Molecular genetic analysis of hybridisation

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Part1. Molecular genetics and the study of hybridisation in natural populations

Geoffrey K. Chambers and Elizabeth S. MacAvoy

Abstract

The range of molecular tools in present use for the study of population genetics include: molecular cytogenetics, allozyme electrophoresis, protein sequencing, restriction fragment length polymorphism (RFLP), DNA fingerprinting, PCR and DNA sequencing, amplified fragment length polymorphism (AmFLP), SSCP, TGGE, DGGE and heteroduplex analysis, randomly amplified polymorphic DNA (RAPD), and microsatellite analysis. Underlying principles, advantages, and disadvantages of these leading and most versatile technologies are examined in the context of two case studies.

This survey will introduce conservation biologists and wildlife managers to the most commonly used techniques and help them to select the most cost-effective molecular genetic tools for conservation study.

1. Background

Recent advances in the development of molecular genetic techniques have provided conservation biologists with a rapidly increasing diversity of powerful laboratory tools. As a result, wildlife managers are now faced with an almost bewildering range of alternatives from which to choose. Making such decisions is difficult since the methods which are currently available differ considerably with respect to versatility, cost, sampling requirements and development time for each new species. In particular, the advent of the polymerase chain reaction (PCR), which makes it possible to obtain highly discriminating analyses from relatively small biological samples has spawned a very wide range of more or less reliable analytical systems within the last ten years. The purpose of this article is to outline the leading characteristics of the most commonly used techniques, as a guide to the selection of the most cost-effective system. This has been done in the context of two specially selected case studies and by referring to other work carried out previously in New Zealand.

2. General concepts

As populations diverge mutations occur in DNA and individuals begin to accumulate genetic changes which eventually come to characterise each population. These changes are recorded in their genomes and may also be expressed in gene products (RNA and protein). Those changes with functional consequences, may give rise to observable morphological and behavioural characteristics. It follows, therefore, that appropriately discriminating molecular analyses of genes and gene products can provide very sensitive tools with which to measure population differentiation, even in the absence of more overt characters. It also follows that the rate of molecular evolution of the genetic target is of critical importance when selecting both the target itself and the method of analysis. Rapidly evolving markers, such as some repetitive DNA sequences, will provide good systems for individual identification, whereas slowly evolving systems are required for high level taxonomy. Measuring the genetic differentiation of populations represents an intermediate between these two extremes. Any molecular study of hybridisation requires that one is able to monitor gene flow accurately between two more or less well differentiated populations. For this reason we elected to analyse DNA sequence variation in mitochondrial DNA (mtDNA) for our study on hybridisation of New Zealand Black and Pied Stilts (MacAvoy and Chambers, 1998 - accompanying paper). We chose two target regions, cytochrome *b* and the control region, which evolve at medium to fast rates relative to most nuclear DNA markers, and which have previously proved useful for this type of work.

3. Survey of genetic targets and analytical methods

The sections below describe in outline the more commonly used molecular techniques. It is intended only as an overview as several good texts are now available for detailed reference, e.g., Avise (1994), Hillis et al. (1996), Majerus et al. (1996) and Smith & Wayne (1996).

Molecular cytogenetics

Changes in chromosome number and shape are detected by chemical banding/ staining or by *in situ* hybridisation with radioactive or fluorescently labelled DNA probes. The latter technique is popularly known as 'FISH' or 'chromosome painting' to its practitioners. All such methods are highly taxon dependent as regards both ease of application and discriminating power and they all require considerable expertise. In general, they have been applied only in cases where a favourable combination of such factors pre-exists.

Allozyme electrophoresis

Changes in the size, shape and charge of enzyme proteins all affect their mobilities in an electric field. These are detected by dye-based chemical staining systems linked to their catalytic activities. The protocols are general across many taxa, quick to develop and provide good data for investigation of species level problems, including hybridisation. The major disadvantages of this method are the requirement for large scale and often destructive sampling of individuals, coupled with the need to freeze samples collected in the field as quickly as possible. Nonetheless, this method has been in routine use for around thirty years and remains the first choice of many investigators. In New Zealand the utility of this set of techniques is well illustrated by the work of Daugherty et al. (1990) on tuatara (*Sphenodon* spp.). The enduring popularity of allozyme electrophoresis is due in part to the factors above, and in part to the view that allozyme electrophoresis samples a wide range of independent nuclear markers. Thus, in the context of hybridisation, data produced in this way reflect gene flow mediated by both males and females.

Protein sequencing

Automated chemical detection of the linear order of amino acid residues reveals the sequence of the protein. Recent advances in this technology mean that such information can be obtained much more rapidly than was previously possible, and from much smaller biological samples. However, this technique cannot yet be applied to population level studies but remains well suited to confirm the results of genetic cloning experiments and of particular value for high level taxonomic questions. Note that protein sequences are increasingly produced by inference from DNA sequences rather than by direct experiment. This practice is not without its pitfalls.

Restriction fragment length polymorphism (RFLP)

Mutational changes in DNA produce nucleotide substitutions which can create or destroy short four or six nucleotide sequence motifs that are recognised by restriction endonucleases. These changes can be revealed as changes in fragment sizes (RFLPs) when DNA is cut with such enzymes and then run out on an electrophoretic gel. The earliest studies of this type were limited to looking at small DNA molecules, such as mtDNA. Despite its general applicability, the method is not especially efficient as a typical single enzyme digest can only probe around 20 to 50 nucleotide positions out of around 16,000 in a typical mtDNA molecule. This means that several enzymes must be used either alone or in combinations to map variation in individual samples. The technique is thus further limited by a requirement for fresh and relatively large (around 1 to 2g) tissue samples, from which to produce sufficient DNA for multiple analyses. This together with the time and expense involved to purify mtDNA, has discouraged many investigators from using these methods. This opinion must, however, be balanced by the observation that RFLP analysis of mtDNA has a distinguished history and has been successfully applied to many population genetic and phylogenetic questions (see Avise 1994 for an account of this era).

DNA fingerprinting

Improved sensitivity and generality in RFLP analysis have been achieved by coupling the basic methodology described above to solid phase DNA-DNA hybridisation detection systems based on radioactively labelled DNA probes. This method also allows analysis of much more complex mixtures of DNA molecules, such as those produced by restriction enzyme digestion of whole genomic DNA. Ultimately these methods have almost all proved to be too time consuming and too expensive for routine use in population genetics. There is one conspicuous exception to this generality; the group of techniques commonly known as DNA fingerprinting (see Chambers 1994). Here data are produced by using a cloned hypervariable minisatellite (also known as a 'variable number of tandem repeats' or VNTR) DNA region as a probe.

