

Cryptic differentiation in the Manx Shearwater hinders the identification of a new endemic subspecies

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Decision date: 06-Sep-2020

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/jav.02633].

Abstract

The taxonomy of Procellariiformes, particularly petrels and shearwaters, is still unresolved. The Manx shearwater *Puffinus puffinus* is one of the best studied seabirds worldwide. Most of the information known on this seabird is focused on the northern core populations where the species is abundant. However, the species shows a high number of peripheral populations, which are extremely small and difficult to study in comparison to central populations. Using an integrative approach, we provided significant evidence of phenological, morphological, acoustic, plumage colour, and genetic differentiation of the Canarian Manx shearwaters (the most southern population) from the northern breeding colonies, which is compatible with a long period of isolation. Birds from the Canary Islands breed around 2-3 months earlier, are smaller and lighter, and show darker underwing plumage than those from northern populations. In addition, Canarian call features are different from the northern populations. Finally, genetic analyses of the mitochondrial control region indicate an incipient genetic differentiation of Canarian Manx shearwaters from the other breeding populations. The Canarian population holds a small number of breeding colonies and it is declining, so accurate taxonomic recognition critically affects conservation efforts. For all the aforementioned reasons, we propose to rank the Canarian breeding population as a new taxon by presenting the formal description of a new subspecies *Puffinus puffinus canariensis* ssp. nova.

Keywords conservation, extinct, genetic structure, island biogeography, Macaronesia, speciation

Introduction

As a consequence of the high number of endemic insular species, islands hold a considerable proportion of the world's terrestrial biodiversity despite representing just the 5.3% of Earth's land area (Tershy et al. 2015). Unfortunately, recent human activities, driving loss of species, population declines and reductions in genetic variation, are more concentrated on islands (Leigh et al. 2019, Howard et al. 2020). In fact, around 90% of the bird extinctions in historical times have occurred on islands, mainly related to the arrival and settlement of humans (Wood et al. 2017b). Conservation actions focused on protecting insular biota demand an accurate taxonomic recognition of all taxa, particularly those threatened. This is challenging when speciation processes are not conspicuous, especially in rare and cryptic taxa (Srinivasulu et al. 2019).

Seabirds, and particularly Procellariiformes, breed mainly on islands and are one of the most threatened groups of birds (Rodríguez et al. 2019, Dias et al. 2019). Seabirds are vagile species foraging over thousands of square kilometres although their breeding colonies are located on small, isolated, and distant islands. Peripheral populations are usually smaller and receive few immigrants from core populations (Channell 2004, Friesen 2015). Under such conditions founder events, selection, inbreeding, and genetic drift processes promote phenotypic and genetic differentiation of peripheral populations (Illera et al. 2016), but also make them more prone to extinction than larger, and genetically connected core populations. Mechanisms of speciation in seabirds are poorly understood (Friesen 2015). Recently, long-term barriers and population persistence have been proposed as mechanisms for genetic structuring and speciation (Lombal et al. 2020), although the high philopatry of seabirds alone could lead to reproductive isolation (Friesen 2015). In addition, allochrony, i.e. sympatric populations that breed in distinct seasons, could also be an important mechanism explaining differentiation and speciation in petrels (Taylor et al. 2019 and references therein).

The taxonomy of many seabird groups, particularly petrels and shearwaters, is still unresolved (e.g. Martínez-Gómez et al. 2015, Wood et al. 2017a, Rodríguez et al. 2019). Cryptic differentiation, that is, unnoticed differentiation of isolated and genetically distinct populations due to similar external appearance, has contributed to the unresolved taxonomy of petrels and shearwaters (Taylor et al. 2019). The lack of clear physical barriers to gene flow, coupled with limited morphological divergence, has hindered recognition of many cryptic petrel taxa (Welch et al. 2011). However, the identification of new cryptic lineages has recently flourished (e.g. Bolton et al. 2008, Pyle et al. 2011, Kawakami et al. 2018, Fischer et al. 2018, Taylor et al. 2019). Recognizing new taxa and evolutionary significant units has implications for biodiversity conservation and management (Moritz 1994, Thomson et al. 2018).

The genus *Puffinus* (Family Procellariidae) includes five extinct and around 19 extant species of small and medium sized black-and-white shearwaters but, for some species, taxonomy is still unresolved (Ramírez et al. 2010, BirdLife International 2020). With a monograph and more than 200 research papers focused on the species, the monotypic Manx shearwater *Puffinus puffinus* is one of the most studied seabirds (Brooke 1990, Lee et al. 2020). Most studies are based on core breeding populations located on United Kingdom and Ireland where more than 90% of the breeding population is concentrated (> 310,000 breeding pairs; BirdLife International 2015). However, peripheral colonies are distributed on other North Atlantic islands, such as Iceland, Faroes, islets off Brittany (France), Newfoundland (Canada), Massachusetts and Maine (USA), Azores, Madeira, and the Canary Islands (Lee et al. 2020). At the southern limit of its breeding distribution, i.e. Azores, Madeira, and the Canaries, we know very little of its ecology and biology because of small population sizes and inaccessibility of nest-burrows, which are located on cliff edges of remote areas (Monteiro et al. 1999, Trujillo 2008, Nunes et al. 2010, Meirinho et al. 2014). No active nest-burrows are currently known in these archipelagos, and our best evidence of successful reproduction comes from fledglings grounded by artificial lights (Rodríguez et al. 2008, Rodríguez and Rodríguez 2009, Nunes et al. 2010). Our knowledge of at-sea distribution is even more limited. While it is well known that European population winter in the Southern hemisphere (Brooke 1990, Guilford et al. 2009), nothing is known about the migration or the foraging areas of these peripheral populations.

Despite being an iconic species in ornithology, neither comparative nor phylogeographic studies of the Manx shearwater have been conducted across its large latitudinal breeding range (Lee et al. 2020). Here, we assess population differentiation across six locations distributed in a latitudinal gradient of 3,800 kilometres, from 28 to 62°N (Figure 1). Specifically, we study breeding phenology, morphological traits, plumage characteristics, flying vocalizations, ring recoveries, and genetic structure (mitochondrial DNA). Our results show that the Canary Islands population is phenotypically distinguishable from other populations in breeding phenology, biometrics, plumage colouration and acoustics traits. In addition, we found an incipient genetic differentiation, representing a new case of cryptic differentiation of peripheral populations in Procellariiformes. For all the aforementioned reasons, we propose that this Canarian

population be recognised as a new taxon with subspecific rank. We perform a formal taxonomic description, and discuss the conservation management implications.

Methods

Breeding phenology

Given the small size and the inaccessibility of nesting grounds of the Macaronesian populations, i.e. those of Azores, Madeira, and the Canary Islands, breeding phenology was assessed via the rescue date of fledglings attracted to artificial lights. Shearwater fledglings are attracted to artificial lights during their maiden flights from nests towards the ocean (Rodríguez et al. 2017). Grounded fledglings disoriented by lights have been considered the best evidence of reproduction for some rare and threatened Procellariiformes species, including the Manx shearwater on some Macaronesian islands (Rodríguez et al. 2008, Nunes et al. 2010). We collected information on breeding phenology from three rescue programmes aiming to reduce light-induced mortality of petrels. Rescue date is considered a proxy of fledging as fledglings are grounded during the first flight from their nests to the ocean. According to that reported for the related Cory's shearwater *Calonectris borealis*, light-grounded fledglings are attracted to and grounded by artificial light the night they fledge (Rodríguez et al. 2015). Although most birds are rescued during the first 24 hours after grounding, some could be rescued until five days after grounding (Rodríguez et al. 2015). However, there is no reason to think that time between grounding and rescue differs among the three rescue programs. Thus, we assume that rescue date is synonymous to fledging date, making our data comparable among the three rescue locations. The most northern rescue programme was conducted in Mallaig (Scotland, United Kingdom –UK–; 25 kilometres away from Rum, the second largest colony of the species) between 2016-2018 fledging seasons (Syposz et al. 2018). The second population studied was in Corvo (Azores) between 2009-2019. The most southern rescue programme was carried out in the Canary Islands on La Palma (2000-2018) and Tenerife (1998-2019).

