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The Mascarene Petrel *Pseudobulweria aterrima* is a criti
cally endangered seabird endemic to Reunion Island,
with an extremely small population suffering several
threats. Fifteen polymorphic microsatellite loci were iso
lated from this species to analyse genetic diversity, esti
mate contemporary effective population size, search for
evidence of a population bottleneck and see whether
results support the hypothesis that life history traits
could preserve allelic diversity in small populations.
Results from 22 individuals found grounded as a conse
quence of light pollution highlight a surprisingly high
genetic diversity, an absence of inbreeding, a contempo
rary effective population size estimated at approximately
1211 individuals and a probable bottleneck around
10 000 generations ago. Additional studies on genetic
diversity and structure from a larger number of samples
are thus required to evaluate the evolutionary potential
of this critically endangered species.

Seabirds are the most threatened birds in the world,
with about 28% of species considered endangered
(Croxall *et al.* 2012). Among seabirds, tropical petrels
(genera *Pterodroma* and *Pseudobulweria*) are among the
least known and most vulnerable (Croxall *et al.* 2012),
with the two main threats being predation by intro
duced mammals (Keitt *et al.* 2002, Le Corre 2008) and
light pollution, especially for young birds (Rodriguez
et al. 2017). These threats may lead to local extinctions
of some populations (Croxall *et al.* 2012, Dias *et al.*
2019).

Pseudobulweria, comprising four species, is the least
known genus of Procellariidae (Warham 1990). Among
them, the Mascarene Petrel *Pseudobulweria aterrima*,
endemic to Reunion Island, is the only representative of
the genus in the Indian Ocean. This species is listed as
Critically Endangered due to an extremely small breed
ing population (~ 100 200 mature individuals) and a
presumed continuing decline related to predation and
light induced mortality (Birdlife International 2018).
With the prominent exception of a seabird rescue cam
paign initiated in 1997 by a local non governmental
organization (Société d'Etudes Ornithologiques de La
Réunion SEOR, Le Corre *et al.* 2003), research and
conservation actions focused on Mascarene Petrels effec
tively started in 2015 with the implementation of the
European LIFE + Pétrels project. Given the recency of
research efforts, the ecology and biology of this species
are still poorly known.

Endangered species typically have small and decreas
ing population sizes and low genetic variability, espe
cially when compared with closely related taxa that are
not threatened (Ardern & Lambert 1997, Spielman
et al. 2004). In small populations, theory also predicts a
loss of genetic variability due to genetic drift and possi
ble inbreeding depression (Nei *et al.* 1975). This is a
major concern in conservation biology, as the loss of
genetic variability may result in reduced survival,
reduced fertility and increased susceptibility to diseases
(Frankel & Soulé 1981, Frankham *et al.* 2002). Many
models and concepts on the loss of genetic diversity are
based only or mainly on genetic drift and mutation (e.g.
Lande 1995). In a small population, genetic drift no
longer has an effect on the loss of diversity, and it is

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demographic and environmental stochasticity that pre vail (Lynch & Gabriel 1990, Lynch & Lande 1998, Masel 2012). Species life history traits may influence the rate of genetic variability decrease (Lippé *et al.* 2006). For example, the loss of genetic diversity could be slo wed down in monogamous species (Kuo & Janzen 2004, Weiser *et al.* 2013). Monogamous breeding will retain genetic diversity more effectively than situations that allow mate choice or intrasexual competition (Gooley *et al.* 2018). Long generation time and overlapping gen erations could also reduce genetic loss. Multiple repro ductive outputs in long lived species result in higher genetic contribution to subsequent generations com pared with one or few reproductive outputs. When a population passes through a bottleneck, reduction of genetic diversity depends on population size and time, measured in number of generations (Hailer *et al.* 2006). Overlapping generations, often associated with a long generation time, thus reduce the loss of genetic diversity by allowing multiple cohorts simultaneously to be part of a genetic pool.

The Mascarene Petrel is a primarily monogamous and long lived species with delayed sexual maturity (Schreiber & Burger 2001) and an estimated genera tion time of 17.7 years according to Birdlife Interna tional. Thus, we can expect that the loss of genetic diversity is less important than expected in this species even if it has a small population size. The objective of this study was to conduct a first assessment of the evolutionary potential of Mascarene Petrels, thereby informing future conservation measures and to see whether the results support the hypothesis that life history traits could preserve allelic diversity in small populations. We identified and characterized polymor phic microsatellite loci in order to investigate: (1) genetic diversity and inbreeding, (2) contemporary effective population size and (3) a potential popula tion bottleneck in this species.

