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Isolation and characterization of 50 microsatellite loci for two shearwater species, *Ardenna pacifica* and *Puffinus bailloni*

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Abstract :

Background

Shearwaters (order Procellariiformes) are an excellent study system to investigate the genetic consequences of the co-called "seabird paradox", as they are able to disperse long distances but many species exhibit natal and breeding philopatry. However, few microsatellite markers are currently available for these taxa, hampering genetic inferences.

Methods and results

In this study, 25 novel microsatellite loci were isolated and characterized for each of two distantly related shearwater species: the wedge-tailed shearwater (Ardenna pacifica) and the tropical shearwater (Puffinus bailloni). Polymorphism tests were performed for a total of 91 A. pacifica individuals sampled at Reunion and Round Island, and 48 P. bailloni individuals from Reunion and Europa Island, in the western Indian Ocean. The analyses revealed 23 polymorphic loci for A. pacifica, with the number of alleles per locus (Na) ranging from 2 to 8 (mean = 3.957 ± 0.364). Nineteen polymorphic loci were found for P. bailloni, with Na varying from two to five (mean = 3.053 ± 0.247). The observed heterozygosity (Ho) was relatively low for the two species, with Ho ranging from 0.022 to 0.725 (mean = 0.326 ± 0.044) for A. pacifica and from 0.021 to 0.688 (mean = 0.271 ± 0.051) for P. bailloni, but comparable to the estimates available for other Puffinus species.

Conclusions

The new microsatellite loci provide a valuable tool for further population genetic studies, and will allow for design of effective conservation and management plans for A. pacifica, P. bailloni and other closely-related species.

Keywords : Seabirds, Wedge-tailed shearwater, Tropical shearwater, Microsatellites, Population genetics, Conservation

Introduction

Seabirds, one of the most diverse group of avifauna, are distributed across all oceans and marine ecosystems of the world, and are renowned for their ability to migrate long distances [1]. Shearwaters (order Procellariiformes) are highly pelagic seabirds, which undertake long-distance non-breeding migrations and large-scale movements when central placed foragers during breeding [2]. In contrast, many shearwater species exhibit natal philopatry and return repeatedly to the same colony for breeding, often close to their natal nest site. This is often described as the "seabird paradox" [3]. Although this behavior may increase the probability that adequate resources and mates are available for reproduction [4], it may also result in limited gene flow between colonies, high levels of endemism, and in an increased risk of inbreeding depression and vulnerability to stochastic demographic fluctuations [5].

The taxonomic history of shearwaters has been revised repeatedly [6–8]. A recent study using genomic data from 25 of the 32 recognized shearwaters species provided the first well-resolved phylogeny for this taxa [9]. The study supported the monophyly of the three genera of shearwaters, *Ardenna*, *Calonectris* and *Puffinus*, and positioned *Ardenna* and *Calonectris* as sister genera [9]. The three genera exhibit differences in species richness [10], and are an ideal study system to investigate the genetic processes underlying species diversification in marine ecosystems. However, molecular nuclear markers are still unavailable for most of the shearwaters species (but see [11–13]), which limits our ability to explore these genetic processes.

In this study, we identify and characterize new polymorphic microsatellite loci for two distantly related shearwater species: the wedge-tailed shearwater (*Ardenna pacifica*, Gmelin 1789) and tropical shearwater (*Puffinus bailloni*, Bonaparte 1857). These microsatellite markers will provide a valuable tool for population genetic studies and allow researchers to identify conservation or management units, made up of one or more connected breeding colonies, and hence develop effective conservation actions for *A. pacifica* and *P. bailloni*, and potentially for other closely-related species.