In its first applications this technique produced a barcode-like pattern of bands (or a 'multilocus' DNA fingerprint) that could be used to identify individuals and to analyse family relationships. Importantly, multilocus probes first developed for humans (e.g., Jeffrey's famous 33.15 and 33.6 systems, see later) were subsequently found to work in a wide range of wild animals, including New Zealand Blue Duck (*Hymenolaimus malachorynchos*); see Triggs et al. (1991 and 1992). Lingering concerns about statistical problems associated with the non-independent data produced by multiple cross sample comparisons have now been shown to be well-founded, but of small magnitude (Call et al., 1998) and fairly easy to correct (Lynch 1990). In some later studies, species-specific single-locus probes were developed, but this requires considerable and uncertain investment in molecular biological expertise and facilities. The method has not found widespread favour among investigators.

PCR and DNA sequencing

The use of the Polymerase Chain Reaction to provide enzyme catalysed amplification of selected DNA target segments coupled with the development of automated, fluorescent dye based DNA sequencer technology has enabled many new laboratories to enter the field of molecular genetics. Reliable data can now be generated quickly and relatively easily from small biological samples. Often it is unnecessary to freeze samples, as DNA containing tissues can be preserved quite well at room temperature in media containing high concentrations of salt or organic solvents. Indeed, production of DNA sequences even from subfossil and dried trace specimens (including museum collections) has become almost routine by using a set of ultra careful laboratory protocols called 'ancient DNA' techniques: e.g., for New Zealand examples refer to Cooper et al. (1992), Lento et al. (1995) and Trewick (1997). Given all of the above advantages it is no wonder that, over recent years, PCR and DNA sequencing has become the pre-eminent methodology for species and population level investigations.

Amplified fragment length polymorphism (AmFLP)

In this technique PCR products are digested with restriction enzymes and the sizes of the resulting DNA fragments determined by electrophoresis. This method is often applied as a coarse grained survey method, in much the same way as RFLP analysis (see above). However, if DNA sequence information is available about the target locus to be analysed, then fine grained diagnostic

systems can be developed to probe for the presence or absence of particular nucleotide substitutions. The efficiency of this approach is well demonstrated by our analysis of cytochrome *b* and control region targets in the mtDNA of various stilt species (MacAvoy & Chambers 1998, this issue, p.15).

SSCP, TGGE, DGGE and heteroduplex analysis

A range of new analytical techniques is also being developed for the detection of nucleotide substitutions in PCR products. These include SSCP (single strand conformation polymorphism), TGGE and DGGE (temperature and denaturing gradient gel electrophoresis, respectively) and heteroduplex analysis. These techniques use a variety of more or less sophisticated electrophoretic methods to survey short single or double stranded DNA molecules for sequence variation. Variants so revealed can then be fully characterised by DNA sequencing.

Randomly amplified polymorphic DNA (RAPD)

Pairs of short synthetic oligonucleotide primers with randomly selected sequences are used to co-amplify multiple genomic targets in PCR reactions and the mixture of products analysed by electrophoresis. The technique has many desirable characteristics. It is reasonably general across taxa, quick to set up, inexpensive to run and returns highly discriminating data. It is for these reasons that many laboratories have been tempted to adopt this method, and it has proved particularly popular, and useful, among plant population geneticists. Regrettably, experience has shown that not only is the method often unreliable in the sense that in many systems individual results are often difficult to replicate exactly from run to run (Pérez et al. 1998), but also that quite large (in the order of 100 or more) samples must be analysed in order to calculate RAPD allele frequencies with requisite precision (Lynch & Milligan 1994).

Microsatellite analysis

Short hypervariable repetitive DNA elements (usually segments of di- and trinucleotide repeats) are amplified by PCR and analysed by electrophoresis, often using automated DNA sequencer technology. This technique has multiple advantages; accurate and reproducible allele frequency data from several highly polymorphic systems can be produced from large numbers of individuals in a relatively short time. For these reasons this technology seems to be rapidly becoming the method of choice, for individual identification, parentage analysis and the study of population structure. However the hypermutable nature of microsatellites which makes them such attractive tools in the first place also means that microsatellites have to be developed and fine-tuned for each species and may not be useful for even closely related species. For each new project, microsatellite loci are isolated by DNA cloning and DNA library screening procedures. Candidate loci must then be characterised by DNA sequencing before methods for their amplification can be developed and optimised. This can take several months, even in the hands of a highly experienced molecular biologist. Hence the requirement for well trained staff and good facilities. These factors coupled with lead-in time and its associated costs, may act as deterrents to some investigators. However, on balance the rapid adoption of this technology is strong testimony to its utility and shows that the investment of time and resources required in the development phase is generally considered worthwhile.

The data produced from microsatellite analysis, genotype and allele frequencies, are similar to those obtained from allozyme electrophoresis and, therefore, amenable to analysis by much the same statistical methods. However, the authors of this article wish to add one note of caution at this point. At present no one has yet described in full the mechanisms by which microsatellites mutate and evolve (see Amos et al. 1996). Thus, it is not yet possible to develop entirely satisfactory mathematical models for prediction of allele frequency spectra in populations (e.g., Kruglyak et al. 1998), or for phylogeny reconstruction (see Goldstein & Pollock 1997 for a review). So investigators may find that they face a less than ideal situation when they come to test various null hypotheses, for example regarding population structure. Again, in balance, we must add that these problems do not affect all applications of microsatellite analysis equally, and may be relatively minor difficulties at worst. To our minds, the current popularity of these systems demonstrates clearly that most analysts do not see this problem as an overly serious concern.

4. Molecular genetic analysis of hybridisation: Two case studies

Here we contrast the methods used and results obtained by Abernethy (1994) in her study on native red deer (*Cervus elaphus*) and introduced Sika deer (*C. nippon nippon*) in Scotland with our own (MacAvoy & Chambers 1999, this issue) on New Zealand stilts (*Himantopus* spp.). In the first case, a small group of Sika deer (approximately 20 individuals) escaped from the Carradale Estate in Kintyre around one hundred years ago and began to mate with the local red deer. Hybridisation is now taking place across a wide zone spreading out from the site of the original introduction. As Sika and red deer differ in a number of morphological characters, such as coat colour and antler size and shape, it is relatively easy to identify the individual species, but only some of their hybrids can be picked by eye in the field. Under the circumstances described it is expected that the feral Sika deer gene pool will contain many red deer markers and that Sika deer genes will be entering the red deer population by introgression across the hybrid zone independently of morphological markers.

