Molecular systematics of New Zealand fairy tern (*Sterna nereis davisae*) based on mitochondrial DNA sequences

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Introduction

Investigation of the taxonomic status of the New Zealand fairy tern (*Sterna nereis davisae*) has been highlighted as a priority item in the Department of Conservation's (DoC) recovery plan for this species (Parrish 1995). For the past two years, staff at the Institute of Molecular Systematics (IMS) at Victoria University of Wellington (VUW) have been applying molecular techniques to this problem. Our strategy has been to analyse nucleotide sequences of parts of the mitochondrial genome (mtDNA) of NZ fairy tern which might then be compared with similar data for Australian fairy tern (*S. n. nereis*). Analyses have concentrated on two target regions of NZ fairy tern mtDNA: the 12S ribosomal RNA and cytochrome *b* genes. This report describes progress to date on what has proved to be a technically demanding challenge.

Materials and methods

Biological samples from NZ fairy tern analysed in this project were collected by DoC filed workers in 1994 and are listed in full in Coddington 1996 Appendix 1 pp 74-76. A limited subset of samples was selected and DNA extracted from them by standard procedures as reported earlier (Chambers 1995). Feathers from preserved specimens of Australian fairy tern were obtained from the Museum of Victoria (see Coddington 1996 pp 77) and processed in a similar fashion (see Coddington 1996 pp 32-33). Samples of enriched mtDNA preparations from *S. n. nereis* were purchased from the South Australian Museum.

Mitochondrial DNA target regions were amplified by means of the polymerase chain reaction (PCR) using synthetic oligonucleotide primers (e.g. see Coddington 1996 pp 33-36 for details of the cytochrome *b* system). When necessary the double-stranded products (dtDNA) obtained were reamplified (Coddington 1996 p 35) to provide sufficient template for DNA sequencing reactions (for methods see Coddington 1996 pp 36-39). Individual nucleotide sequences were read from autoradiographs, saved as computer files and assembled into consensus sequences for analysis (see Coddington 1996 p 39 and Chambers, 1995).

Results

Preliminary trials were carried out on DNA extracted from samples FT1 and FT2 (see Chambers 1995). The first target to be examined was the 12S rRNA gene because it had proven to be reliable in many earlier studies (extensive unpublished data from IMS workers). Overall, a 382 nucleotide segment of the 12S gene sequence was obtained (E S MacAvoy unpublished data - not

shown) which showed many similarities to the 12S genes of other avian species (Cooper 1994; Evans 1994).

These first observations serve as a powerful positive control experiment demonstrating that authentic mtDNA sequences can be produced from the material supplied a dead embryo and tissue scraped from an egg shell. However, without other 12S sequences from closely related species we could not be absolutely certain that the data obtained had originated from *S. n. davisae* rather than from some contaminating microorganism. Further, based on experience with other closely related pairs of endemic NZ bird species and subspecies (e.g. see Evans 1994), it was judged unlikely that the 12S gene would be evolving quickly enough to provide sufficient discriminating characters on which to form an opinion with respect to the central question. It was, therefore, decided to attempt analysis of a more rapidly evolving mtDNA target the cytochrome *b* gene.

Preliminary data from the initial phase of this study were reported by Chambers (1995). In short, FT1 and FT2 yielded a composite 257 nucleotide partial cytochrome *b* sequence which showed every sign of being authentic. A second phase of work began in 1996 as a BSc (Hons) project (Coddington 1996). At the outset it was decided to proceed directly with a comparative analysis of DNA from Australian fairy tern. In doing so we hoped to learn as quickly possible if cytochrome *b* was likely to be a useful tool for this level of taxonomic inquiry.

