SHORT COMMUNICATION

Economical genotyping of little blue penguin (*Eudyptula minor*) clades from feather-based DNA

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Abstract: Determination of clade membership is a crucial requirement for many research questions addressing phylogeography, population structure, mating patterns, speciation, and hybridisation. The little blue penguin (*Eudyptula minor*) can be separated into two deeply divergent clades. However, assigning clade membership in little blue penguins requires molecular methods. Genetic sequencing can be used to identify clade membership but is expensive. Here, we present an economical alternative to the use of sequencing to determine little blue penguin clade membership. We extracted DNA from feathers using a method that produced reasonable quantities of DNA. We then amplified the D-loop section of the mitochondrial control region from total genomic DNA extracts, using the primers 'C L-tRNA^{glu}' and 'D H-Dbox' followed by digestion with the restriction enzyme AluI. When visualised on a gel, distinctive banding patterns clearly indicated clade membership. We sequenced a subset of our samples and verified the accuracy of this method. The methods we present should facilitate little blue penguin research through a cost-effective approach to clade analysis as well as a successful technique to extract DNA from feathers when blood or tissue samples are not available.

Keywords: clades; DNA extraction; enzymes; feathers; restriction RFLP

Introduction

Determination of clade membership is required for many research questions addressing phylogeography, mating patterns, speciation, and hybridisation, as well as wildlife management and conservation (e.g. Irwin 2002; Ruegg 2008; Dammannagoda et al. 2011; Webb et al. 2011). However, in many taxonomic groups clade membership can be difficult to determine based on morphology or behaviour alone. In such cases, molecular methods can provide the basis for determinating clade membership. Here, we present a cost-effective approach based on restriction fragment length polymorphism (RFLP) for the identification of clade membership of individual little blue penguins (*Eudyptula minor*), a species with cryptic clades.

An RFLP-based approach for determining clade membership is a well-established method used in multiple taxonomic groups including microbes (Jameson et al. 2008), parasites (Su et al. 2012), plants (Garrick et al. 2008), amphibians (Patrelle et al. 2011), mammals (MacKay et al. this issue), and birds (Rohwer et al. 2001). Here, we present the first use of RFLP to distinguish between clades of little blue penguins.

Little blue penguins, also known as blue penguins, fairy penguins, little penguins, and kororā, are found in New Zealand and southern Australia. Kinsky and Falla (1976) divided this species into six subspecies based on feather colour and morphological patterns: five subspecies in New Zealand and one in Australia. Multiple studies have attempted to use plumage and morphological variability to distinguish these six subspecies (e.g. Meredith & Sin 1988; Hocken 1997), but with little success.

More recent molecular analyses have not supported the six subspecies classification of Kinsky and Falla (1976) and showed that little blue penguins can be separated into two deeply divergent clades based on mitochondrial DNA from the small ribosomal subunit (12S), cytochrome oxidase *b*, and control regions (Banks et al. 2002, 2008; Peucker et al. 2009). One clade is comprised of penguins from Australia and south-east New Zealand, which Peucker et al. (2009) termed the ASENZ clade, while the other clade is comprised of penguins from New Zealand only, which Peucker et al. (2009) termed the NZO clade. Both clades breed at colonies in Oamaru (Banks et al. 2008; Peucker et al. 2009) and on Motunau Island (Peucker et al. 2009).

Despite the deep divergence between the two little blue penguin clades based on mitochondrial DNA markers, the morphology and colouration of individuals within these clades is not sufficiently distinctive to allow definitive classification through casual visual inspection or even through multiple morphological measurements (Banks et al. 2002; Peucker et al. 2009). While Banks et al. (2002) could not reliably distinguish between the two clades based on vocalisations, Mason (2011) and Mason and Waas (unpubl. data) found evidence of clade-based differences in little blue penguin braying vocalisations, analysing a different set of call characteristics and using a larger sample.

Cryptic clades present a problem that is often solved by sequencing portions of an individual's genome, which is a relatively expensive process. Here, we present a cost-effective method to assign little blue penguins to either the NZO or ASENZ clade based on variation in the mitochondrial D-loop region.