In New Zealand stilts an almost reversed situation exists. In a similar timeframe the self-introduced pied stilt has virtually succeeded in replacing the native black stilt over its entire range. The remnant black stilt population is now critically endangered by both hybridisation with pied stilts and by a variety of environmental factors. Here the expectation is that New Zealand pied stilt gene pool will contain black stilt markers that differentiate them from their source population of Australian black winged stilts. This should reflect the degree of interbreeding that occurred historically between black and pied stilts. In fact, this may not be very great given the reported ecological preferences of the two species combined with the contemporary observations of declining black stilt

numbers during the establishment phase of pied stilt colonisation. The present black stilt population is intensively managed and its gene pool is, therefore, expected to contain a minimal number of pied stilt markers due to prior introgression.

Abernethy (1994) used three methods of molecular analysis to examine interspecific breeding dynamics in red and Sika deer; allozyme electrophoresis, AmFLPs in mtDNA (termed RFLPs in the actual report) and microsatellites. Useful species specific diagnostic markers were found in all three systems reflecting a considerable degree of evolutionary divergence between the two species. The results of the molecular genetic analyses are clear and in accord with expectations; allele (allozymes and microsatellites) and haplotype (mtDNA) frequency clines are observed across the zone of active hybridisation. Phenotypically Sika-like deer near to Carradale show significant frequencies of red deer alleles. Equally Sika mtDNA and nuclear markers have penetrated morphologically red-like deer populations beyond the hybrid zone. Interestingly, the Sika deer nuclear markers were found to have introgressed further than their maternally inherited mtDNA haplotypes. This process is termed 'cytonuclear disequilibrium' and reflects the greater dispersal ability of Sika deer stags over Sika deer hinds.

New Zealand black and pied stilts are very closely related as is reflected in Green's (1988) allozyme study. Her data may also perhaps be interpreted to suggest that the New Zealand pied stilt is a genetic intermediate between Australian black winged stilt and New Zealand black stilt (presumably mediated by post-colonisation interbreeding with black stilts). However, data from much more highly discriminating loci (such as microsatellites) will be required to test this hypothesis thoroughly. Our own analysis of mtDNA haplotypes shows no differentiation whatsoever between Australian black winged and New Zealand pied stilts. Our DNA sequences from the control region of the mitochondrial genome do allow the New Zealand black stilts to be distinguished easily from the other two populations. The study also shows no introgression of mtDNA markers from pied to black stilts. We originally selected mtDNA analysis as an investigative tool because it generally reveals greater levels of genetic variation than allozyme analysis (a necessary prerequisite in this case), but also because 'universal' primer systems (Kocher et al. 1989) are available for studies on birds. This reduces the lead in time required to develop molecular analysis systems (relative to microsatellites, for example). Further, the opportunity to develop AmFLP assays diagnostic for mtDNA haplotypes provides an added advantage. We believe that our choice of method is entirely vindicated by the data obtained.

We must also acknowledge two ways in which our study could be improved and extended. Both stem from considerations arising from the strictly maternal inheritance of mtDNA markers. Our data on hybrid node birds show definitively that black stilt females are participating in the hybridisation process. However, because field observations suggest that hybridisation is a sex-biased process; i.e., that matings between black stilt males and pied stilt females are more common than those between black stilt females and pied stilt males (Pierce 1984) there is a real possibility of cytonuclear disequilibrium in this case too. This would have practical consequences for the black stilt conservation programme (Pierce 1996). If this process is going on then pied stilt alleles may

be introgressing into the black stilt gene pool undetected by either morphological scrutiny or mtDNA analysis. In the longer term, the danger is that what now seems to be a morphologically defined **and** genetically well differentiated species, the New Zealand black stilt (*Himantopus novaezelandiae*) could, as a result of undetected nuclear gene flow, become genetically degraded to what is simply a dark colour morph of the New Zealand pied stilt (*H. h. leucocephalus*).

There are two ways in which to resolve these problems. First by examining a greater number of hybrid individuals using the existing technology the extent of female mediated gene flow may be defined more accurately than at present. If, as seems likely, the cross over between Black and Pied Stilt mtDNA haplotypes lies somewhere in the middle of the hybrid plumage nodes, then the Black Stilt nuclear genome may still be relatively uncontaminated. The apparently biased pattern of male mating behaviour will be expected to produce a greater flow of nuclear genes from Black to Pied Stilts rather than vice versa. Second, a sufficiently large number of unlinked nuclear genes should be examined over large numbers of samples for each plumage node. Microsatellite DNA analysis is the only method presently capable of producing the data required. This technique was not adopted for the present study due to constraints on time and other resources. It remains, in our opinion, the most attractive option for any future genetically-based management strategy for conservation of this species.

5. Concluding remarks

This commentary has attempted to explain the underlying principles, advantages and disadvantages of a wide range of molecular tools presently used in the study of population genetics. The applications of a few of the leading and most versatile technologies have been examined in the context of two case studies. We hope that this will introduce wildlife conservationists to these techniques and help them to select molecular genetic tools for conservation study.

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Part 2. Mitochondrial DNA sequence from black and pied stilts

Elizabeth S. MacAvoy and Geoffrey K. Chambers

Abstract

The utility of mitochondrial DNA analysis as a tool for genetic assessment of hybridisation between New Zealand black stilt (*Himantopus novaezelandiae*) and New Zealand pied stilt (*Himantopus himantopus leucocephalus*) has been examined using polymerase chain reaction amplification of two target genes, cytochrome *b* and the control region. Two methods, DNA sequencing and restriction enzyme digestion, have been evaluated for their analysis. The data obtained show that mitochondrial DNA analysis is a powerful tool for stilt conservation, revealing species specific markers for both birds and that it can be used to monitor hybridisation between them.

1. Introduction

Hybridisation between New Zealand black stilt (*Himantopus novaezelandiae*) and New Zealand pied stilt (*Himantopus himantopus leucocephalus*) poses an immediate threat to the survival of the black stilt as a distinct species (Pierce 1996). It is important for conservation agencies to have a detailed understanding of this process if they are to manage the recovery of New Zealand black stilt. Mitochondrial DNA (mtDNA) analysis offers one of the best means to assess the genetic status of the two parent species and the extent and directionality of the hybridisation process because mtDNA is maternally inherited and shows no recombination.

2. Background

The New Zealand black stilt (NZBS), *H. novaezelandiae*, is a critically endangered endemic species. Its long and well recorded history of interbreeding with the New Zealand pied stilt (NZPS), *H. h leucocephalus*, has resulted in well defined hybrids. Pierce (1984) has subdivided these into 10 nodes based on

adult plumage. Among these, node A is considered to represent the 'pure' pied stilt genotype and node J the 'pure' black stilt genotype. All departures from these two plumage types, nodes B to I, are thought to reflect variable combinations of the two extreme genotypes. These intermediate nodes are assumed to reflect the compositions of underlying nuclear genetic loci responsible for morphological characteristics and allozyme markers (Pierce 1984, Green 1988).