Materials and methods

A. pacifica ranges throughout the tropical and subtropical waters of the Indian and Pacific Oceans (latitudes 35°N and 35°S) and is known to breed on a large number of oceanic islands

and on the east and west coast of Australia [14]. In contrast, the congener *P. bailloni* is widely distributed in the western tropical Indian and Pacific Oceans. In the western Indian Ocean the main colonies are in the Seychelles, the Chagos Archipelago, Reunion and Europa Island [15]. For the present study, biological samples were collected in two study sites for each species between 2009 and 2019. *A. pacifica* individuals were sampled in Reunion (21°70 S, 55°31 E) and Round Island (Mauritius; 19°51 S, 57°47 E), in the western Indian Ocean. *P. bailloni* were sampled in Reunion and Europa Island (southern Mozambique channel; 21°21 S, 40°22 E). Two distinct breeding colonies were selected for each species to allow for potential variation in microsatellite genotypes. Shearwaters were captured at breeding colonies by hand. Additionally, samples from all *P. bailloni* from Reunion and some *A. pacifica* were collected from dead birds, fatally injured as a consequence of light pollution [16]. Blood samples (approx. 0.5 ml) were collected from live birds from the medial metatarsal or basilic veins, while muscle samples (approx. 10 g) were collected from dead animals. All samples were preserved in 70% ethanol. A total of 91 *A. pacifica* (55 from Reunion and 36 from Round Island) and 48 *P. bailloni* (30 from Reunion and 18 from Europa Island) samples were available for genetic analyses. Additionally, three *A. pacifica* and five *P. bailloni* blood samples from Cousin Island (Seychelles; 4°20 S, 55°40 E) were available from previous field expeditions and included in the microsatellite library.

A microsatellite library was designed and established for each shearwater species by GenoScreen (Lille, France). Genomic DNA was extracted from whole blood and muscle samples using the NucleoSpin Tissue kit (Macherey-Nagel), following the manufacturer's protocol. A total of nine individuals for *A. pacifica* and ten for *P. bailloni* were equimolarly pooled and 1µg of DNA was used for the development of the genomic library. The libraries were analyzed on an Illumina MiSeq platform to generate 250bp paired-end reads using the MiSeq Reagent Nano Kit v2. A total of 1,128,338 reads were obtained for *A. pacifica* and 1,211,564 for *P. bailloni*. The resulting sequences were assembled using Usearch software, producing 131,356 contigs for *A. pacifica* and 161,218 contigs for *P. bailloni*. The bioinformatics program QDD v3 [17] was used to analyze the sequences. All bioinformatic steps from the raw sequences until obtaining the primers were performed using this software, including the removal of adapters/vectors, the detection of microsatellites, the detection of redundancy/possible mobile element association, the selection of sequences with target microsatellites, and the primer design using BLAST, ClustalW and Primer3

117 software. A total of 2,524 primer sets were finally designed on suitable microsatellite flanking
118 regions for *A. pacifica* and 3,102 for *P. bailloni*. Of these, 421 primer pairs were selected for *A.*
119 *pacifica* and 507 for *P. bailloni*, by keeping only perfect di/tri/tetra motives, with A and B quality
120 design (from internal parameters of QDD), and with at least 20bp between primer and
121 microsatellite. Finally, 95 microsatellite loci were randomly selected for preliminary molecular
122 tests for each shearwater, using DNA of eight individuals. Thereafter, a subset of 48 microsatellite
123 loci were tested for polymorphism for each species, using DNA from an additional fifteen
124 individuals (2 from Round Island, 10 from Reunion and 3 from Cousin Island for *A. pacifica,* and
125 one from Europa Island, 9 from Reunion and 5 from Cousin Island for *P. bailloni*). Polymerase
126 Chain Reaction (PCR) was performed for each microsatellite locus in a 10 µl reaction containing
127 0.5 U of TAQ DNA polymerase, 6 pmol of dNTP, 37.5 pmol of $MgCl_2$, 10 pmol of each forward
128 and reverse primer, and 1 µl (~ 10 ng) of DNA template. PCR amplifications were carried out
129 using the following thermal conditions: initial denaturation step at 95 ºC for 15 min, followed by 40
130 cycles of 95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 60 s, and a final extension step at 72 ºC for
131 10 min. PCR products were separated on an ABI 3730XL DNA analyzer (Applied Biosystems)
132 and sized with GeneScan-500 LIZ size standard. All molecular analyses were performed by
133 GenoScreen (Lille, France).
134 Microsatellite alleles were scored using GeneMapper® v4.0 (Applied Biosystems). The
135 twenty-five best microsatellite loci for each study species (di- and trinucleotide repeats) were then
136 selected for multiplex development, based on the allelic diversity, fragment size and peak patterns
137 in the electropherograms. Five multiplex reactions were developed for each shearwater species
138 with the help of the Multiplex Manager v1.2 [18]. Each multiplex included primers that amplify
139 fragments of different size and were labelled with four fluorescent dyes (6-FAM, PET, VIC, NED)
140 to enable fragment analysis multiplexing. Multiplex conditions were optimized using seven
141 individuals per study species (5 individuals from Reunion and 2 from Cousin Island for *A. pacifica*,
142 and 7 individuals from Reunion for *P. bailloni*). PCR were subsequently performed for the entire
143 *A. pacifica* (n = 91) and *P. bailloni* (n = 48) dataset, considering the same conditions and thermal
144 cycle as for the simplex PCR, but with an uneven quantity of primer per multiplex (see Table 1 for
145 details).

