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The evolution of north-east Atlantic gadfly petrels using statistical phylogeography

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Abstract

Macaronesia (north-east Atlantic archipelagos) has been host to complex patterns of colonization and differentiation in many groups of organisms including seabirds such as gadfly petrels (genus *Pterodroma*). Considering the subspecies of widely distributed soft-plumaged petrel for many years, the taxonomic status of the three gadfly petrel taxa breeding in Macaronesia is not yet settled, some authors advocating the presence of three, two or one species. These birds have already been the subject of genetic studies with only one mtDNA gene and relatively modest sample sizes. In this study, using a total of five genes (two mitochondrial genes and three nuclear introns), we investigated the population and phylogeographical histories of petrel populations breeding on Madeira and Cape Verde archipelagos. Despite confirming complete lineage sorting with mtDNA, analyses with nucDNA failed to reveal any population structuring and Isolation with Migration analysis revealed the absence of gene flow during the differentiation process of these populations. It appears that the three populations diverged in the late Pleistocene in the last 150 000 years, that is 10 times more recently than previous estimates based solely on one mtDNA gene. Finally, our results suggest that the Madeira petrel population is ancestral rather than that from Cape Verde. This study strongly advocates the use of nuclear loci in addition to mtDNA in demographical and phylogeographical history studies.

Keywords: intron, mitochondrial DNA, petrel, procellariiformes, seabird

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Introduction

Processes of population differentiation, potentially leading to speciation, have been particularly well studied in birds in the last 50 years (Price 2008). Some bird species have, however, been less studied than others: oceanic seabirds, for instance, have been poorly studied, and the most recent major review of speciation in birds provides only seven examples from this group, concerning only three genera (but see Friesen 2007; Friesen *et al.* 2007a; Price 2008). Pelagic seabirds that usually breed on oceanic islands are regarded as challenging models when investigating the processes of population differentiation. They are indeed highly mobile organisms, and physical barriers to their dispersal are virtually nonexistent, which is expected to enhance high gene flow between populations. However, although rare, physical barriers to gene flow can affect the population structure and differentiation of pelagic seabirds. The best example is probably the Isthmus of Panama, which has been identified as the main cause of isolation between populations of pantropical seabirds such as boobies (Steeves *et al.* 2003, 2005a; but see Hailer *et al.* 2011). In

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regions where such physical barriers are absent, it has been shown that nonvisible barriers such as oceanic fronts can act as barriers to gene flow (e.g. in *Calonectris* shearwaters, Gomez-Diaz *et al.* 2006). Furthermore, non-physical causes such as the discrete location of their breeding localities associated with their extreme philopatric behaviour can enhance population differentiation in seabirds (e.g. in boobies, Steeves *et al.* 2005b). Processes acting on seabird population genetics are therefore particularly complex. The petrels (order Procellariiformes), containing 113 recognized species (BirdLife International 2012), are probably the most extreme seabirds in this regard. First, they are the most pelagic seabirds, with almost all species breeding on oceanic islands (Brooke 2004). Second, they also show an extreme degree of philopatric behaviour (Austin 1996; Brooke 2004). Third, because many species show nocturnal habits on the breeding grounds, their communication system (and presumably behavioural pre-isolating mechanism: Bretagnolle 1996) almost never rely on visual cues, and many taxa show cryptic and highly convergent plumages. This has resulted in obscure taxonomy, many cases of phylogenetic uncertainties, including cryptic species with unclear phylogeographical histories, which causes frequent revisions of their taxonomy (Brooke 2004). The recent use of phylogenetic information has indeed led to several taxonomic revisions (Abbott & Double 2003; Burg & Croxall 2004; Austin *et al.* 2004; Bolton *et al.* 2008), or the discovery of cryptic species and strong genetic population structuring (Friesen *et al.* 2006, 2007b; Smith & Friesen 2007; Bolton *et al.* 2008).

Perhaps one of the best examples of Procellariiformes phylogenetic complexity exists in the north-east Atlantic islands, where gadfly petrels (genus *Pterodroma*) breeding in Madeira and Cape Verde archipelagos have led to never-ending taxonomic debates (reviews in Zino *et al.* 2008 and Shirihai *et al.* 2010). Three *Pterodroma* taxa breed in these islands: one on Madeira Island itself (Zino's petrel; taxon *madeira*), one on Bugio Island (Deserta petrel; taxon *deserta*) just 40 km south-east of Madeira and one in the Cape Verde archipelago (Cape Verde petrel; taxon *feae*) about 2000 km to the south, on four distinct islands (Fig. 1). The three taxa are of conservation concern (Shirihai *et al.* 2010) and show low population sizes (500–1000 pairs for Cape Verde Islands; 150–180 pairs for Bugio and 65–80 pairs for Madeira Island; BirdLife International 2012). These populations hardly differ morphologically, in colouration and vocalizations (Zino & Zino 1986; Bretagnolle 1995; Zino *et al.* 2008; Jesus *et al.* 2009; Shirihai *et al.* 2010). The populations on Madeira and Bugio Islands have been intensively managed and monitored in the past 30 years with all known birds being ringed, and no