The integrity of the New Zealand black stilt as a recognisable biological entity or species is threatened by this ongoing hybridisation process and future conservation management rests upon the ability to identify and increase the breeding population of 'pure' black stilt (Reed et al. 1993). This requires investigation of a special type of genetic system, mitochondrial DNA (mtDNA), which unlike nuclear genes does not undergo recombination.

3. Objectives

The objectives listed below are presented exactly as given in New Zealand Department of Conservation (DOC) Grant #2240.

1. To seek species-specific markers for Australian pied stilts, 'pure' New Zealand pied stilts and 'pure' New Zealand black stilts using restriction-enzyme analysis of mtDNA.
2. To identify which haplotypes are present in individual stilts classified as hybrids on the basis of plumage characteristics.
3. To evaluate the extent to which there may be 'cryptic' hybrids within the 'pure' black stilt and 'pure' New Zealand pied stilt phenoytpes.
4. To use the results to infer the directionality of hybridisation between black stilt and pied stilt in New Zealand.

4. Materials and methods

4.1 MATERIALS

Muscle tissue samples from 19 New Zealand black stilts were received from the DOC, Twizel Field Centre in April 1997. These samples were from juveniles not yet showing adult plumage, but since the plumage nodes to which each of their parents belonged is also known, individuals can be selected as possible sources of 'pure' New Zealand black stilt DNA. All the female parents were of node J except one, leg banded as YR-RBk which belonged to plumage node I. The male parents were mainly node J, with one node G and two node H individuals. Samples of liver and muscle from 5 Australian black-winged stilts (ABWS) and

from New Zealand pied stilts and New Zealand hybrid stilts (NZHS) representing plumage nodes A and B-I respectively were available from frozen tissue owned by DOC and stored at the Institute of Molecular Systematics (IMS), Victoria University of Wellington (VUW). These are the same samples that were previously used in an allozyme study at VUW by Green (1988).

4.2 METHODS

DNA was extracted from liver and muscle tissue by SDS-proteinase K digestion, purified by phenol and phenol/chloroform extraction (Sambrook et al. 1989) and concentrated using disposable microconcentrators (Centricon: Amicon Inc.). Target areas of mtDNA, were amplified by means of the Polymerase Chain Reaction (PCR) using synthetic oligonucleotide primers (Table 1) and the double-stranded products (dsDNA) were used for DNA sequencing and restriction enzyme digests.

TABLE 1. PRIMERS FOR PCR AND SEQUENCING.

L14841	5' —AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA —3'	(Kocher et al. 1989)
H15149M	5'—AAACTGCAGCCCCTCAGAATGATATTT—3'	(present study)
CH16746L	5'—ACCCCAAGGACTACGGCTTGAA—3'	(Wenink et al. 1994)
TS400H	5'—GTGAGGAGTCCGACTAATAAAT—3'	(Wenink et al. 1994)
TS1271H	5'—AGCTTGGCATCTTCAGTGCCA—3'	(Wenink et al. 1994)
SCR1	5'—TCAACTACAAGAACAACCCATTA—3'	(present study)
SCR2	5'—TTTCTCGAGTGGCCAATCATAGTGTGC—3'	(present study)

DNA sequencing

The amplified target DNA was purified by electrophoresis on low melting point agarose gel. Then the pure dsDNA obtained from gel slices was either used directly for manual double-stranded DNA sequencing (Sequenase Version 2.0 Sequencing Kit, USB) or it was purified with a Prep-a-Gene Kit (BioRad) and used for automatic sequence analysis (ABI Prism Model 377 Automatic DNA Sequencer) with dye terminator technology.

Restriction enzyme digestion

Amplified target DNA was purified and then digested with a number of restriction enzymes (separately) and the products separated on 2% agarose gels and visualised with ethidium bromide staining.

4.3 SELECTED GENETIC TARGETS

(1) Cytotchrome *b*

This target area was amplified using primer L14841 (Kocher et al. 1989) and a modified version of H15149 (Kocher et al. 1989), named H15149M, which has seven 3'terminal nucleotides omitted relative to H15149 (see Table 1 above). These primers amplify a 376bp fragment. A total of twenty six amplified DNA products were sequenced: 3 ABWS, 9 NZPS, 4 NZHS and 10 NZBS (* in Table 2). Products from nineteen individuals were used to test the restriction enzyme analysis methods (+RE in Table 2). Fewer NZHS were analysed than was originally planned; see Results for explanation.

(2) Control region

Seven synthetic oligonucleotide primers used in a study of Turnstone and Dunlin control regions (Wenink et al. 1994), were tested on stilt DNA to establish if any would support PCR amplification of the control region in stilt species. One pair CH16746L/TS400H amplified a product of 455nt, but the yield from different samples was variable. New primers were designed to anneal within this target area and these primers, SCR1 and SCR2, reliably amplified a product of 350bp. DNA samples from twenty six stilts were sequenced (* in Table 2) and nineteen of these also tested by restriction enzyme digestion (+RE Table 2). As a further check the whole control region was amplified (using primers CH16746L and TS1271H, Wenink et al. 1994,) and sequenced in one individual to establish that the internal primers used (SCR1 and SCR2) were indeed amplifying a target lying within a mtDNA nucleotide sequence with control region characteristics.

(3) Long PCR target

Cytochrome *b*—control region. To be certain that the two short targets examined above in experiments (1) and (2) were present on a contiguous stretch of DNA, primers L14841 and SCR2 were used to amplify a full length 2 Kb target. This was purified by gel electrophoresis, the band cut from a low melting point agarose gel and purified using a Prep-a-gene Kit (Biorad). If the yield was higher than 10ng/ml then the DNA product was sequenced directly from each end using the external primers. If the yield of DNA was below 10ng/ml, then the product was used as the template to re-amplify cytochrome *b* and the control region targets separately using primer pairs L14841/H15149M and SCR1/SCR2 as above. Sixteen DNA samples were sequenced for both targets: 6 NZPS and 10 NZBS (including female YR-BkY).

4.4 SEARCH FOR RESTRICTION ENZYME MARKERS

The DNA sequences obtained from target areas of the cytochrome *b* gene and the control region were examined, using the DNAStar software package, for restriction enzyme sites which would act as markers for DNA sequence haplotypes.

TABLE 2. DISTRIBUTION OF CYTOCHROME *b* AND CONTROL REGION HAPLOTYPES ACCORDING TO PLUMAGE NODES OF SAMPLES.