The early results from this study were encouraging (Coddington 1996 pp 60-61) and show that DNA extracted from the 43 year old museum specimens was capable of supporting specific PCR-catalysed amplification of mitochondrial cytochrome *b* targets. However, the DNA sequence data reported by Coddington (1996) for cytochrome *b* from Australian fairy tern (Coddington 1996 pp 62-65) do not match those previously obtained. It was clear that the Australian fairy tern sequence data reported in Coddington (1996) are not authentic because the nucleotide insertions and deletions in this sequence relative to that of the NZ fairy tern cytochrome *b* sequence would be expected to cause frameshift errors and result in a nonfunctional protein product. Two explanations were considered. First, that errors had been introduced into the sequence when reading from the rather faint autoradiographs. Second, that a non-mitochondrial target (e.g. a nuclear pseudogene homologue) had been amplified inadvertently (see Zhang and Hewitt 1996). We judged that both artefacts were potential contributors to the data.

Our next step was to consult Dr Stephen Donnellan of the South Australian Museum who has worked on Australian populations of the little tern (*Sterna albifrons*): see Donnellan (1996). He explained that his own staff had found that this work presented 'unexpected technical difficulties' and had, therefore, taken longer than they had originally anticipated. Two problems were encountered. First, using routine procedures involving 'universal' PCR primers (Kocher *et al.* 1989) DNA extracted from Australian little tern blood samples frequently returned multiple sequences due to co-amplification of nuclear and mtDNA homologues of target gene regions. This was overcome by using a special purification technique (caesium chloride density gradient centrifugation) to prepare enriched mtDNA isolates. The IMS has obtained two

such preparations for Australia fairy tern from Dr Donellan's laboratory. These should provide valuable comparative data for our New Zealand study, but have not been analysed yet. Second, Dr Donellan and coworkers found quite low levels of genetic variation in the mtDNA regions that they originally selected for analysis: NADH dehydrogenase subunit 6 (ND6) and cytochrome *b*. They solved this problem by analysing a more rapidly evolving region of the mitochondrial genome: the control region (CR). In our experience this can be a tricky system to get up and running and we have not attempted to amplify CR targets from NZ fairy tern samples.

We then compared our partial cytochrome *b* sequence for NZ fairy tern reported earlier (Chambers 1995) with that for Australian fairy tern (Donellan, 1996 and Donellan unpublished data) as shown in Figure 1. There is only one nucleotide substitution (a pyrimidine transversion at position 24) between this pair of 252 nucleotide long sequences. This may be compared with 11 fixed differences in 314 nucleotide positions between Australian representatives of *S. nereis* and *S. albifrons*. A further 4 nucleotide positions are polymorphic within *S. albifrons* populations in Australia. In summary, our preliminary and rather fragmentary data indicate that the cytochrome *b* genes of *S. n. davisae* and *S. n. nereis* show a level of divergence less than or equal to that shown by local populations of *S. albifrons*, which in turn is at least tenfold lower than that between the two tern species *S. nereis* and *S. albifrons*.

Conclusions

Our preliminary investigation of the genetics of the New Zealand fairy tern has shown this to be a technically demanding problem. First, biological samples collected in the field and from museum specimens are limited in number and generally of low quality, and thus require special handling. Nonetheless, it has been possible to obtain some preliminary, but authentic, mitochondrial DNA sequence data from them. Examination of these sequences and comparison with those obtained by Dr S Donellan and co-workers support the proposition that *S. n. davisae* from New Zealand is an extremely close relative of *S. n. nereis* from Australia. The present dataset is much too limited, both in terms of the number of nucleotide positions and the number of individual birds surveyed, to decide if there really are fixed genetic differences between the two subspecies. However, given that one potentially discriminating nucleotide substitution has been observed, it would be prudent to retain the present biological classification thereby regarding them as potential 'evolutionary significant units' (ESU) and practical to regard the New Zealand fairy tern as a 'management unit' (MU) *sensu* Mortiz (1994). During 1997, the collection and analysis of cytochrome *b* data will be continued at IMS in order to confirm and extend the above findings, but it is already clear that a more extensive study will be required to resolve this question with confidence.

