.8	1.5	0.8					
11. *Garrodia nereis* AF076056 *[a]	9.0	9.9	5.3	11.2	9.1	7.6	9.6	5.3	6.1	10.1				
12. *Oceanites gracilis galapagoensis* JN587553 [b]	9.8	9.8	8.3	9.1	9.8	11.4	9.8	8.3	7.6	8.3	10.6			
13. *Oceanites gracilis gracilis* JN587554 [b]	10.6	10.6	9.1	9.8	10.6	12.1	10.6	9.1	8.3	9.1	11.4	2.3		
14. *Oceanites oceanicus* AF076062 *[a]	9.5	9.3	9.8	9.6	11.4	12.9	9.4	9.8	9.1	8.9	10.8	4.5	3.8	
15. *Fregetta* sp. AMNH 194110	1.3	2.2	5.3	6.3	6.8	8.3	7.9	8.3	9.1	8.3	9.2	9.1	9.8	9.7

* no voucher specimen; [a] Nunn & Stanley (1998); [b] Robertson *et al*. (2011).

protocols, we also sequenced one individual of *Fregetta grallaria titan* from Rapa, Austral Islands (from a feather obtained by B. Fontaine in December 2002 on Tarakoi Islet), in order to obtain a longer sequence than that available on Genbank (JN587557, only 132 bp). Several primers for the cytochrome-*b* gene were designed specifically for this study (Table 1). PCR amplifications were performed in 25 μl reactions with 2 μl of template and 0.4 μM final concentration for primers, using Qiagen Taq (no PCR additives). The thermo-cycling procedure commenced with an initial denaturation of three minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 40 seconds at annealing temperature (46–50°C depending on the primer) and 40 seconds at 72°C for elongation. PCR products were purified using a Qiagen QIAquick purification kit and sequenced in both directions at a contract sequencing facility (Macrogen, Seoul, South Korea) on an ABI3730 XL automatic DNA sequencer, using the same primers as used in PCR. Contiguous sequences derived from the set of sequence fragments were created using Sequencher (Genecodes, Ann Arbor, MI). Sequences were aligned to all storm petrel cytochrome-*b* sequences available on GenBank as well as those of four other Procellariformes (two albatrosses, one *Pterodroma* petrel and one *Puffinus* shearwater) and two Sphenisciformes from the following studies: Nunn *et al*. (1996), Nunn & Stanley (1998), Slack *et al*. (2006), Watanabe *et al*. (2006), Robertson *et al*. (2011). All species of Oceanitidae were represented in the dataset except Polynesian Storm Petrel *Nesofregetta fuliginosa*, for which no cytochrome-*b* sequence was available. The two Sphenisciformes were used as outgroup, following the phylogeny in Hackett *et al*. (2008). The data were subjected to Bayesian inference using MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003), with models selected using MrModeltest 2.3 for each codon position, using the AIC criterion (Nylander 2004). We conducted two independent runs of four Markov chains for one million generations each. Markov chains were sampled every 1,000 generations, with a 10% burn-in period.

Results and Discussion

Partial cytochrome-*b* gene sequences of 557 bp were obtained for *Fregetta grallaria titan* and for AMNH 194110, deposited in GenBank under accession nos. KP857579 and KP857580, respectively. The alignment was straightforward with no indels, as expected for a protein-coding gene. We translated the nucleotide sequences to proteins using Mega (Tamura *et al*. 2013) and found no stop codons. We detected no contamination in the negative controls. Results from the AIC criterion in MrModeltest supported the GTR + I for the first codon position, and the GTR + I + G model for the second and third codon positions (General Time Reversible, Proportion Invariant, and Gamma: Lanave *et al*. 1984, Rodríguez *et al*. 1990). In the phylogenetic tree obtained using MrBayes (Fig. 2), the two families Oceanitidae and Hydrobatidae were found to be monophyletic, but because a single gene was used, the relationships between families within Procellariformes were poorly supported (i.e. posterior probabilities <0.95) and lie beyond the scope of this paper. Within Oceanitidae, *Garrodia* and *Pelagodroma* were sister taxa, as in Robertson *et al*. (2011), but the remaining relationships between genera were not supported. At species level, AMNH 194110 belonged with strong support to genus *Fregetta*, and more specifically formed a clade with *F. grallaria titan* and *F. grallaria* ssp. from the East Pacific (Genbank sequence AF076054 without voucher specimen; G. Nunn pers. comm.). The lowest sequence divergence between AMNH 194110 and any other individual was with *F. g. titan* (1.3%, vs. 2.2% for *F. grallaria* ssp. AF076054; Table 2), and the two are sister taxa, albeit without strong support (posterior probability of 0.65). However, another individual supposedly of the same species, *F. g. leucogaster* (JN587556 from Gough Island; sequences from a toe pad of NHMUK 1953.55.101; Robertson *et al*. 2011), did not belong to this group and clustered with strong support with *F. tropica*. Finally, we found strong support for the two *Oceanites* as sister species, and for the group formed by the three *Pelagodroma marina* individuals.