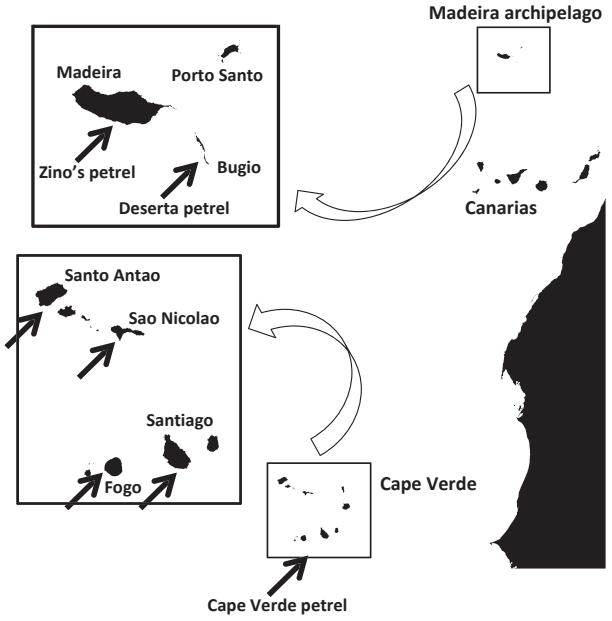

Fig. 1 Macaronesian archipelagos and breeding localities of Macaronesian *Pterodroma* populations: Madeira Island (Zino's petrel), Bugio Island (Deserta petrel), Fogo and other Cape Verde Islands (Cape Verde petrel).

migration and interbreeding have ever been observed despite the geographical proximity of the breeding colonies and the overall similarity in plumage and calls in these populations. The taxonomic treatment of these taxa has been greatly challenging. First considered subspecies of widely distributed soft-plumaged petrel, *P. mollis*, the three forms were subsequently considered as one distinct species with three subspecies (Bretagnolle 1995), two species with one subspecies (Bourne 1983; Zino *et al.* 2008) or three distinct species (Robb & Mullarney 2008; Jesus *et al.* 2009). Currently, Birdlife International recognizes two species, *P. madeira* and *P. feae*, the latter with two subspecies, *P. f. feae* and *P. f. deserta*. Phylogenetic analyses based on mtDNA have represented a major step in these proposed splits. Zino *et al.* (2008) showed that nucleotide sequence of cytochrome b (*cytb*) of 26 birds from Madeira and Bugio differs by 2.2%. They concluded that the two forms separated some 2.5 million years ago (i.e. late Pliocene); though, Sangster *et al.* (2002) suggested a more recent split (840 000 years ago, Early Pleistocene) based on unpublished *cytb* data. Similarly, Jesus *et al.* (2009), also based on *cytb*, compared 35 individuals from Bugio and Cape Verde and found that the average K2P sequence divergence between populations was 1.58% (2.4% and 2.3%, respectively, for Madeira and Bugio, and Madeira and Cape Verde divergences). The split between Bugio and Cape Verde was suggested at 1.75 million years ago by these last authors. In both studies (Zino *et al.*

2008; Jesus *et al.* 2009), the three populations were reciprocally monophyletic, and no *cytb* haplotypes were shared. Although this is not what Jesus *et al.* (2009) conclude, trees recovered in their study indicate that Zino's petrel is basal to the sister taxa Cape Verde and Deserta petrels. Although never tested accurately, the acknowledged historical scenario suggests that the Madeiran archipelago might have been colonized twice – Zino's petrel evolving when the climate was cooler and wetter in the Early Pleistocene, and petrels from Cape Verde colonizing Bugio and potentially other islands more recently when the climate became warmer and dryer (Bourne 1983). This could explain the differences between Madeira and Bugio birds despite the close proximity of these islands; though in his scenario, Bourne (1983) did not explicitly suggest that birds from Cape Verde colonized both Madeira and Bugio.

The separation of the three Macaronesian gadfly petrel taxa into three species, supported by the *cytb* data of Zino *et al.* (2008) and Jesus *et al.* (2009), is currently uncorroborated by other criteria such as morphology (Shirihai *et al.* 2010) or vocalizations (Bretagnolle 1995; but see Robb & Mullarney 2008). It is known that few migrants per generation can prevent population differentiation (Wright 1931) and even limited male-mediated gene flow might explain this lack of morphological and behavioural differentiation between the three taxa in spite of apparent complete lineage sorting in mtDNA. Disentangling patterns of divergence between populations can have broad implications both for our understanding of divergence processes and for defining species boundaries in threatened taxa. It is therefore necessary to further investigate these three petrel populations of the north-east Atlantic by other means than mitochondrial DNA.