DNA extracted from feathers is generally of lower quality than DNA extracted from avian blood or tissue (Bush et al. 2005; Harvey et al. 2006; see discussion in McDonald & Griffith 2011). However, feathers may be the only available, or permitted, source of DNA for researchers. We present the results of our search for a kit-based DNA extraction technique that produces DNA from plucked feathers, sufficient for most genotyping procedures.

Materials and methods

Study areas

We collected feathers from little blue penguins at four colonies on the east coast of New Zealand's South Island with the goal of sampling from genetically homogeneous colonies of NZO and ASENZ clade penguins as well as from two colonies at Oamaru previously shown to contain individuals of both clades (Banks et al. 2008; Peucker et al. 2009).

For the NZO clade colony, we collected feather samples from 35 individuals at the Pohatu Penguin Colony at Flea Bay on Banks Peninsula (43º52′05″ S, 173º00′19″ E). For the ASENZ clade colony, we collected feather samples from 31 individuals at the Penguin Place colony on Otago Peninsula (45º48′02″ S, 170º44′21″ E). For the mixed-clade colonies, we collected feather samples from 141 individuals at the Oamaru Quarry colony (45º06′38″ S, 170º58′47″ E) and from 62 individuals at the Oamaru Creek colony (45º06′07″ S, 170º58′21″ E). The two Oamaru colonies are approximately 1 km apart.

DNA extraction

We focused our sampling efforts just prior to the 2011 breeding season on individuals that were previously banded and in the same nest box as another individual. We plucked two feathers from the centre of the upper back of focal penguins at the Banks Peninsula colony and three feathers from focal penguins at the Otago Peninsula colony and the two Oamaru colonies. Approximately 2–3 mm of the basal tip of each feather's calamus was removed and placed for storage in a microcentrifuge tube (1.5 ml or 2.0 ml) containing 95% ethanol.

Because DNA extracted from feathers is generally of lower quantity and quality, we made several modifications to the manufacturer's protocol for using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, USA) to extract DNA from tissue samples (e.g. Bush et al. 2005). We obtained the best results from the method suggested by Gebhardt and Waits (2008), which used increased incubation (i.e. incubating samples for 16–20 h at 56°C in Buffer ATL and Proteinase K, incubating at 70°C in Buffer AL for 45 min, incubating in 100 µl of Buffer AE at 70°C for 15 min), recycling the Buffer AE through the silica columns with an additional 5 min incubation at 70°C and decreased elution volumes of 100 µl. We compared the effect of extracting from two versus three feathers by measuring the DNA concentrations of a subset of our extractions, using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific, USA).

Clade analysis

To evaluate the clade to which an individual belonged, we used PCR (polymerase chain reaction) to amplify the D-loop section of the mitochondrial control region from total genomic DNA extracts. The D-loop was amplified using the primers C L-tRNA[glu], CCT GCT TGG CTT TTY TCC AAG ACC and D H-Dbox, CTG ACC GAG GAA CCA GAG GCG C (Roeder et al. 2002) following Banks et al. (2002). This general method was used successfully to separate the two clades of little blue penguins in previous research (Banks et al. 2002, 2008; Roeder et al. 2002; Overeem et al. 2008; Peucker et al. 2009).

For PCR, 10.0 µl of Maxime PCR PreMix (i-Taq) (iNtRON BioTechnology, Korea), 0.5 µl of each primer (10 mM), 2.0–3.0 µl of DNA, and 6.0–7.0 µl of water were used for each reaction for a total reaction volume of 20 µl. Negative controls were incorporated in each amplification round using water rather than DNA. All PCRs were carried out using Eppendorf thermal cyclers (Eppendorf MasterCycler, Germany) with an amplification profile of 94°C for 4 min, 40 cycles of 94°C for 10 s, 65°C for 10 s, 72°C for 35 s, and a final step of 72°C for 5 min.

Given the large number of samples in this study, we reduced costs by using an alternative to sequencing each sample to identify clade membership. We aligned D-loop sequences from both clades of little blue penguins, downloaded from GenBank using Clustal X (Thompson et al. 1997), and then used the 'find restriction sites' function in Geneious 5.0.2 (Drummond et al. 2010) to identify restriction enzymes that would cut the PCR product from the D-loop into fragments that would distinguish individuals from the two clades. AluI (Promega, USA), a restriction enzyme that recognises the nucleotide sequence AGCT and cuts between the guanine and cytosine nucleotides, was selected as it produced fragments that would clearly differentiate the two clades.

