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Progress of the development of the DNA identification for the southern albatross bycatch in longline fishery

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Abstract

Species identification by external anatomy or physical appearance of albatrosses in the southern hemisphere is often difficult because the species groups show considerable overlap in both plumage score and morphology (Cuthbert et al. 2003). Therefore we investigated a molecular biological approach for the taxonomy of those species. Firstly, a sampling protocol was developed for an observer to easily collect the necessary samples. Secondly, species or species group identification was performed using photographs. And thirdly, Alderman's method (Alderman 2003), using RFLP methods, was employed for two different types of samples: known species and known species group. The DNA taxonomy method needs to be relatively inexpensive and simple as it needs to be used by several countries with different technical resources. Using Alderman's RFLP method seven of 13 species in this study could be identified. We also suggest some improvements are required such as (1) the need to differentiate some of the wandering albatross group species by visual identification by electrophoresis, (2) quantifying intraspecific polymorphism in the grey-headed albatross, and (3) assessing levels of intraspecific polymorphism in Atlantic yellow-nosed albatrosses. At the present time, this method needs further development for practical application.

Introduction

Bycatch is one of the causes of population declines in seabirds (Brothers 1994). Since albatrosses in the Southern Hemisphere (southern albatrosses) are listed as vulnerable species, mitigation measures for seabird bycatch have been discussed and implemented in tuna RFMOs. Species information would help to develop and assess the effectiveness of the bycatch mitigation measures as the movement, diet and the distribution vary among species. It would also help with the development of risk assessments to determine the vulnerability and/or the bycatch rate in each species.

Species identification by external anatomy or physical appearance is often difficult as the species show considerable overlap in both plumage score and other morphological characters (Cuthbert et al. 2003) and thus it becomes necessary for identification to depend on DNA analysis. The method of DNA species identification needs to be relatively inexpensive, accurate and simple because it needs to be used by several countries with different capacities as a general method, including a simple sampling protocol. However, a simple molecular biological method

for southern albatrosses has not yet been developed. Efficient molecular methods to distinguish these species are required. Alderman's RFLP analysis (Alderman 2003) can distinguish 20 albatross species and species group including species in the wandering albatross and yellow-nosed albatross groups, which are difficult to differentiate using morphological characteristics. Since RFLP analysis allows the identification by gel electrophoresis, it is relatively economical and does not require special equipment (e.g. DNA sequencer). Using DNA, correct assignment is high, for example 87-90% in wandering albatrosses (Burg 2008); however for other species such as the yellow-nosed albatross molecular information is lacking (but see Chambers et al. 2009). In addition to the missing information from several species, the intraspecific polymorphism and the intraspecific polymorphism in mitochondrial cytochrome *b* makes it difficult to estimate applicability of the DNA identification in southern albatrosses.

The aim of this study is to investigate an economical, accurate and simple method to identify bycatch southern albatrosses. Firstly, a sampling protocol was developed. Secondly, each sample was identified to the species group level, using the 1990 Sibley and Montroe classification level, based on a photo. Thirdly, DNA analyses were performed. The inter/intra-specific polymorphism, nucleotide and haplotype divergence were examined to assess the applicability of using DNA methods in southern albatrosses. We examined whether Alderman's RFLP method can identify bycatch samples only by electrophoresis without sequencing and assessed levels of intraspecific polymorphism. From these examinations, improvements of the molecular taxonomy, implementation for future management in terms of the practicality of DNA information were discussed.

Materials and Methods

1) Sampling

Bycatch samples: bycatch albatrosses with rings had been gathered from the observer program and onboard research programs by pelagic longliners from 1997 to 2014 were autopsied, and morphologically identified. The pectoral muscles were sampled from each individual and stored at -25 ºC.

Known provenance samples (base samples): the wandering albatross species (*Diomedea exulans antipodensis*, *gibsoni*, Burg and Croxall (2004)) which have been collected from known colonies (Adams Island, Antipodes Island, Bird Island, Crozet Island) from 1997 to 1998 were used for the analysis. A blood sample was collected from each specimen and ethanol-preserved at room temperature. The pectoral muscle obtained from bycatch birds which had been banded (or ringed) were used as known provenance samples.

2-1) Evaluation of Alderman (2003) by DNA sequences analysis

We obtained DNA sequences for 58 specimens, mainly from known provenance samples (Table 1) and examined the intra/inter-specific genetic distance and polymorphism.

2-2) DNA extraction, Polymerase Chain Reaction (PCR) amplification, restriction enzyme fragmentation and calculation of the fragment length

For DNA extraction, DNeasy Tissue Kit (QIAGEN, Netherlands) was used and done according to the DNeasy Tissue Kit protocol. The primers, H15915v2 (5'-gtcttgtaaaccaaagaatgaagac-3') and L14863v2 (5'-ttcgccctatccatcctcat-3'), which are newly designed in this study, were used for PCR of the mitochondrial cytochrome *b* gene. PCR conditions were 98℃ for 30sec, followed by 30 cycles at 98℃ for 10 sec, 55℃ for 30 sec, 72℃ for 60 sec, and a final extension at 72℃ for 2 min and TaKaRa Ex Taq Hot Start version (TaKaRa Co., Ltd.) was used.

Amplified DNA fragments were purified by GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, USA), and subsequently sequenced in both direction using BigDye Terminator cycle sequencing kit v3.1 and ABI3500xl sequencer (Life Technologies, USA). Sequences were visually aligned using DNASIS Pro V2.2 (Hitachi Software Engineering Co., Ltd, Japan). Sequence divergences were calculated using the Kimura two parameter (K2P) distance model. After the sequence was read, the fragment lengths of the *Hinf I*, *HaeIII*, *Alu I* and *Mbo I* digestion products were calculated.

3) Evaluation of Alderman (2003), by RFLP

To examine whether Alderman's RFLP method could be used for the scientific research program such as the observer program and for general-purpose, the combination of photo identification and electrophoresis method were tested.

3-1) Species group identification using photographs

Species identification using photos allows us to narrow down the list of enzymes and select the appropriate combination to confirm the species identification. This approach reduces electrophoresis and screening by 62.5% compared to applying all enzymes in each species.

As part of the Japanese National Observer Program, photographs were taken on board for species identification by experts. Japan developed the original species identification method (Kiyota and Minami 2000) and has been trying to improve the accuracy (Inoue et al. 2011, 2012). The identification method has improved as collaboration with BirdLife International (Inoue et al. 2011, 2012) and with use of the Seabird Bycatch Identification Guide (ACAP Secretariat and National Research Institute of Far Seas Fisheries 2015, Beck et al. 2013). The identification methods used in this study are outlined in Beck et al. (2013). As the identification error was only 1.3% (25 misidentifications per 1916 individuals checked by second person, Inoue et al. 2011), it demonstrated that the identification to species group is highly accurate in the Japanese National Observer Program. The bycaught birds were brought back from onboard research vessels, autopsied and photographed. With this photo id method, albatrosses were identified to at least species group, which corresponds to the species prior to the major taxonomic revisions in the 1990s. Sample sizes in each species group are shown in Table 2.