Indeed, the supremacy of mitochondrial DNA (mtDNA) in vertebrate phylogenetic and phylogeographical studies has recently been challenged by the increasing use of, and the sometimes conflicting results that emerged from, nuclear genes (Edwards *et al.* 2005; Zink & Barrowclough 2008; Edwards & Bensch 2009). It thus appears that complex phylogeographical patterns lying between intraspecific population genetics and interspecific phylogenetics cannot be described with the use of a single mitochondrial gene. Nuclear genes were not available until fairly recently (Lee & Edwards 2008), and mitochondrial DNA was mostly used, given its nonrecombining properties and its high mutation rate (Avise *et al.* 1987; Zink & Barrowclough 2008). In addition, because of its lower effective population size (Ne) compared to nuclear DNA (approximately 4 Ne), lineage sorting will occur faster for mtDNA than for nucDNA, thus allowing the detection of more recent split events (Zink & Barrowclough 2008). However, with the increasing use of nuclear markers along with mtDNA, a growing body of evidence has indicated that inferences based on mtDNA should be regarded with caution (Funk & Omland 2003; Chan & Levin 2005). Many studies revealed discrepant results between mtDNA and nuclear genes and even sometimes incongruences between the two types of markers (e.g. Shaw 2002; Spinks & Shaffer 2009; see also Zink & Barrowclough 2008 and Lee & Edwards 2008 for a review and a recent example in birds). Such discrepancies or incongruences have been explained by lower mutation rate of the nuclear genes, incomplete lineage sorting and possibly more complicated evolutionary scenarios including hybridization or natural selection that may actually act on mtDNA (Avise *et al.* 1987; Edwards *et al.* 2005; Bazin *et al.* 2006; Spinks & Shaffer 2009). Although the respective merits of nuclear vs. mtDNA markers are disputed in regard to disentangling the pattern and process in evolution and speciation (see Zink & Barrowclough 2008; Edwards & Bensch 2009), consensus has eventually emerged that their combination provides powerful tools for phylogeographical investigations. In addition, the recent advances in methodology have provided analytical frameworks to investigate the origin and evolution of taxa by integrating simultaneously several genes histories, for example multilocus phylogenetic inference (Heled & Drummond 2010) and statistical phylogeography (Hey 2010).

In this study, we used a statistical phylogeography method with two mitochondrial markers and three nuclear introns to investigate (i) whether nuclear DNA confirms the reciprocal monophyly of the three taxa observed with mtDNA (Zino *et al.* 2008; Jesus *et al.* 2009) and the evolutionary relationships among them, (ii) the timing of divergence in these threatened seabirds and whether divergence occurred with or without gene flow and (iii) whether further genetic data can resolve current uncertainties around the species status of these three petrel populations.

Materials and methods

Sample collection and laboratory methods

Because of the taxonomic uncertainties surrounding the three taxa under study, we refer to them hereafter by their taxon or vernacular names as opposed to their potential species names. Blood samples were obtained from the island of Bugio (Desertas islands, off Madeira), Madeira and two islands of the Cape Verde archipelago, Fogo and Santo Antão Islands (Fig. 1). Blood was collected from the veins on the leg or wing using insulin syringes and microcapillaries and stored

in 70% ethanol and then frozen at −20 °C until processing.

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. Cytochrome oxydase 1 gene (CO1) was amplified using primers F1B 5′-AACCGATGACTATTYTCAACC-3′ and R1B 5′-TACTACRTGYGARATGATTCC-3′ (Gangloff *et al.* 2012). The PCR consisted of 37 cycles at 94 °C for 30 s, 51 °C for 40 s and 72 °C for 50 s. Cytochrome b gene was amplified using primers L14987 5′-TATTTCTGCTTGATGAAACT-3′ and H16025 5′-CTAGGGCTCCAATGATGGGGA-3′ (Jesus *et al.* 2009) and 40 PCR cycles consisting of 30 s at 94 °C, 50 s at 58 °C, 50 s at 72 °C. Primers FIB-BI7U 5′-GGAGAAAACAGGACAATGACAATTCAC-3′ and FIB-BI7L 5′-TCCCCAGTAGTATCTGC-CATTAGGGTT-3′ (Prychitko & Moore 1997) were used to amplify the beta-fibrinogen intron 7 (βFibint7). We ran thirty-nine PCR cycles consisting of 1 min at 94 °C, 40 s at 58 °C and 50 s at 72 °C. For these three markers, PCR cycles were preceded by an initial denaturation step of 4 min at 94 °C and were followed by a 5 min final extension step at 72 °C.

PCR amplification of the nuclear cold shock domain containing E1 (CSDE1) and PAX interacting protein 1 (PAXIP1) introns followed protocols and used primers described by Kimball *et al.* (2009).

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.

Before conducting analyses, mitochondrial origin of mitochondrial gene sequences was confirmed by translating these to check for the presence of stop codons and other indices of their potential nuclear origin.

Preliminary analyses indicated no differentiation between the two colonies within Cape Verde, and therefore, we pooled the data from Fogo and Santo Antão Islands. Sequencing of PAXIP1 and CSDE1, initially not planned, was carried out opportunistically after the analyses had started, by randomly selecting a small number of individuals of the three populations, explaining the unbalanced sample size between these two markers and the three others (Table 1). To estimate genetic diversity and genetic structure, we used all available sequences for each gene. For the analysis of phylogenetic relationships and estimates of gene flow and divergence times, to reduce computational time, we used all available sequences for PAXIP1 and CSDE1 and subsampled randomly eight individuals (Felsenstein 2006; Kuhner 2009) from each taxon for the three other genes (CO1, *cytb* and βFibint7). We repeated this subsampling and analyses three times; results were similar among replicates for all analyses, so results of one replicate are presented here. Prior to species tree and Isolation with Migration analyses, recombination in nuclear loci was tested with the four-gamete test (Hudson & Kaplan 1985) implemented in DnaSp v5 (Librado & Rozas 2009). In addition, because the Isolation with Migration analysis required having known phase for nuclear sequences, we determined the gametic phase using the program PHASE (Stephens *et al.* 2001) implemented in DnaSp v5. Phased haplotypic data of nuclear introns were then used in analyses, individuals thus being represented by two sequences.