Evidence of null alleles, large-allele dropout, and stutter bands were examined for each locus with MicroChecker 2.2.3 [19]. Genepop 4.7.5 [20, 21] was used to test for deviations from Hardy–Weinberg equilibrium (HWE) for each locus, and to test for linkage disequilibrium for each locus-pair combination. The p-values were adjusted using the Benjamini and Yekutieli correction method for multiple comparisons [22, 23]. The mean observed number of alleles per locus (Na), observed heterozygosity (H_O), and unbiased expected heterozygosity (uH_e) estimated according to Nei [24] were computed using GenAlEx 6.5 [25]. To ensure that the set of microsatellite loci could reliably identify unique genotypes for the two species, the probability of identity (pID; [26]) was computed using GenAlEx 6.5 [25].

Results and discussion

The initial microsatellite primer screening revealed twenty-three polymorphic loci for *A. pacifica* (Table 2) and nineteen for *P. bailloni* (Table 3), which amplified a total of 91 and 58 alleles for each study species. Despite the uneven sample sizes (n = 91 for *A. pacifica* and n = 48 for *P. bailloni)*, the number of alleles per locus (Na), and observed (H_O) and expected unbiased (uH_e) heterozygosities were comparable for the two shearwater species. The Na for *A. pacifica* ranged from two to eight (mean = 3.957 ± 0.364), and the Na for *P. bailloni* from two to five (mean = 3.053 ± 0.247). For *A. pacifica*, the H_O varied from 0.022 to 0.725 (mean = 0.326 ± 0.044) and the uH_e from 0.022 to 0.730 (mean = 0.343 ± 0.044; Table 2). Likewise, the H_O for *P. bailloni* ranged from 0.021 to 0.688 (mean = 0.271 ± 0.051) and the uH_e from 0.021 to 0.709 (mean = 0.298 ± 0.053; Table 3). No heterozygote deficit was detected when combining the two study sites for each shearwater species (i.e.; global F_{IS} was not significantly different from zero for both *A. pacifica* and *P. bailloni*), suggesting that we did not violate the assumption of population panmixia in the present study.

No large-allele dropout and stutter peaks were detected for the two shearwaters. However, two loci (Pb72 and Pb83) exhibited null alleles for *P. bailloni* and three loci (Ap19, Ap23, Ap36) for *A. pacifica*. No significant linkage disequilibrium was detected among any locus-pairs for the two study species, after correcting for multiple tests comparison. Significant deviations from HWE were found for three loci (Ap19, Ap21, Ap36) for *A. pacifica,* after Benjamini and Yekutieli correction (p-value > 0.05). All the loci developed for *P. bailloni* were in HWE (Table 2

and Table 3). The probability of identity (pID) using the present set of loci was 9.27×10^{-9} for *A. pacifica* and 3.01×10^{-6} for *P. bailloni*, suggesting that the probability of two unrelated individuals drawn randomly from our dataset sharing the same multilocus genotype is approximately zero.

Overall, the genetic diversity indices for *A. pacifica* and *P. bailloni* were comparable to previous estimates for other *Puffinus* species (e.g., $H_O = 0.377 \pm 0.241$ for *Puffinus mauretanicus* [11]; $H_O = 0.436 \pm 0.257$ for *Puffinus yelkouan* [11]; $H_O = 0.549 \pm 0.175$ for *Puffinus carneipes* [12]), but lower than those reported for other local seabirds using microsatellites datasets (e.g., *Onychoprion fuscatus* and *Pseudobulweria aterrima*; [27, 28]).