Morphometric measures

Given the small size and the inaccessible nesting locations of southern populations, we could not capture adults. Thus, morphometric measures were collected from 102 rescued fledglings in the three rescue programmes mentioned above in Mallaig (2019), Azores (2017-2019) and the Canary Islands (2017-2019). In addition, we conducted a separate morphological analyses using the information on wing length compiled by Zonfrillo (2001) for 512 individuals and six breeding colonies (Canary Islands, Madeira, Bardsey, Copeland, Rum, and Mykines) across the breeding range in a latitudinal gradient of 34 degrees. Finally, we compiled skeletal measurements of 12 individuals hosted at the collections of the Tring Natural History Museum, UK, and 3 individuals (holotype and paratypes 1 and 2) hosted at the collection of Doñana Biological Station EBD-CSIC, Spain.

From live birds, we recorded body mass (g), wing length, tarsus length, skull length (S), and four bill morphometric measurements: culmen, from the base of forehead feathers in centre of nasal tube to distal part of the curve of the hooked bill; bill length at nostril, from centre of dorso-medial part of tube to distal part of the curve of the hooked bill; bill depth, from of base of forehead feathers to ventral surface of lower mandible; and bill depth at nostril, from the base of nasal tube at nostrils to ventral surface of bill. We measured 11 skeletal traits following McMinn et al. (1990) from specimens hosted at zoological collections (length of skull, mandible, keel, humerus, ulna, radius, carpometacarpus, femur, tibiotarsus and tarsometatarsus, and sternum width). The biometrics were taken using a spring balance (± 5 g), a ruler (± 1 mm) and an electronic calliper (± 0.01 mm). All length measurements were taken in mm. Differences among populations were analysed through a multivariate analysis of variance (MANOVA) for the fledglings. We conducted post-hoc Tukey tests to identify differences between pairs of populations for each variable. In addition, we explored variation in the morphological traits among locations performing a principal component analysis (PCA). Finally, we ran a ANOVA and Post-hoc Tukey tests with the means, standard deviations and sample sizes on wing length provided by Zonfrillo (2001). We do not know the age of these birds as it is not reported by Zonfrillo (2001). The low sample size of skeletal traits precluded us to use statistical analyses. Instead, we plotted the data to visually assess the distribution of the Canarian birds (types) in relation to Manx shearwaters from northern populations.

Plumage colouration

To assess differences in plumage colouration of adults, we conducted an online search on the main birding websites and personal blogs looking for at-sea photographs showing underwing of Manx shearwaters. We only considered at-sea photographs with date and location (around the Canary Islands,

Madeira, Azores, and UK). We only used pictures taken during the breeding season of each population. With such a design, we are confident that photographs did not correspond with migrants from other breeding colonies (Dataset S1). For juveniles, we used in-hand photographs of fledglings rescued after being attracted by artificial night lights in Mallaig, Azores, and the Canary Islands. We scored the amount of dark colouration of axillary underwing and undertail covert feathers. Regarding the axillary underwing feathers, we grouped individuals into four categories: 1) birds showing a clean white underwing, 2) birds showing a dark spot in the articulation, 3) birds with a dark spot and a diagonal axillary bar, and 4) birds with a dark spot in the articulation, a diagonal axillary bar and a conspicuous dark flank bar (Figure S1). To score undertail covert feather colouration, we noted the presence or absence of black spots only in handled juveniles, as it is a trait hard to quantify using free bird photographs (pers. obs.). We performed contingency table analyses to assess the association of plumage colouration to each population.

Acoustic trait analyses

We used flying male calls at breeding colonies to assess variation among populations with the program LUSCINIA (Lachlan 2007). We compiled recordings from online recording repositories (Macaulay Library, Cornell Lab of Ornithology, www.macaulaylibrary.org, and Xeno-canto, www.xeno-canto.org) and audio-books (Moreno 2000, Roché and Chevereau 2001, Robb et al. 2008). In addition, we used our own unpublished recordings (Dataset S1). We only used recordings of similar quality and completeness, which were evaluated visually on sonograms (Figure S2). Noise reduction, signal improvement by altering the reverberation or dynamic range, or high or low threshold filter were not applied to recordings. We used only song phrases of calling males in flight (Figure S2), as their vocalizations are highly stereotypical (James 1985, Robb et al. 2008). We included six phrases on average (range 1-23) from each colony and sampling night. With such a design it was not possible to know the exact number of individuals recorded because birds could not be unequivocally identified at night. We manually identified the elements in each recording and composed a database of acoustic traits. To describe calls, we used 12 out of the 15 acoustic parameters calculated by LUSCINIA: mean frequency, peak frequency, peak frequency change, fundamental frequency, fundamental frequency change, harmonicity, Wiener entropy, frequency bandwidth, amplitude, vibrato rate, vibrato amplitude, and vibrato asymmetry (Lachlan 2007). Differences between vocalizations of northern populations (including those from Iceland, Ireland, and UK) and Canarian population were analysed through a multivariate analysis of variance (MANOVA). We also explored variation in the acoustic traits performing a PCA with those variables showing significant differences between populations.

Genetic analyses

We collected Manx shearwater tissue samples (blood or muscle) from six locations: Faroe Islands (Denmark, $n = 10$), Mallaig (UK, $n = 9$), Skomer (UK, $n = 14$), Corvo (Azores, $n = 25$), Madeira ($n = 2$), and La Palma and Tenerife (Canary Islands, $n = 12$). No birds were killed to get genetic samples. Macaronesian birds were captured during the rescue programmes, thus we can confirm that they were hatched at these archipelagos. Mallaig birds were light attraction fatal victims assumed to come from the closest and large Rum colony (Syposz et al. 2018). Faroe birds were fledglings legally hunted for human consumption by Skúgvoy residents. We also sampled individuals admitted to Canarian wildlife rehabilitation centres during September-October when northern populations migrate through the Canaries to the southern hemisphere (Brooke 1990, Guilford et al. 2009). These last birds were considered migrants, that is, individuals not breeding in the Canary Islands. We also sampled three and two birds hosted at the Zoological Collection of Doñana Biological Station (EBD-CSIC), Seville, Spain, and Museo de Naturaleza y Arqueología, Santa Cruz de Tenerife, Canary Islands, Spain, respectively (see details in Dataset S1).

DNA was extracted from blood or muscle samples using a robotic (Freedom EVO 100; Tecan) magnetic bead protocol. We amplified a 319 base pairs (bp) fragment of the hypervariable Domain I of the mitochondrial control region by using the primers PpufF 5'-CATCCATTCAACTTAGG-3' and PpufR 5'-GGTCCTGACGCTAGTAATAC-3' -designed for this study from those used by Gómez-Díaz et al. (2006). We followed volumes and PCR conditions used by Gómez-Díaz et al. (2006). A 16 capillary sequencer ABI PRISM 3130xl (Applied Biosystems) based on fluorescence was used for sequencing. Chromatograms were carefully inspected by eye and sequences edited using Geneious Pro (v. 5.3.6; Biomatters, Auckland).

We estimated the genetic diversity estimated per population using the DnaSP programme (v 5.10.01; Librado and Rozas 2009). We calculated the number of haplotypes, haplotype and nucleotide diversities and theta ($2Ne\mu$). In addition, we assessed population differentiation using pairwise F_{ST} values and tested their statistical significance by performing 10,000 permutations using the ARLEQUIN programme (v 3.5.1.2; Excoffier and Lischer 2010).

We aligned the sequences using the program MAFFT (v 7; Katoh et al. 2009) using a FFT-NS-I strategy (with an iterative refinement method). We used two datasets. Dataset 1 included all sampled sequences of *P. puffinus* of this study (GenBank accession numbers: MT796218-58) plus three sequences of *P. mauretanicus*, a close relative to *P. puffinus* (Ramirez et al. 2010), obtained from GenBank used as outgroups (GenBank accession numbers: DQ230184, DQ230146, and DQ230179). Dataset 2 included dataset 1 plus all sequences of *Puffinus* spp. available in GenBank (retrieved through BLAST; Morgulis et al. 2008), plus 2 sequences of *P. baroli* and 1 sequence of *Ardenna gravis* obtained in this study (GenBank accession numbers: MT796259-61). Dataset 1 allowed us to assess the phylogenetic structure within *P. puffinus*, while dataset 2 allowed us to place the phylogenetic divergences existing within *P. puffinus* in the context of the divergences existing among valid species of the genus *Puffinus*. We evaluated phylogenetic relationships among haplotypes using maximum likelihood and Bayesian inferences. Maximum likelihood inference was performed with the software IQtree (Nguyen et al. 2015) for dataset 1. We ran the analysis in the IQtree web server (<http://iqtree.cibiv.univie.ac.at/>; Trifinopoulos et al. 2016) using the best-fit substitution model and the “ultrafast” strategy described in Minh et al. (2013) to compute 1,000 bootstrap replicates to assess support. The Bayesian inference was performed with the programme BEAST2 (v 2.52; Bouckaert et al. 2014). The BEAST analyses, used in dataset 2, relied on two independent MCMC chains that ran for 30 million generations, in which we enforced a strict clock and the best-fit substitution model according to the software Partitionfinder (Lanfear et al. 2012). Convergence of the MCMC chains was assessed with TRACER (v 1.7; Rambaut et al. 2018). Both runs converged on similar posterior estimates and sampled trees were combined in a summary tree computed using the software TreeAnnotator (included in BEAST2). Finally, we constructed a statistical parsimony haplotype network using sequences of *P. puffinus* (dataset 1) using TCS v.1.21 with 90 % connection limit (Templeton et al. 1992, Clement et al. 2000). This network was edited and visualized with tcsBU (Múrias dos Santos et al. 2016).

