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RESEARCH ARTICLE

The complete phylogeny of *Pseudobulweria*, the most endangered seabird genus: systematics, species status and conservation implications

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Abstract *Pseudobulweria* is one of the least known and most endangered of all seabird genera. It comprises six taxa, of which two are extinct, three are critically endangered and one is near threatened. Phylogenetic relationships between these taxa and position of the genus in the Order Procellariiformes have never been studied, and the taxonomic status of several taxa remains unsettled. Conservation management of *Pseudobulweria* taxa will be enhanced if these uncertainties are resolved. We used a multilocus gene tree approach with two mitochondrial DNA markers (cytochrome oxidase subunit 1 and cytochrome *b* gene) and one nuclear intron (β Fibrinogen intron 7) to investigate phylogenetic relationships within the genus using sequences from all taxa. We combined gene trees to estimate a phylogeny of the genus using a multispecies coalescent methodology. We confirmed the link between *Pseudobulweria* and a clade comprising *Puffinus* and *Bulweria* genera. The Fiji petrel's status, as belonging to the genus, is confirmed, as is the specific status of newly rediscovered Beck's petrel. Maintenance of the two subspecies of Tahiti petrel as currently described is not supported. Discovering the breeding grounds of all taxa is the key for their conservation, which is vital to both the marine and fragile insular tropical ecosystems where *Pseudobulweria* are apical predators.

Keywords Conservation · Procellariiformes · *Pseudobulweria* · Seabird · CO1 · Cytochrome *b* · β Fibrinogen

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Introduction

Specific status is the most recognised unit used by conservation organisations and international and/or national government agencies to determine conservation policy and actions (Drummond et al. 2009; Farrier et al. 2007; Posigham et al. 2002). As resources (both human and financial) are limited, all conservation organisations need access to the most accurate systematic and taxonomic analyses of all taxa, threatened or not, including if possible their evolutionary history. Seabirds, and more particularly petrels (Order Procellariiformes), have traditionally been difficult to study because much of their life is spent at sea coupled with the remoteness of their breeding grounds, usually on isolated oceanic islands (Brooke 2004; Friesen et al. 2007). In consequence, even today, detailed knowledge of their taxonomy, phylogeography and conservation status is scant and controversial (Brooke 2004). Though there has been considerable improvement in the past twenty years through the development of molecular biology, many groups in this Order remain poorly known, the systematics of many taxa

and even genera is still debated, and their phylogenetic relationships unsettled (Brooke 2004; Mayr 2009). This is particularly disturbing at a time where many Procellariiformes taxa are in decline and to some degree threatened with extinction (IUCN 2010). The genus *Pseudobulweria* (family Procellaridae) is a good example of such phylogenetic uncertainties and extinction threats. Of six taxa that compose the genus, two are already extinct, three are critically endangered and one is near-threatened (IUCN 2010), making *Pseudobulweria* the most threatened seabird genus in the world. In addition, it remains one of the least known and most controversial petrel genera (Bretagnolle et al. 1998; Brooke 2004; Shirihai 2008). Indeed, the mere existence and validity of the genus remains controversial, its relationships with other petrels are contradictory, the number of species is debated, and species relationships have never been investigated.

First proposed by Mathews (1936) for the Fiji petrel (*P. macgillivrayi*), the genus was later merged within genus *Pterodroma* (Jouanin and Mougin 1979; Del Hoyo et al. 1992; Warham 1990) before being reinstated (Imber 1985; Sibley and Monroe 1990). Based on molecular data from only two taxa of the genus, Bretagnolle et al. (1998) confirmed its validity. Yet, its position within the Procellariiformes continues to be debated according to four recent phylogenetic analyses. Bretagnolle et al. (1998) found that *Pseudobulweria* was closely related to genus *Puffinus* (and next to *Bulweria* and *Procellaria*) rather than *Pterodroma*, but did not analyse *Pachyptila*. Nunn and Stanley (1998) also found that *Bulweria* was closest to *Procellaria* with which it formed a sister clade to *Halobaena/Pachyptila*, next to *Puffinus*, though they did not include any *Pseudobulweria* in their analysis. Subsequently, Kennedy and Page (2002) used two methods to analyse Procellariiformes phylogeny with partial trees from various studies (including the two former ones): a supertree approach using a matrix representation with parsimony (MRP) methodology, and a mtDNA supermatrix approach. While their MRP supertree placed *Pseudobulweria* taxa as a sister clade to *Puffinus* and as close relatives to *Bulweria* and *Procellaria*, their mtDNA supermatrix placed *Pseudobulweria* as a sister clade to the *Pachyptila/Halobaena* clade, and not to *Procellaria*, *Puffinus* and *Bulweria*. Kennedy and Page (2002) concluded that the MRP supertree was the best estimate of Procellariiformes phylogeny. Then, Penhallurick and Wink (2004), used Cytb as Nunn and Stanley (1998), to analyse the taxonomy in this Order (note critical views in Rheindt and Austin 2005), but did not include sequences from Bretagnolle et al. (1998) in their phylogenetic analyses. These authors found that *Procellaria/Bulweria* were a sister clade of the *Pachyptila/Halobaena* clade.

Furthermore, the number of species within the genus is still uncertain. Five *Pseudobulweria* taxa (four living species, of which one has two subspecies, and one extinct) are currently known and a further extinct one still remains to be named (Worthy and Tennyson 2004). The phylogenetic relationships between these are unknown (Brooke 2004; Shirihai et al. 2009) and even the precise number of species within the genus is not settled. The extinct taxon *P. rupinarum* is known only from St Helena Island in the Atlantic Ocean, where it was numerous, and was probably extirpated after 1502 (Olson 1975). From bone remains used to describe the taxon, it appeared to be slightly larger than Mascarene petrel *P. aterrima*. Another extinct taxon was breeding in the southern Tuamotu archipelago, Pacific Ocean, being apparently very abundant, and was the size of Beck's *P. becki* and Fiji petrels (Worthy and Tennyson 2004). All surviving taxa now live in the Indian and Pacific Oceans (Fig. 1) and breeding colonies are unknown for all but Tahiti petrel (*P. rostrata*). Until recently, three taxa were known by no more than two (Beck's, and Fiji petrels) and seven (Mascarene petrel) specimens (Attié et al. 1997; Bretagnolle et al. 1998; Shirihai 2008; Shirihai et al. 2009). Hence, given the paucity of data and poor number of specimens held in museums, systematic studies on this genus were so far mostly based on scant morphological data and Tahiti petrel is the only taxon that has been studied in some detail (Villard et al. 2006). Furthermore, phylogenetic relationships between taxa have been investigated solely between Tahiti and Mascarene petrels, with the validity of the two *P. rostrata* subspecies still being questioned (Bretagnolle et al. 1998; Villard et al. 2006) and the phylogenetic relationships of Beck's and Fiji petrels having never been investigated. In addition, the taxonomic status of *P. becki* is still uncertain.