METHODS

Total genomic DNA was extracted from whole blood samples of 22 Mascarene Petrels found grounded during the seabird rescue campaign on Reunion Island from 2008 to 2018. Blood samples were stored in 70% etha nol, and DNA was then extracted with the QIAmp Blood & Tissue kit (Qiagen).

A genomic library was constructed using DNA of one bird. Fifteen microsatellite sequences were isolated by Ecogenics GmbH (Zurich, Switzerland; Data S1).

A 3 primer PCR approach using an M13 tail (5' CACGACGTTGTAAAACGAC 3') for the forward pri mer was used for microsatellite loci amplification of DNA from all 22 birds, following Schuelke (2000). Four different dyes (6 FAM, PET, VIC, NED) were used for

the universal M13 forward primer to enable fragment analysis multiplexing. Simplex PCR amplifications, tar geting one locus at a time, were performed using a Gen eAmp PCR System 9700 (Applied Biosystems) in a 10 μL reaction volume containing 5 μL of MasterMix Applied 2x (Applied Biosystems), 0.025 μM of the for ward primer with M13 5' tail, 0.25 μM of the reverse primer, 0.25 μM of dyes and 2 μL of genomic DNA (15 ng/μL). PCR amplifications were carried out under the following conditions: an initial denaturing step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, annealing temperature for 30 s (Table S1) and 72 °C for 30 s, and a final elongation at 72 °C for 20 min. Two different simplex PCR plates, each with a different dye, were mixed and PCR product sizes were determined using a 3730XL DNA analyser (Applied Biosystems) and sized with a LIZ (500) standard using GeneMapper (Applied Biosystems).

For each locus, evidence for null alleles, large allele dropout and stutter peaks were examined using Micro Checker (Van Oosterhout *et al.* 2004). The test for link age disequilibrium among loci was conducted with GENEPOP (Rousset 2008) using a Benjamini and Yeku tieli correction (Benjamini & Yekutieli 2001, Narum 2006). Diversity indices were calculated with GenAlex (Peakall & Smouse 2012). Hardy Weinberg Equilibrium (HWE) was examined with GENEPOP (Rousset 2008) using an exact test with 100 batches and 1000 iterations per batch for each locus and a Chi square test for global HWE. Contemporary genetic effective population size (Ne) was estimated using the VarEff 1.2 R package (https://forge dga.jouy.inra.fr/). In addition, past changes in effective population size from microsatellite data (Nikolic & Chevalet 2014) were estimated with the approximate likelihood Markov chain Monte Carlo (MCMC) approach in the VarEff 1.2 R package (https://forge dga.jouy.inra.fr/). The mutation rate of microsatellite markers used in theVarEff 1.2 R package was calculated according to the method of Storz and Beaumont (2002) implemented in the MSVAR 1.3 pro gram. The likelihood frequency of bottleneck events from the time to the most recent common ancestor (TMRCA) detected has been estimated with a new function source (BTLIKE, with free access and help sup port on the website https://forge dga.jouy.inra.fr/). Finally, we used Bottleneck 1.2.02 (Piry *et al.* 1999) to test for recent changes in Ne using three different tests based on expected heterozygosity excess: Wilcoxon sign rank test (two tailed for heterozygosity excess or defi ciency), sign test and standard difference test (100 000 iterations; Cornuet & Luikart 1996), and under two mutation models: a two phase (TPM; non step wise 5%, variance 12; typical values for many microsatellite markers; Busch *et al.* 2007) and a stepwise mutation model (SMM).

RESULTS

Only one locus (Pseate 7805) had null alleles. Analyses were performed without this locus and these did not differ from analyses with all markers, so it was kept in this study. No large allele dropout and no stutter peaks were detected for any of the loci. No significant linkage disequilibrium was observed among the loci ($P > 0.05$). The number of alleles per locus (Na) ranged from 3 to 16, with an average of 7.27 (Table 1). The mean number of effective alleles (Nef) (from Kimura & Crow 1964) was 4.01 per locus and was always lower than Na, highlighting the presence of rare alleles. The observed (Ho) and expected (He) heterozygosities had means of 0.68 and 0.67, respectively. Ho reached 1 for locus Pseate 5563, which is 100% heterozygous for this locus. No significant deviation from HWE was found in the population and the mean Fis was negative (0.051) and non significant (P 0.213). With regard to loci, only locus Pseate 7805 showed significant deviation from HWE (P 0.001).