Long-distance movements based on ringing data

To study long-distance inter-colony movements of Manx shearwater, we asked for ringing recoveries to the EURING Data Bank (<http://www.euring.org/edb>). We requested birds ringed as nestling or fledglings at their breeding colonies and recovered in any state. Then we filtered for recoveries further than 100 kilometres from the natal colony. We selected those birds recovered at colonies; i.e. code ‘Status’ equal to N (nesting or breeding) or K (in colony -not necessarily breeding but not pullus). To study long-distance movements between northern colonies (>48 degrees in latitude North) and peripheral colonies, i.e. those locates on Azores, Madeira, Canaries, and islets of the East coast of North America, we selected any ‘Status’ code at recovery.

Taxonomic score

To address the inherent subjectivity of assigning taxonomic rank to allopatric forms (Patten 2015), we followed the integrative taxonomic system based on phenotypic differentiation (Tobias et al. 2010, Sangster 2018). Briefly, we scored differentiation in biometrics, acoustics, colour of plumage, ecology and behaviour, and geographic relationships. For variables that were quantified, i.e. biometrics and acoustics, we calculated effect sizes (Cohen’s *d* statistics). For qualitative traits (colour of plumage, ecology and behaviour, and geographic relationships), we scored them according to the criteria proposed by Tobias *et al.* (2010).

Unless otherwise noted, statistical analyses were conducted in R (v 4.0.0; R Core Team 2020). We used the packages ggplot2 and effsize for data analyses and graph preparation (Wickham 2016, Torchiano 2019).

Results

Breeding phenology

According to the rescue date of fledglings grounded by artificial lights, birds from the three populations (Mallaig, Azores and the Canary Islands) fledged in different periods. The 90% fledging periods (0.05-0.95 quantiles) for each population were from 5 to 30 September in Mallaig (n = 627), from 12 August to 8 September on Azores (n = 81), and from 6 July to 13 August on Canary Islands (n = 76). The Canarian birds fledged on average 31 days earlier than Azorean birds and 52 days earlier than the Mallaig birds (Figure 2).

Morphological differentiation

The MANOVA analysis identified significant morphological differences among the Manx shearwaters fledglings grounded by artificial lights in Mallaig (n = 28), Azores (n = 27), and the Canary Islands (n =

10) (Wilk's Lambda = 0.16; $d.f.$ = 16, 110; $P < 0.001$). Significant differences were identified for weight, wing, bill length at nostril, bill depth at nostril, and bill depth (Figure 3). Canary birds were lighter and showed significantly shorter wings than birds from northern populations (Figure 3). The ANOVA on wing length compiled by Zonfrillo (2001) also showed that the Canary and the Madeiran birds have the shortest wing lengths (mean \pm SD: 227.2 ± 5.9 mm and 234.8 ± 4.3 mm, respectively) as compared with the remaining populations (Figure S3). This was also supported by our limited compilation of skeletal measurements. The Canary types showed the shortest bone measurements of the extremities, particularly for humerus, ulna, radius and carpometacarpus (wing bones), and femur, tibiotarsus and tarsometatarsus (leg bones) (Figure S4).

The PCA performed with the eight biometric variables (weight, wing, tarsus, skull and the four bill measurements) reduced the number of variables to three principal components explaining 67.3% of the total variance. PC1 explained 33.9%, and showed moderate positive weighting for all variables indicating that it can be considered a proxy of size. PC2 explained 19.2% of the variance and showed a moderate positive weighting for culmen length, and a negative weighting for weight. PC3 explained 14.2% of the variance and showed a high negative weighting for bill depth and bill depth at nostril (see factor loadings in Table S1). PCA plots depicted most Canary individuals segregated from Mallaig individuals (Figure 4). Azorean individuals showed intermediate values between Canary and Mallaig individuals.

Plumage colouration

We studied photographs of 186 individuals (117 adults and 61 fledglings) from the Canary Islands, Madeira, Azores, and UK. Axillary underwing pattern of adults and fledglings were different among populations ($\chi^2 = 79.94$; $d.f.$ = 6; $P < 0.001$; $\chi^2 = 33.54$; $d.f.$ = 4; $P < 0.001$ for adults and fledglings, respectively), being paler in the UK birds and darker in the Canary individuals (Figure 5). Undertail covert feathers were also darker in Canary fledglings, but differences were marginally significant ($\chi^2 = 5.49$; $d.f.$ = 2; $P = 0.064$; Figure 5).

Acoustic trait analyses

The MANOVA analyses of 115 vocalizations from northern and Canary populations showed significant differences for nine out of the 12 acoustic variables (Wilk's Lambda = 0.41; $d.f.$ = 12, 102; $P < 0.001$; Figure 6). The PCA performed with the nine significant acoustic variables reduced the number of variables to three principal components explaining 79.5% of the total variance (see factor loadings in Table S2). PCA plot depicted differences in flying calls traits between northern and Canary populations (Figure 4).

Genetic analyses

We recovered 33 haplotypes from 80 birds (Table S3). Genetic diversity reached the highest values in the northern populations and the minimum in the Canary Islands, which corresponds with the southern peripheral population of the Manx shearwaters. Control Region pairwise F_{ST} values showed a moderated level of differentiation among populations (range $0.005 < F_{ST} < 0.4$). Differentiation among Azores, Skomer, Mallaig, and Faroes were lower than any of the pairwise comparisons of these populations with the Canary Islands. In general, F_{ST} values are the highest in the most distant populations, suggesting longer periods of isolation. Importantly, all pairwise comparisons including the Canary population were significant, being the only population where such a result was obtained (Table 1). However, haplotype network analysis did not show a strong genetic structure among populations (Figure S5). Azores and European populations shared most of haplotypes. Despite lack of structure, 8 out of 12 Canary birds grouped together as they shared 4 haplotypes, separated by, at least, 5 and 6 nucleotide substitutions to its closest haplotype in the Azores or Mallaig populations, respectively. The remaining four Canary individuals were distributed within the network, but recovered as singleton haplotypes (Figure S5). Finally, birds collected in the Canaries during September-October were genetically more similar to northern populations than the Canary breeding population (Table 1). Such a result confirms that these birds were migrant individuals.

Our phylogenetic inferences consistently grouped most of the sequences sampled in the Canary Islands (8 out of 12) in a highly supported clade (bootstrap $> 80\%$, posterior probabilities > 0.95 ; Figures 7 and S5). Genetic divergences between the Canary clade and the other populations were below the divergence level expected among valid *Puffinus* species, however, our phylogenetic results indicate an incipient genetic differentiation (Figure S6).

Ringing recovery data

According to the EURING database, just nine out of >5,500 birds ringed as nestlings and recovered at any circumstances have been found nesting (Status code = N) in a different colony further than 100 km from their natal colony. Numbers could increase to 56 if we assume as breeders those birds older than five years and found in the colony, but not necessarily breeding (Status code = K). All these recoveries corresponded with British, Irish and French breeding areas (Figure S7). With regards to longer movements, i.e. from European colonies (Iceland, Faroe Islands, Ireland, and UK) to the peripheral colonies in Macaronesia and the East coast of North America, no birds were reported as breeders. However, older birds, that is, individuals with a higher chance to recruit into a breeding population given that first breeding mostly began when birds are 5-6 years old, were found around Newfoundland (Canada), in contrast to those reported around the Canary Islands (Figure S8). Interestingly, a bird hatched at Skokholm (UK) was recovered when it was 8 years old in Azores on 25 April, a date when Azorean Manx shearwaters have already laid their eggs.