The recent rediscovery of Beck's petrel (Shirihai 2008) and the first observation at sea of Fiji petrel (Shirihai et al. 2009) threw new light on the conservation status of this genus. In this article we use three different genes obtained from all extant taxa in order to investigate species limits and species validity, species relationships, genus monophyly and genus position within the other petrels. In particular we use newly developed species tree inference tools based on Bayesian statistics and multispecies coalescent theory (Heled and Drummond 2010; Liu et al. 2009a, b; O'Meara 2010; Yang and Rannala 2010). As even the most probable gene trees topologies are not necessarily congruent with species trees (Degnan and Rosenberg 2009; Nichols 2001), as seen in pines (Syring et al. 2007), grasshoppers (Carstens and Knowles 2007), finches (Jennings and Edwards 2005) or hominids (Ebersberger et al. 2007), implementation of such multilocus approaches allows inference of phylogenies when there are conflicting

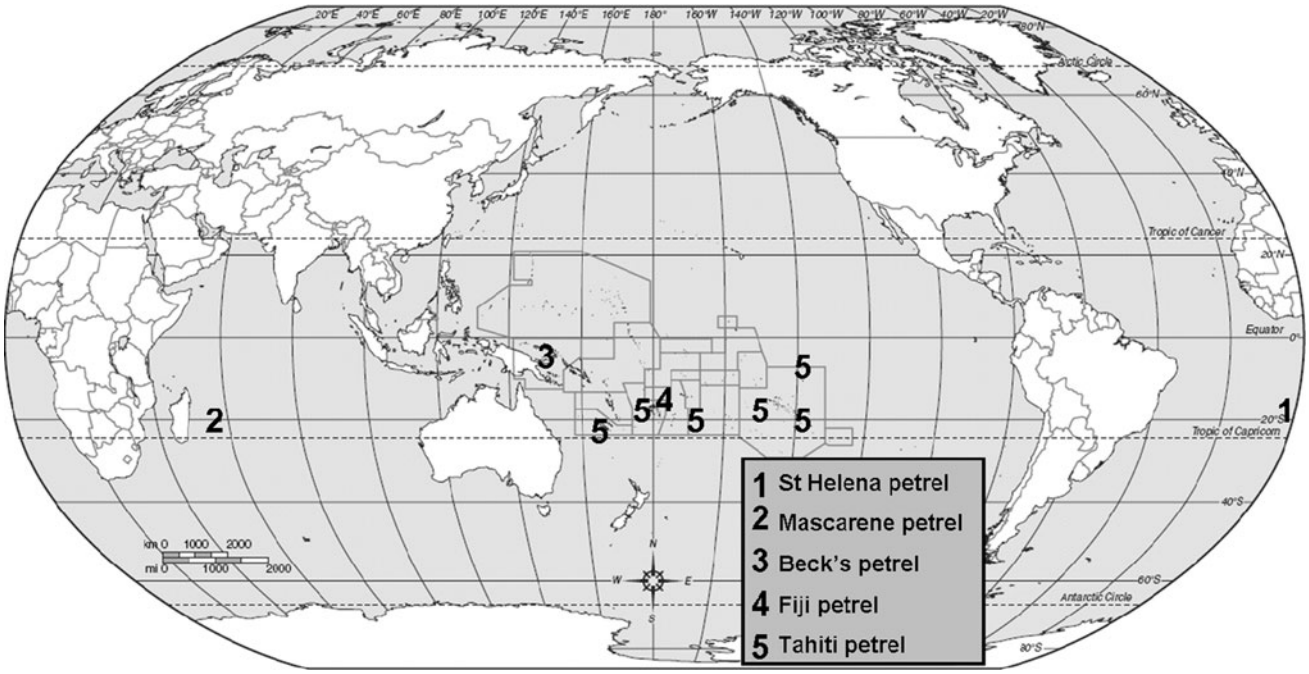

Fig. 1 Distribution of *Pseudobulweria* taxa, including extinct St Helena petrel *P. rupinarum*

branching patterns between different genes (Degnan and Rosenberg 2009; Heled and Drummond 2010).

Materials and methods

Samples

Table 1 summarises the origin of *Pseudobulweria* samples used. For the type specimen of *P. becki* and for one specimen of *P. aterrima*, a 1 mm piece of skin from the foot pad was collected without damaging the specimen. Total genomic DNA was extracted using the DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions except that we increased the time of proteinase digestion to 2 h. Since samples were mostly collected on dead or old museum birds, DNA was degraded and fragment sizes for amplification were mostly small (<200 bp) and proved difficult to amplify and sequence. Thus we failed to obtain useable gene sequences for some samples, and for others we could amplify and sequence only fragments of the total gene sequences. We sequenced two mitochondrial genes and one nuclear intron. Primers used for sequencing are shown in Table 2.

Cytochrome oxydase 1 gene (CO1) was amplified using PCR consisting of 37 cycles following a hot start at 94°C and a 4 min initial denaturation step at 94°C. Cycles, i.e. 94°C for 30 s, 55°C for 40 s and 72°C for 50 s, were completed by a final extension at 72°C for 5 min.

Cytochrome *b* gene was amplified with 40 PCR cycles consisting of 30 s at 94°C, 50 s at 58°C, 50 s at 72°C. These cycles followed a 4 min initial denaturation step at 94°C and were completed by a final extension at 72°C for 5 min.

Primers FIB-BI7U 5′-GGAGAAAACAGGACAATGA CAATTCAC-3′ and FIB-BI7L 5′-TCCCCAGTAGTATC TGC-CATTAGGGTT-3′ (Prychitko and Moore 1997) were used along with other primers specifically designed (Table 2) for Beta Fibrinogen intron 7 (*β*Fibint7) amplification. We ran thirty-nine PCR cycles consisting in 1 min at 94°C, 40 s at 58°C and 50 s at 72°C preceded by an initial denaturation step of 4 min at 94°C. These cycles were followed by a 5 min final extension step at 72°C.

For all genes, sequencing was conducted under BigDye™ terminator cycling conditions at the "Genoscope-Centre National de Séquençage", France. DNA sequences were aligned using CodonCode Aligner 3.0.3 (CodonCode Corporation 2009) and ClustalW (Thompson et al. 1994) as implemented in Mega version 4 (Tamura et al. 2007) and checked by eye.