The deficit of heterozygotes observed in *P. bailloni* may reflect the smaller distribution of the species and the potential isolation of the colonies, as *Puffinus* are known to be extremely philopatric [11]. The results are, however, remarkable for the widely-distributed and abundant *A. pacifica*. It is important to emphasize that the polymorphism of each 25 microsatellite loci was evaluated with individuals from only two study sites per species. Microsatellite loci may exhibit new alleles when genotyping individuals from other geographic areas and, consequently, display a higher genetic variance than revealed in this study.

Altogether, the newly developed microsatellite loci provide a valuable tool to investigate genetic diversity, population genetic structure, population connectivity and reconstruct the demographic history of *A. pacifica* and *P. bailloni* at a large geographic scale. Although the new primer pairs have not been tested for cross-amplification in both of our study species or in other seabird species, there is potential application of these markers in other closely related species, for which genetic studies are a priority and microsatellite markers are not yet available (e.g., [27, 29]). Future molecular studies are needed to investigate the cross-species transferability and genetic variability of the newly described microsatellites loci in other shearwater species.

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Author Contributions: The study was conceptualized and designed by Laurence Humeau and Matthieu Le Corre. Field work was organized by Matthieu Le Corre, Vikash Tatayah, Nirmal Jivan Shah and Malcolm Nicoll. Data collection were performed by Matthieu Le Corre, Audrey Jaeger, Arthur Choeur, Merlène Saunier, François-Xavier Couzi, Vikash Tatayah, and Naïs Avargues. Data analysis was performed by Helena Teixeira and Laurence Humeau. The first draft of the manuscript was written by Helena Teixeira and all authors revised the manuscript. All authors read and approved the final manuscript.

Ethics approval: Bird capture, handling and sample collection were approved and carried out in concordance with the principles of the Centre de Recherche sur la Biologie des Populations d'Oiseaux (PP 616 and banding authorization 44 of MLC; CRBPO, National Museum of Natural History, Paris). In addition, sample collection in Europa was conducted under the approval of the Terres Australes et Antarctiques Françaises (TAAF). Sample collection and exportation permits at Round Island were granted by the National Parks and Conservation Service (NPCS) (Government of Mauritius). Sample collection and exportation permit at Cousin Island were granted by the Seychelles Bureau of Standards.

Data availability: Sequences of the twenty-five microsatellites markers developed for *Ardenna pacifica* and *Puffinus bailloni* are publicly available in GenBank under the accession numbers ON854684 – ON854733.

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327 **Table 1.** Characteristics of the five multiplexes developed for *Ardenna pacifica* and *Puffinus*
328 *bailloni*.

Ardenna pacifica				*Puffinus bailloni*			
Locus	**Multiplex**	**Dye**	**Quantity (pmol)**	**Locus**	**Multiplex**	**Dye**	**Quantity (pmol)**
Ap19	M1	6-FAM	1.6	Pb53	M1	6-FAM	5.2
Ap83	M1	VIC	0.8	Pb26	M1	6-FAM	2.6
Ap06	M1	VIC	2.5	Pb80	M1	VIC	1.2
Ap26	M1	NED	1.0	Pb22	M1	NED	1.8
Ap58	M1	PET	2.5	Pb56	M1	PET	1.5
Ap07	M2	6-FAM	2.6	Pb06	M2	6-FAM	1.6
Ap03	M2	VIC	0.8	Pb43	M2	VIC	5.2
Ap08	M2	VIC	3.0	Pb05	M2	VIC	3.4
Ap73	M2	NED	1.4	Pb28	M2	NED	1.0
Ap92	M2	PET	4.0	Pb40	M2	PET	2.2
Ap21	M3	6-FAM	2.0	Pb46	M3	6-FAM	2.0
Ap23	M3	VIC	1.0	Pb72	M3	VIC	5.8
Ap32	M3	NED	0.8	Pb90	M3	NED	0.8
Ap36	M3	PET	5.6	Pb48	M3	NED	2.0
Ap09	M3	PET	5.2	Pb66	M3	PET	2.6
Ap48	M4	6-FAM	1.6	Pb84	M4	6-FAM	2.0
Ap15	M4	6-FAM	5.0	Pb16	M4	VIC	1.6
Ap29	M4	VIC	1.6	Pb64	M4	NED	1.0
Ap79	M4	PET	1.4	Pb83	M4	NED	1.0
Ap57	M4	PET	3.0	Pb93	M4	PET	1.6
Ap25	M5	6-FAM	3.0	Pb55	M5	6-FAM	1.6
Ap44	M5	6-FAM	8.0	Pb63	M5	6-FAM	5.4
Ap27	M5	NED	2.0	Pb21	M5	VIC	1.4
Ap37	M5	NED	6.4	Pb92	M5	NED	1.2
Ap10	M5	PET	1.4	Pb82	M5	PET	1.6