Taxonomic score

The largest effect sizes in biometrics were reached in body mass (Mallaig-Canary Islands, decrease: $d = 3.40$; 95% CI = 2.40, 4.40) and culmen (Azores-Canary Islands, increase: $d = -0.28$; 95% CI = -0.95, 0.41). For acoustic traits, the largest effect size was reached in frequency bandwidth (northern populations-Canary Islands: $d = 1.42$; 95% CI = 0.99, 1.85). Canarian shearwaters showed a minor difference in plumage colour, showing darker axillary underwings and undertail coverts (Figure 5). With regards to ecology and behaviour, we highlighted differences in breeding phenology and breeding habitat. Thus, our taxonomic final score was 6 (Table 2).

Discussion

The Manx shearwater is one of the most studied seabirds in Europe, an iconic species for European and, particularly, British ornithology (Brooke 1990, Lee et al. 2020). However, comparative studies between populations are strikingly absent (Lee et al. 2020). Such a circumstance could be explained because the core population breeds in large numbers on accessible islands around Britain and Ireland, while southern peripheral populations are patchily distributed and difficult to sample. Our results reveal that birds of the Canarian population breed around 1-2 months earlier, are smaller, weight less, and show darker underwing plumage than northern birds. Preliminary analyses also show some level of differentiation in nine acoustic traits. Genetic results of the mitochondrial control region indicate an incipient differentiation of Canarian Manx shearwaters from their northern counterparts, which is supported by the highest estimates of pairwise differentiation with any other population. Such a recent isolation could explain the lack of monophyly of the Canarian sequences, which show a typical scenario of incomplete lineage sorting recorded in other seabirds species (e.g. Gómez-Díaz et al. 2006). The Canarian Manx shearwaters reached a taxonomic score of 6 or 5, if we exclude the acoustic traits as Tobias et al. (2010) did in their subspecies comparison. These values are higher than the mean value found among 23 allopatric subspecies of the Western Palearctic avifauna (mean and SD = 4.1 ± 1.8) (Tobias et al. 2010). In addition, Canarian Manx shearwaters occupy a distinct breeding geographic area, which fits within the subspecies definitions compiled by Remsen (2010). For all the aforementioned reasons, we propose to rank it as a new taxon. The formal description of this new subspecies is presented in the section taxonomic account.

Differentiation

Allochrony is a particular case of sympatric speciation, where the change of breeding schedules promote isolation and differentiation of populations over time (Taylor and Friesen 2017). Allochrony has been identified as one important factor fuelling differentiation in Procellariiformes (Taylor et al. 2019 and references therein). Our findings suggest that allochrony could be the mechanism leading to differentiation of the Canary Islands Manx shearwater. The peaks of rescue date of fledglings grounded by lights show that Canarian and Madeiran birds have an earlier breeding season than British populations (Brooke 1990, Rodríguez et al. 2008, Nunes et al. 2010). Canarian birds start visiting colonies as early as 15 January (pers. obs.), when European Manx shearwaters are still wintering in the Patagonian Shelf off (Argentina) (Guilford et al. 2009). While northern European shearwaters arrive near breeding colonies around 20-25 March after pre-nuptial migration, the Canarian shearwaters are already incubating. Assuming incubation and rearing chick periods of 51 and 70 days (Lee et al. 2020), Canarian shearwaters should lay on average around 26 March, which agrees with anecdotal information reported by other authors (Bannerman 1914, Bannerman and Bannerman 1965, Nunes et al. 2010). In addition, post-nuptial

migration routes of European populations pass through Madeira and Canary Islands on September-October (Guilford et al. 2009), when the Canarian breeding population has departed (Nunes et al. 2010; pers. obs.). During pre-nuptial migration, European shearwaters fly past the eastern coast of America taking advantage of dominant oceanic current systems, i.e. far away from the Macaronesian archipelagos (Guilford et al. 2009). Thus, seasonal differences in breeding phenology, and routes and timing of migration could enhance reproductive isolation between northern populations and Madeiran and Canarian populations. As a consequence, Canarian and Madeiran populations might use different migration routes and wintering areas than those used by northern populations, although more research on at-sea distribution is needed.

Several authors had noted that birds in southern populations average smaller in body size than in northern populations (Brooke 1990, Zonfrillo 2001). We have statistically analysed such differences. Body mass at fledging and wing length are the most different morphological traits between northern and Canarian populations. Also, Canarian birds show darker underparts, with darker axillary underwing and undertail covert feathers than northern populations, which is consistent with the described by other ornithologists (Gil-Velasco et al. 2015). These findings on size and colouration are in line with the Bergmann and Gloger rules, but more specific research is required (Salewski and Watt 2017, Delhey 2019).

Mating calls are considered a prezygotic reproductive barrier in birds (Price 2007, Uy et al. 2018) and this trait could fuel differentiation in nocturnal species such as procellariiformes, since most species are nocturnal at their nesting colonies. So, their calls could be likely a good indicator of reproductive isolation (Bretagnolle 1995). Our acoustic analyses showed differences in nine variables describing spectral cues (Figure 6). Playback experiments are now needed to shed light on vocal recognition among breeding populations (Curé et al. 2010, 2012). This information will be decisive to evaluate the effect of vocal divergence on population recognition and reproductive isolation.

Ring recoveries indicate that Manx shearwater interchange between colonies is scarce, but it might still be sufficient to allow gene flow within the European colonies (Harris 1972; Brooke 1990; this study). In fact, we have demonstrated the existence of shared haplotypes within European colonies and between Azores and European colonies, which could be explained by gene flow (Figure S5). The recovery of an eight year old individual ringed in Skokholm (UK) and recovered in Azores during the breeding season, as well as sporadic breeding attempts on other Azorean islands (Bried et al. 2007), support our genetic findings, that is, the low genetic differentiation between European and Azorean breeding colonies. Interestingly, all sequences ($n = 8$) of birds collected in Canary Islands during September-October, i.e. out of the breeding period, were nested in other clades out of the Canarian clade (Figure 7), which was also supported by the low F_{ST} differentiation of these birds with the northern populations (Table 1). These findings confirm that the European Manx shearwaters migrate through the Canary Islands in their post-nuptial migration (Brooke 1990, Guilford et al. 2009), and highlight the importance of the sampling dates to elucidate the origin of the birds.

The small fragment of mitochondrial gene used in our study, and the limited phylogenetic resolution prevent us from further exploring the origin or dating the divergence found. A high genomic coverage in combination with a wide taxon sampling is now required to unravel the evolutionary history of *P. puffinus* and to assess the influence of introgression or incomplete lineage sorting. However, our results show an incipient process of differentiation of the Canarian population. On one hand, the estimates of pairwise differentiation of the Canary Islands with any other population are the highest and significant (note that we have to be cautious with Madeiran results due to our small sample size). In addition, the main Canary Island group recovered in our network is separated at least by five nucleotide substitutions from its closest haplotype in Azores. Following this theme, Taylor et al. (2019) proposed several cryptic *Hydrobates* species, which showed less differentiation for the same control region gene. On the other hand, four out of 12 Canary Islands sequences nested in other clades. Incomplete lineage sorting and/or introgression could explain this result. Introgression has been already recorded in the closely relative Mediterranean *Puffinus* species, the Balearic and Yelkouan shearwaters, that can hybridise (Genovart et al. 2007). Importantly, we did not find any sequence belonging to the Canarian clade in other colonies, which could indicate some kind of site fidelity for the Canarian birds. The differences in breeding phenology, vocalizations, and allopatry could enhance reproductive isolation and, consequently, fuel the speciation process. Finally, regardless of the mechanisms explaining the process of incipient genetic differentiation here reported, we suggest recognition of the Canarian breeding population as a subspecies, as by definition, subspecies can mix among them (Remsen 2010). In this sense, genetic distances between Canarian Manx shearwaters and Manx shearwaters from northern populations are smaller than among recognised *Puffinus* species (Figure S6).