3-2) DNA extraction, PCR, restriction enzyme fragmentation, and electrophoresis

Total DNA was extracted from each specimen with using NucleoSpin Tissue kit (TaKaRa Bio Inc., Japan) and following the manufacturer's protocol. Following the approach outlined in Alderman (2003), the primers, CB ALBH (5'-gtatcttgttttctaggg-3'), and L14863 (5'-tttgccctatctatcctcat-3') were used to amplify the mitochondrial cytochrome *b* and its flanking regions. The PCR amplification condition consisted of 1x PCR buffer, 0.6 µl dNTPs (), 0.2 µl primers (25 pmol/µl), 0.05µl Ex Taq (TaKaRa Bio Inc.), and 1 uL template DNA (approximately 16 ng on average). PCR cycles were 90 seconds at 94 °C, 35 cycles of 30 seconds at 94 °C, 30 seconds at 54 °C, 60 seconds at

72 ºC and one cycle of 180 seconds at 72 ºC. PCR products were directly digested with four restriction endonuclease: *Hinf I*, *Hae III*, *Alu I* and *Mbo I* at 37 ºC for at least 1 hour in a reaction volume of 12 µl. Digested products were analyzed on an agarose gel (KANTO HC, Kanto Chemical Co., Inc, Japan) and NuSieve 3:1 agarose (Lonza, Switzerland) with 100 bp DNA ladder marker (TaKaRa Bio Inc., Japan). Conditions for each restriction enzyme and electrophoresis are shown in Table 3.

Results

1) Evaluation of the Alderman (2003) by sequencing

Inter/Intra-species genetic distance and diversities

The inter-species genetic distance between *T. chlororhynchos* and *T. carteri*, and between *D. gibsoni* and *D. exulans* were relatively small (0.35% and 0.5% respectively) compared to the average pairwise distance of 6.5% among all species pairs (Table 4). *T. melanophris* (n=8) and *D. epomorpha* (n=2) showed no intraspecific variation and intraspecific variation in the three other species for which multiple samples were sequenced ranged from 0.06-0.12%. The intracolony genetic distances were not less than the between colony genetic distances nor are they consistently higher or lower than the within *D. exulans* distance.

D. exulans, *D. gibsoni* and *T. chrysostoma* had high haplotype diversities (0.60, 1.0 and 0.80 respectively), but low nucleotide diversity (Table 5). Intra-colony haplotype diversities in *D. exulans* were high in Bird Island and Crozet (0.72 and 1.00 respectively; Table 6).

Application of Alderman's RFLP method

A new primer set was used for sequencing in this study with the forward (L14863v2) and reverse (H15945v2) primers designed at 40 bp upstream and 29 bp downstream of those in Alderman (2003) respectively. As a result, the sizes of restriction fragments were adjusted in this study for comparative data analyses and discussion.

For examination of Alderman's RFLP method by estimating the fragment length in *Hinf I*, *Hae III*, *Alu I* and *Mbo I* from the sequence data, fragment lengths of *D. exulans* digested in each enzyme matched to Alderman (2003). Similarly, fragment lengths in *D. gibsoni* (N=2), *D. epomophora* (N=2), *T. carteri* (N=1), *T. impavida* (N=1), *T. melanophris* (N=8), *T. cauta/steadi* (N=1), *T. bulleri bulleri* (N=1), *T. chrysostoma* (N=6 + one GenBank AP009193) matched to Alderman (2003). However, while the fragment lengths in *Hinf I* and *Hae III* digests for *T. chlororhynchos* were consistent with Alderman (2003), the *Alu I* fragments were not (497, 429, 228 *c.f.* Alderman 497, 393, 228) and matched *T. carteri* instead. The fragment length in *Mbo I*, 550,358,143, also did not match Alderman (2003), but matched with *P. nigripes* instead. Thus, the result is not consistent with Alderman's result in *T. chlororhynchos*.

2) Evaluation of the Alderman (2003) with the RFLP with agarose gel electrophoresis

2-1) Production of sampling protocol in the Japanese scientific observer program

Simple muscle sampling protocol for DNA analysis was provided as part of the Pelagic Longline Fisheries

Scientific Observer Program Research Manual (NRIFSF 2014). Disposable biopsy punches (Kai Industries Co., Ltd, Japan) were used for the tissue sampling since the equipment could obtain a sample from the muscle relatively easily (Figure 1). The sample collection procedure was done by cutting the breast of the bird to expose the pectoral muscle and then the biopsy punch is inserted at the incision and rotated. If the pectoral muscle could not be exposed, the sampling could be done by placing the biopsy punch directly to the armpit where the feathers are relatively sparse (Figure 2).

2-2) Evaluation of the RFLP with agarose gel electrophoresis

Selecting the subset of restriction enzymes best suited to identify each species group is the most efficient and economical method for species identification and appears to work for 7 species groups. As the fragment sizes differ for each enzyme set, gel concentration and running time were decided for each species groups. This procedure reduced the electrophoresis.

Wandering albatross group (*D. dabbenena, antipodensis/gibsoni, exulans*)

Because the three restriction enzymes, *Hinf I, Hae III* and *Mbo I*, show the same patterns among these three species (Table 9, Alderman 2003), species identification was done using *Alu I*. *Alu I* digestion products differ for each of the three species: 497, 237, 173 bp for *D. dabbenena*, 497, 237, 156 bp for *D. antipodensis/gibsoni* and 497, 393, 228 for *D. exulans*, differences between 173 and 156 bp should be distinguished on an agarose gel. The *Alu I* digestion products were electrophoresed for 240 minutes at 50V on 3% Nusieve 3:1 agarose to distinguish these species; however, the bands below 200 bp were too weak to distinguish (Figure 3). The products were electrophoresed at 100V on 4.5% agarose gel to increase the resolution of the smaller bands (Figures 4a and 4b). The bands < 200 bp and 200-500 bp level were observed after 20 and 40 minutes of electrophoresis, respectively, but the products specific for *D. dabbenena* (173 bp) and *D. antipodensis/gibsoni* (156 bp) could not be differentiated from one another. However, the differences between the products in 200-500 bp range were identified visually (Figure 4a and 4b), suggesting that *D. exulans* and the other wandering albatross species could be distinguished using this method.

Also the single result of *Alu I* showed no evidence that they are not the species other than wandering albatross group, thus the digestion products of *Hinf I* were electrophoresed. *Hinf I* could distinguish wandering albatross group from other species. The result showed the identification of *D. exulans* or *D. gibsoni/antipodensis/dabbenena*. Intraspecific polymorphism was not observed in any of the 125 samples. The samples in wandering albatross group were assigned into 63 *D. exulans*, 14 *D. dabbenena/gibsoni/antipodensis* and 48 were not assigned (Table 8).

Royal albatross group (*D. epomophora/sanfordi*)

While *D. epomophora* could not be distinguished from *D. sanfordi* by Alderman (2003), *D. epomophora/sanfordi* did have a unique restriction pattern for *Mbo I* (Table 9) allowing identification of the royal albatross group from other species. As such, the digestion products of *Mbo I* were electrophoresed (Figure 5). The 27 samples out of 35 were assigned into *D. epomophora/sanfordi* and 8 samples could not be assigned. No irregular fragment lengths were observed through the examination of 35 samples.