Table 1 Diversity estimates for three Macaronesian *Pterodroma* populations. Number of individuals sequenced (N), number of polymorphic sites (Np), number of haplotypes (Nh), haplotype diversity (h), nucleotide diversity (π, in%, i.e. 0.001 = 0.1%), average number of nucleotide differences (k)

	Population	N	Np	Nh	h	π%	k
CO1	Deserta petrel	89	5	4	0.474	0.07	0.534
	Cape Verde petrel	58	10	9	0.687	0.15	1.095
	Zino's petrel	57	11	9	0.778	0.23	1.683
cytb	Deserta petrel	94	5	6	0.219	0.03	0.229
	Cape Verde petrel	59	11	11	0.556	0.11	0.963
	Zino's petrel	59	12	11	0.535	0.09	0.788
CSDE1	Deserta petrel	6	0	1	0	0	0
	Cape Verde petrel	5	0	1	0	0	0
	Zino's petrel	6	3	4	0.45	0.1	0.5
PAXIP1	Deserta petrel	5	4	4	0.53	0.3	1.4
	Cape Verde petrel	7	15	10	0.95	0.7	3.56
	Zino's petrel	7	12	8	0.87	0.5	2.58
βFibint7	Deserta petrel	76	2	3	0.039	0.005	0.039
	Cape Verde petrel	28	6	5	0.507	0.083	0.662
	Zino's petrel	29	2	3	0.068	0.009	0.069

Genetic diversity and structure

Nucleotide diversity (π) and haplotype diversity (h) were calculated with Arlequin v3.5 (Excoffier & Lischer 2010).

To investigate whether populations at Cape Verde, Madeira and Bugio are differentiated, we conducted an exact test of population differentiation (Raymond & Rousset 1995) for each genetic marker. Proportion of genetic variation explained by interpopulation

differences was investigated for each marker with an analysis of molecular variance (AMOVA). AMOVA allows estimating the proportion of total variance accounted for within and among populations. Statistical significance of the variance components was determined by 1000 permutations of genotypes. All these analyses were performed in Arlequin v3.5

Evolutionary relationships estimation

For all five markers, phylogenetic relationships between haplotypes were inferred using haplotype network reconstruction, with the median-joining networks method implemented in NETWORK v4.5.1 (Bandlet *et al.* 1999). Haplotype networks, contrary to phylogenetic tree reconstruction, do not impose bifurcating relationships between sequences and are well suited for intraspecific investigations (Posada & Crandall 2001; Forister *et al.* 2008).

To estimate the phylogenetic relationships among the three populations, we used the *BEAST method (Heled & Drummond 2010) implemented in the software BEAST v. 1.6.1 (Drummond & Rambaut 2007). This method, using the multispecies coalescent (Degnan & Rosenberg 2009), models the lineage sorting process between taxonomic units for groups of individuals not connected by gene flow above, at or below the species level and does not require inclusion of an outgroup (Heled & Drummond 2010). Four runs of 5×10^7 generations, sampling every 1000 generations and with a burnin of 1.25×10^7, were performed and then combined in LogCombiner v.1.6.1 (Heled & Drummond 2010). Nucleotide substitution models were determined with jModelTest v0.1.1 (Guindon & Gascuel 2003; Posada 2008), and we used a relaxed clock model with an uncorrelated lognormal distribution. We used a mutation rate fix mean value of 0.794 ± 0.115% per million year for CO1 (Pereira & Baker 2006), 1.89 ± 0.35% per million year obtained for Procellariiformes *cytb* (Weir & Schluter 2008) and 0.36% per million year for nuclear introns (Axelsson *et al.* 2004). We specified a Yule process species tree prior under a continuous population size model. We used the software TRACER v1.5 (Drummond & Rambaut 2007) to visualize the results of the runs as well as for checking effective sample size of each parameter.

Estimation of gene flow and population divergence

We estimated gene flow and divergence time among Macaronesian petrel populations using IMa2 (Hey 2010). This software, using the Isolation with Migration model (Hey & Nielsen 2007), tests whether or not populations diverged in the presence of gene flow, but requires an *a priori* tree topology of the populations under study. We thus used the species tree obtained with *BEAST to provide this required topology. We first ran an analysis with the 'M mode' applying the infinite sites mutation model for the nuclear introns and HKY model for the mitochondrial data. We used 40 independent Markov chain with a geometric heating scheme (ha = 0.975, hb = 0.75). Runs consisted of 1.10^6 sampled steps following a discarded burnin of 1.10^6 steps. Because posterior probabilities of gene flow between populations all peaked at zero, we then ran an M mode analysis adding a 'no gene flow' prior. Procedures for this analysis were similar to the previous M mode analysis.

To scale parameters in demographical units, a range of mutation rates can be given as prior to the analysis. For mitochondrial data, we used the estimates of 0.794×10^{-8} and 1.89×10^{-8} substitutions/site/year estimated for Procellariiformes for CO1 (Pereira & Baker 2006) and *cytb* (Weir & Schluter 2008), respectively. For introns, we used a mutation rate of 3.6×10^{-9} substitutions/site/year found by Axelsson *et al.* (2004) for birds from 33 autosomal loci. In addition, evaluating the degree of gene flow, divergence time and population sizes in a demographical unit is possible provided that a generation time is available for the studied taxa. *Pterodroma* petrels are long-lived seabirds, but no accurate estimation of generation time currently exists for these species. However, assuming a stable population, it is possible to estimate this parameter based on age at maturity and adult survival as $T = A + p/(1\text{-}p)$ (1) with T the generation time, A the age at maturity and p the adult survival rate (Lande *et al.* 2003; Saether *et al.* 2004). Available estimated adult survival rate in other *Pterodroma* is 0.93 (Simons 1984; Brooke *et al.* 2010), and age of sexual maturity in other *Pterodroma* is approximately 6 years of age (Simons 1984; Warham 1990). Thus, using values of 0.93 and 6 for p and A, respectively, equation (1) gives a generation time T of approximately 20 years. This value was also found by Cuthbert (2004) in Atlantic petrel (*Pterodroma incerta*), and here, we used an approximate generation time of 20 years. Runs were monitored by observing effective sample size (ESS) values and inspecting parameter plots for trends (see manual recommendations). Analyses were run three times to ensure convergence, and because all results were similar, only one is presented here.