The 15 microsatellite markers shared a similar range of mutation rate of around 3×10^{-4} (sd 0.0004) with MSVAR estimates. The estimate of contemporary effective population size (Ne) from 22 stranded individuals was 1211 individuals in harmonic mean (95% confidence interval (CI) 489 7999) from VarEff analysis. Estimates of the posterior distribution of past effective population sizes revealed a relatively large ancestral population size, three times larger than the current estimate (current theta index 4.405, past theta index 12.886). Analysis of the posterior distribution of the TMRCA showed a clear peak about 10 000 generations ago and confirmed a decrease in population size in the past, with a smaller peak at about 500 generations (Figure S1). The two bottlenecks detected had high confidence intervals of 94% but the beginning of the oldest peak interval (10 000 generations) was unclear due to the mixed signals of overlapping peaks. Bottleneck analysis based on expected heterozygosity excess also showed clear support for a recent population contraction. Both TMP and SSM models were consistent with the scenario of a bottleneck according to the results of Wilcoxon sign rank test, sign test and standard difference test (TPM: P 0.041, 0.002 and 0.073, respectively; SSM: P 0.011, 0.000 and 0.030, respectively).

DISCUSSION

These results show the importance of understanding genetic diversity in rare and critically endangered species. Indeed, our results suggest that an assumption of low genetic diversity in very rare species is not always supported and that conservation of the Mascarene Petrel may be successful despite a very small population size. It is interesting to note that Mascarene Petrels have a relatively high genetic diversity (highlighted by the analysis of Na and Nef values) for a rare and threatened species that appears to have suffered a recent bottleneck (Ardern & Lambert 1997, Spielman *et al.* 2004). *Nef*

Table 1. Results of initial microsatellite primer screening for Mascarene Petrels.

Locus	n	Na	Nef	Ho	He	Fis	HWE test
Pseate 143	20	9	2.996	0.600	0.666	0.099	0.052
Pseate 255	22	8	5.469	0.864	0.817	0.057	0.647
Pseate 321	22	6	3.025	0.773	0.669	0.154	0.926
Pseate 1317	22	7	3.546	0.591	0.718	0.177	0.453
Pseate 1500	22	3	1.865	0.500	0.464	0.078	1.000
Pseate 1594	22	5	3.315	0.727	0.698	0.041	0.748
Pseate 1603	22	12	4.914	0.773	0.796	0.030	0.050
Pseate 1837	22	4	1.537	0.409	0.349	0.172	1.000
Pseate 3421	22	4	1.624	0.455	0.384	0.183	1.000
Pseate 3689	22	4	2.127	0.636	0.530	0.201	0.702
Pseate 5557	22	3	2.451	0.727	0.592	0.229	0.611
Pseate 5563	22	16	12.253	1.000	0.918	0.089	0.966
Pseate 7441	22	13	6.127	0.909	0.837	0.086	0.133
Pseate 7805	22	9	5.319	0.636	0.812	0.216	**0.001**
Pseate 8002	22	6	3.639	0.727	0.725	0.003	0.437
All loci							
Mean	21.867	7.267	4.014	0.688	0.665	0.051	0.213
se	0.133	1.012	0.698	0.043	0.044	0.035	--

Fis coefficient of consanguinity (Wright 1931); *He* expected heterozygosity; *Ho* observed heterozygosity; *n* number of individuals; *Na* total number of alleles per locus; *Nef* number of effective alleles per locus; HWE test *P*-values of the Hardy Weinberg equilibrium exact test (significant values in bold, *P* < 0.05).

values are also lower than *Na* values, which may suggest the presence of rare alleles in this population. Inbreeding and substructuring (Wahlund effect) have not been detected in the population, as indicated by the absence of a significant deviation from HWE (Wright 1969, Gar nier Géré & Chikhi 2001).

Assuming an average generation length of 17.7 years for this species (Birdlife International 2018), the two peaks observed with the VarEff package would then indicate two population bottlenecks about 8850 and 177 000 years ago. The first event was consistent with the tests for heterozygosity excess in the Bottleneck pro gram, which is detectable over a relative short period $2Ne$ $4Ne$ generations in the past (Cornuet & Luikart 1996), and both analyses (VarEff package and Bottle neck) converged towards a reduction in population size.

Despite these findings, we interpret our results with caution because our sample size is small and estimates of the time of the demographic event could be more biased and subject to higher error than are estimates of *Ne* (Cabrera & Palsbøll 2017). Cornuet *et al.* (2008) suggested that the poor estimation performance in time of the demographic event is caused by insufficient data. Moreover, the signal for a population decrease could also be due to population structure, which is known to generate spurious signals of bottlenecks (Nikolic & Che valet 2014, Mazet *et al.* 2015). VarEff can be used to detect the effect of structure by varying the sampling strategy (Nikolic & Chevalet 2014, Salmona *et al.* 2017) but we were limited in this study by the small number of individuals sampled.