Yellow-nosed albatross group (*Thalassarche carteri* and *T. chlororhynchos*)

The restriction patterns of *Hinf I* and *Mbo I* were reported to show no difference between samples (Table 9, Alderman 2003). The patterns of fragment lengths of *Alu I* are 497, 429, 228 in *T. carteri* and 497, 393, 228 in *T. chlororhynchos* (Table 9), thus the difference between 429 and 393 bp fragments should be distinguished on agarose gel. In addition, the length of *Hae III* digestion products are known to be 305, 234, 174, 153 in *T. carteri* and 305, 175, 153 in *T. chlororhynchos* creating different banding profiles (Table 9). Thus, the combination of the digestion products of *Alu I* and *Hae III* should allow the clear resolution of these two species. All 14 samples that successfully amplified matched the banding pattern of *T. carteri* (Table 8, Figure 6). Irregular fragment lengths were not observed through the examination of 14 samples. Two samples failed to amplify.

Shy albatross group (*T. cauta/steadi, salvini, eremita*)

The restriction patterns of *Mbo I* of *T. cauta/steadi* are known to be distinguished from that of *T. eremita* and *T. salvini*, and the *Hinf I* banding patterns of *T. eremita* are different from *T. salvini* and *T. cauta/steadi* (Table 9, Alderman 2003). The combination of the two enzymes should allow resolution into three groups. The Shy albatross group show an exclusive restriction pattern with *Hae III* to all other species except *T. carteri*, but the visual appearance of *T. carteri* is very different from that of shy albatross group. Therefore, if those species are identified by photo id in combination with the unique banding profile for *Mbo I*, *Hinf I* and *Alu I*, members of the shy albatross group can be identified. The digestion products of *Hinf I*, *Hae III*, and *Mbo I* were electrophoresed (Figure 7). All 16 samples were identified as *T. cauta/steadi* (Table 8) and no irregular fragment lengths were observed.

Black-browed albatross group (*T. impavida, melanophris*)

The two species in the black-browed albatross group (*T. impavida* and *T. melanophris*) can be distinguished by *Mbo I* (Table 9, Alderman 2003). As the banding pattern of *T. melanophris* is different from *T. impavida* (Figure 8), black-browed albatross group could be clearly assigned to 9 *T. melanophris* and 7 *T. impavida* (Table 8). No irregular banding pattern observed in either species.

Grey-headed albatross (*T. chrysostoma*)

As grey-headed albatross (*T. chrysostoma*) is known to have exclusive restriction pattern in *Alu I* from other species (Table 9, Alderman 2003) digestion products of *Alu I* were electrophoresed (Figure 9). In one of the 16 samples, shorter fragment length appeared in the banding pattern (Table 8; Figure 9) and it appears to match that predicted for *T. melanophris* and *T. impavida*. The photos of No.471 matched *T. chrysostoma*. Others were all assigned into *T. chrysostoma*.

Buller's albatross group (*T. bulleri bulleri/platei*)

As Buller's albatross (*T. bulleri bulleri/platei*) is known to have exclusive restriction pattern in *Hae III* (Table 9, Alderman 2003), the digestion products of *Hae III* were electrophoresed (Figure 10). In 16 samples, the bands appeared around 175 and 156 bp and the samples were assigned to Buller's albatross (Table 8). Intraspecific

polymorphism was not observed through the examination of 16 samples.

Dark colored albatross group (*Phoebetria fusca, palpebrata*)

The restriction pattern in *Hinf I* allows identification of each of these species from each other and from all other species (Table 9, Alderman 2003). As such, the species identification in this group could be performed using only *Hinf I*. The digestion products of *Hinf I* were electrophoresed (Table 3) and the banding patterns were examined for those two species (Figure 11, Table 8). The samples in *Phoebastria* albatross group were assigned into 16 *P. fusca* and 15 *P. palpebrata* and one sample failed to amplify (Table 8). Intraspecific polymorphism was not observed in any of the 31 samples.

Discussion

In this study, we indicated an economical molecular biological approach for the identification of southern albatrosses, which can be used by international research programs such as Regional Observer Program of tuna-RFMO. We also examined one of the molecular biological taxonomic approaches, Alderman's RFLP analysis, for the southern albatrosses to investigate identification of albatrosses in the hopes that it can eventually be utilized on bycatch samples. Though more samples should be examined to determine whether there are any irregular fragment lengths caused by intra-species variation, it was suggested that seven species out of 13 species in this study could be identified by Alderman's RFLP analysis. We also found that there are some improvements such as (1) the impossibility of visual identification by electrophoresis in some of wandering albatross group species, (2) intraspecific polymorphism in the grey-headed albatross, and (3) intraspecific polymorphism in Atlantic yellow-nosed albatrosses.

Evaluation of Alderman's RFLP method: possible cause and provision of the identification error

One identification difficulty and two apparent identification errors were indicated in our study. At the present time, Alderman's RFLP method is unlikely to be able to be applied for practical use by international research programs and there is a need to find a better marker for the wandering albatross group and a means for reducing error to be developed. Inter-species genetic distances were relatively small (6.5% on average) compared to other bird species (8% Johns and Avise 1998). The inter-species genetic distances were particularly small between *T. chlororhynchos* and *T. carteri*, and between *D. gibsoni/antipodensis* and *D. exulans*, which might lead to identification error. Considering those genetic distances and diversities, it is expected that the error might increase in the course of this examination. In addition, haplotype diversities of *D. exulans*, and *T. chrysostoma* were high in this study, suggesting the difficulty of the investigation of the species-specific sequences.

Alderman's RFLP method was employed in our study because it was supposed to allow differentiation of species within each of the wandering albatross group and yellow-nosed albatross group. However, it requires improvement for those two species groups. Either result of 20 and 40 minutes running at high electrical current or long-running electrophoresis at low electrical current could not distinguish the three wandering albatross species used in this study. The method could be improved by: 1) use of polyacrylamide gels to resolve small size differences in

the DNA fragments, or 2) increase the amount of DNA/PCR product used in the digest and on the gel. Another solution would be to develop species-specific DNA markers. Though we only had one known origin Atlantic yellow-nosed albatross sample, the banding pattern differed from Alderman (2003) even after accounting for differences in the primers. Chambers et al. (2009) indicated that genetic distance of cytochrome *b* sequence between two yellow-nosed albatross species is only 0.35%. The genetic distance between *T. carteri* and *T. chlororhynchos* in our study showed a similar difference (0.35%), though we only sequenced one individual from each species. In the situation where there is an only a subtle difference between those species, the restriction fragment length might not reflect the species-specific sequence. If so, the new species-specific sequence would need to be found. Also, Chamber et al. (2009) suggested that it is not sufficient in isolation to justify splitting the yellow-nosed taxon pair. And our study suggested that the genetic distance between them were relatively small compared to others in the south albatross group. Taxonomy of those two species might be needed to be evaluated.