Conservation implications

As in other islands around the world (Wood et al. 2017b), a considerable reduction of avifauna diversity has occurred in the Canary Islands following human colonisation, and Procellariiformes are not an exception (Illera et al. 2012, 2016). Three out of ten species recorded in the Canaries are extinct (*Pterodroma* spp., *Puffinus holeae*, and *Puffinus olsoni*; Ramirez et al. 2010, Illera et al. 2016). Worldwide the breeding population of the Manx Shearwater has been estimated to be 680,000-790,000 mature individuals, and it is considered of “Least Concern”, with a global trend unknown (BirdLife International 2020). The status of this new taxon in the Canaries is largely unknown, although population size has been tentatively estimated to be 250-1,000 pairs (BirdLife International 2015). The species was very abundant in the past, at least, on La Palma (Canary Islands), where historical chronicles indicate they were annually harvested by dozens (Martín et al. 1989, Medina 2007, Trujillo 2008). However, the low number of birds recorded annually grounded by lights suggests that the population trend is dramatically decreasing (BirdLife International 2015). Currently, nesting sites are located on a few locations in the Canaries (Medina 2007, Trujillo 2008), and thus, our best evidence of successful reproduction comes from fledglings grounded by artificial lights (Rodríguez et al. 2008). Despite this lack of information on the biology of this taxon, according to scientific reports, our experience, and threats to other related taxa (Duffy 2010, Raine et al. 2017), we identify four main threats on land: 1) predation by introduced rodents (Martín et al. 1989, Hernández et al. 1990, Nunes et al. 2010); 2) attraction and disorientation by artificial lights (Rodríguez et al. 2008, Rodríguez and Rodríguez 2009); 3) collision with powerlines (Trujillo 2010); and 4) competition for nest burrows with the larger and more abundant Cory’s shearwater *Calonectris borealis* (Bolton et al. 2004, Trujillo 2008, pers. obs.).

The taxonomic identification of the Canarian Manx shearwater as a new subspecies should lead to prioritisation of its conservation through an action plan. The main objective of the action plan should be obtaining critical information on its biology and ecology, especially on population size, distribution of breeding colonies, and foraging areas. Such information would let us classify this taxon within the Spanish list of threatened species, which will promote specific actions to ameliorate its unfavourable conservation status. We consider that, at least, two identified land threats can be controlled with local and regional support: 1) control or exclusion of introduced predators (rats and cats), and 2) installation of nest-boxes whose entrance would exclude the larger species. Meanwhile, management on power lines and lighting systems, i.e. shielding luminaries, restricting emissions in the shorter wavelengths, and reducing timing and intensity of lighting, as well as an enhancement of rescue programmes can already be conducted in those areas where fallout is concentrated (Rodríguez et al. 2008, Rodríguez and Rodríguez 2009). Regarding at-sea threats where there is no specific information, it seems reasonable to speculate that, as other shearwaters, plastic ingestion, oils spills, food depletion, and interaction with fisheries could severely affect their populations (Cardoso et al. 2014). Thus, a better knowledge on foraging areas during breeding, migration stopovers and wintering areas, is now needed to identify its marine threats.

Taxonomic account

Genus: *Puffinus* Brisson 1760

Species: *Puffinus puffinus* Brünnich 1764

Puffinus puffinus canariensis ssp. nova.

Diagnosis:

a) Appearance

Puffinus puffinus canariensis is in appearance similar to *Puffinus puffinus puffinus* from northern populations. However, *P. p. canariensis* are smaller in size (wing length) and body mass than *P. p. puffinus*. With regards to colouration, *P. p. canariensis* shows darker axillary underwing and undertail feathers than *P. p. puffinus* from northern populations (Figure S9).

b) Holotype and paratypes

Holotype (EBD 30790A): Adult female (skin and skeleton) from La Orotava (Tenerife Island, Canary Islands) admitted to the Wildlife Rehabilitation Center La Tahonilla on 6 March 2009.

Paratype1 (EBD 30792A): Immature female collected on Los Tilos waterfall, La Palma, and admitted to Cabildo de La Palma, on 15 June 2018.

Paratype2 (EBD 30793A): Adult male collected on San José, Breña Baja, La Palma, admitted to Cabildo de La Palma, on 28 February 2018.

Paratype3 (TFMCVA337). Adult female collected by J. Leal in Bco. Los Tilos, La Palma, on April 1987.

Paratype4 (TFMCVA376). Fledgling (?) female collected by Dirección General de Medio Ambiente in Barlovento, La Palma, on 12 August 1988. No age data is recorded at the hosting collection.

Fresh measurements were taken by BRM, and bone measurements were taken by Carlos Urdiales, the curator of Zoological Collection of the Doñana Biological Station (ZC EBD-CSIC) in Seville, Spain, as described in McMinin et al. (1990). The skins and the skeleton of types (EBD 30790A; EBD 30792A; EBD 30793A) are deposited at the Zoological Collection of the Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas, Sevilla, Spain, and types (TFMCVA337; TFMCVA376) at Museo de Naturaleza y Arqueología, Cabildo de Tenerife, Santa Cruz de Tenerife, Canary Islands, Spain. Mitochondrial control region sequences are deposited at the National Center for Biotechnology Information (NCBI) gene bank database. For accession numbers and morphological measurements see Table 3.

Bone measurements (in mm) of the holotype: skull maximum length 78.65, premaxillar length 38.60, 24.70, mandible length 66.70, humerus 75.75, ulna 68.65, radius 66.85, carpometacarpus 39.95, femur 29.70, tibia 73.35, tarsometatarsus 41.45, corucoid 24.50, scapula 39.15, sternal keel length 57.50.

c) Status

Extant

d) Etymology:

The subspecies name *P. p. canariensis* reflects the origin of holotype and paratypes specimens.

e) Breeding distribution

The new taxon breeds on La Palma and Tenerife, Canary Islands, and maybe on La Gomera and El Hierro as well (Medina 2007). The Manx shearwater population breeding on Madeira Island could also belong to this subspecies, but its taxonomic identity should be clarified in the future.

f) Marine habitat

The taxon is a pelagic seabird. Foraging and wintering areas are unknown as no tracking studies are available. Adults are not present at sea around colonies from late July to mid-January.

g) Terrestrial (nesting) habitat

Contrary to the grassy slopes used as nesting habitat by the nominal subspecies *P. p. puffinus* (Lee et al. 2020), the nesting habitat of *P. p. canariensis* is located on cliff edges and inaccessible ledges of ravines within laurel forest, which is typically located on north-facing slopes influenced by north-easterly humid trade winds (Martín et al. 1989, Hernández et al. 1990, Zonfrillo 2001, Trujillo 2008, Nunes et al. 2010). According to local people, the species was more common in the past when nesting burrows were also located at lower elevations and out of laurel forest domains (Aníbal Álvarez González, pers. comm.).

Declarations

Acknowledgements – We are grateful to all participants in the rescue programmes, and many colleagues, particularly Martyna Syposz, Gustavo Tejera, Enrique Sacramento, Rubén Barone, Domingo Trujillo, Sandra Hervías, Bárbara Ambrós, Sérgio Marín, Ana Isabel Fagundes, Cátia Gouveia, and students of Anglia Ruskin University. We also thank the collaboration of Corvo Natural Park (specially to Rui Pimentel), Madeira Institute of Forestry and Nature Conservation, and Funchal Ecological Park. Zac Hinchcliffe courteously provided a photograph. We thank the European Union for Bird Ringing (EURING) which made the recovery data available through the EURING Data Bank and to the many ringers and ringing scheme staff who have gathered and prepared the data. We are grateful to Carlos Urdiales, the curator of the Zoological Collection of Doñana Biological Station CSIC who provided some samples and prepared skin specimens, to Judith White, the curator of the collections of the Natural History Museum, Tring, and Esther Martín, the curator of the collections of the Museo de Naturaleza y Arqueología, Santa Cruz de Tenerife, Canary Islands. We thank the subject editor and two anonymous reviewers for their comments on this paper.

Funding – This study was partially funded by the Canary Islands' Ornithology and Natural History Group (GOHNIC). JCI was funded by the Spanish Ministry of Science, Innovation and Universities (PGC2018-097575-B-I00) and by a GRUPIN research grant from the Regional Government of Asturias (IDI/2018/000151).

Author contributions – AR and BR conceived and designed the study. AR, BR, TP, MC, JD, JN, CS, PG, and FMM collected field data and samples. AR carried out the statistical analyses. JGP, TM and JCI conducted genetic analyses. AR, BR and JCI wrote the manuscript. All authors critically revised the manuscript and gave final approval for publication.

Conflicts of interest – AR and BR are consultants on the species to the Spanish Ministry of Ecological Transition.

Permits – Permission to sample birds were granted by the Regional and Local governments of the Canary Islands (no. 2018/6842, AFF 94/18, AFF273/18), Scotland (no. A5429), Azores (samples were

granted by Regional Directorate of Environment and Regional Directorate for Sea Affairs; no. 70/2017/DRA; no. 60/2018/DRA) and Madeira. No permit was required for the Faroe Islands samples. **Data accessibility** – All data supporting the findings of this study are included in the Supplementary material. The new subspecies proposed have been registered in ZooBank (<http://zoobank.org/>), the proposed nomenclatural registry for zoology. The ZooBank Life Science Identifier (LSID) is urn:lsid:zoobank.org:pub:6289E285-A2D6-43B6-82D4-6E2CFC48AE77 (study). Unique haplotypes have been deposited in the NCBI gene bank database (accession numbers MT796218–61). Holotype and paratypes are hosted at the Zoological Collection of Doñana Biological Station CSIC, Seville, Spain (EBD30790A, EBD30792A, and EBD30793A), and Museo de Naturaleza y Arqueología, Santa Cruz de Tenerife, Canary Islands, Spain (TFMCVA337 and TFMCVA376).