To cut the PCR product, we incubated 6.0 µl of PCR product, 2.9 µl of water, 1.0 µl of 10X Buffer B, 0.1 µl (10 µg/µl) of BSA, and 0.5 µl (5 units) of AluI at 37°C for 60–75 min. Restriction products were run on a 1.0% agarose gel (Ultrapure, Invitrogen, USA) containing ethidium bromide (1.0 mg/L), and visualised on an AlphaImager 2000 (Alpha Immotech, USA). Fragment size was estimated using a TrackIt 100 bp DNA ladder (Invitrogen, USA).

The PCR primers C L-tRNA[glu] and D H-Dbox amplify an approximately 656 nucleotide portion of the D-loop (Roeder et al. 2002; Peucker et al. 2009). The *in silico* digestion of the GenBank sequences found that PCR product from the D-loop for individuals in the ASENZ clade would be cut by AluI at position 71, giving two segments approximately 71 bp and 585 bp in length (Fig. 1). In the NZO clade, AluI would cut the D-loop at positions 71, 152, and 365, giving four segments approximately 71 bp, 81 bp, 213 bp, and 291 bp in length. To test the reliability of our restriction enzyme procedure to distinguish accurately between NZO and ASENZ clades, we sequenced the PCR products from a subsample of 84 individuals that included 24 samples from the Banks Peninsula colony, 19 samples from the Otago Peninsula colony, and 41 samples from the two Oamaru colonies. All resulting sequences were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) and assigned accession numbers JX130593–JX130676.

Selection of individuals from each site for sequencing was random, except that we included the mates of any birds from the Oamaru sites that the RFLP procedure showed to be from the NZO clade. We removed unincorporated primers and dNTPs from PCR products, using the ExoSAP-IT® (USB Corp., USA) procedure following the manufacturer's recommendations. Cleaned PCR products were sequenced on an ABI 3730 automated sequencer (Applied Biosystems Inc., USA) in one direction, using the D H-Dbox primer for sequencing because a poly C region prevents successful sequencing using the C L-tRNA[glu] primer from the opposite

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     CTGACCGAGG  AACCAGAGGC  GCAAAAGCGC  AAGTTGGGCT  AGGGTGTAGG

 50  GGGAAAGAAT  GGGCCTGAAG  CTAGTAGCAT  GGGATCTTTC  ATACGCCGGG

100  TTGCTGATTT  CACGTGAGAA  GTCAAATCAA  TAGATAACCT  AATCCCTGAA

150  GCTGGCGCCC  CGAGAAGTTA  TTGGATATTA  AGCGCTCCTC  CATTAAGATC

200  AAGTAGCCAG  ACATATCCGT  ACCTACCACA  GATAAGCAAT  GCACACACCA

250  TTAAAAGCAT  GGGCTTAGTC  CTCTTAACGG  GACAGAAAGC  AATTACTACA

300  GTTCATAACA  GTATCATTTC  CCAGATTCAC  TACATAACAT  TCGTTTAGCA

350  CGAGAGTAGT  AAAGCTAATC  CGTATATCAT  TACTTAGTTA  ATCCTTGAAA

400  GTATTACTCT  ATCAATCTCT  AAAGCATATC  CGTATACAAG  AACATTTTCA

450  TTTAACCCTA  CAGTAATAAT  TGTAACACAT  ACATTCAAAT  TAATGCCCTA

500  GCACTAACAT  AATGTATTAT  GTACATAGAG  TGAATGCACG  CCATACATGG

550  GGGGGTAGGG  GGGGGTAGGA  GGAGAGTATT  TTTAGGGAGT  TCCTGTGGTT

600  GAGTCAGACA  ACGGCGGCTT  TTCAGGTCGC  AGGTCTTGGA  GAGAAGCCAA

650  GCAGG
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Figure 1. Restriction sites in the mitochondrial D-loop region of a little blue penguin (Slack et al. 2003; GenBank accession number AF362763) from the New Zealand only clade showing fragment sizes of 70, 81, 213 and 291 bp. AGCT is the nucleotide sequence recognised by the restriction enzyme AluI, cutting between the guanine and cytosine nucleotides. Birds from the Australia and south-east New Zealand clade have a single restriction site at positions 68–71 because of sequence differences between the two clades.

end of the fragment (Banks et al. 2002; Roeder et al. 2002), yielding sequences of approximately 380 nucleotides.