High genetic diversity has already been observed in some vulnerable procellariiform species. Hale *et al.* (2015) reported high genetic diversity in two relict Hut ton's Shearwater *Puffinus huttoni* breeding populations. They found an average of 6.89 alleles/locus with an average *Ho* (observed heterozygosity) of 0.49 in the first population (*n* 39), and 7.33 alleles/locus with *Ho* 0.45 for the second population (*n* 30). Gravley *et al.* (2019) also found high genetic diversity in two subspecies of Northern Fulmar (*Fulmarus glacialis rodger sii* and *Fulmarus glacialis auduboni*). They found an aver age of 5.0 alleles/locus with an average *Ho* of 0.40 (*n* 39) for the first species (*F. g. rodgersii*), and an average of 6.1 alleles/locus with an average *Ho* of 0.45 for the second (*F. g. auduboni*) (*n* 30). Loss of genetic diversity should occur whenever a population goes through a bottleneck (Nei *et al.* 1975) and relatively high genetic diversity in a rare species can indicate that decline in population size is recent (Moritz 2002).

Population contraction over a large number of gener ations may be required for loss of genetic diversity but this effect could be influenced by a species' life history traits, such as long generation times, delayed sexual maturity and/or the rate of demographic fluctuation (Kuo & Janzen 2004). This phenomenon has been observed in other bottlenecked populations (Hailer *et al.* 2006, Pickles *et al.* 2012) and supports the hypothesis that monogamy and long generation time could slow genetic diversity loss after the detected bottleneck in the Mascarene Petrel population. For example, Hailer *et al.* (2006) argue that the long generation time of White tailed Eagles *Haliaeetus albicilla* has acted as an intrinsic buffer against loss of genetic diversity in a bottlenecked population. Weiser *et al.* (2013) compared the loss of genetic diversity following a bottleneck in three species with different life history traits (monogamous/polyga mous, long/short generation time); they showed that a monogamous species with a long generation time had the highest probability of retaining rare alleles without immigration in the population. Furthermore, Milot *et al.* (2007) simulations on Wandering and Amsterdam alba trosses (*Diomedea exulans* and *Diomedea amsterdamen sis*), which have respectively a large and small population size, support the hypothesis that genetic diversity in these two long lived species was already depleted prior to their divergence. Amsterdam Albatross would be able to maintain its genetic variation over a very long period of time, due to its life history traits: long life span, high reproductive success, inbreeding avoidance behaviours (e.g. Amos & Balmford 2001) and odour based individual recognition (e.g. Bonadona & Nevitt 2004). Although there are some limitations in comparing genetic diversity across microsatellite studies, our study adds to a body of work that suggests that long lived species may be able to maintain their genetic variability, a key factor enabling survival of a natural population (Frankham 2005).

Our results suggest that there is still high genetic diversity in the Mascarene Petrel, supporting the argu ment that conservation efforts are not too late for this species. Further studies of diversity and genetic structur ing, associated with other markers such as single nucleotide polymorphisms (SNPs), and of additional individuals sampled from the newly discovered breeding sites could help to better understand this species and contribute to more informed conservation planning.

AUTHOR CONTRIBUTION

Jade Lopez: Formal analysis (equal); Investigation (equal); Validation (equal); Visualization (equal); Writing original draft (equal); Writing review & editing (equal). **Natacha Nikolic:** Formal analysis (equal); Investigation (equal); Methodology (equal); Software (equal); Validation (equal); Visualization (equal); Writing review & editing (equal). **Martin Riethmuller:** Resources (equal). **Jerome Dubos:** Resources (equal); Writing review & editing (equal). **Patrick Pinet:** Funding acquisition (equal); Project administration (equal); Supervision (equal); Writing review & editing (equal). **Patxi Souharce:** Resources (equal). **François-Xavier Couzi:** Resources (equal). **Matthieu Le Corre:** Conceptualization (equal); Funding acquisition (equal); Investigation (equal); Project administration (equal); Supervision (equal); Validation (equal); Writing original draft (equal); Writing review & editing (equal). **Audrey Jaeger:** Investigation (equal); Supervision (equal); Validation (equal); Writing original draft (equal); Writing review & editing (equal). **Laurence Humeau:** Conceptualization (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Project administration (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing original draft (equal); Writing review & editing (equal).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are present in Table S2; genotype of the 22 individuals of Mascarene Petrel (*Pseudobulweria aterrima*) for the 15 microsatellite markers used.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Methods for the development of the microsatellite markers by Ecogenics GmbH (Zurich, Switzerland).

Table S1. Characteristics of 15 microsatellite markers developed for Mascarene Petrels. Annealing temperature (Ta).

Table S2. Genotype of the 22 individuals of Mascarene Petrel (*Pseudobulweria aterrima*) for the 15 microsatellite markers used.

Figure S1. Posterior distribution of the time to most recent common ancestor allele (TMRCA). Each peak represents a potential bottleneck.