Phylogenetic analyses

Genetic distances

For the three genes, corrected genetic distances (K2P) between taxa were calculated with Mega v.4 (Tamura et al. 2007) using the pairwise deletion option to allow for

Table 1 *Pseudobulweria* taxa, identifying codes, origins, and tissue sources analyzed

Taxon	Code	Locality	Tissue source	CO1	Cytb	βFibint7
P. aterrima	MNHN 1995-165	Reunion Island	Muscle	NA	1102	975
P. aterrima	MNHN 1970-102	Reunion Island	Skin (from palm)	526	NA	NA
P. becki	AMNH 235376	Bismarck sea	Skin (from palm)	721	145	NA
P. becki	BMNH 2008-1-1	New Ireland	Liver	717	799	934
P. macgillivrayi	NA	Gau Island, Fiji	Neck tissue	580	683	975
P. macgillivrayi	NA	Gau Island, Fiji	Neck tissue	432	881	NA
P. rostrata rostrata	GenBank U70482[a]	Gambiers	Blood	684	496	NA
P. rostrata rostrata	MHNG P08-30	Marquesas	Muscle	723	514	952
P. rostrata rostrata	MHNG P08-31	Marquesas	Muscle	736	481	955
P. rostrata rostrata	MHNG P08-32	Marquesas	Feather	NA	409	NA
P. rostrata rostrata	NA	Tahiti	Blood	695	NA	957
P. rostrata rostrata	FM 170141	Vatuira, Fiji	Muscle	694	410	540
P. rostrata trouessarti	GenBank U70493[a]	New Caledonia	Blood	694	496	NA
P. rostrata trouessarti	NA	New Caledonia	Feather	695	NA	957
P. rostrata trouessarti	NA	New Caledonia	Feather	694	NA	957
P. rostrata trouessarti	NA	New Caledonia	Feather	695	NA	NA

Museum samples came from Paris National Museum of Natural History (MNHN), New York American Museum of Natural History (AMNH), Geneva Natural History Museum (MHNG), Fiji Museum (FM) and the British Museum of Natural History (BMNH). For each gene, total number of nucleotide sequenced is shown. NAs denote that DNA amplification or sequencing failed despite repeated attempts

[a] GenBank accession number refers to Cytb sequence from Bretagnolle et al. (1998)

comparison of complete gene sequences with sequences in which fragments were missing due to poor DNA quality. This measure of genetic distance is widely used in avian studies, in particular those using CO1 (e.g. Hebert et al. 2004; Kerr et al. 2007; Johnsen et al. 2010), and thus it allows comparisons among taxa and studies.

Gene trees inference

For each of the three genes, phylogenetic relationships of *Pseudobulweria* taxa within the order and with other petrel orders belonging to three families within Procellariiformes (i.e. Hydroabatidae, Diomedeidae and Procellariidae) were estimated using Bayesian Markov Chain Monte Carlo (MCMC) phylogenetic analyses with MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). jModelTest v0.1.1 (Guindon and Gascuel 2003; Posada 2008) was used to evaluate the fit to the data of 24 models of nucleotide substitution implemented in MrBayes. We used Bayesian Information Criterion (BIC) to evaluate which model best fitted our data (Sullivan and Joyce 2005). For all Bayesian analyses, default priors of MrBayes 3.1.2 were used for MCMC parameters. We used three heated chains and one cold chain for all analyses and runs were started with random trees. Two independent MCMC runs were conducted with 4×10^6 generations each. Trees and parameters were sampled every 100 generations. Standard deviations of split frequencies were used to assess stationarity (a cut off value of 1% was used), with the average standard deviation of split frequencies expected to approach zero when the two runs converge onto stationarity distribution (Ronquist et al. 2005). Additionally the potential scale reduction factor should approach one when runs converge. For each run the first 25% were discarded as burn-in. Since order Sphenisciformes is supposed to be the closest relative to Procellariiformes (Brooke 2004) and because sequences from all three genes were available on GenBank for this taxon, we used Humboldt penguin *Spheniscus humboldti* as an outgroup to root the gene trees. Within family Procellariidae, contrary to CO1 and Cytb, no βFibint7 sequences were available on GenBank for genera *Pachyptila*, *Halobaena*, *Bulweria*, *Calonectris*, *Macronectes* (Table 3) and the gene tree with this marker was therefore built without these genera. GenBank accession numbers and Barcode Id numbers of sequences retrieved in these databases are shown in Table 3.

Species tree inference

In "species tree", we here use "species" following the terminology of Heled and Drummond (2010), i.e. it is not necessarily referring to the taxonomic rank, but designates any group of individuals that have diverged sufficiently to no longer have breeding history with individuals outside

Table 2 Primers used in amplification of CO1, Cytb and βFibint7 genes in *Pseudobulweria* petrels

Primers CO1	Primers Cytb	Primers βFibint7
F1B 5′-AACCGATGACTATTYTCAACC-3′	L14987 5′-TATTTCTGCTTGATGAAACT-3′	F21 5′-TGACAATTCACAATGGCATGTTCT-3′
R1B 5′-TACTACRTGYGARATGATTCC-3′	H16025 5′-CTAGGGCTCCAATGATGGGGA-3′	R158 5′-ACCACGACATGCTGTGAAAACT-3′
R141 5′-AGCATGGGCGGTGACGATT-3′	F13 5′-CTTCGAAAGTCCCACCCCT-3′	F72 5′-GATGGTACGTACTTGCATTAGACA-3′
F78 5′-ACTTATTCGTGCAGAACTTGGTC-3′	R142 5′-TGGCTAGTAGGAGGCCGGT-3′	R210 5′-TGCATGGACGTTCAGCTGGT-3′
R208 5′-AGGGGGACTAGTCAGTTTCC-3′	F103 5′-GGATCCCTCCTAGGCATCTGT-3′	F160 5′-TTTTCACAGCATGTCGTGGT-3′
F150 5′-CGCCCATGCTTTCGTAATAATTT-3′	R202 5′-ACATTTCGGCAGGTGTGAGC-3′	R277 5′-ACTTGGCTGTGGAGCAGCA-3′
R254 5′-AGCTTATGTTGTTTATACGTGGGA-3′	F167 5′-ACACAGCTGACACAACCTTAGC-3′	F244 5′-GCCAAGGGCAGGTAAAACT-3′
F207 5′-TGGAAACTGACTAGTCCCCCT-3′	R297 5′-CCGTAGTAGAATCCCCGTCCG-3′	R380 5′-TGCCACCATCAGTCTCTGACA-3′
R323 5′-ACCTGCTCCTGCTTCTACGG-3′	F249 5′-ACATGCAAACGGAGCCTCA-3′	F347 5′-ACAAATCAGCAAATCTGGATGCAA-3′
F288 5′-ACCTCCGTCCTTCCTCCTAT-3′	R369 5′-ACGAAGGCAGTTGCCATAAGA-3′	R462 5′-CCTGTCTCTTTCCTCAGGACCCA-3′
R416 5′-CCTGCCAGGTGGAGGGAGA-3′	F314 5′-ACGGCTCCTACCTATACAAAGAG-3′	F411 5′-CCACTGACTTGCTTAAGTAGGAA-3′
F377 5′-ATCTAGCCCATGCCGGAGC-3′	R457 5′-TGGCCAATGTAGGGGATGGC-3′	R522 5′-ACAATTGAGCTCCTGTCTTCTG-3′
R502 5′-AAGGGGGTTTGGTACTGTGA-3′	F418 5′-TCATTCTGAGGTGCGACAGTCA-3′	F476 5′-AGAGACAGGTAGCATGTCCTATT-3′
F453 5′-GGCAATCAACTTCATTACAACAGC-3′	R529 5′-AGTGTAGGGCGAAGAATCGGGT-3′	R638 5′-TGAGAACTGTACATCTTCCCCAA-3′
R581 5′-AGCATGGTGATGCCTGCGG-3′	F473 5′-GCCAAACCCTTGTAGAATGAGCC-3′	F574 5′-ACTATGTGCTATGTCTTTCTCT-3′
F537 5′-ACTCATCACTGCCGTCCTAC-3′	R595 5′-AGCCAGATTCGTGGAGGAAGGT-3′	R722 5′-GTCTACCGATTGTAGTCTAACTT-3′
R678 5′-TGGGTGGCCGAAGAATCAG-3′	F553 5′-CTCCTACCTTTTGCAATCACAGGA-3′	F641 5′-GGGAAGATGTACAGTTCTCATTGT-3′
F642 5′-TGGCGGAGGAGACCCAGTC-3′	R674 5′-AGCCTAGGATATCTTTTAAGGTGA-3′	R796 5′-GCACTTGGAAGGTGAAGCAGC-3′
	F630 5′-TGGTGTCGTATCAAACTGCGA-3′	F756 5′-TCCGAAAGAGATGCAGCTAAA-3′
	R758 5′-CGCTGGAGTAAAGTTTTCTGGGT-3′	R852 5′-AAATCCTCCCTGAACTTTCTGT-3′
	F711 5′-TCTCCCACTAACAGCCCTAGCT-3′	F807 5′-TTCCAAGTGCACTGTGTAGCA-3′
	R836 5′-GGAATTGAGCGTAGGATGGCGT-3′	R938 5′-GAGTGGCAGATGAACTGTAAGCA-3′
	F793 5′-ACACCTCCCCATATTAAACCAGA-3′	F900 5′-TCAGTACAGGGGCAGGTGTA-3′
	R915 5′-TGGCTTTATGGAGGAATGGA-3′	R1048 5′-GGGTTGGCTGAGTGGCAGC-3′
	F885 5′-AGCTGCCTCAGTATTGATCCTA-3′	
	R1012 5′-ACTGGCTGGCTGCCTACTCA-3′	