329 Multiplex, multiplex mix in which the locus was amplified; Dye, fluorescent label of the microsatellite primers;
330 Quantity, Quantity of primers used per PCR reaction.

331

Table 2. Results of the initial microsatellite primer screening in *Ardenna pacifica*. Analyses were performed using 91 individuals from Reunion and Round (Mauritius) Island.

Locus	Accesion no.	Primer sequences (5' – 3')	Repeat motif	Fragment size (bp)	N	Na	Ho	uHe	HWE	Null alleles
Ap19	ON854691	F: ACCAGCCTGCAGATTTAGGA R: TGAAACAGGCAAATGAGCGC	(AC)10	140 - 142	91	2	0.088	0.161	0.010**	Yes
Ap83	ON854707	F: GGGTGCAAAGGACCTTCAGT R: AGGATCTTTGTTCCCTCAAGCA	(AC)7	200 - 204	90	3	0.067	0.065	1.000	No
Ap06	ON854685	F: GTGAGGTGTTCTCCAGGAGG R: GAGAAGTGAGGAGATGGGCT	(AG)13	301 - 321	91	7	0.495	0.562	0.945	No
Ap26	ON854695	F: ACAGAGGACAAAGCAAATATTAGCC R: ACACTGAATTGTTTGTACAGGCC	(AG)9	197 - 203	91	3	0.495	0.475	1.000	No
Ap58	ON854704	F: TCCATGGATTTGTTACAGGAGT R: ACAGAGAATGCCTGACTTTGGT	(AGC)8	204 - 207	91	2	0.352	0.357	1.000	No
Ap07	ON854686	F: ACCTGACCAACTTCGAAGCA R: GCATCTGTGCCAGTGAGATT	(AG)13	267 - 275	87	5	0.678	0.638	1.000	No
Ap03	ON854684	F: AGTGCCTACGCCATCATAGC R: TGGAGATTTGCACCCTTCCC	(AAC)16	168 - 189	91	8	0.725	0.730	1.000	No
Ap08	ON854687	F: ACTGTGAAGTGAGCACTGAGA R: GGAACACCCTACAGCAGATCA	(AC)12	238 - 248	91	5	0.341	0.295	1.000	No
Ap73	ON854705	F: AGTAGAGGAATATGCACCTGGT R: CGCTTCCGTGTACTTCTTCC	(AG)8	218	91	1	–	–	–	–
Ap92	ON854708	F: CCTCCTCTTAAGATTACCTACCAAT R: GCCCACCATGATAATGCGGT	(AT)7	163 - 189	82	7	0.305	0.332	0.302	No
Ap21	ON854692	F: TCCTCCCAGATCTTCTCCGG R: AGAATTGCGAGCCTGGATCC	(AC)10	231 - 239	91	5	0.505	0.453	0.000***	No
Ap23	ON854693	F: GAACAGATTATTCCTTTCCACTCTGC R: GTTGACCAGCTGGAGAGTCC	(AC)9	144 - 148	90	3	0.300	0.407	0.056	Yes