SAMPLE	PLUMAGE NODE	CYTOCHROME *b* HAPLOTYPE	CONTROL REGION HAPLOTYPE
*156	ABWS	A	A1
*161	ABWS	A	A4
*195	ABWS	A (+RE)	A1 (+RE)
*1	A	A	A1
*10	A	A (+RE)	A2
14	A	A	A2
21	A	A	A1
*27	A	A	A1
30	A	A	A1
*34	A	A (+RE)	A1
36	A	A	A3
*37	A	A (+RE)	A1
53	A	A	A1
78	A	A	A1
*99	A	A	A1 (+RE)
*100	A	A	A1 (+RE)
*101	A	A	A1
*102	A	A	A5
*63B	D1D2	A	A1
*96	EF	A	A1
*97	EF	B	B1
*80	GHI	B (+RE)	B1
*YR-BkR	J	C (+RE)	B2 (+RE)
RW-RR	J	C (+RE)	B2 (+RE)
*RW-YW	J	B (+RE)	B1
*WG-YR	J	C	B2
*WBk-WBk	J	B	B1 (+RE)
WBk-WG	J	C (+RE)	B2 (+RE)
RY-YW	J	B (+RE)	B1 (+RE)
RW-GG	J	B	B1 (+RE)
*RW-RY	J	B	B1 (+RE)
RY-YY	J	B (+RE)	B1 (+RE)
RY-GG	J	B (+RE)	B1 (+RE)
*RW-GY	J	B (+RE)	B1
WR-RR	J	B (+RE)	B1 (+RE)
*WBk-RBk	J	B (+RE)	B1 (+RE)
*YR-BkY	J	C (+RE)	B2 (+RE)
*WBk-GR	J	C (+RE)	B2 (+RE)
*WBk-GW	J	C	B2 (+RE)
YR-BkW	J	B (+RE)	B1 (+RE)
RBk-GBk	J	C (+RE)	B2 (+RE)

* Samples sequenced using two separate targets.

4.5 PHYLOGENETIC ANALYSIS

Sequences from cytochrome *b* and the control region were aligned in XEsee version 3.1 (Cabot 1989). Aligned sequences were combined and analysed using PAUP version 4.0b1 (Swofford 1998). Maximum parsimony and neighbour-joining methods were used to search for the shortest trees. The data set was bootstrapped (1000 replications) to estimate support for internal branches.

5. Results

5.1 DNA EXTRACTION

DNA extracted from Green's (1988) samples was found to be partially degraded but useable. The NZBS muscle samples from Twizel consistently yielded good quality DNA (data not shown).

5.2 CYTOCHROME *b* SEQUENCES

The 376 bp cytochrome *b* target was successfully amplified from forty one samples (both short and long targets) and the dsDNA products obtained proved suitable for both manual and automatic DNA sequencing. Sequences obtained (Figure 1) are considered to represent authentic mtDNA because they contain no stop codons, and when translated they encode a protein (not shown) with the expected characteristics of an avian cytochrome *b*.

Among the nucleotide sequences of the 314 bp central portion of the cytochrome *b* genes (the area between the primers) three haplotypes (designated A, B and C) can be distinguished (Figure 1). These differ by transition substitutions at three positions, 41, 70 and 170 (Table 3). The transitions T to C at positions 41 and 170 occur at first codon positions and at position 70 at a third codon position. The transition substitution at position 170 results in an amino acid substitution. The ABWS (N = 3) and NZPS (N = 15) analyses all exhibit Haplotype A. The NZBS (N = 19) displayed either Haplotype B (11 individuals) or Haplotype C (8 individuals). Two NZHS, node EF, were

TABLE 3. NUCLEOTIDE POSITIONS WHICH DIFFER BETWEEN CYTOCHROME *b* HAPLOTYPES.

	NUCLEOTIDE POSITION		
	41	70	170
Haplotype A	T	T	T
Haplotype B	T	C	C
Haplotype C	C	T	C

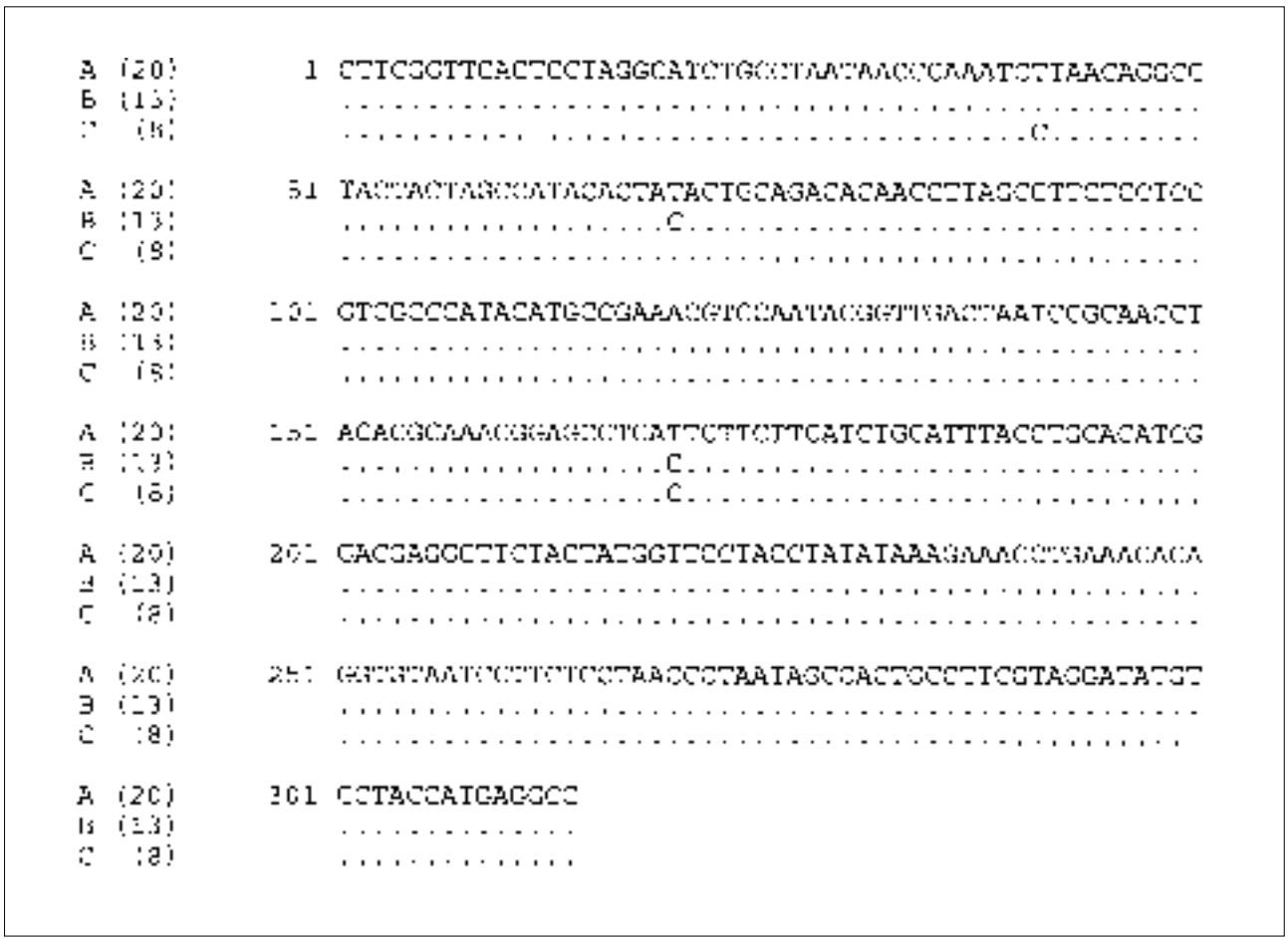

Figure 1. Representative DNA sequences from target regions of *Himantopus* mtDNA for cytochrome *b*. Positions of sequence identity are shown as dots and nucleotide substitutions by single letter IUB code.