All primers were designed for this study, except for L14987/H16025 from Jesus et al. (2009)

that group, and thus it can include taxonomic rank or any "diverging population structure". We found different placements of genera, and variations in taxa positions within the *Pseudobulweria* clade in gene trees although incongruent patterns were mostly unsupported (posterior probabilities <95%; see Results). Potential gene trees incongruences with species trees have been known for decades (Pamilo and Nei 1988). A common practice to avoid the problem consists in concatenating data assuming that all data have evolved under a single evolutionary tree. However, this method was recently shown to result potentially in support of incorrect species trees (Kubatko and Degnan 2007). Here we used the *BEAST methodology (Heled and Drummond 2010) as applied in the software BEAST v1.5.4 (Drummond and Rambaut 2007) to reconstruct *Pseudobulweria* species tree.

In order to precisely identify the phylogenetic relationships of Procellariiformes genera, we ran an analysis with all sequences used previously to build phylogenetic gene trees with MrBayes, first with both mitochondrial genes in order to include genera *Pachyptila*, *Halobaena*, *Bulweria*, *Calonectris* and *Macronectes* for which we did not have data for βFibint7 (Table 3). We then ran an analysis using the three genes using data from genera for which sequences were available for βFibint7 to confirm results from the mitochondrial analysis. We specified genera (*Pseudobulweria*, *Puffinus*, *Fulmarus*, *Pterodroma* etc.) as "units" to build the species tree. Because we had different datasets between genes (i.e. different number of sequences per taxon and sequences from different individuals between genes; Table 1) we chose not to link trees of the two mitochondrial genes. Thus the analysis was run with unlinked substitution and clock models and unlinked trees. For each gene, we used a relaxed clock model with an uncorrelated lognormal distribution of the substitution rate with a fix mean value of 0.794 ± 0.115% per million year for CO1 (Pereira and

Table 3 Outgroup taxa and sequences GenBank accession numbers, BarCode Id number (marked with an asterisk)

Family	Genus	Taxon		Sequence Code
Procellariidae	Pachyptila	Pachyptila turtur	CO1	BROMB324-06*
			Cyt*b*	AF076070
	Halobaena	Halobaena caerulea	CO1	BROMB700-07*
			Cyt*b*	AF076057
	Bulweria	Bulweria bulwerii	CO1	BROMB697-07*
			Cyt*b*	AJ004156
				U74341
	Puffinus	Puffinus tenuirostris	CO1	DQ434025
				DQ434027
			Cyt*b*	U74352
			*β*Fibint7	AY695220
		Puffinus lherminieri	CO1	DQ434015
			Cyt*b*	AF076085
			*β*Fibint7	DQ881991
	Calonectris	Calonectris diomedea	CO1	DQ432808
				DQ433417
			Cyt*b*	AY139626
				U74356
	Fulmarus	Fulmarus glacialis	CO1	DQ433651
				DQ432933
			Cyt*b*	AJ004178
				U74348
			*β*Fibint7	EF552765
				DQ881958
	Macronectes	Macronectes giganteus	CO1	FJ027768
			Cyt*b*	U48941
				AF076060
	Pterodroma	Pterodroma hasitata	CO1	DQ434001
			Cyt*b*	EU167017
		Pterodroma cahow	CO1	KKBNA075-04*
			Cyt*b*	U74331
		Pterodroma cookii	CO1	ROMC325-07*
			Cyt*b*	U74345
		Pterodroma ultima	CO1	JF522137
			Cyt*b*	JF522109
			*β*Fibint7	JF522119
		Pterodroma neglecta	CO1	JF522135
				JF522136
			Cyt*b*	U74341
				GQ328985
			*β*Fibint7	JF522120
Diomedidae	Phoebastria	Phoebastria nigripes	CO1	DQ433934
				DQ433935
			Cyt*b*	U48950
				EU166988
			*β*Fibint7	EU739406
				EF552760

Table 3 continued

Family	Genus	Taxon		Sequence Code
Hydrobatidae	Oceanites	Oceanites oceanicus	CO1	DQ433048
				DQ433049
			Cyt*b*	AF076062
			*β*Fibint7	EU739449
	Hydrobates	Hydrobates pelagicus	CO1	AY567885
			Cyt*b*	AF076059
				AJ004182
			*β*Fibint7	DQ881965
	Oceanodroma	Oceanodroma leucorhoa	CO1	AY666284
				DQ434684
				DQ434685
			Cyt*b*	AF076064
			*β*Fibint7	AY695221
Spheniscidae	Spheniscus	Spheniscus	CO1	AY567888
		Humboldti	Cyt*b*	DQ137220
			*β*Fibint7	DQ881996

Baker 2006; Table S4, Supplementary Material 2, for Procellariiformes) and 1.89 ± 0.35% (obtained for Procellariiformes) for Cytb (Weir and Schluter 2008). Since *β*Fibint7 lacks a well calibrated substitution rate, we did not enter this parameter. We used HKY model, with a discrete approximation of the gamma-distributed rate of heterogeneity (four rate categories, Yang 1994) for CO1 and *β*Fibint7, and a GTR+G model for Cytb following jModeltest analysis. We specified a Yule process species tree prior under a continuous population size model. The analysis was run for 200×10^6 generations. The run was sampled every 1,000 generation, with the initial 50,000 steps being discarded as burn in. We used the software TRACER v1.5 (Drummond and Rambaut 2007) to visualize the results of the run as well as for checking effective sample size of each parameter. Second, we investigated the inter-taxa species tree within *Pseudobulweria* with the three genes using only *Pseudobulweria* sequences. The same specifications than the previous analysis were used.