Ap32	ON854698	F: AAGCAGCTTCTAATGCAGGT R: AACCTTGCGTGTTGTAGGCT	(AC)9	151 - 155	89	3	0.225	0.252	0.991	No
Ap36	ON854699	F: TTGCCTTTCTTCTCTGCTTCTG R: AACTGCATAAGTATAGAACATTGTGC	(AG)8	194 - 200	91	4	0.275	0.401	0.010**	Yes
Ap09	ON854688	F: CCAAGTTTCAGTTACCTATCTGGG R: TCCTTGGAGATCTTGTGAACAGT	(AC)11	295 - 299	89	3	0.056	0.076	0.465	No
Ap48	ON854702	F: TGGGTAACAGGTCTCCAGGT R: CTGGCCGAGAAAGCATACCA	(ACC)8	169	91	1	–	–	–	–
Ap15	ON854690	F: TCCTGCAACAGAACGTCTCA R: TGCTGTGACTTTACAAGAACTGG	(AG)10	295 - 299	90	3	0.267	0.292	0.485	No
Ap29	ON854697	F: CAAGCTCAGTTGAAACGGGC R: TAGACCAAGCCCTGACCCTT	(AC)9	224 - 228	90	3	0.544	0.565	0.868	No
Ap79	ON854706	F: CCTGGAGTGAGCAGATAGGTG R: GCCAAATTAAGGCCAACTGCA	(AC)7	140 - 142	90	2	0.433	0.451	1.000	No
Ap57	ON854703	F: GAGGGCAAAGGCAATTTCGG R: GGAACACTCTTCTGGCTCCC	(AC)8	235 - 237	90	2	0.044	0.044	1.000	No
Ap25	ON854694	F: TGTCATGGCATTGAACTGCTG R: AGGAAGACTCTGTCTATATGCCT	(AC)9	184 - 194	91	4	0.154	0.166	0.945	No
Ap44	ON854701	F: TCCCATGGATAACTGCACGC R: GGCGTAAACACTGGCAACTT	(AAT)8	291 - 318	90	5	0.156	0.148	1.000	No
Ap27	ON854696	F: CTCCTTAATAGGCTATCGCAGTCC R: AGGAATTGTCTTGATTTCGGTGT	(AC)9	243 - 253	91	6	0.670	0.713	1.000	No
Ap37	ON854700	F: AAGAGCTAGTGCAGAAGTGAC R: ACATCCTGGAGTCAGAGATGA	(AC)8	303 - 307	91	3	0.022	0.022	1.000	No
Ap10	ON854689	F: GCTCACCACCATTTATCCACC R: GCAACTGGGAGGAACACCAT	(AC)11	221 - 225	91	3	0.297	0.275	1.000	No

F, sequence of forward primer, R, sequence of reverse primer; Fragment size, observed allele size range; N, sample sizes per locus; Na, number of alleles per locus across samples; Ho, observed heterozygosity; uHe, unbiased expected heterozygosity; HWE, p-value for departure from Hardy–Weinberg equilibrium test; Null alleles, presence (Yes) or absence (No) of null alleles.

* p-value < 0.5, ** p-value < 0.1, *** p-value < 0.01

339 **Table 3.** Results of the initial microsatellite primer screening in *Puffinus bailloni*. Analyses were performed using 48 individuals from Reunion and Europa

340 Island.

Locus	Accesion no.	Primer sequences (5' - 3')	Repeat motif	Fragment size (bp)	N	Na	Ho	uHe	HWE	Null alleles
Pb53	ON854720	F: TGAATTTGGGTTGTCTTGGTCA R: CAGCTTTACGCATGCACTGT	(AT)8	244 - 248	48	3	0.458	0.424	1.000	No
Pb26	ON854714	F: GGGAAGCTGGGAAGATGAAAGA F: ACAGAGCTGATATAAGGTGCTAAA	(AC)9	286 - 296	48	4	0.271	0.259	0.440	No
Pb80	ON854727	F: TCAAGCAACACAGGGTACGG R: AGATTCTGTGCTTCTGCCCA	(AG)7	189	48	1	–	–	–	–
Pb22	ON854713	F: AGCTGGTTGGCATCTCACAA R: GTGCACAAGTCCAGCAATGG	(AC)9	248	48	1	–	–	–	–
Pb56	ON854722	F: CCCAGAGAATACCACTGACCG R: CCACTGGAGAAGGTTGCAGA	(AC)8	250 - 258	48	3	0.458	0.503	0.829	No
Pb06	ON854710	F: GGCTTCCTAGGAACACCTGA R: AGCATCCCTATCAGAATGGCC	(AC)12	153 - 161	48	5	0.125	0.121	1.000	No
Pb43	ON854717	F: TCTTTGGGTTACGGAGAATTCA R: AGACCCACAGGCTCTGATGT	(AG)8	246	48	1	–	–	–	–
Pb05	ON854709	F: GGGTTACCATGTCTGAGACCA R: TGCTCTACCTGGGAATGGGA	(AC)13	289 - 301	48	5	0.688	0.666	1.000	No
Pb28	ON854715	F: AGCTGTGCTCTGTTAGGTCTC R: GCGTGCACAAAGGCTGTAAG	(AC)9	126 - 128	48	2	0.083	0.118	0.440	No
Pb40	ON854716	F: GGGTCACGTAAAGTATCTCCTAACA R: CCTAGTCTTCATGGTGCCCT	(AG)8	271 - 273	48	2	0.042	0.041	1.000	No
Pb46	ON854718	F: GCCCAGAATGCTGAAACTGT R: TGGAGATCAGTAAGTGTCTGTCA	(AG)8	144	48	1	–	–	–	–
Pb72	ON854726	F: TTCCTTCAGCTGCCTTGGTG R: TGGCTTTCAGTTTAGGTGACCA	(AC)7	281 - 303	48	3	0.021	0.062	0.188	Yes