In this study, an irregular banding pattern was found in one sample of grey-headed albatrosses. This sample was identified two times by the expert's identification using photos and by the autopsy. Adult grey-headed albatrosses would be rarely misidentified from their particular appearance. Thus, it is unlikely to be misidentification. Burg and Croxall (2001) suggested that average levels of mitochondrial control region sequence divergence were higher in grey-headed albatrosses than in the black-browed albatross group (2.99% compared to 1.80-2.06%). And also, in our study nucleotide and haplotype diversity in grey-headed albatross were relatively high and intra-species genetic distance was high. Thus, the restriction fragment length might not reflect the species-specific sequence in grey-headed albatross like the Atlantic yellow-nosed albatross. In this case, the new species-specific sequence would need to be found. Also, it is possible the sample doesn't belong to the bird in the photo because of mistaken identification. Analysis should be continued to find whether this is a miss-taken sample or caused by inraspecific polymorphism by increasing the sample size. In addition, we should sequence the sample which shows an unfamiliar band to confirm if it is unique profile for *Alu I* or not.

For practical conservation management

Considering the practical issues, cost is the largest problem with use of the molecular biological approach. The cost is high even in the equipment and machines for the DNA extraction and amplification and RFLP analysis. Therefore, a center which has equipment and machines for DNA analysis is needed to conduct the molecular biological taxonomy applied for bycatch species. On the other hand, detail information would be needed to introduce effective seabird bycatch mitigation measures. To solve the dilemma between lack of information and the measurement against fishery, one practical approach would be identification to species group by photos.

As shown in this study, southern albatross identification with molecular methods is still at the development stage for assigning each species accurately and easily. In order to preserve the southern albatrosses, management actions need to be those that most countries with fisheries are able to report because effectiveness of mitigation measures is evaluated based on the feedback. Thus, identifying southern albatrosses to species level might be impractical for management or evaluation purposes, at this time.

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Figure 1: Biopsy Punch for sampling albatross pectoral muscle (modified from Japanese pelagic longline fisheries scientific observer program, research manual).

Method sampling pectoral muscle

⓪ Prepare label and cutting knife and break a seal of biopsy punch. Keep the encasement of biopsy punch for storing the samples.

① Make a small slit in either the left or right breast of the bird, and expose the pectoral muscle under the feather and fat.

② Stick the biopsy punch on the slit of pectoral muscle, rotate the biopsy punch, and sample pectoral muscle.

① Replace the biopsy punch with pectoral muscle in the case of the biopsy punch, put them in the sampling plastic bag with the label for storage.

② Fill 「1」 at the column of muscle in the field note.

If you do not want to cut the bird breast muscle, may stick the biopsy punch directly at armpit skin where the feathers are relatively sparse.

Figure 2: The protocol for the sampling pectoral muscle in the Japanese scientific observer program (modified from the Japanese pelagic longline fisheries scientific observer program manual).

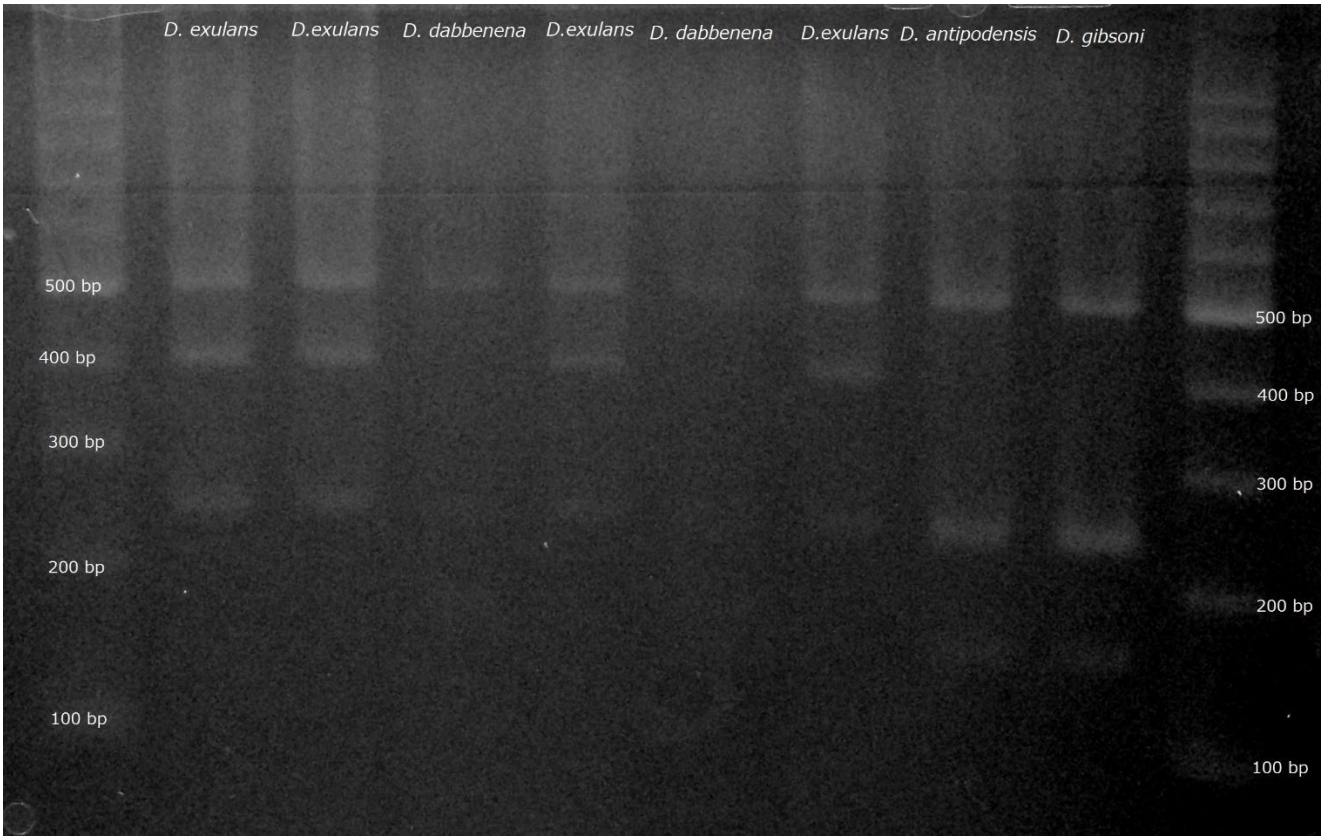

Figure 3 The banding pattern of the restriction fragments in wandering albatross group run in 3% NuSieve 3:1 agarose for 240 minutes.