All *BEAST analyses were run on the BioHPC compute cluster at the Cornell University Computational Biological Service Unit (http://cbsuapps.tc.cornell.edu).

Tahiti petrel haplotype network

Since we were subsequently able to obtain additional CO1 sequences (GenBank accession numbers JF522138–JF522194) for *P. rostrata trouessarti* from New Caledonia and *P. rostrata rostrata* from Tahiti and Marquesas (for a total Tahiti petrel sample size of 68), we investigated the relationship between the specimens belonging to these two recognised subspecies of Tahiti petrel. We built a median-joining haplotype network using the software Networks v4.5.1 (Bandlet et al. 1999) after identifying haplotypes with DnaSP v5 (Librado and Rozas 2009), using 68 sequences of Tahiti petrel and two sequences of Beck's petrel.

Results

Total numbers of nucleotides sequenced per gene for each *Pseudobulweria* sample for gene and species trees analyses are shown in Table 1. Some samples failed to give useable sequences for one or two genes despite numerous PCR attempts and therefore do not appear in corresponding gene trees. Sequences are deposited in GenBank under accession numbers JF522101–JF522108, JF522110–JF522118, JF522121–JF522134. Due to the limited number of nucleotide sequenced for cytochrome *b* gene with Beck's petrel sample AMNH235376 (<150; Table 1), this fragment could not be deposited on GenBank, but is available on demand to the corresponding author.

The genus *Pseudobulweria*: monophyly, and relationships with other petrels

With all three genes, though tree topologies differ somewhat in the placement of genera (Fig. 2), *Pseudobulweria* appears to be strictly monophyletic. Although we could not sequence *P. rupinarum* (only bones are available), plates provided in Olson (1975) clearly suggest that *P. rupinarum*

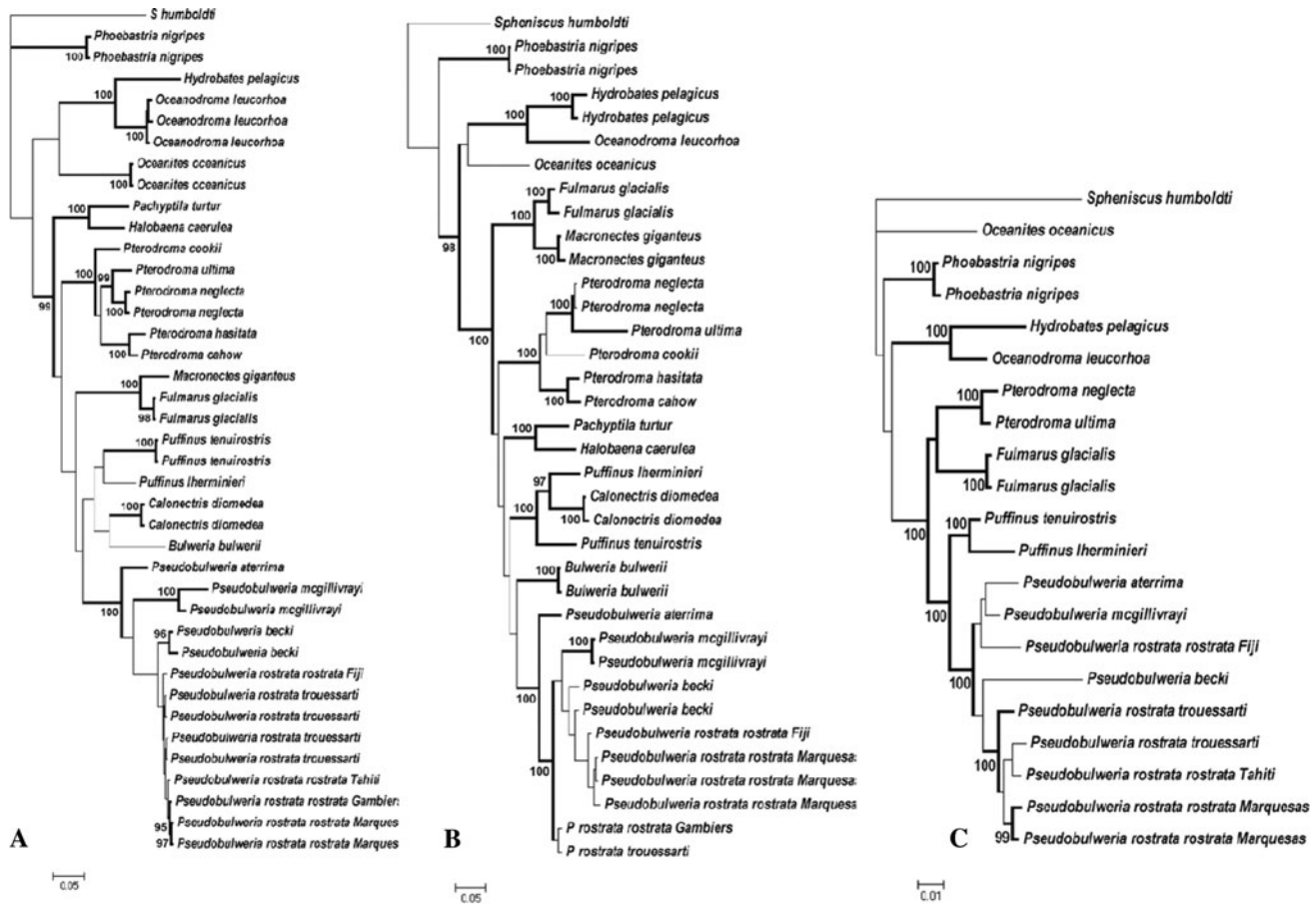

Fig. 2 Bayesian inference phylogenetic tree within Procellariiformes obtained with CO1 (**a**), Cytb (**b**) and βFibint7 (**c**) genes with MrBayes. Node with posterior probabilities superior or equal to 95% are indicated by *bold lines* and *labels* indicate posterior probabilities

also belongs to *Pseudobulweria* (based on characters on skull and especially bill angle in comparison to skull).

With CO1, the gene used for the BarCode of Life in birds, *Pseudobulweria* appears as a sister clade of a clade containing *Puffinus*, *Calonectris*, *Bulweria*, *Fulmarus* and *Macronectes* (Fig. 2). Node support between these two clades, however, is not strong (0.88). In Cytb, *Pseudobulweria* is a sister group of *Bulweria*, although with posterior probability inferior to 0.8 (Fig. 2). With this gene, different subclades within family Procellariidae are weakly supported, with the exception of the *Fulmarus/Macronectes* group. In βFibint7, *Pseudobulweria* is the sister group of *Puffinus* with strong support (posterior probability of 100%; Fig. 2). However we lack data from genera such as *Bulweria* with this nuclear intron.