Results and discussion

DNA extraction

We successfully extracted DNA from feathers obtained from all sampled penguins. However, our final DNA extractions from Banks Peninsula penguins, from which we plucked two feathers versus three feathers from Oamaru and Otago Peninsula penguins, contained significantly lower concentrations of DNA (Banks Peninsula colony: 11.8 ± 2.3 ng/μl, range $4.3 – 45.0$ ng/μl, $n = 19$; Oamaru and Otago Peninsula colonies: 71.4 ± 11.6 ng/μl, range $5.24 – 235.9$ ng/μl, $n = 25$; $t = 5.03$, $P < 0.0001$). Given this result, we recommend using a minimum of three plucked feathers for DNA extraction.

While we were successful in extracting useable DNA from plucked feathers, we would caution that DNA extracted from blood samples may be a superior choice for some research questions. The additional steps we took to increase the quantity of DNA in our extractions greatly increased the time and effort needed to extract DNA. The concentrations of DNA from our extractions were highly variable, and poor quality or dilute DNA can produce spurious alleles or allelic dropout when analyses are conducted with microsatellite markers (McDonald & Griffith 2011). Further, should DNA extraction from feathers fail, no re-extraction is possible unless the sampled individual can be re-located and additional feathers plucked.

Extracting DNA from shed feathers is potentially even less invasive than using plucked feathers. However, our research required certainty of DNA source origin, so we could not use shed feathers. Even so, DNA extracted from shed feathers is generally of even lower quality and quantity than that from plucked feathers (Hogan et al. 2008; Gebhardt et al. 2009).

We support the goal of reducing the invasiveness of scientific research and believe the use of feathers as a source of DNA sometimes can be the best or only available option. In such cases, the methods we present here should improve the ability of researchers to address genetic, ecological, and behavioural questions using DNA extracted from feathers.

Clade analysis

Of the 84 samples we sequenced, 81 (96.4%) conformed to one of the two band patterns expected from the AluI restriction enzyme procedure. The Banks Peninsula samples included three individuals that did not produce the expected pattern for either the NZO or ASENZ clades (e.g. see two of the three in lanes 8 and 11, Fig. 2). Sequencing of the three anomalous samples showed that each sample had lost the restriction site at position 365, giving three segments approximately 70 bp, 81 bp, and 504 bp. Sequencing showed all three belonged to the NZO clade.