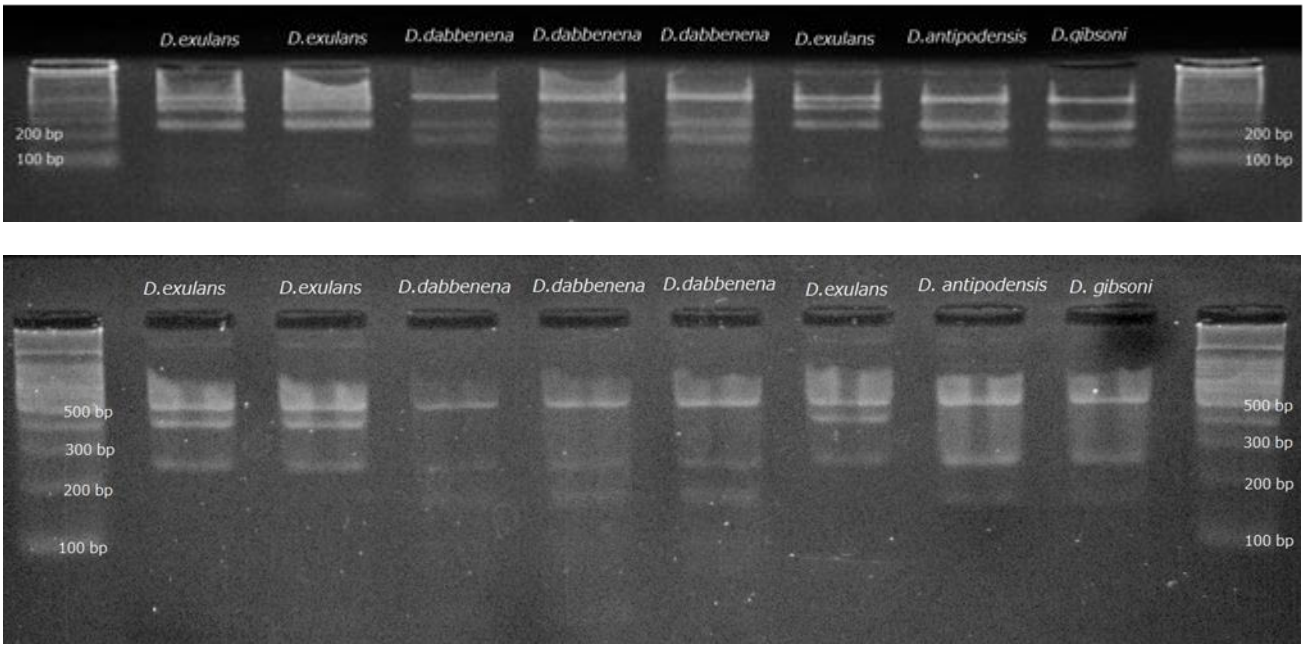

Figure 4a The banding pattern of the restriction digest in wandering albatross group run in 4% agarose gel KANTO HC for 20 minutes (upper). **Figure 4b.** The banding pattern of the enzyme fragment length in wandering albatross group run for 40 minutes (lower).

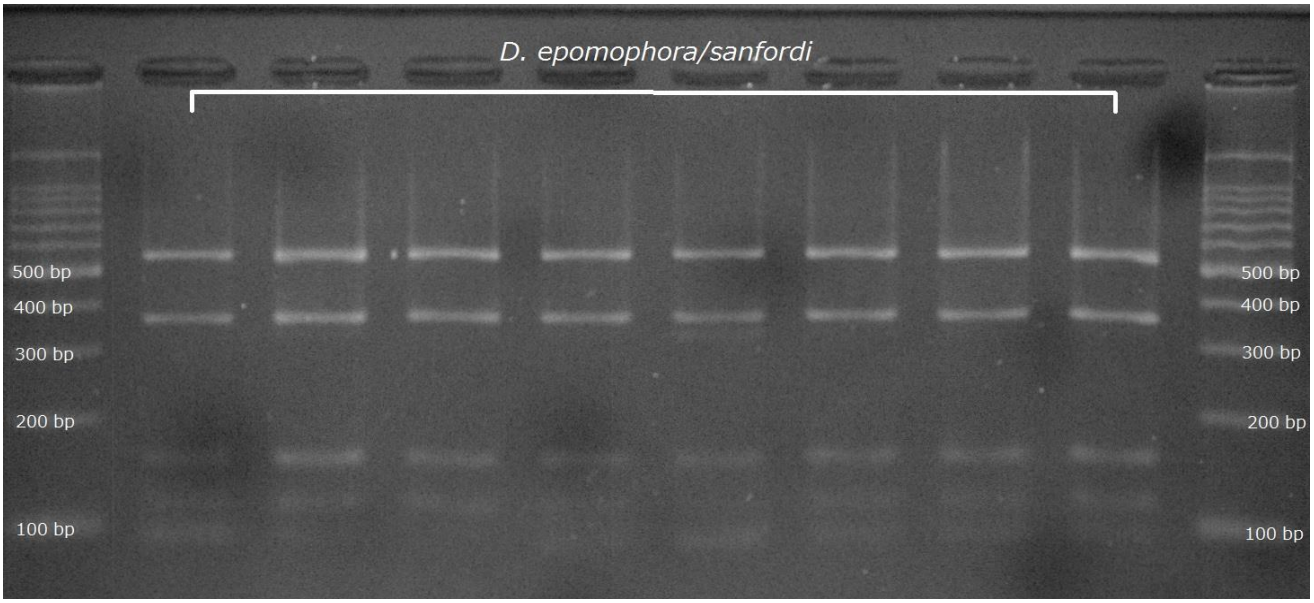

Figure 5 The band pattern of the enzyme fragment length in royal albatross group (*D. epomophora/sanfordi*) run in 4.5% agarose gel KANTO HC for 45 minutes.

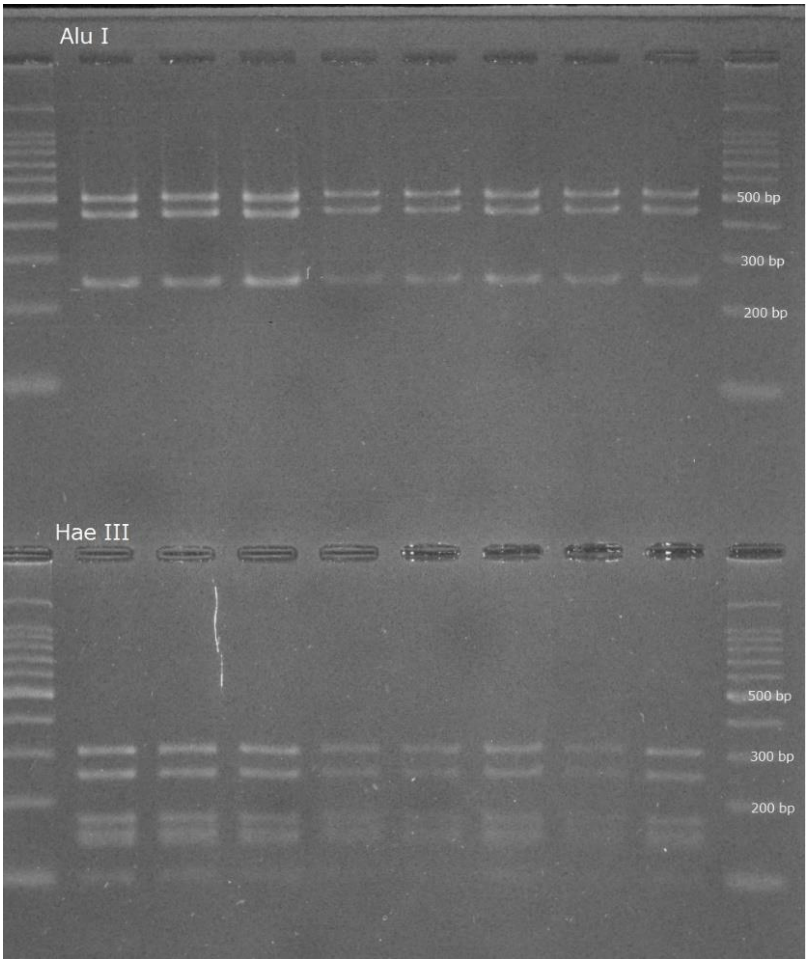

Figure 6 The band pattern of the enzyme fragment length in yellow-nosed albatross group run in 4.5% agarose gel KANTO HC for 80 minutes. Top row shows *AluI* fragments and bottom row *Hae III* digest. All samples were

identified as *T. carteri*.