With both data sets (i.e. mitochondrial and nuclear + mitochondrial), clades recovered with species tree analyses on genera data recovers mostly weakly supported clades. These analyses indicate the presence of two main clades: one made of family Hydrobatidae and one clade made of families Diomedeidae and Procellariidae (Fig. 3). In the latter clade, *Pseudobulweria* is the sister group to a sub-clade composed of *Bulweria*, *Calonectris* and *Puffinus* (Fig. 3) or *Puffinus* alone (with the nuclear + mitochondrial data set; Fig. 3) though the separation is very weakly supported (Posterior probability 0.66 and 0.81 in mitochondrial and mitochondrial + nuclear analyses, respectively; Fig. 3). In both analyses time to the most recent common ancestor of *Puffinus* and *Pseudobulweria* was estimated at about 13 millions years ago (95% HPD: respectively 11–23 and 5.5–21.4 million years ago in mitochondrial and mitochondrial + nuclear species tree analyses).

Genetic distances and taxa relationships within genus *Pseudobulweria*

With mitochondrial gene CO1 inter-taxa divergences (K2P corrected distances) in the genus ranged from 1.21% between *P. rostrata trouessarti* and *P. becki* to 7.4% between this latter taxon and *P. aterrima*. Fiji petrel showed the lowest divergence with Beck's (6.31%) and the

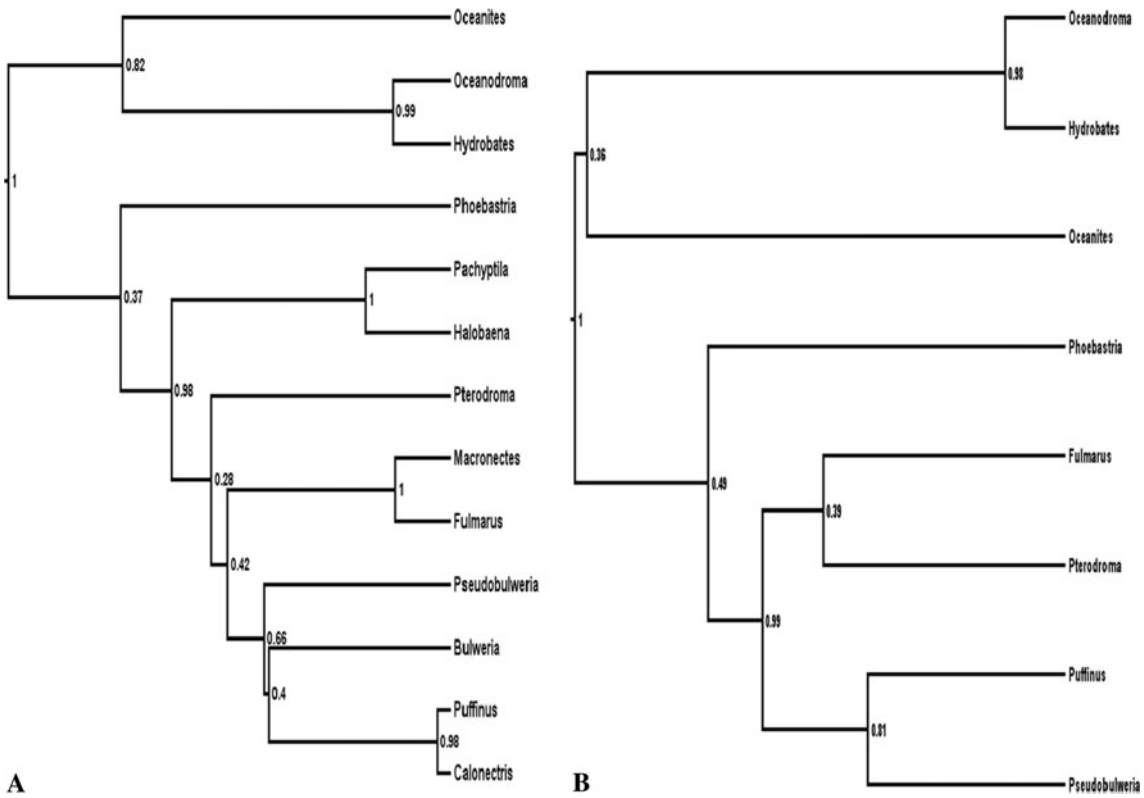

Fig. 3 Species tree reconstructed for genera within Order Procellariiformes with *BEAST, using two mitochondrial genes (CO1, Cytb; tree A) and two mitochondrial genes and one nuclear intron (βFibint7; tree B). *Node labels* indicate posterior probabilities

greatest with Mascarene (7.15%) petrels, respectively. Sequence divergence between the two Tahiti petrel taxa (i.e. *P. rostrata rostrata* and *P. rostrata trouessarti*) was 0.12%.

Mean Fiji petrel divergence was the greatest with *P. rostrata rostrata* (7.99%) and the smallest with *P. rostrata trouessarti* (4.99%) with mitochondrial gene Cytb. With this marker, the two forms of Tahiti petrel showed a genetic distance of 1.73%.

Using βFibint7, the greatest divergence was between Beck's and Fiji petrels (2.46%), the latter being closest to Mascarene petrel (7×10^{-4}%). For the two forms of Tahiti petrel, sequence divergence was 0% with this nuclear intron.

Within genus *Pseudobulweria*, with CO1 *P. aterrima* and *P. macgillivrayi* seem the first to have evolved in the sampled taxa, and *P. becki* is slightly differentiated from *P. rostrata* (Fig. 2). However, contrary to the monophyly of the genus that is well supported, nodes within the genus are poorly supported. The situation is different with Cytb. With this gene, *P. macgillivrayi* is embedded in a group also containing *P. becki*, *P. rostrata rostrata* and *P. rostrata trouessarti*, while *P. aterrima* is placed outside this group with strong node support. With the nuclear intron βFibint7, one well supported subclade exists within *Pseudobulweria* made of all *P. rostrata* but the Fiji specimen from the other taxa. Relationships between the latter are poorly supported but seem to indicate that *P. becki* is more closely related to *P. rostrata* although node support is poor (Fig. 2). Thus the relationship between the two Tahiti petrel taxa is unclear when using βFibint7, and unresolved with the two mitochondrial markers.

The species tree analysis finds support for the hypothesis that *P. becki* and *P. rostrata* are each others closest relatives and, within that group, that *P. becki* differs from the two Tahiti petrel taxa (Fig. 4). This tends to confirm the full separation of the *P. becki* lineage from *P. rostrata*. According to this analysis, the dark (*P. aterrima*, *P. macgillivrayi*) and white vented (*P. becki*, *P. rostrata*) *Pseudobulweria* taxa diverged around six-seven millions years ago (95% HPD, range: 3.2–10.75).

Finally, haplotype network reconstruction with CO1 using Tahiti and Beck's petrel sequences (Fig. 5) recovers a clear separation of *P. becki* from the two Tahiti petrel subspecies (11 mutations). Within Tahiti petrel, birds from Gambier, Tahiti and Marquesas are differentiated from New Caledonian birds. Birds from Fiji, although having a different haplotype from the New Caledonian haplotypes, are related to this latter taxon rather than to birds from Polynesia (Marquesas, Gambiers, Society) (Fig. 5).