Future prospects

The data to hand are only just beginning to throw some light on this important conservation problem, but do provide a reasonable basis for future strategic decisions. It is clear that a rapidly evolving segment of the mitochondrial genome, e.g. the control region, should be analysed from as large a number of individual birds as is practical. For the New Zealand fairy tern this number is probably quite small, perhaps ten to twenty birds. However, it is equally, if not more important, to have good representation from as many populations of Australian fairy tern as can be managed. This is necessary in order to decide if the mtDNA haplotypes observed in *S. n. davisae* are unique to this subspecies, or if they simply represent a limited subset of *S. n. nereis* haplotypes. It would also probably be prudent to examine a set of nuclear DNA markers such as microsatellites.

Recommendations

- That New Zealand and Australian populations of the fairy tern (*Sterna nereis*) continue to be known as the subspecies *S. n. davisae* and *S. n. nereis* respectively.
- That the New Zealand fairy tern be regarded as a MU for conservation purposes and as a potential ESU for future conservation and taxonomic purposes.
- That DoC give consideration to further molecular genetic analysis work on these birds in the context of conservation management needs.
- That any future genetic survey of these subspecies be carried out following the comments made in this report with regard to sampling strategy, target systems for analysis and the used of experienced staff: see Appendix 1.

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NZFT                                TTGACACAGATCCTAACAGGACTCCTACT 29
OZFT        TCTCTCCTAGGCATCTGTCTG.......................T.....
OZLT        TCTCTCCTAGGCATCTGTCTGC.A..T..A...................

NZFT        AGCCATACACTACACTGCAGATACAACCCTAGCTTTTTCATCTGTGGCCC 79
OZFT        ...................................................
OZLT        ...T.............................C.........R......

NZFT        ACACATGCCGAAATGTACAGTATGGTTGATTAATCCGTAACCTCCATGCA 129
OZFT        ...................................................
OZLT        ...................................................

NZFT        AACGGAGCATCATTCTTCTTCATTTGCATCTATCTACACATTGGACGAGG 179
OZFT        ...................................................
OZLT        ....................................T..............

NZFT        ATTCTACTACGGTTCATACCTGTACAAAGAAACCTGAAACACCGGAGTTA 229
OZFT        ...................................................
OZLT        ............B.....................................G

NZFT        TCCTTCTACTAACCCTAATAGCCA                           253
OZFT        ........................CTGCCTTCGTAGGATACGTCCTG
OZLT        ....C..............R...CTGSCTTCGTAGGATATGTCCTG
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Figure 1: Partial DNA sequences of the mitochondrial Cytochrome b gene from three terns; NAFT, New Zealand fairy tern, composite DNA dta (two individuals) from IMS experiments after Chambers (1995) but with four less certain residue positions trimmed from the ends; OZFT, Australian fairy tern, data from Donellan (1996); and OZLT, Australian little tern, data from Donellan (1996), composite data for four individuals from four different populations.

Appendix I

Sampling regimes and sample sizes

In any study of this type it is always necessary to compromise between the ideal requirements of the laboratory analyst and what can be most readily obtained and preserved by field workers. Indeed, when a species is so rare as the New Zealand fairy tern, any biological material must be considered to be of value.

The best avian tissues to collect for mitochondrial DNA analysis are breast muscle, heart or liver. These are preferably collected fresh and immediately stored frozen in liquid nitrogen. Blood samples taken in syringes containing anticoagulant (EDTA is preferred over heparin) are a good source of nuclear DNA and do not require the sacrifice of individuals. Blood samples are often not good starting material for mitochondrial DNA analysis. They contain large numbers of nucleated red blood cells and consequently bulk DNA extracted from such sources has an unfavourably low ratio of mtDNA to nuclear DNA. This can lead to preferential and inadvertent amplification of nuclear homologues of mtDNA targets, e.g. as was Donellan's experience with Australian little tern.

Workers in the IMS laboratory have often found that freshly plucked feathers are the best source of mtDNA from endangered bird species. Samples can be obtained easily when birds are handled for banding or weighing, and although they do not yield large quantities of bulk DNA, the ratio of mtDNA and nuclear DNA is sufficiently high to ensure amplification of correct target sequences. Feathers collected in the field can be saved on file cards under adhesive tape and do not need to be frozen immediately, provided that they can be shipped to the analytical laboratory promptly.