Pb90	ON854731	F: GGAGTCAGCTGCATCTCGAG R: GTGTCTAGTTCCTGGACCGC	(AC)7	221 - 223	48	2	0.021	0.021	NA	No
Pb48	ON854719	F: GGTATGCATTGCTAATGTGCCT R: TCTCCTCATTCTCTTCTCTGCA	(AC)8	296 - 300	48	3	0.500	0.509	1.000	No
Pb66	ON854725	F: ACGGACGTGAATTTCTTCCT R: GCATATGCATTGAGCTGAAGC	(AC)7	187	48	1	—	—	—	—
Pb84	ON854730	F: CAGAGGACACAGATGTTGCA R: CTGCTGTCAGTTTCTCTGGA	(AG)9	250 - 524	48	2	0.021	0.021	NA	No
Pb16	ON854711	F: TGGTTGAATGTCAGAGAAATACAGA R: TGTACTTGACCTGCCATCGG	(AC)10	201 - 207	48	4	0.646	0.709	0.188	No
Pb64	ON854724	F: TGCCAAATTTATGTGTCTGATGT R: AGGTTGGGTCTTCTAGCACA	(AT)8	133 - 135	48	2	0.208	0.281	0.411	No
Pb83	ON854729	F: AGAAGCCAGGTTCCCAACAC R: TCCGTTTATGTTATCAGCAGATCCT	(AC)10	277 - 287	48	4	0.354	0.493	0.214	Yes
Pb93	ON854733	F: CAAGCCAGACCTTGCTGAGA R: CGTGAGGCAATTTGATAGGACC	(AG)7	239 - 243	48	2	0.021	0.021	NA	No
Pb55	ON854721	F: CCAAGTGACTGTGTCGGGTT R: TCAGCCTGACACTGAAGTCG	(AC)8	189 - 195	48	4	0.417	0.455	0.290	No
Pb63	ON854723	F: AGTGACCAGGCTTGTGTCAC R: CTTCCACACCGTAGCAGGAG	(AC)8	272 - 274	48	2	0.354	0.468	0.414	No
Pb21	ON854712	F: GCTAAGAAGCTCCTCCAGTCT R: GGCTTGGGATACATAGGCACA	(AC)9	145 - 151	48	4	0.375	0.410	1.000	No
Pb92	ON854732	F: GCTCAGAACTGGCTAGAGGC R: AGGGATCGCGATAGATGGGT	(AG)7	300	48	1	—	—	—	—
Pb82	ON854728	F: CAGCTGGCAAGACCTTGAGA R: GATGGCAGGACACGTACCTC	(AC)11	216 - 2018	48	2	0.083	0.081	1.000	No

F, sequence of forward primer, R, sequence of reverse primer; Fragment size, observed allele size range; N, sample sizes per locus; Na, number of alleles per locus across samples; Ho, observed heterozygosity; uHe, unbiased expected heterozygosity; HWE, p-value for departure from Hardy–Weinberg equilibrium test; Null alleles, presence (Yes) or absence (No) of null alleles.

* p-value < 0.5, ** p-value < 0.1, *** p-value < 0.01