Although a single mitochondrial gene is clearly insufficient to elucidate with certitude relationships within Oceanitidae, these results suggested several directions where future molecular research should be directed. First, placement of AMNH 194110, a bird with dark belly streaking, closely related to the white-bellied subspecies *titan* from Rapa, might support Murphy & Snyder's (1952) conclusion that this specimen is closely related to, and might be, *F. grallaria*. According to Murphy & Snyder (1952), wing length of AMNH 194110 (165 mm) is intermediate between those of *titan* and nominate (184 mm and 156 mm for females, respectively). Consequently, although our results assign AMNH 194110 to *F. grallaria*, its attribution at subspecies level is uncertain, pending further sampling of *grallaria* at other localities (e.g., *F. g. segethi*, breeding on the Juan Fernández and Desventuradas Islands, was not included in our analysis). One also cannot eliminate the possibility that this specimen belongs to another taxon, closely related to but distinct from *F. grallaria*. This female, possibly breeding (according to its enlarged gonads, as reported on the specimen's label), was collected in waters off the Marquesas Islands, 2,070 km from the closest known breeding areas on Rapa, suggesting either long-distance dispersal or the possibility of another breeding population somewhere in the Marquesas. The presence of subfossil bones attributed to *Fregetta* on two Marquesan islands (Ua Huka and Tahuata; Steadman 2006), provides support for past presence of this taxon in the archipelago.

Second, *F. grallaria*, as currently defined, may not be a monophyletic taxon. In our cytochrome-*b* phylogeny, the individual of *F. g. leucogaster* from Gough Island (NHMUK 1953.55.101: Robertson *et al*. 2011) is closely related to two *F. tropica*: JN587555 from South Island, New Zealand (no voucher specimen: Robertson *et al*. 2011) and AF076053 from Marion Island, south-west Indian Ocean (no voucher specimen: Nunn & Stanley 1998; G.

Nunn pers. comm.). This group differs by *c*.9% from sequences of other *F. grallaria* (Table 2), a degree of divergence much greater than that typically reported among Procellariform species (Austin *et al*. 2004). This result was previously found by Robertson *et al*. (2011; their Table 1), but they did not include *leucogaster* in their phylogenetic tree and did not mention it in their discussion. As noted above, the taxonomic status of *Fregetta* species breeding on Gough Island is poorly understood, with two species with white bellies (*F. tropica melanoleuca* and *F. grallaria leucogaster*) that might breed in sympatry (Brooke 2004, Flood & Fisher 2011).

Finally, the fifth streaked specimen of the '*pealea*' series (Murphy & Snyder 1952) is now in the Smithsonian Institution, Washington DC, collection (USNM 15713; Fig. 1c); it is the type of *Thalassidroma lineata* Peale, 1848, which was synonymised with *F. tropica* by Murphy & Snyder (1952). Although we did not sample that bird genetically, its wing length is similar to that of AMNH 194110 (166 mm *per* Murphy & Snyder 1952). Thus both are smaller than *titan* but much larger than *F. maoriana* (*c*.148 mm: Stephenson *et al*. 2008). They share with the latter a streaked belly, but we found that AMNH 194110 is distinct genetically from *F. maoriana*. These two '*lineata*' specimens, similar in size and coloration, could therefore represent a distinct taxon within *Fregetta*, but the discovery of additional individuals will be necessary to support this hypothesis.

Our evaluation of the identity of one specimen, based on a single mitochondrial gene, employs an approach similar to barcoding (Frézal & Leblois 2008). Because the cytochrome-*b* phylogeny was consistent with the nuclear tree in Robertson *et al*. (2011), we consider that using this gene to assess the identity of this storm petrel is appropriate. However, we cannot eliminate the possibility of hybridisation between closely related storm petrels, leading to the introgression of mitochondrial genes from one species to another (Rheindt & Edwards 2011), and causing for example the polyphyly of *F. grallaria*. Alternatively, the taxonomic definition of *F. grallaria*, and perhaps *F. tropica*, a species with considerable individual variation in plumage, might not be accurate (Brooke 2004). Further sampling of individuals from multiple locations, as well as a wider array of genes, is clearly required to decipher relationships within these two species.

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Addresses: Alice Cibois, Dept. of Mammalogy and Ornithology, Natural History Museum of Geneva, CP 6434, 1211 Geneva 6, Switzerland, e-mail: alice.cibois@ville-ge.ch. Jean-Claude Thibault, ISYEB (Institut de Systématique, Evolution, Biodiversité), UMR 7205 CNRS MNHN UPMC EPHE, Muséum national d'Histoire Naturelle, CP 51, 55 rue Buffon, F-75005 Paris, France, e-mail: jncldthibault@aol.com. Mary LeCroy, Ornithology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA, e-mail: lecroy@amnh.org. Vincent Bretagnolle, CEBC-CNRS, 79360 Beauvoir sur Niort, France, e-mail: breta@cebc.cnrs.fr