Results

We obtained samples from 98 petrels from Bugio, 84 petrels from Madeira and 59 from Cape Verde. We sequenced 794, 481 and 478 bp for βFibint7, CSDE1 and

PAXIP1, respectively, and 732 and 872bp for CO1 and *cytb*, respectively. Numbers of individuals sequenced per population for each gene are shown in Table 1. No recombination was detected in the nuclear intron data. After translation of mitochondrial sequences, no nonsense or stop codons were found and no insertion or deletions were observed in these sequences. Furthermore, DNA was amplified with specific primers designed for Procellariiformes (Jesus *et al.* 2009; Gangloff *et al.* 2012) rather than universal primers, which is supposed to reduce the risk of Numts amplification (Sorenson & Quinn 1998). In addition, *cytb* sequences obtained were similar to those obtained with the same taxon previously (Zino *et al.* 2008; Jesus *et al.* 2009). Finally, we also checked forward and reverse sequences and checked for the presence of double peaks that are characteristics of Numts. No duplications were detected, and true mitochondrial origin of obtained sequences is very likely. Mitochondrial data contained 54 polymorphic sites (26 and 28 for CO1 and *cytb*, respectively; Table 1), while combined nuclear data exhibited 44 variable sites (10 in βFibint7, 3 in CSDE1, 31 in PAXIP1; Table 1).

These variable sites defined a total of 22 and 28 haplotypes in CO1 and *cytb*, respectively, while after phasing the nuclear intron data, 6, 22 and 11 haplotypes were found in CSDE1, PAXIP1 and βFibint7, respectively (Table 1).

With all five markers, petrels from Bugio showed lower haplotypic diversity (range 0 in CSDE1 to 0.53 in PAXIP1) than Cape Verde and Madeira (range 0 in CSDE1 for Cape Verde petrel to 0.95 in PAXIP1 again for Cape Verde petrel) despite having a sample size 1.5-2 times greater in CO1, *cytb* and βFibint7. The trend was similar with nucleotide diversity (Table 1).

Population structure

Mitochondrial and nuclear markers exhibited discrepant pattern in terms of population structure. Exact test of population differentiation indicated a significant differentiation among the three populations taken together ($P < 0.001$) in mitochondrial markers. With pairwise comparisons, the pattern was similar, all pairwise tests indicating statistically significant differentiation (all $P < 0.001$) between pairs of populations in mitochondrial data. Besides, with these markers, most of the genetic variation was observed among the three populations rather than within population (see AMOVA tests), the former accounting for 91.3% and 93.4% of the total variation in CO1 and *cytb*, respectively (Table 2). These results are fully consistent with previous analyses carried out on *cytb* only (Zino *et al.* 2008; Jesus *et al.* 2009). The pattern was quite different, however, with nuclear markers. With these introns, Raymond and Rousset test failed to detect any differentiation when looking at the three populations together (all three $P > 0.2$) and no differentiation between any pair of population was detected with this test (all $P > 0.08$). Furthermore, in all three nuclear introns, in contradiction with mitochondrial markers, more than 80% of the genetic variation was found within rather than among populations (83%, 100% and 93% in βFibint7, CSDE1 and PAXIP1 respectively; Table 2).

Phylogenetic relationships and population divergence

With both mitochondrial genes, the networks indicate complete lineage sorting among the three taxa. In sharp contrast, the three nuclear introns' lineages are not sorted, as indicated by the haplotype networks that confirmed the lack of population structure in Macaronesian petrels with nuclear lineages (Fig. 2). In these introns, the three taxa shared a common dominant haplotype and showed few private haplotypes. In PAXIP1, birds from Madeira and Bugio did not share any haplotype other than the dominant one found in all three taxa, while on the other hand, Cape Verde petrels shared haplotypes with the two other taxa. In CSDE1, only Madeira birds presented private haplotypes. With

Table 2 Genetic variation within and among the three *Pterodroma* petrel populations breeding in Macaronesia (AMOVA)

		d.f.	Sum of squares	Variance components	Percentage of variation	*P* values
CO1	Among populations	2	704.787	5.3	91.28	< 0.001
	Within populations	201	101.835	0.51	8.72	
cytb	Among populations	2	1079.63	7.85	96.39	< 0.001
	Within populations	209	61.407	0.29	3.61	
βFibint7	Among populations	2	3.62	0.022	17.3	< 0.001
	Within populations	263	28.446	0.108	82.7	
CSDE1	Among populations	2	0.162	0.00	0	> 0.5
	Within populations	31	2.75	0.09	100	
PAXIP1	Among populations	2	5.324	0.11	7.5	< 0.05
	Within populations	35	46.229	1.32	92.5	