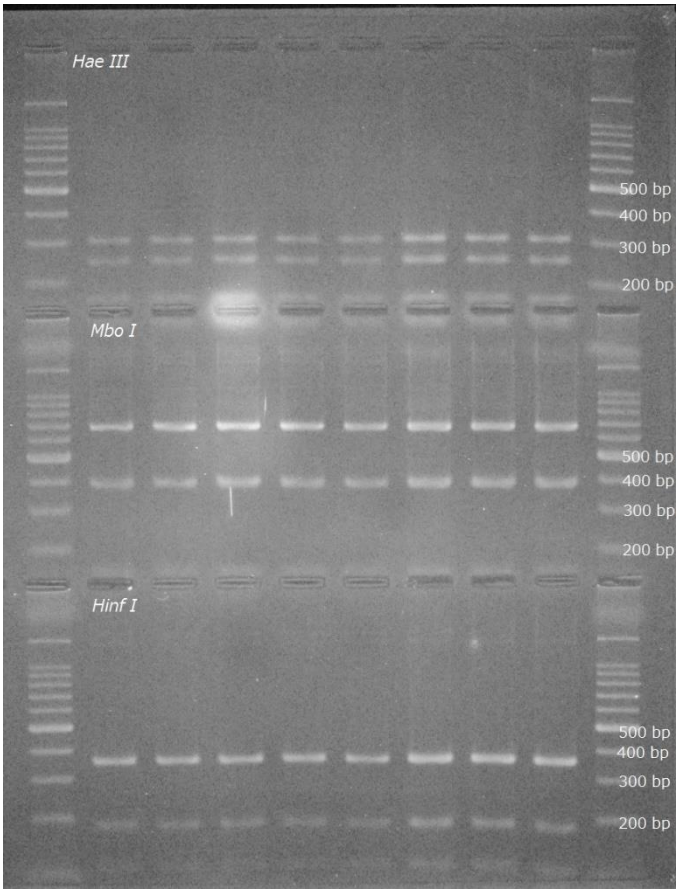

Figure 7 The banding profile in shy albatross group on a 4% agarose gel KANTO HC run for 80 minutes. Upper set of bands show *Hae III* digest, middle bands are *Mbo I* digest, and the lower bands show *Hinf I* digest for the same set of eight samples. All samples were identified as *T. cauta cauta/steadi*.

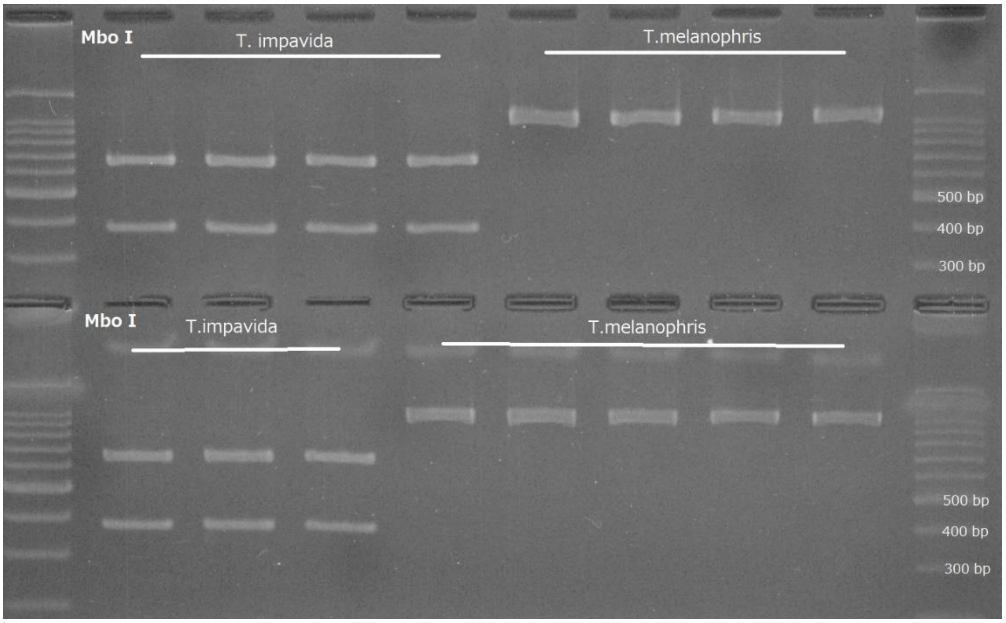

Figure 8 *Mbo I* restriction digest of 16 samples in the black-browed albatross group run in 4% agarose gel KANTO HC for 40 minutes. Samples were assigned into *T. melanophris and T. impavida*.

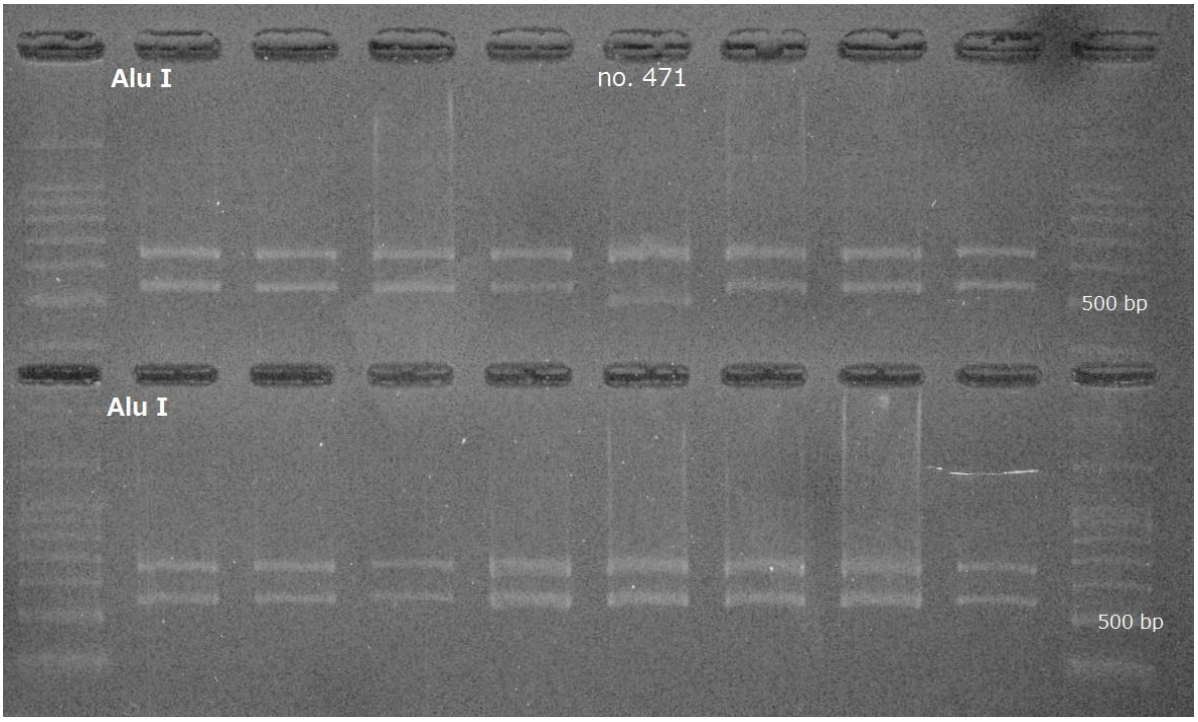

Figure 9 The banding pattern of the *AluI* digested cytochrome b fragment in grey-headed albatross run in 4% agarose gel KANTO HC for 70 minutes. In those 16 samples, one sample, no 471 shows a banding pattern uncharacteristic of grey-headed albatross.