Finally, any other biological samples which can be collected in the field, including dead embryos, egg shells with membrane attached, or dead chicks, can serve as a source of DNA for analysis. Results are much less predictable using DNA extracted from such sources. They also require special handling in the laboratory to avoid inadvertent amplification of contaminating DNA from microorganisms and human operators. The same considerations also apply to tissues taken from preserved museum specimens, which represent an additional but generally rather restricted resource for sampling. A most important consideration when collecting any biological sample for DNA analysis, but particularly for those containing trace amounts or ancient DNA, is to avoid contamination from sample to sample or from operator to sample. This is best achieved by using disposable surgical gloves, scalpel blades and syringes.

The numbers of individuals required to be examined in any conservation genetics study is again largely determined by what is possible in practice. Clearly the numbers of New Zealand fairy tern samples should be as large as possible. This would enable DoC not only to resolve the central systematic question, but also to estimate genetic diversity within the population. It is equally, if not more, important to collect good representative samples from as many Australian (and New Caledonian) fairy tern populations as possible. In my

opinion, a sampling regime might consist of five individuals from each of five widely spaced populations. Finally, outgroup samples from other, more or less closely related tern species would be required. However, here samples would only be required from one or two individuals from two or three outgroup species, e.g. Australian little tern.

Protocol for field collections

The exact protocol of choice depends, first on what tissue is being collected, and second on what can actually be achieved in practice. Some generalisations can be made: samples stored frozen are much better than those which are just kept cold (e.g. in a refrigerator) or held at room temperature, and if samples absolutely must be maintained at room temperature, it is necessary to add a preservative or at least to keep them dry (or desiccated). These precautions are designed to minimise post mortem deterioration, especially degradation due to the action of contaminating microorganisms.

Blood and tissue samples should be transferred to labelled plastic cryotubes (e.g. Nunc brand) and placed directly into liquid nitrogen or liquid air. It is often most convenient to leave large containers of liquid nitrogen at a base camp and carry one or two small thermos flasks into the field site. Alternatively, samples can be stored on ice and transferred to liquid nitrogen later, e.g. at the end of the day, but this is not always a successful procedure. It is also possible to store whole birds frozen and to dissect them later, preferably without thawing. Good quality ethanol (i.e. molecular biology grade) is a good preservative. Adding two volumes of ethanol to a tissue sample results in a 70% ethanol preserving solution. Samples preserved in this way can be held at room temperature for several days or even weeks, but should be transferred to ultracold storage as soon as this can be achieved. A variety of other preservative cocktails have also been used by field workers with varying degrees of success. For recipes and references I recommend two general reviews of field collection and sample preservation methods for molecular systematics Cann *et al.* (1993) and Dessauer *et al.* (1996).

Analytical systems

From the existing molecular genetic data on NZ fairy tern it is becoming clear that rapidly evolving (highly variable) systems should be analysed. Donellan's (1996) experience with Australian little tern shows that the control region of mtDNA in this species is more than three times as variable as cytochrome *b* (47/271 versus 15/314 substituted nucleotide positions). This is clearly the place to begin any future study on NZ fairy tern. Based on our present data analysis of cytochrome *b*, the control region of the *Sterna nereis* genome might be expected to return up to six polymorphic nucleotide positions upon which to test the proposition that there are fixed genetic differences between the mitochondrial genomes of *S. n. davisae* and *S. n. nereis*. This will be an even more technically challenging project than the cytochrome *b* analysis.

In the longer term it will probably be desirable to examine highly polymorphic nuclear markers such as microsatellite systems. However, this represents an altogether more complex project requiring special cloning and NDA sequencing work for each new bird species examined.

Recommendations

- That the New Zealand Department of Conservation commission a DNA sequence analysis of the control region of the mitochondrial genome of ten or more individual New Zealand fairy tern (*S. n. nereis*) individuals (minimum = five) and populations (maximum = five) as can be conveniently obtained.

- That when this initial project has been completed and reported, due consideration be given to a full microsatellite analysis of *S. n. davisae* and *S. n. nereis* as a collaborative venture between IMS and DoC.

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