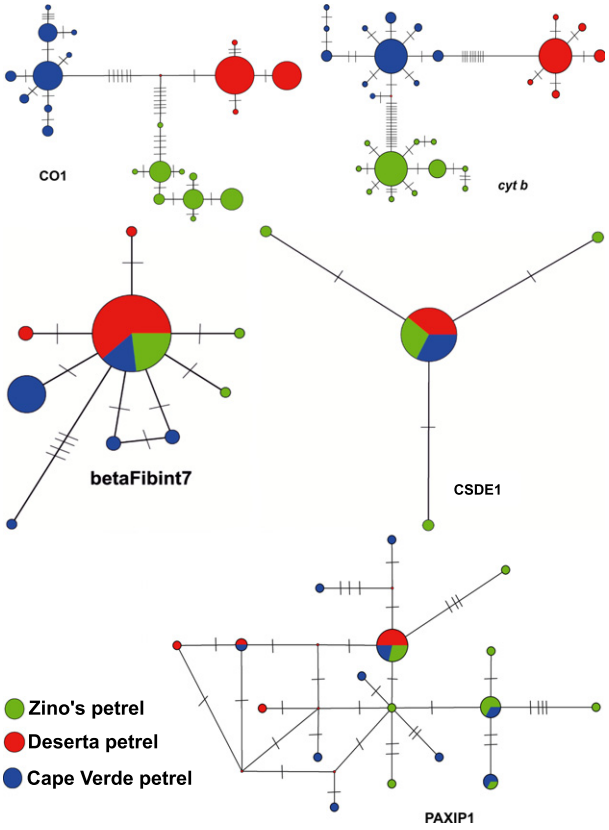

Fig. 2 Haplotype networks obtained with CO1, cytb, PAXIP1, βFibint7 and CSDE1 loci with Macaronesian *Pterodroma* petrels. Size of circles is proportional to the number of individuals possessing this haplotype.

βFibint7, apart from the dominant haplotype, taxa did not share any haplotype and Cape Verde petrels presented a greater diversity of haplotypes than the two other taxa, reflecting the differentiation detected in pairwise exact tests of population differentiation.

The *BEAST analysis provided a credible set of trees that included three rooted topologies. Two of them represented <3% of the total trees. The third topology shows strongly supported nodes (Fig. 3). According to this latter tree, petrels from Madeira are ancestral and Cape Verde and Bugio birds are sister taxa that diverged later.

In the analysis of Isolation with Migration, posterior distributions of migration parameters all peaked at zero, indicating that the three petrel populations differentiated without gene flow. We therefore re-ran an analysis with all five genes with a prior specifying no gene flow. With this analysis, the split Cape Verde/Bugio is estimated around 32 000 years ago (95% HPD 9000–153 000), and the differentiation between the ancestral Cape Verde/Bugio on one hand and Madeira on the other hand occurred about 153 000 years ago (95% HPD 47 000–359 000; Fig. 4). Effective population sizes of the three extant Macaronesian petrel taxa were estimated by the IM analysis to be comprised between 1887 and 12 867 individuals (Table 3).

Discussion

This is the first study on the phylogeography of north-eastern Atlantic gadfly petrels that uses nuclear genes in addition to mitochondrial ones. Results from our two mitochondrial genes basically agree with previous studies exclusively based only on one mtDNA marker and reduced sample sizes (Zino *et al.* 2008; Jesus *et al.* 2009). However, we found that nuclear genes provided a strikingly different picture compared with mtDNA with no clear population structure and no lineage sorting. Our results suggest that petrels from Madeira are the ancestral taxon, not Cape Verde petrels, in line with phylogenetic trees obtained by previous authors with *cytb* gene only (Jesus *et al.* 2009). Our study shows that divergence of the three populations occurred without gene flow and times of divergence between any pair of taxa are about 10 times more

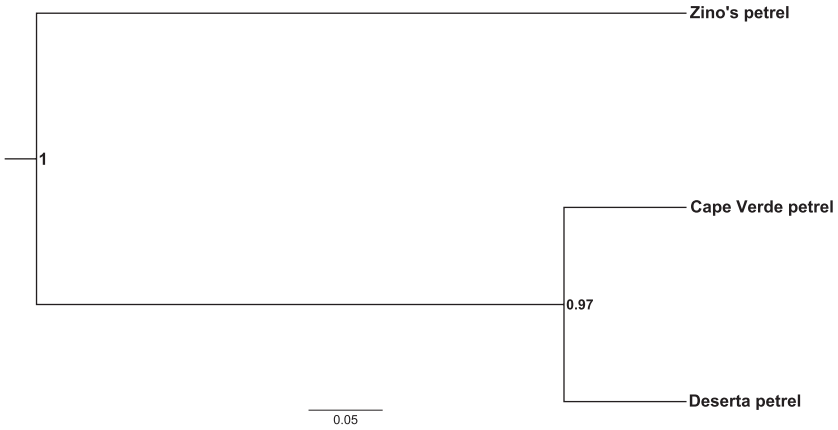

Fig. 3 Species tree reconstructed for Macaronesian *Pterodromas* with *BEAST, using two mitochondrial genes (CO1 and cytb) and three nuclear intron (βFibint7, CSDE1 and PAXIP1). Node labels indicate posterior probabilities.