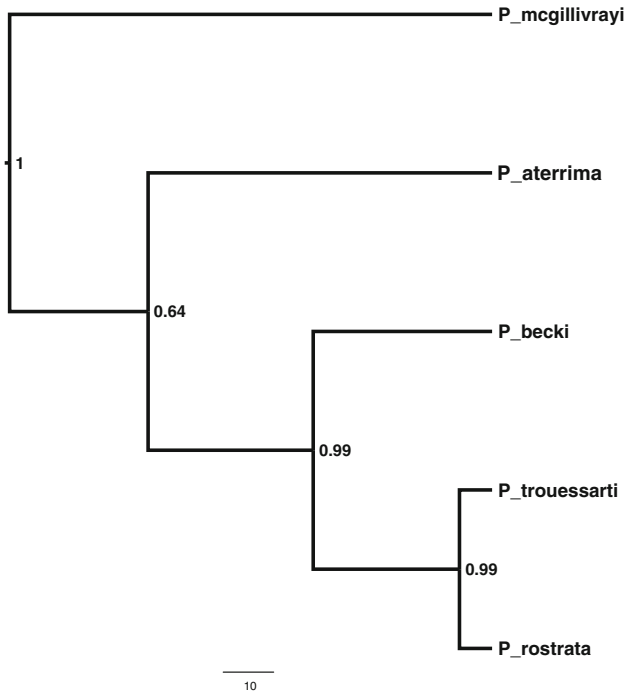

Fig. 4 *Pseudobulweria* species tree reconstructed with *BEAST, based on two mitochondrial loci (CO1, Cytb) and one nuclear marker (βFibint7). *Node labels* indicate posterior probabilities

Discussion

Position of *Pseudobulweria* within Procellariiformes

With Cytb, Bretagnolle et al. (1998) found that *Pseudobulweria* was part of a clade containing both *Puffinus* and *Bulweria*, with a more direct relationship to *Puffinus*. Here, with the same gene *Pseudobulweria* was more directly related to *Bulweria* than to *Puffinus* though with low support. With CO1, the relationship of *Pseudobulweria* was found in a clade including both *Puffinus* and *Bulweria*. Our species tree analyses, based on two and three genes simultaneously, support a link between a *Puffinus/Bulweria* clade and *Pseudobulweria*, *Halobaena* and *Pachyptila* forming a clade more distantly related within Procellariidae. These results thus confirm the MRP supertree result of Kennedy and Page (2002) and the findings of Bretagnolle et al. (1998) but contradict the mtDNA supermatrix of Kennedy and Page (2002). Thus, despite the lack of nuclear intron data from *Bulweria* and *Procellaria*, we believe that our study, by integrating information from several markers, supports the conclusion that *Pseudobulweria* affinities are to be found with *Puffinus/Bulweria* rather than with *Pachyptila/Halobaena* or *Procellaria*.

Subspecies of Tahiti petrel

The traditional view considers that birds from New Caledonia belong to *trouessarti* subspecies, while birds from Polynesia (Marquesas and Society islands) belong to *rostrata*. The sub-specific status of birds from Gambier, American Samoa and Vanuatu remain unclear, and these populations were assigned to a sub-species based on geographic distance mainly. Evidence from individual genes used in this study is somewhat confusing regarding the degree of divergence of the two traditional subspecies. CO1 node supports were low and corrected divergence was 0.12%, well under the average intraspecific divergence found among 260 North American bird taxa by Hebert et al. (0.27%; 2004). In addition, species tree reconstruction separated these two taxa from *P. becki* but does not

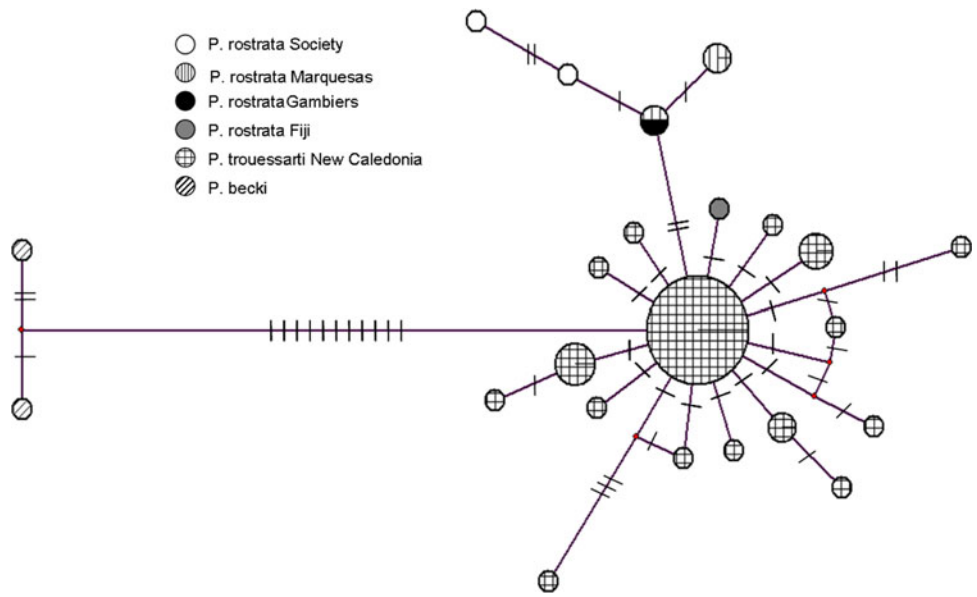

Fig. 5 Haplotype network built with 68 sequences of Tahiti petrel and two sequences of Beck's petrel with the mitochondrial gene CO1. *Bars* across *lines* connecting different haplotypes indicate the number of mutations between haplotypes. *Small dots* between some haplotypes indicate putative missing haplotypes. *Circles sizes* are proportional to number of individuals

allow concluding on their status and degree of divergence. An important limitation however remains: species tree analysis (such as in *BEAST) requires that the taxa investigated do not exchange genes. However, in the case of *P. rostrata rostrata* and *P. rostrata trouessarti*, gene exchanges cannot be ruled out, certainly not on the basis of our results since we have too few sequenced individuals from taxon *P. rostrata rostrata*. Thus, the genetic difference between these taxa appears very small, a result also found when a Cytb sequence of a bird from American Samoa was obtained (M. Rauzon, S. Olson and R. Fleischer personal communication). In addition, inconclusive morphological differences were found between New Caledonian and Polynesian birds (Villard et al. 2006). Birds from Fiji, belonging presumably to subspecies *rostrata* on the basis of geographic distance, are actually closer to birds from New Caledonia (spp. *trouessarti*) in the CO1 haplotype network. Morphologically, these birds are also much smaller than any other *rostrata* (V. Bretagnolle unpublished data), thus raising additional issues with regard to subspecies geographic delineation. Therefore, given the lack of specimens and genetic data from Vanuatu and, to some extent American Samoa, it is not possible to conclude about the validity of the two subspecies of Tahiti petrels because they are currently ill-defined geographically.