tested, one displayed Haplotype A and one Haplotype B. One node D1D2 was Haplotype A and one node GHI was Haplotype B (see Table 2 above).

5.3 RESTRICTION ENZYME MARKERS FOR CYTOCHROME *b* HAPLOTYPES

Haplotype A has no restriction enzyme recognition site around nucleotide position 170 whereas the C for T substitution present in Haplotypes B and C creates an *Ear* I site. This can be used to distinguish between ABWS/NZPS and NZBS. The substitution of C for T at position 41 in Haplotype C destroys an *Mse* I site which can be used to distinguish it from Haplotypes A or B (Table 4). Illustrative results for an experiment in which various cytochrome *b* products representing all three haplotypes have been cut with the two enzymes above are shown in Figure 2 (next page). The haplotypes of all the samples tested agreed with the sequenced haplotype. Double digests were not successful as the buffer and reaction conditions required for each restriction enzyme were different. Ultimately it was judged easier to undertake a series of single digests rather than to change buffer systems and perform multiple digests.

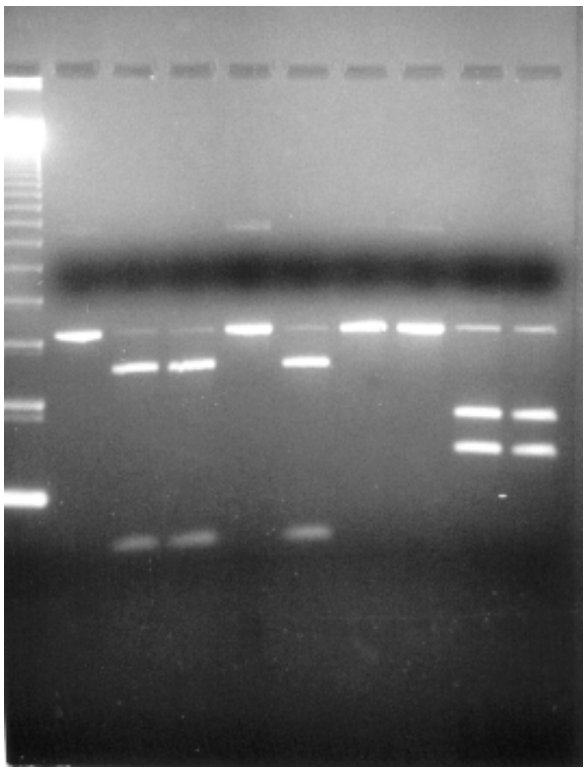

Figure 2. Electrophoretic analysis of cytochrome *b* amplification products following restriction enzyme digestion. Lanes (numbered left to right) contain: (1) 123bp molecular size marker; (2) uncut dsDNA amplification product; (3–6) digested with *Mse* I; and (7–10) digested with *Ear* I. Samples (haplotypes) are: ABWS 195 (Haplotype A) in lanes 3 and 7; NZPS 10 (Haplotype A) in lanes 4 and 8; NZBS YR-BkR (Haplotype C) in lanes 5 and 9; and NZBS WB-RBk (Haplotype B) in lanes 6 and 10. Digestion product sizes are given in Table 4.

TABLE 4. LIST OF USEFUL RESTRICTION ENZYMES SITES IN CYTOCHROME *b* WITH BAND SIZES.

ENZYME	HAPLOTYPE A		HAPLOTYPE B		HAPLOTYPE C	
	# SITES	BAND SIZES	# SITES	BAND SIZES	# SITES	BAND SIZES
Mse I	1	76, 300	1	76, 300	0	376
Ear I	0	376	1	165, 211	1	165, 211

5.4 CONTROL REGION SEQUENCES

A search was made for primer pairs which would support amplification of a selected area of the control region. Of the primer pairs tested only two pairs gave products of the expected size. When these products were investigated further one primer set was found to correctly target a 455bp region at the 5' end of the control region (established by sequencing the whole control region) and the other to amplify a unique 350bp target but one whose sequence unexpectedly did not match that of 3' end of the control region. A total of forty one amplification products from the 5' region were sequenced (Figure 3) and found to fall into two main haplotype classes (A and B). Haplotypes A1 and B1 differ by 15 nucleotide substitutions and one deletion in Haplotype B1 (Table 5). Haplotype Class A has 5 subtypes, A2 to A4 differ from A1 by one substitution and A5 by three substitutions. Haplotype Class B has two subtypes (Haplotypes B1 and B2) which differ by one nucleotide substitution, T for C at nucleotide position 271.