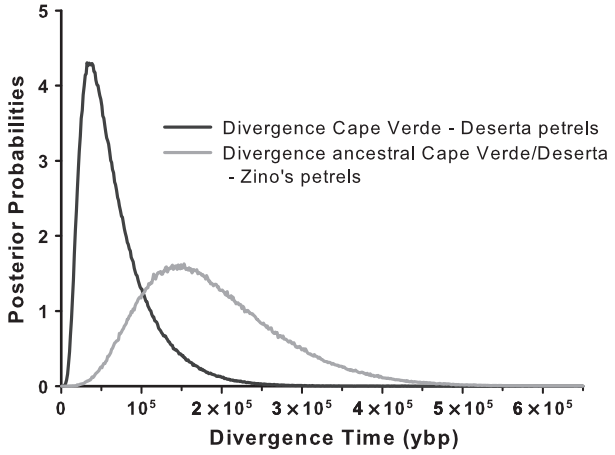

Fig. 4 Population divergence time estimation under IM model with IMa2 for the three populations of Macaronesian petrels.

Table 3 Effective population size estimates at time of divergence obtained with IMa2 for the three populations of Macaronesian gadfly petrels

	Population size estimation (95% highest posterior density interval)
Deserta petrel	1887 (515–6005)
Cape Verde petrel	3260 (1201–9093)
Zino's petrel	1887 (858–6348)
Ancestral Cape Verde/Deserta	12 867 (2917–320 655)
Ancestral population	4289 (0–14 926)

recent than previous estimates, one being estimated <40 000 years ago.

Lack of population structure and reciprocal monophyly in nuclear intron, as opposed to strong structure, lineage sorting and monophyly in mitochondrial markers, as found in this study is not a unique case in birds (Zink & Barrowclough 2008). For instance, in Procellariiformes, Welch *et al.* (2011) identified the same pattern in *Pterodroma* petrels breeding in Hawaiian and Galapagos archipelagos. Lee & Edwards (2008), finding similar results in their study of red-backed (*Malurus melanocephalus*) and white-winged (*Malurus leucopterus*) fairy wrens in Australia, argued that such pattern can be expected to be common for bird species that recently diverged or had large ancestral populations. This can be due to the differences in coalescence time for nuclear and mitochondrial loci (Moore 1995; Palumbi *et al.* 2001) and their different effective population sizes (Zink & Barrowclough 2008) leading to incomplete lineage sorting. Our data suggest that ancestral effective population sizes were not especially high (Table 3), but divergence times in NE Atlantic gadfly petrels are indeed extremely recent. Alternatively, the discrepancy between mitochondrial and nuclear markers could also be explained by extreme female philopatry associated with male-mediated nuclear gene flow (Zink & Barrowclough 2008). However, male-biased dispersal has never been documented in petrels and, in birds in general, female dispersal is dominant (Newton 2003). In addition, populations of *Pterodroma* on Madeira and Bugio Islands have been intensively monitored in the last three decades and no interbreeding between Zino's petrel and Deserta petrel has ever been observed. Furthermore, the Isolation with Migration analysis indicated an absence of gene flow and migration between diverging populations, thus allowing rejection of a model of differentiation with migration and supporting an allopatric pattern of differentiation (Hey 2006; Gaggioti 2011). This leads to the conclusion that the lack of reciprocal monophyly and population structure in nuclear introns is probably due to an incomplete sorting of nuclear lineages because of very recent divergence of Macaronesian petrel populations, a pattern similar to that of Hawaiian and Galapagos petrels (*P. sandwichensis* and *P. phaeopygia*; Welch *et al.*, 2011).

Using three nuclear introns in combination with two mitochondrial genes with *BEAST provided a well-supported species tree, and IMa2 analysis provided divergence time estimates. It should, however, be kept in mind that given the low phylogenetic signal observed in the nuclear introns (Fig. 2), the topology of the species tree is probably driven mostly by the mitochondrial data. Taxon *madeira* diverged from the ancestral population about 153 000 years ago (95% HPD 47 000–359 000). This divergence was then followed later on by the divergence of taxa *feae* (Cape Verde) and *deserta* (Bugio), about 32 000 years ago (95% HPD 9000–153 000). These divergence estimates are far more recent than previous estimates based solely on *cytb* and different methodologies (Sangster *et al.* 2002; Zino *et al.* 2008; Jesus *et al.* 2009). They are also much more recent than the 550 000 years before present divergence estimates of morphologically similar Hawaiian and Galapagos petrels (Welch *et al.*, 2011). It could be questioned how mtDNA lineages can show reciprocal monophyly with such recent divergence, in particular between Madeira and Bugio. We suggest that the reason lies in effective population size. Indeed, for mitochondrial DNA, the time (in generations) required for a gene tree to show reciprocal monophyly is about twice the female effective population size (2 Nef), and in species with equal sex ratio and equal reproductive success for males and females (which is the case in petrels in general), 2 Nef ~ Ne (McKay & Zink 2010). In the case of Bugio and Madeira populations, mitochondrial reciprocal monophyly could hence be observed after

approximately 1887 generations (range 515–6005 and 858–6348 for Bugio and Madeira birds, respectively) based on IMa2 Ne estimates (Table 3). It appears that divergence time for these two populations was estimated at 32 000 years ago (95% HPD 9000–153 000), that is approximately 1600 [450–7650] generations. There is therefore an agreement between the estimated time since divergence for Bugio and Madeira birds and the time necessary for their mitochondrial lineages to reach reciprocal monophyly. It is therefore probably that we are looking at populations that have been isolated for approximately just over 2 Nef generations, giving enough time to mtDNA lineages to sort completely.