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Figure Legends

Figure 1 Map

Figure 1. Breeding distribution (red areas) of the Manx shearwaters *Puffinus puffinus*, and colonies sampled (red dots). The red dot outside of the Canary Islands breeding distribution indicates the sampling of migrant individuals. Breeding distribution range taken from BirdLife International (2015).

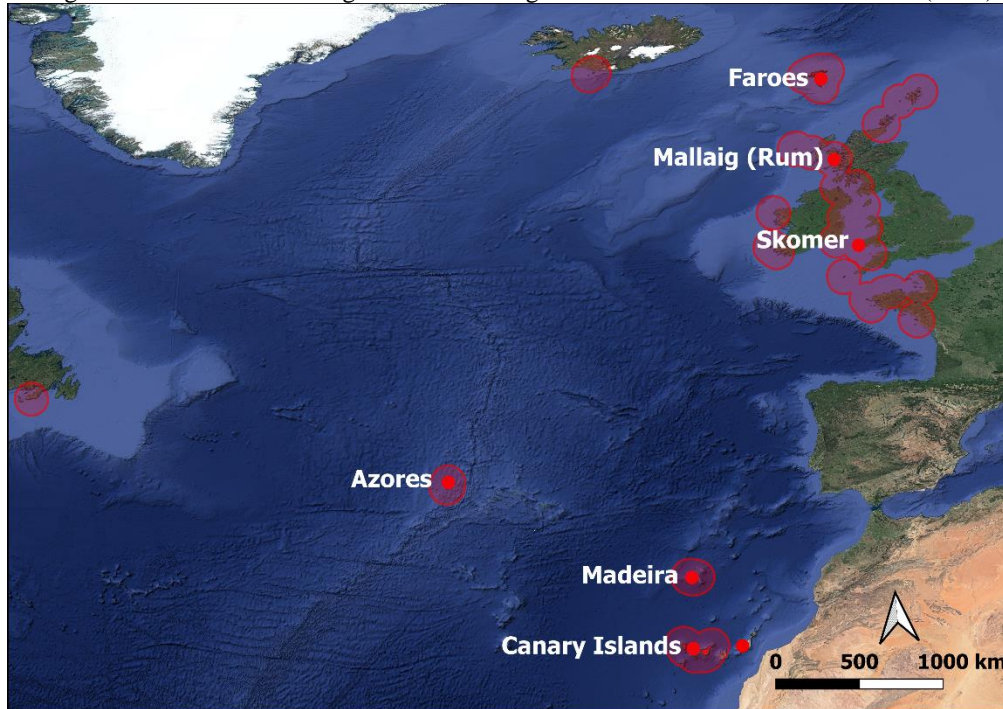


Figure 2 Phenology

Figure 2. Histograms of rescue date of Manx shearwater *Puffinus puffinus* fledglings grounded by artificial lights and collected at three rescue programmes, Mallaig (Scotland), Corvo (Azores), and Tenerife and La Palma (Canary Islands). Vertical lines indicate the average rescue date for each location (Canary Islands = 25 July; Azores = 25 August; Mallaig = 15 September).

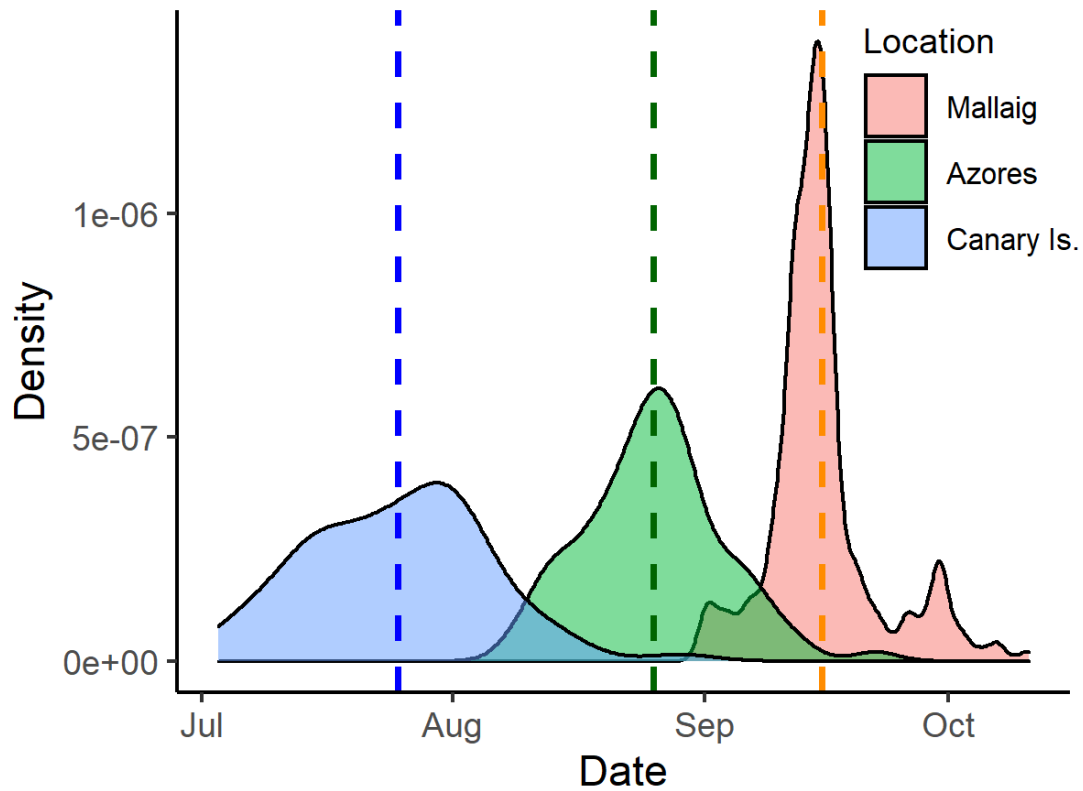


Figure 3 Biometrics

Figure 3. Biometrics of Manx shearwater *Puffinus puffinus* fledglings rescued at three rescue programmes to mitigate mortality induced by artificial lights: Mallaig, United Kingdom (red); Corvo, Azores (green), and La Palma and Tenerife, Canary Islands (blue). Different letters indicate significant differences ($P < 0.05$) among groups. No letters depicts non-significant differences among groups.