Our method is potentially affected by substitutions at the restriction sites, but we believe that these substitutions would almost certainly produce anomalous restriction patterns rather than cause misclassification of individuals. For example, for an ASENZ bird to be misclassified to the NZO clade, the bird would have to have substitutions of the correct bases at the correct sites that resulted in the gaining of two restriction sites for AluI and that then produced fragments corresponding to the fragment sizes of NZO birds, which seems highly improbable. The possibility of misclassifying birds can be reduced by sequencing any individuals with anomalous gel banding patterns. We also suggest using higher concentrations of agarose in the gels and running the gels for longer to increase the resolution of the gels and thus increase the probability of detecting anomalous band patterns. Alternatively, Metaphor[TM] high resolution agarose or polyacrylamide gels would also allow small differences in fragment lengths to be discriminated.
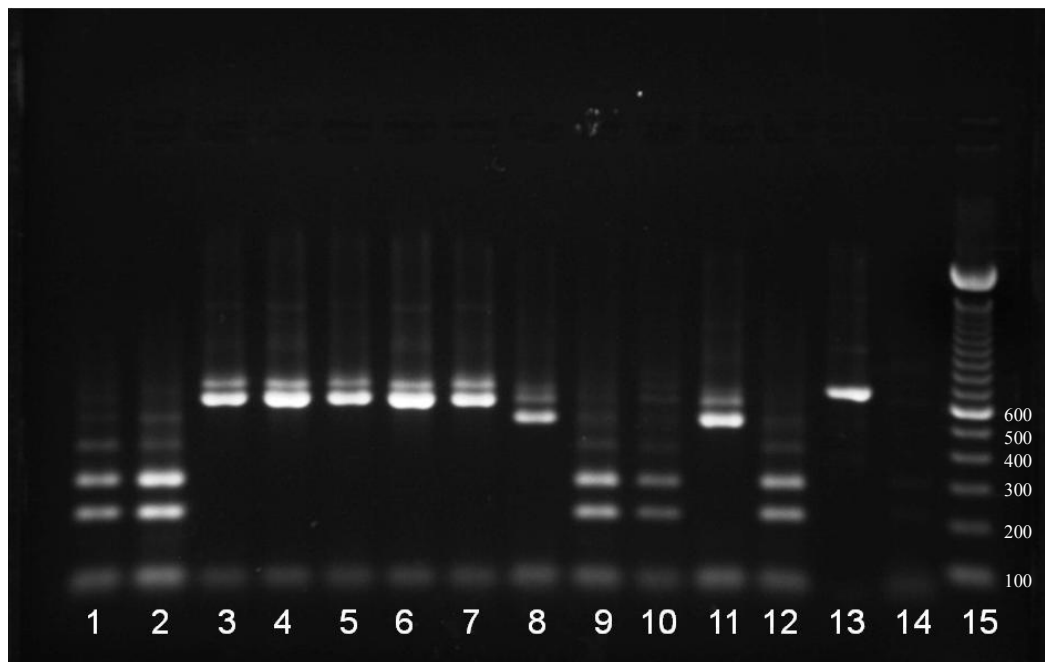
Figure 2. PCR (polymerase chain reaction) products of primers C L-tRNA^{glu} and D H-Dbox on DNA extracted from little blue penguin feathers followed by RFLP analysis through digestion with the AluI restriction enzyme. Lanes 1, 2, 9, 10 and 12 are NZO-clade individuals. Lanes 3–7 are ASENZ-clade individuals. Lanes 8 and 11 are also NZO-clade individuals, but these samples have lost one restriction site. Lane 13 is undigested PCR product, lane 14 is the no-template control, and lane 15 is the ladder (TrackIt, Invitrogen, USA).

Based on the restriction enzyme procedure, we classified 31/35 (88.6%) of sample penguins from the Banks Peninsula colony as NZO clade and 1/35 (2.9%) as ASENZ clade (Fig. 2). We could not classify 3/35 (8.6%) of sample penguins from this colony. Sequencing revealed that these individuals had lost a restriction site and classified them as NZO clade. We classified 31/31 (100.0%) of sample penguins from the Otago Peninsula colony as ASENZ clade. At the Oamaru Creek colony, we classified 60/62 (96.8%) of sample penguins as ASENZ clade and 2/62 (3.2%) as NZO clade. At the Oamaru Quarry colony, we classified 128/141 (90.8%) of sample penguins as ASENZ clade and 13/141 (9.2%) as NZO clade.

Based on results from previous studies (Banks et al. 2008; Peucker et al. 2009), we expected NZO penguins to make up a greater proportion of the populations at the two Oamaru colonies. Of the 203 penguins we sampled at the Oamaru colonies, we documented only 15 (7.4%) NZO clade penguins. Banks et al. (2008) found 3/10 (30.0%) and Peucker et al. (2009) 3/13 (23.1%) penguins at Oamaru were of the NZO clade. The small samples sizes of the Banks et al. (2008) and Peucker et al. (2009) studies could explain this difference.

The main advantage of the RFLP method to distinguish taxonomic groups with cryptic clades is the reduced cost compared with Sanger sequencing. Identifying morphologically cryptic clades allows researchers to eliminate a potentially confounding factor from a wide range of comparative studies – biogeographical, ecological, and toxicological. For example, MacKay et al. (this issue) used an RFLP approach to investigate differences in susceptibility of morphologically cryptic house mouse clades to rodenticides and McCormick (2011) used RFLPs to identify zones of hybridisation between mouse subspecies. Better knowledge of the taxonomy of study animals ensures that comparisons are made at a level that is appropriate for the research question, and more economical molecular methods to identify cryptic taxa will increase the accuracy of many of these comparisons.

Research is needed to determine the range of the ASENZ clade and mating patterns between NZO and ASENZ individuals in colonies where they are sympatric. No information is currently available on whether the ASENZ clade population is stable, contracting, or expanding. The relatively economical method of genotyping clades we present here should help researchers address these and other research questions.

Acknowledgements

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