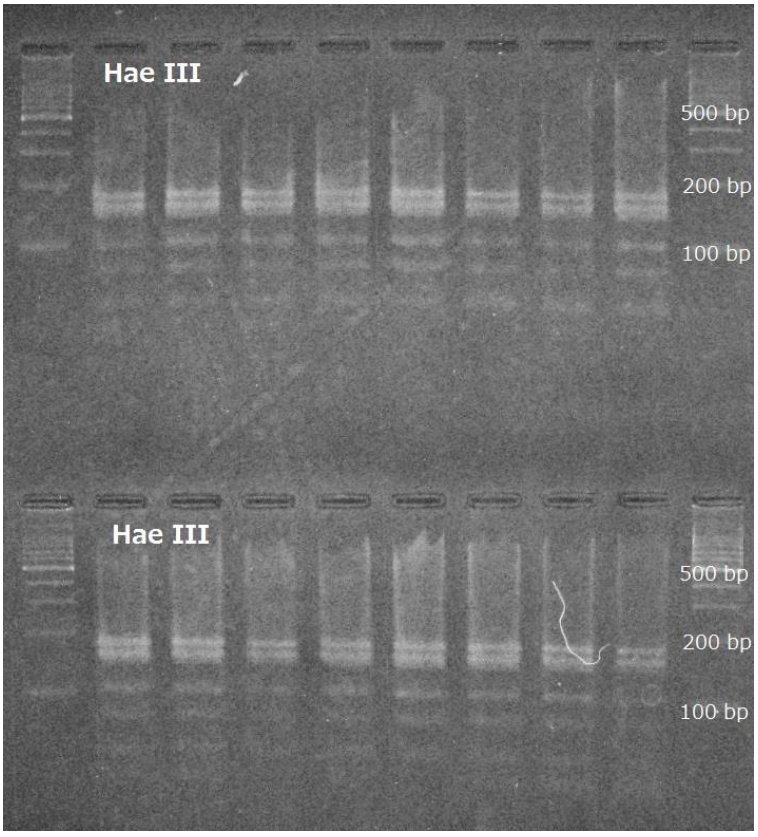

Figure 10 The banding pattern of the enzyme *HaeIII* in Buller's albatross group run in 4.5% agarose gel KANTO HC for 70 minutes.

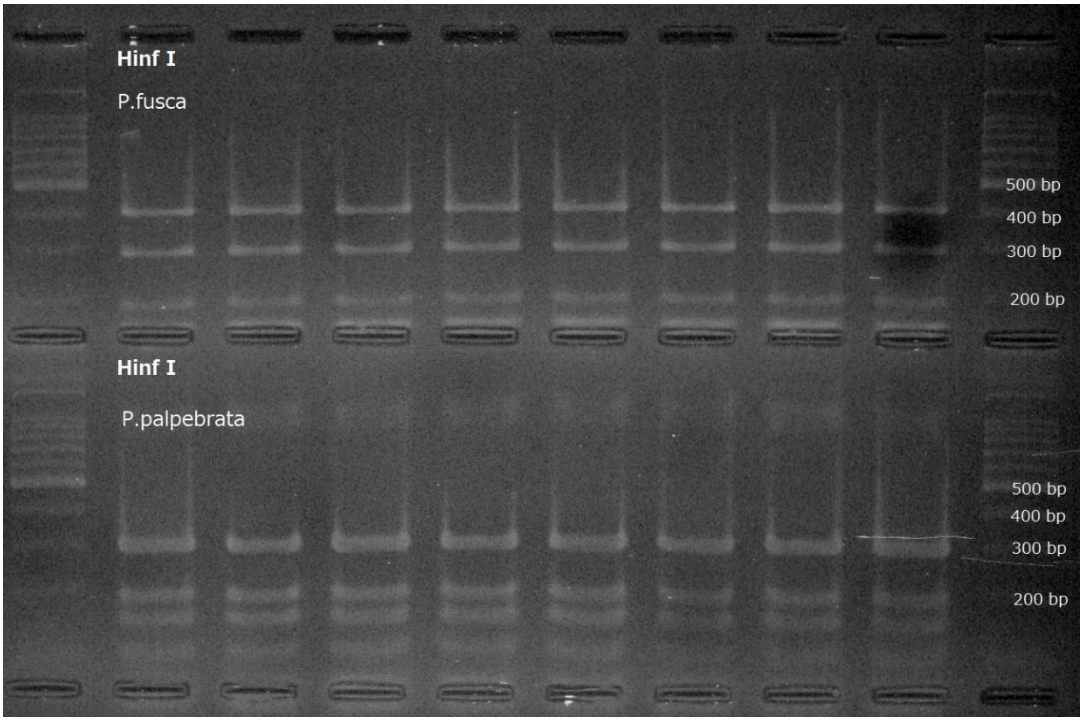

Figure 11 The banding pattern of the *HinfI* enzyme digest in *Phoebetria* albatross run in 4% agarose gel KANTO HC for 60 minutes. Samples were assigned to *P. fusca* (top row) and to *P. palpebrata* (bottom row).

Table 1 Sample sizes and species used in DNA sequencing

Species name	Sample size
D. epomophora	2
T. impavida	1
T. melanophris	8
T. carteri	1
T. chlororhynchos	1
T. bulleri bulleri	1
T. cauta cauta	1
D. gibsoni	2
T. chrysostoma	6
D. exulans	35
Total	58

Table 2 Samples used in restriction digests to identify species of albatrosses. Samples were classified into groups based on photo id.

species group	composition of species	Sample size
Wandering albatross group	*D. exulans/dabbenena/gibsoni/antipodensis*	125
Royal albatross group	*D. epomophora/sanfordi*	35
Black-browed albatross group	*T. melanophris/impavida*	16
Shy albatross group	*T cauta/steadi/salvini/eremita*	16
Black-colored albatross group	*P. fusca/palpebrata*	32
Yellow-nosed albatross group	*T. chlororhynchos/carteri*	16
Buller's albatross group	*T. bulleri bulleri/platei*	16
Grey-headed albatross	*T. chrysostoma*	16

Table 3 Table shows the species group and used restriction enzyme and the condition of electrophoresis.