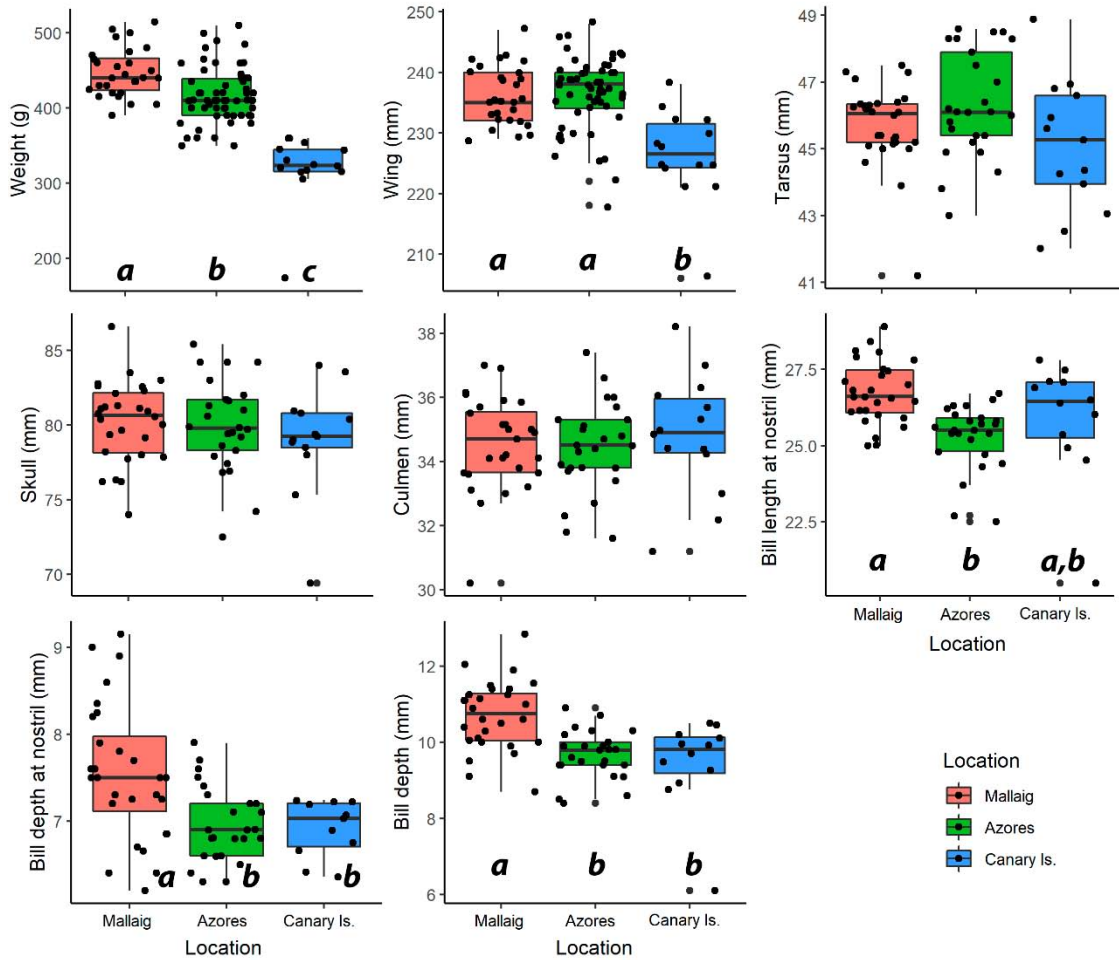


Figure 4 PCAs

Figure 4. Principal component analysis (PCA) plots for the morphometric and acoustic variables. For morphometrics, we measured fledglings grounded by artificial lights in Mallaig (red), Azores (green), and the Canary Islands (blue). PCA includes the following variables: body mass, wing, tarsus, skull, culmen, bill length at nostril, bill height, and bill height at nostril. For acoustics, we analysed calls from northern populations (red) and Canary Islands (blue). PCA includes the following variables: mean frequency, peak frequency change, fundamental frequency, harmonicity, Wiener entropy, frequency bandwidth, amplitude, vibrato amplitude, and vibrato asymmetry. Crosses indicate means and standard deviations.

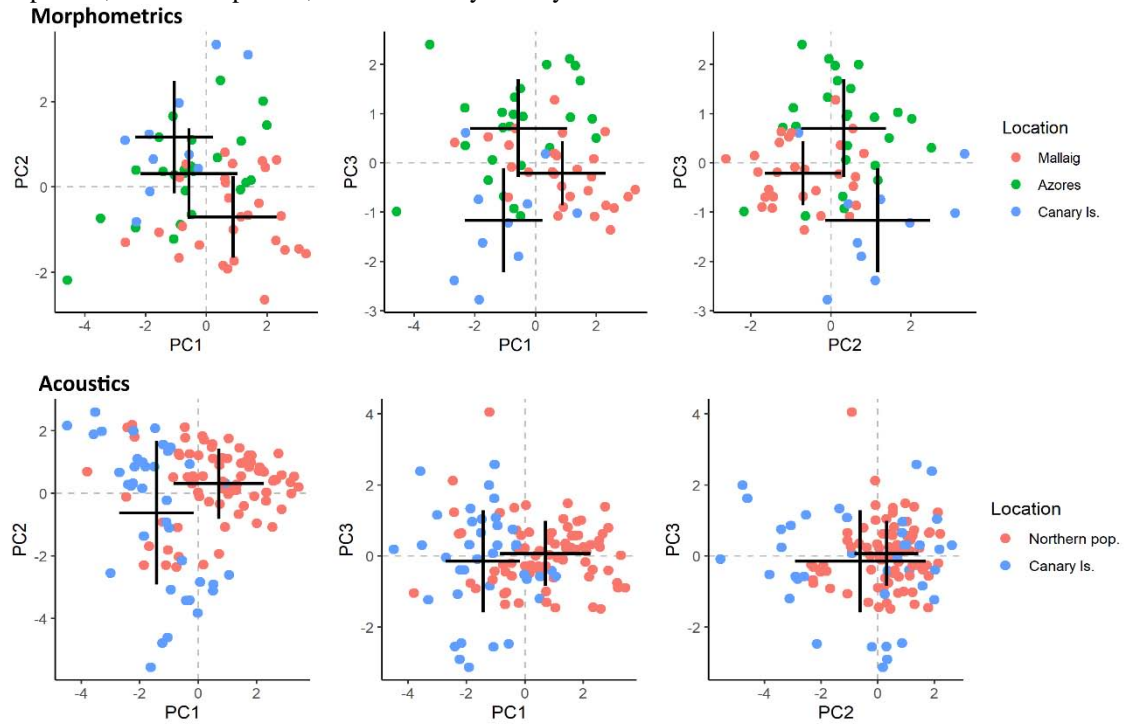


Figure 5 Colouration

Figure 5. Percentages of individuals showing variation in plumage colouration (axillary underwing and undertail covert feathers) of the Manx shearwaters. White numbers indicate the numbers of birds assessed in each population. UK = United Kingdom.

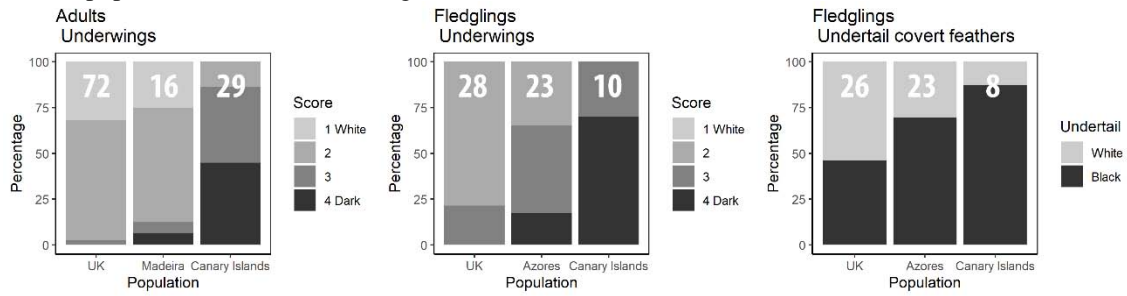


Figure 6 Acoustics

Figure 6. Acoustic variables of Manx shearwater (*Puffinus puffinus*) flying calls from northern populations (including those from Iceland, Ireland and United Kingdom) and Canary Islands, Spain. Variables with significant differences between groups are highlighted (y-axis label in bold and axis lines in black). See main text for analyses and sampling details.