Philopatry, breeding allochrony and distribution during the nonbreeding season are factors promoting seabird population differentiation (Steeves *et al.* 2005b; Friesen *et al.* 2007a,b; Rayner *et al.* 2011). In Cook's petrel (*Pterodroma cooki*), Rayner *et al.* (2011) identified that interaction between different nonbreeding distributions, allochronic breeding and natal philopatry was restricting gene flow between populations and that adaptation to different habitats, both in the breeding and nonbreeding periods, could lead to ecologically driven population divergence. The three taxa studied here are allochronic breeders: Cape Verde birds lay in December–February (Bannerman & Bannerman 1968; Cramp & Simmons 1977), Bugio birds lay in July–August (Zino & Zino 1986; Zino *et al.* 2001, 2008), and Madeira birds lay in May–June (Zino & Zino 1986; Zino *et al.* 2001, 2008). In addition, oceanic conditions in the Atlantic Ocean have been greatly affected by Pleistocene climatic variations (Lambeck *et al.* 2002; Bintanja *et al.* 2005) that have been repeatedly outlined in population structuring and differentiation in Procellariiformes (e.g. Austin *et al.* 2004; Cagnon *et al.* 2004; Gomez-Diaz *et al.* 2006). Moreover, the biogeographical cohesion of Macaronesian archipelagos, including Madeira and Cape Verde, is based on the similarity of flora and fauna across them, but this similarity does not match the marine biomes and provinces defined from an oceanographic perspective. Nowadays, NE Atlantic oceanography remains highly complex (e.g. Johnson & Stevens 2000; Sangra *et al.* 2009), but there is a clear oceanographic divide between Cape Verde and the rest of Macaronesian archipelagos (Longhurst 1998), which is consistent with a divergence between Cape Verde and Deserta petrels through local adaptation to different marine habitats. Although nonbreeding distribution at sea of our study birds is currently unknown, it is possible that past and present local adaptations to habitat conditions in this period may have played a role in the differentiation of the populations.

Because seabirds, contrary to terrestrial taxa, can be extirpated and recolonize an island many times, establishing a scenario of colonization of the north-east Atlantic archipelagos is difficult. In this respect, it is notable that bones of *Pterodroma* have been found on several islands of northern Europe. In the absence of genetic investigation, these bones have been attributed to Cape Verde petrel based on size (Serjeantson 2005; see Robb & Mullarney 2008 for a review). Another population was apparently breeding in the Canaries at least until 2500 BP; though, its taxonomic identity is uncertain (Rando 2002). Palaeontological surveys have also found *Pterodroma* bones in Selvagens, Madeira, Porto Santo and Açores (J. C. Rando, personal communication). The presence of these bones, thus, indicates a former breeding range much larger than the current one and supports a former higher diversification for *Pterodroma* populations in Macaronesia. Given these data, in the absence of ancient DNA investigation on bones available, it remains extremely difficult to propose a scenario explaining the presence and distribution of Macaronesian petrel taxa.

Regardless of the underlying causes and scenario, lack of male-mediated gene flow and reciprocal monophyly in mtDNA indicate that the three current petrel populations have significantly diverged recently and seem to evolve independently from each other. Whether this degree of divergence is enough to consider these population different species can, however, be debated. Considering that (i) no interbreeding has ever been observed between populations breeding on Madeira, Bugio and Cape Verde Islands so far, (ii) Isolation with Migration indicates that no gene flow occurred during their differentiation, and (iii) the three populations are allochronic breeders, a character that can act as a prezygotic barrier, we may argue that the three taxa warrant species status under a classical Biological Species Concept (Mayr 1942). However, testing for effective reproductive isolation among these three populations is hardly possible due to the breeding localities and life history of these birds. Furthermore, the lack of current identification cues (Shirihai *et al.* 2010) especially between taxa *feae* and *deserta* makes the specific distinction between the three taxa rather useless in practice. Alternatively, using species concepts based on monophyly (e.g. the Phylogenetic Species Concept, Cracraft 1983) provide opposite results when applied to mitochondrial and nuclear genes, while concept based on the presence of independently evolving lineages (e.g. the Evolutionary Species Concept, Wiley 1978; deQueiroz 2007) may provide support for the separation of the three populations. These three populations, thus, represent a case in point where the process of speciation is currently in progress, and for which unambiguous taxonomic decision is impossible to reach

and will depend on species concept (i.e. which character is prioritized). We can, however, state that (i) evolutionary lineages are reciprocally monophyletic for one class of genes but not for another (nuclear genes), because time of divergence is too short; (ii) there is no gene flow since divergence started, this being probably reinforced by geographical (distribution of breeding colonies and, to some extent, distribution at sea) as well as temporal isolation (in breeding phenology); (iii) divergence did not lead (yet) to morphological or behavioural divergence and (iv) taxa *madeira* and *deserta* are more divergent than taxa *deserta* and *feae*.

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Data accessibility

DNA sequences are available on GenBank under accession numbers: JX 674073 to JX 674826. GenBank accession numbers for all sequenced individuals uploaded as online Table S1 (Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 GenBank accession numbers for all the sequenced individuals.