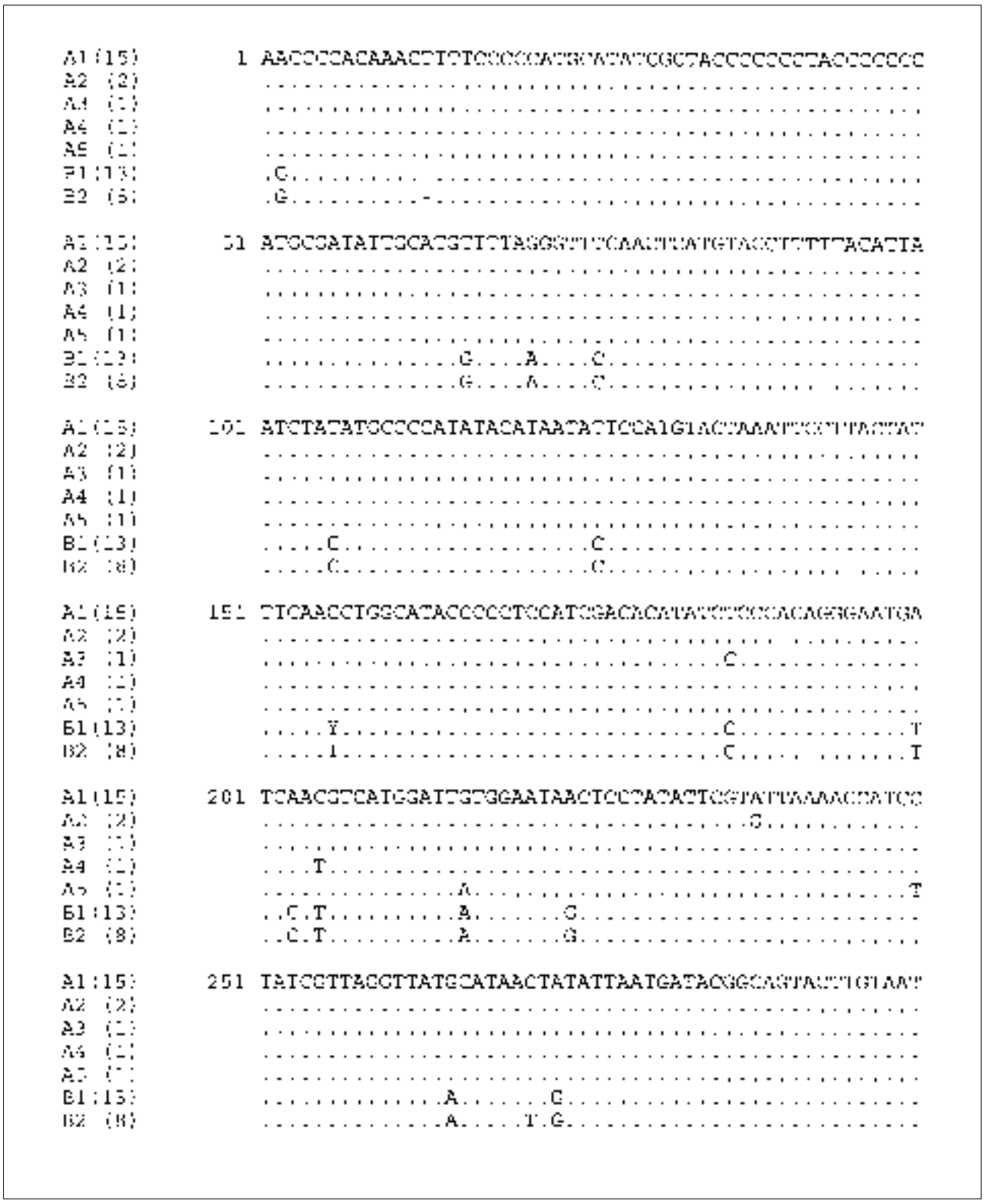

Figure 3. Representative DNA sequences from target regions of *Himantopus* mtDNA for control region. Positions of sequence identity are shown as dots and nucleotide substitutions by single letter IUB code.

TABLE 5. NUCLEOTIDE POSITIONS WHICH DIFFER BETWEEN CONTROL REGION HAPLOTYPES.

	NUCLEOTIDE POSITION																
	2	13	66	71	76	106	126	156	186	200	203	205	216	224	265	271	273
Haplotype A1	A	T	T	G	T	T	T	C	T	A	A	C	G	A	G	C	A
Haplotype B1	G	—	G	A	C	C	C	Y	C	T	C	T	A	G	A	C	G
Haplotype B2	G	—	G	A	C	C	C	T	C	T	C	T	A	G	A	T	G

The distribution of control region haplotypes according to plumage nodes is shown in Table 2. The Haplotype Class A is characteristic of ABWS and NZPS and Haplotype Class B of NZBS. Those NZBS individuals which exhibit Haplotype B1 also have cytochrome *b* Haplotype B, while those with cytochrome *b* Haplotype C have control region B2. Two NZBS with cytochrome *b* Haplotype C were initially determined to have control region Haplotype A when a short template was sequenced, but when a long PCR product was subsequently investigated they were found to have Haplotype B2.

5.5 RESTRICTION ENZYME MARKERS FOR CONTROL REGION HAPLOTYPES

Numerous restriction enzymes sites can be identified in the control region sequences. The most useful enzymes are those which cut once or twice resulting in bands over 60 nucleotides long which could be well separated and visualised on a 2% agarose gel (Table 6). Single digests using these enzymes can be used to positively distinguish between the two Haplotypes classes A and B but not to the level of haplotype class subtype.

TABLE 6. LIST OF USEFUL RESTRICTION ENZYMES SITES IN CONTROL REGION WITH BAND SIZES.

ENZYME	HAPLOTYPE CLASS A		HAPLOTYPE CLASS B	
	# SITES	BAND SIZES	# SITES	BAND SIZES
Alu I	0	455	1	167, 287
Hae III	1	75, 380	1	75, 379
Nsi I	2	89, 122, 244	1	89, 365
Sau 3A I	1	193, 262	0	454
Ssp I	1	188, 267	0	454

Fragments are listed in order of decreasing size and these are not necessarily in sequence order 5' to 3' and band sizes are given in bp.

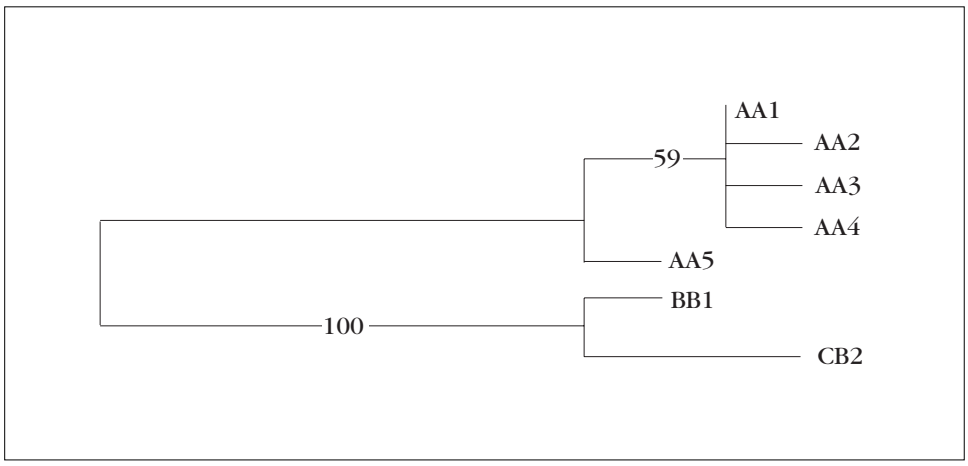

Figure 4. Maximum parsimony phylogram of stilt haplotypes based on sequence from combined cytochrome *b* and control region. An exhaustive search recovered three shortest trees, which differed by minor rearrangements of AA1, AA2, AA3, and AA4. A bootstrap search recovered the same tree. Bootstrap values have been transposed onto the phylogram.