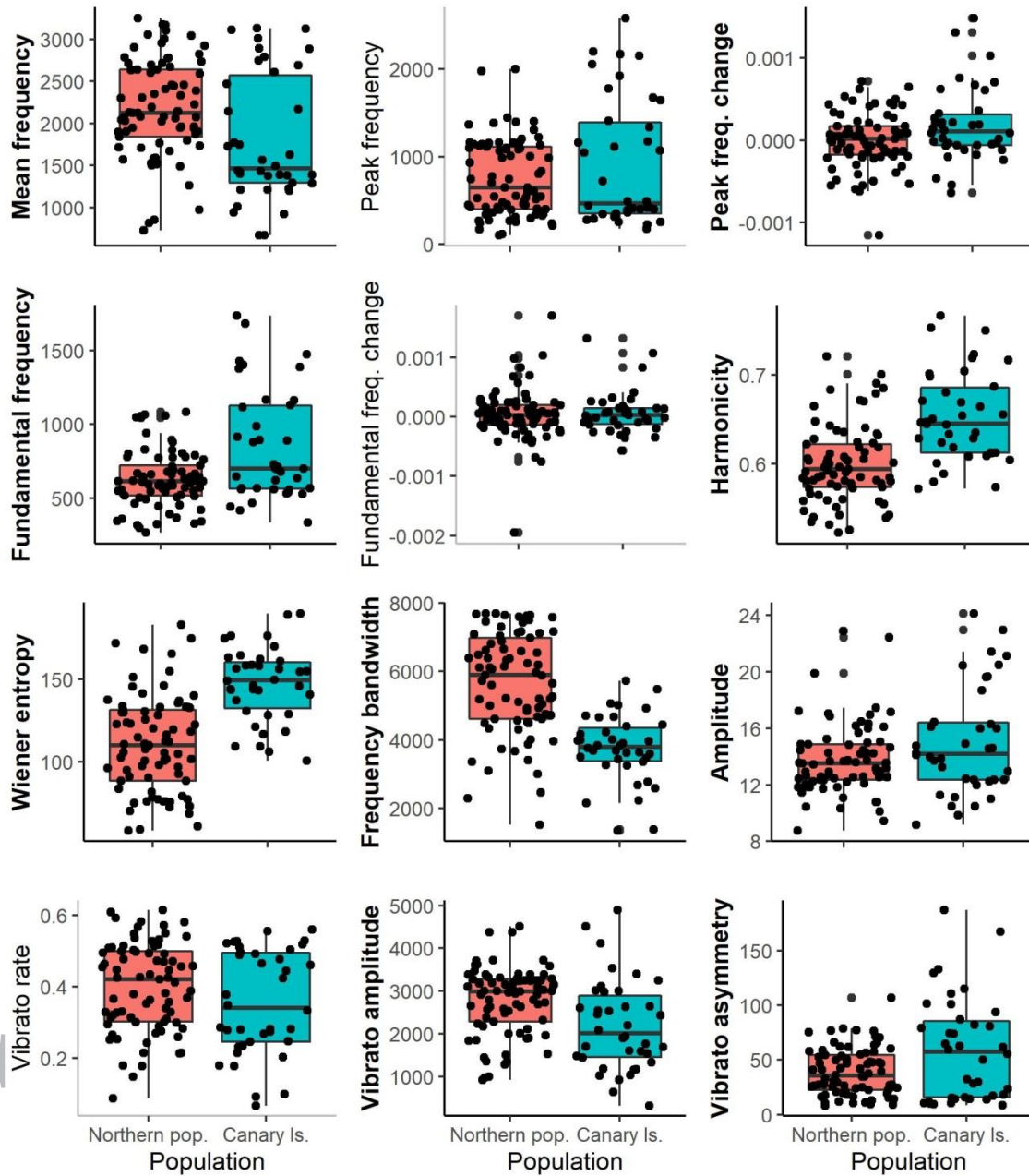


Figure 7 Tree

Figure 7. Maximum likelihood phylogenetic tree based on a 319 base pairs (bp) fragment of the hypervariable Domain I of the mitochondrial control region, including *Puffinus puffinus* sequences and three outgroup sequences of *Puffinus mauretanicus* obtained from the GenBank. Numbers below nodes indicate bootstrap support $\geq 70\%$. Canaryian individuals are highlighted in blue.

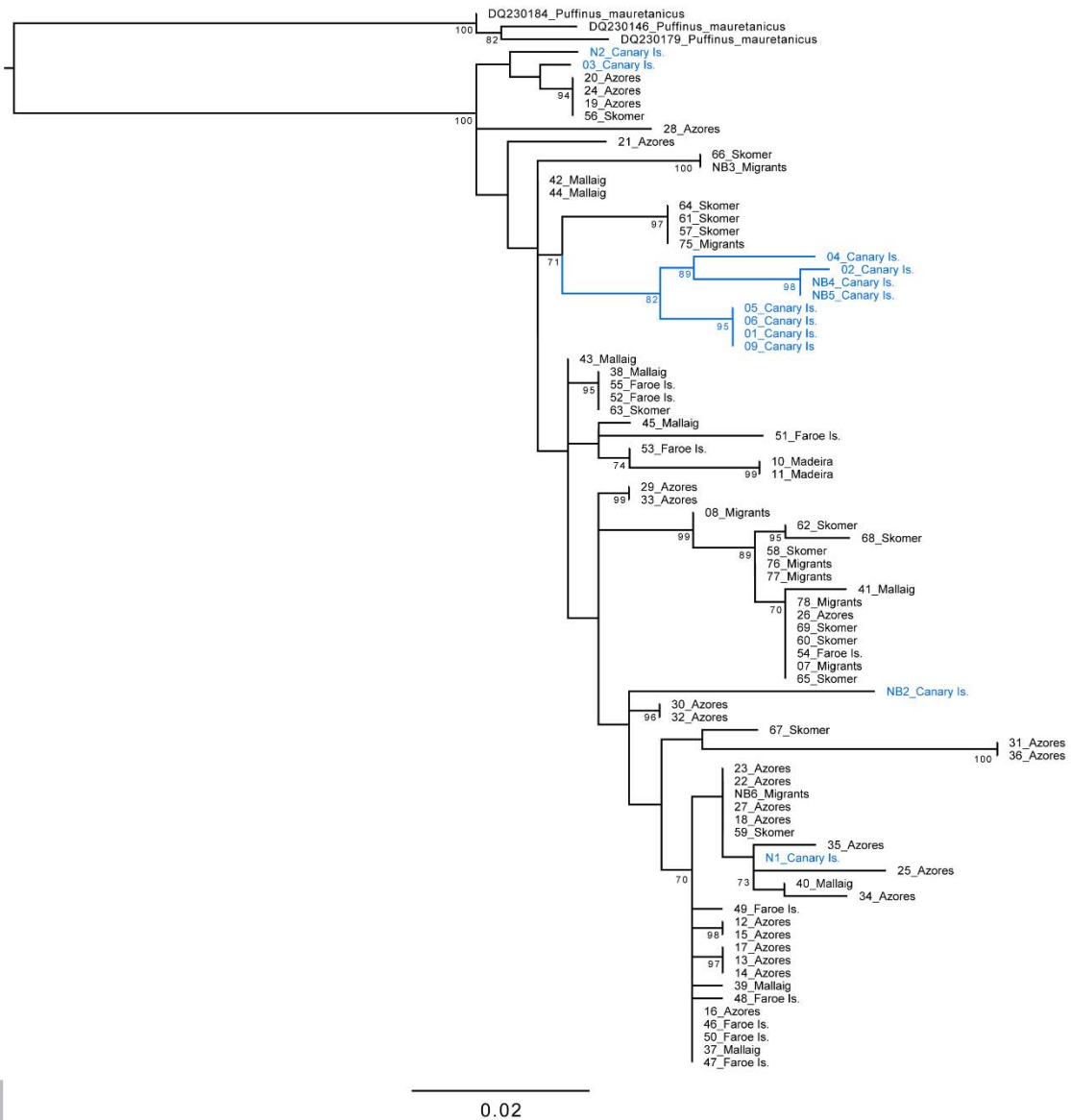


Table Legends

Table 1. Estimates of genetic differentiation (F_{ST}) among breeding populations and migrant birds crossing the Canary Islands. Migrants were birds collected in the Canary Islands during September-October. Significant F_{ST} P -values (< 0.05) are highlighted in bold. Asterisk denotes marginally significant comparisons.

Populations	Canary Is.	Madeira	Azores	Skomer	Mallaig	Faroes
Madeira	0.320					
Azores	0.084	0.257				
Skomer	0.112	0.290	0.050			
Mallaig	0.113	0.334*	0.035	0.081		
Faroes	0.090	0.295	0.005	0.032	0.036	
Migrants	0.164	0.391	0.090	0.041	0.140	0.068

Table 2 Score allocation based on taxonomic characters proposed by Tobias *et al.* (2010).

Trait type or context	Frequency of scoring	Measure	Score
Biometrics	Strongest increase and strongest decrease only	Effect size: $d = 3.40$ Body mass $d = -0.28$ Culmen	2 1
Acoustics	Strongest temporal and spectral character only	Effect size: Temporal: Not assessed Spectral: $d = 1.42$ Frequency bandwidth	- 1
Plumage	Three strongest characters	A slightly different wash or suffusion to all or part of any area	1
Ecology and behaviour	Once	Non-overlapping differences in breeding phenology and habitat	1
Geographic relationship	Once	Allopatry	0

Table 3. Morphometric measures, host museums and GenBank accession numbers of the holotype and paratypes. All data in mm, except body mass in grams.

Type	Weight	Wing	Tarsus	Skull	Culmen	Bill length at nostril	Bill depth at nostril	Bill depth	Museum and accession number	Genbank numbers
Holotype	218	229	43.13	80.00	34.82	27.62	10.57	7.46	EBD 30790A	MT796218
Paratype1	286	239	46.14	83.08	37.30	27.61	11.31	7.98	EBD 30792A	MT796225
Paratype2	234	236	44.32	80.29	35.85	27.73	10.57	7.87	EBD 30793A	MT796225
Paratype3	335	226	46.8	77.91	33.2	24.4	7.40	10.1	TFMCVA337	N/A
Paratype4	324	229	44.5	76.96	36.5	26.3	6.69	9.6	TFMCVA376	MT796224