Beck's petrel taxonomic status

Originally described by Murphy (1928) as warranting full specific status (though at that time he placed *becki* within genus *Pterodroma*) because of its smaller size (about 10–15%, with no overlap) compared to Tahiti petrel, Beck's petrel taxonomic rank has subsequently been debated and challenged, and the taxon was considered either a subspecies of Tahiti petrel (Imber 1985; Jouanin and Mougin 1979; Warham 1990) or a full species (Collar and Andrew 1988; Sibley and Monroe 1990). In addition to biometrics, at-sea behavioural differences led Shirihai (2008) to advocate for full specific status, though acknowledging that no single criteria (except size if judged correctly) could allow separating both forms at sea. We found that *P. becki* was consistently separated from *P. rostrata* in all three loci used. The separation of the two taxa in the species tree analysis was supported by a posterior probability of 0.99, giving credit to the full specific status of Beck's petrel. Despite being well supported, the genetic distance is however small. Using CO1, a divergence of 1.21% was detected. Hebert et al. (2004) found that among 260 North American taxa, maximum average intraspecific divergence was 1.24% with an average value of 0.27%. Our value is therefore just below this average maximum divergence. Interestingly, Hebert et al. (2004) also found 13 species that showed interspecific distances lower than 1.25%. Thus, genetic divergence found with CO1 (as well as the two other genes) seems to indicate the presence of two distinct species, albeit only recently separated. Thus, we provisionally suggest that the two taxa should be considered as fully distinct species. We expect other important species isolating characters such as calls (Bretagnolle 1995) to confirm this separation when *P. becki* breeding colonies are discovered and birds recorded. Such pre-mating isolating trait is likely to be important in those taxa since it is rather likely that Tahiti petrels also breed in close vicinity to Beck's petrel breeding colonies and even possibly together (HS personal observation).

Fiji petrel

The phylogenetic relationship of *P. macgillivrayi* with other members of *Pseudobulweria* had never been studied before. However, based on skull characters (Olson 1975), and at-sea behaviour and flight (Shirihai et al. 2009), there was little doubt that Fiji petrel was a member of the genus. Our genetic data fully confirm the pattern, both gene trees and species tree analyses placing this taxon within *Pseudobulweria*. The exact placement within the Genus varied slightly with the different genes. The species tree topology, suggests that *P. becki* and *P. rostrata* branched apart from *P. aterrima* and *P. macgillivrayi* around 6–7 Myrs ago, in parallel with the colourations of the four species (*P. rostrata* and *P. becki* are white vented, while the other two are entirely dark).

Timing of divergence

Values found in these analyses need to be taken with caution given the overall poor node supports and also because we used only one estimation rate per marker and no fossil calibration.

The separation between Mascarene/Fiji and Tahiti/Beck's petrels estimated around 6–7 millions years ago corresponds to the end of Miocene, a time of marked ecological change (Janis 1993). The Messinian stage of the end of Miocene was characterised by important sea-level regression that were subsequently followed by sea transgression in the early Pliocene (Haq et al. 1987). Such pattern could have increased the available habitat on several islands in the Indo-Pacific region, thus promoting the colonisation of new breeding locations by *Pseudobulweria* common ancestors. New populations would then be isolated due to sea-level increase, promoting the divergence of these taxa and their colouration change by fixation of alternative alleles of the melanocortin-1 receptor gene (*MC1R*). This gene is known to affect colourations in several bird lineages through a single non-synonymous change, as well as a wide range of other organisms, from lizards to mammals (Mundy

2005). Alternatively, changes in oceanic conditions could have driven some birds to modify their foraging habits thus promoting the differentiation of these lineages through different foraging patterns and at-sea behaviour. Indeed, colourations in Procellariiformes were suggested to be linked to feeding strategies and selective pressures such as competition and predation (Bretagnolle 1993).

Similarly, the split between *Puffinus/Bulweria* and *Pseudobulweria* around 13 millions years ago correspond to the mid-Miocene climate transition, a period that experienced sharp oceanic temperature drops (Shevenell et al. 2004) and oceanic current changes (Miller and Fairbanks 1983). It was suggested that this period might have offered improved ecological conditions due to increased oceanic productivity resulting from the cooling of ocean surface temperatures thus possibly promoting taxonomic diversification of seabirds (Warheit 1992, 2002). This transition however remains one of the least understood of such events in the last 34 millions years (Lewis et al. 2008) and its importance in promoting seabird lineages diversification remains to be investigated.

Why are these petrels so rare?

Pseudobulweria taxa apparently exhibit poor resilience to human presence and its accompanying invasive predators (e.g. Olson 1975). However, the recent rediscovery of Beck's petrel and the survival of Mascarene and Fiji petrels on islands that suffered important human alteration and where many introduced predators now roam freely, show that these taxa can still survive for a while in adverse conditions. We suggest that surviving taxa have probably been saved by their formerly very large populations rather than the difficult access of their breeding sites. In comparison to *Pterodroma* that often breed in cliffs or top of active volcanoes, *Pseudobulweria* usually breed on more gentle slopes, at medium to low altitudes and even in some cases on the sea-shore. In addition, breeding sites of *P. rostrata* and *P. aterrima* at least are close to inhabited areas (e.g. in Marquesas, or Reunion Island; VB, personal observation). Despite this, and probably because these petrels breed on inhabited islands, their future survival is strongly impeded, and urgent action is required to save at least three taxa from a likely extinction in the near future.

Conservation implications

Given the current biodiversity crisis and financial limitations, investments may be justified in the conservation of distinct taxa, ecosystems or evolutionary units that are likely to produce future biodiversity (Bowen 1999). The conservation of *Pseudobulweria* is unlikely to promote future biodiversity. Despite this, we believe that in addition to being important systematics conservation target (Bowen 1999), these birds also constitute good conservation objectives in an "ecologist perspective" (Bowen 1999). Their conservation, both on land and at sea, would include highly diverse and fragile insular and marine ecosystems. For instance, investing in Beck's petrel conservation would mean investing in the protection of whole forest ecosystems in one of the world's most biodiversity rich region (Papua-New Guinea area), which might be at risk of excessive logging and/or forest clearance for oil-palm plantations, particularly in New Britain (Buchanan et al. 2008; Shirihai 2008). Similarly, the conservation of Fiji petrel on Gau Island, Fiji, by promoting the control of populations of introduced predators such as cats *Felis catus*, rats *Rattus* spp. or pigs *Sus scrofa* (Priddel et al. 2008) will benefit both native plant and animal communities in remnant forest patches. In conclusion, conservation of these taxa is important both for their intrinsic evolutionary and taxonomic values, as well as for the wider ecosystems of which they are a part. In addition to current action in face of light-induced mortality (LeCorre et al. 2002, 2003; Priddel et al. 2008; P. Raust personal communication), conservation of all critically endangered species of the genus, i.e. Mascarene, Beck's and Fiji petrel, urgently requires the discovery of their breeding colonies for predator control, and possibly translocation operations, as it is unlikely that predator removal will be feasible.

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