Group	Species names	First step			Second step			Third step		
		Restriction enzyme	Gel concentration	Running time	Restriction enzyme	Gel concentration	Running time	Restriction enzyme	Gel concentration	Running time
Wandering albatross group	*D. exulans, D. antipodensis/gipsoni, D. dabbenena*	Alu I	4.5%, 3%	20, 40, 240 min.	Hinf I	4.5%	40 min.			
Royal albatross group	*D. epomophora/sanfordi*	Mbo I	4.5%	45 min.						
Black-browed albatross group	*T. melanophris, T. impavida*	Mbo I	4.0%	40 min.						
Shy albatross group	*T. cauta/steadi, T. salvini, T. eremita*	Hae III	4.0%	80 min.	Mbo I	4%	80 min.	Hinf I	4%	80 min.
Black-colored albatross group	*P. fusca, P palpebrata*	Hinf I	4.0%	60 min.						
Yellow-nosed albatross group	*T. carteri, T. chlororhynchos*	Alu I	4.5%	80 min.	Hae III	4.5%	80 min.			
Buller's albatross group	*T. bulleri/platei*	Hae III	4.5%	80 min.						
Grey-headed albatross group	*T. chrysostoma*	Alu I	4.0%	70 min.						

Table 4 Inter/intra-species genetic distances for cytochrome *b* in southern albatross species. Intraspecific variation for five of the species (xxxxx) could not be calculated as a single sample was sequenced.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1 D.epomophora	0									
2 T. impavida	0.11157	xxxxx								
3 T. melanophris	0.11264	0.00439	0							
4 T. carteri	0.11744	0.0287	0.03147	xxxxx						
5 T. chlororhynchos	0.11854	0.02685	0.02962	0.00351	xxxxx					
6 T. bulleri bulleri	0.11174	0.02681	0.03141	0.03431	0.03245	xxxxx				
7 T. cauta cauta	0.1077	0.02319	0.02594	0.02788	0.02788	0.01506	xxxxx			
8 D. gibsoni	0.03476	0.1075	0.10857	0.10683	0.11004	0.10979	0.10259	0.00088		
9 T. chrysostoma	0.1139	0.01836	0.01927	0.03121	0.02936	0.02745	0.02292	0.10979	0.00117	
10 D. exulans	0.03554	0.1084	0.10947	0.10779	0.11095	0.11069	0.10354	0.00519	0.1107	0.00066

Table 5 Haplotype (*h*) and nucleotide (π) diversities for each albatross species.

Species names	N	*h*	π	s.d.
D. exulans	35	0.59664	0.00066	0.00014
D. gibsoni	2	1.00000	0.00087	0.00044
D. epomophora	2	0.00000	0.00000	0.00000
T. impavida	1	xxxxx	xxxxx	xxxxx
T. melanophris	8	0.00000	0.00000	0.00000
T. carteri	1	xxxxx	xxxxx	xxxxx
T. chlororhynchos	1	xxxxx	xxxxx	xxxxx
T. bulleri bulleri	1	xxxxx	xxxxx	xxxxx
T. cauta cauta	1	xxxxx	xxxxx	xxxxx
T. chrysostoma	6	0.80000	0.00117	0.00039
Total	58	0.83545	0.04861	0.00476

Table 6 Haplotype (*h*) and nucleotide (π) diversities for each colony of *D. exulans*.

	N	h	π	s.d.
Bird Island	22	0.72294	0.00081	0.00015
Crozet	2	1.00000	0.00087	0.00044
Kerguelen	3	0.00000	0.00000	0.00000
Marion Island	8	0.25000	0.00044	0.00032
Total sample	35	0.59664	0.00066	0.00014

Table 7 Genetic distance among colonies in *D. exulans*.

	1	2	3	4
1 Bird Island	0.00081			
2 Crozet	0.00088	0.00088		
3 Kerguelen	0.00044	0.00044	0	
4 Marion Island	0.00062	0.00066	0.00022	0.00044

Table 8 The species composition assigned using Alderman's RFLP method. The species group assignment based on photo id is shown in the column and DNA assignments in rows.

	Wandering albatross group	Royal albatross group	Black-browed albatross group	Shy albatross group	Black-colored albatross group	Yellow-nosed albatross group	Buller's albatross group	Grey-headed albatross
T.melanophris	0	0	9	0	0	0	0	0
T.impavida	0	0	7	0	0	0	0	0
T.carteri	0	0	0	0	0	14	0	0
D.exulans	63	0	0	0	0	0	0	0
D. dabbenena/gibsoni/antipodensis	14	0	0	0	0	0	0	0
D. epomophora/sanfordi	0	27	0	0	0	0	0	0
T. bulleri bulleri/platei	0	0	0	0	0	0	16	0
T. cauta/steadi	0	0	0	16	0	0	0	0
T. chrysostoma	0	0	0	0	0	0	0	16
P.fusca	0	0	0	0	16	0	0	0
P.palpebrata	0	0	0	0	15	0	0	0
unassigned	48	8	0	0	1	2	0	0

Table 9 The fragment length in each species for each restriction enzyme used in this study (from Alderman 2003). Actual restriction sites at first row on the table are *Hae III* cuts at GG/CC, *Alu I* at AG/CT, *Mbo I* at /GATC and *Hinf I* at G/ANTC.

Alu I: 5' - GG↓CC - 3'		*Mbo I:* 5'-AG↓CT-3'		*Hinf I:* 5'-↓GATC- 3'		*Hae III:* 5'-G↓ANTC-3'	
albatrus	926 228	*albatrus*	550 263 198 143	*albatrus*	373 345 257	*albatrus*	539 174 153
irrorata	926 145	*irrorata*	550 461 143	*irrorata*	390 348 257	*irrorata*	325 155
nigripes	533 393 228	*nigripes*	550 358 143	*nigripes*	348 257	*nigripes*	305 234 174 153
immutabilis		*immutabilis*	693 461	*immutabilis*		*immutabilis*	480 276 153
epomophora/ sanfordi	470 393 228	*epomophora/ sanfordi*	550 360 143	*epomophora/ sanfordi*		*epomophora/ sanfordi*	
dabbenena	497 237 173	*dabbenena*		*dabbenena*	354 285 183	*dabbenena*	
antipodensis/ gibsoni	497 237 156	*antipodensis/ gibsoni*	550 461 143	*antipodensis/ gibsoni*		*antipodensis/ gibsoni*	488 174 153
amsterdamensis		*amsterdamensis*		*exulans*		*amsterdamensis*	
impavida	621 497	*exulans*		*amsterdamensis*	390 285 183	*exulans*	
melanophrys		*impavida*		*impavida*		*melanophrys*	305 175
chrysostoma	621 533	*bulleri/platei*		*melanophrys*		*bulleri/platei*	175 156
carteri	497 429 228	*carteri*	653 358 143	*chrysostoma*		*carteri*	
bulleri/platei		*cauta/steadi*		*carteri*	348 183	*cauta/steadi*	305 234 174 153
cauta/steadi		*chlororhynchos*		*cauta/steadi*		*salvini*	
chlororhynchos	497 393 228	*melanophrys*		*bulleri/platei*		*eremita*	
eremita		*chrysostoma*	1011 143	*chlororhynchos*		*chrysostoma*	
exulans		*eremita*		*salvini*		*impavida*	305 175 153
salvini		*salvini*		*eremita*	348 228	*chlororhynchos*	
fusca		*fusca*	540 510 143	*fusca*	285 183 134	*fusca*	480 469
palpebrata	533 393 228	*palpebrata*		*palpebrata*	390 285 183 134	*palpebrata*	