5.6 PHYLOGENETIC ANALYSIS

Phylogenetic trees were constructed by maximum parsimony methods using 614 sites of which 593 were constant, 15 informative and 6 non-informative. An exhaustive search recovered 3 shortest trees which differed only in the order of the AA2, AA3 and AA4 haplotypes. The trees were rooted using the midpoint method (Figure 4). A bootstrap search (x1000) recovered the same tree as the consensus of the three shortest trees. The trees show a relatively deep separation between black and pied stilts (bootstrap support 100%) compared to shallow divergences between haplotypes within the ABWS/NZPS and NZBS. The AA5 haplotype diverged before other NZPS and ABWS haplotypes (bootstrap support 59%). Neighbour-joining analysis results gave essentially the same tree, differing only by minor re-arrangements of AA1, AA2, AA3 and AA4 haplotypes.

5.7 SAMPLE NUMBERING IN TISSUE COLLECTIONS

During the course of our investigations it became clear to us that the identifying numbers written on frozen sample tubes in the DOC tissue collection were not the same as those recorded in Green's (1988) thesis. We took steps to remedy this situation by consulting Ms B.S. Green and Dr M. Williams (DOC, Wellington). Together, they were able to recover Ms Green's original records on magnetic tape and decode them with the help of IBM Inc., Wellington. Note that it was only possible to classify the frozen samples of NZHS to compound plumage node classes, e.g., node class EF, and not to individual plumage nodes as in Green (1988). To date we have not been able to locate any key relating sample numbers to the individual identification numbers and specific plumage nodes given in Green (1988).

6. Discussion

The data presented above illustrate the utility of molecular biological analysis in wildlife conservation work to good advantage. It has been possible to extract DNA of sufficient quality and purity from very small quantities of biological material, even from samples that had been stored frozen for more than a decade. The lower quality of DNA obtained from this latter source can be attributed to damage which occurred during the freezing, thawing and homogenisation process required for allozyme electrophoresis carried out by Green (1988). Despite the observed degradation, most of these DNA samples served as good templates for the amplification of small target regions of the mitochondrial genome.

Standard methods for the amplification and analysis of two mtDNA targets, cytochrome *b* and control region (separately), have been successfully adapted for use with *Himantopus* spp. Comparison of sequences obtained reveal three haplotypes for cytochrome *b* and two haplotype classes for the control region. Cytochrome *b* sequences differ at three positions reflecting the relative evolutionary conservatism of this locus, while control region sequences show numerous substitutions and a deletion reflecting a more variable locus. Four of the pied stilts had control region sequence which differed by one or three nucleotides from all the others. Since the area of the control region sequenced is one of the most variable regions in the mitochondrial genome it is not unexpected that some degree of polymorphism will be found in this area. Such variants may be inherited and eventually increase in frequency to form a new major haplotype but in this particular case the observed changes probably represent relatively recent mutations as most of the samples are of the A1 type.

When the two target areas were sequenced separately all the ABWS and NZPS birds had a composite haplotypes AA (cytochrome *b* is given first) and all but two of the NZBS were scored as either BB1 or CB2. Because two NZBS individuals initially showed a CA haplotype, it was thought that the control region primers might have annealed to a nuclear pseudogene in preference to the authentic mitochondrial DNA target sequence. To test this idea long PCR was used to amplify a long 2Kb target with both target areas on the same DNA strand. This was followed by DNA sequencing of internal subtargets, which ultimately proved to be quicker and more reliable than amplification of the two targets separately. This method was then used to analyse more samples and to reanalyse a number of those which had already been characterised, including the two black stilts which displayed the ambiguous CA composite haplotype (see above). Using the new long PCR technique both of these black stilts were both found to have the CB2 haplotype. This suggests that individual amplification of the separate targets may, in these cases, have resulted in amplification of a genuine mitochondrial cytochrome *b* and a nuclear pseudogene copy of the control region. The finding of nuclear pseudogene copies of mtDNA genetic targets is an increasingly reported phenomenon (Zhang & Hewitt 1996, Quinn 1997) and for certain targets particularly including those which are highly variable, such as the control region, it is very likely for the pseudogene copies to be amplified preferentially, because the sequence of the primers will be expected to have greater complementarity to that of the older and more slowly evolving pseudogene se-

quence. All other samples which were reanalysed using the long PCR method yielded data which agreed with our previous results obtained by analysing the two mtDNA targets individually.

Examination of the distribution of individual haplotype combinations revealed complete linkage disequilibrium between the markers. The ABWS and NZPS birds were all of composite haplotype AA and all NZBS were scored as either BB1 or CB2. These data show marked differentiation between the mitochondrial genomes of NZBS and NZPS, but not between those of NZPS and ABWS. The phylogenetic tree resulting from analysis of the sequence data suggests that the NZBS have been evolving separately from the NZPS/ABWS for a long time and that NZPS arrived too recently to have diverged much from its Australian source population. The difference between AA5 and the other ABWS and NZPS haplotypes suggests there were at least two types of female founders from Australia but it is unknown if the single substitutions in haplotypes AA2 and AA3 occurred in Australia and represent different founder types or if these have occurred in the New Zealand population. Investigation of more Australian stilt populations would need to be undertaken to establish if these haplotypes are present there. Although hybridisation between NZPS and NZBS is well known, it does not seem to have resulted in widespread introgression of mtDNA from NZBS into the NZPS population. However, there has been a significant transfer of nuclear markers between NZBS and NZPS, as Green's allozyme study showed NZPS are genetic intermediates between NZBS and ABWS (Green 1988).

Biased bi-directional hybridisation is known to be actively occurring in stilt populations in the Mackenzie Basin management area as is evident from the existence of the hybrid plumage nodes (NZHS) and from field data (Pierce 1984, Reed et al. 1993). This is supported by our observation of two composite haplotypes (see above) in NZHS individuals belonging to plumage node EF. It is now of considerable interest and conservation importance to examine more NZHS individuals in order to learn more about the genetic consequences and directionality of hybridisation (see above, section 3. Objectives).

The DNA sequencing protocols that we developed have proved to be effective tools and have provided species-specific markers for Australian and 'pure' New Zealand pied stilt which distinguish them from 'pure' New Zealand black stilt. However, these protocols are both time-consuming and expensive. Moreover, they require considerable expertise both in the laboratory and in data processing. Our investigations of restriction enzymes which are capable of distinguishing the mtDNA haplotypes that we have described (Tables 4 and 6) promise a cost-effective alternative. Our preliminary demonstration of restriction fragment length polymorphisms in amplified cytochrome *b* dsDNA products (Figure 2) shows how this might be put into practice.

The results of restriction enzyme digests agreed with the more detailed sequencing results. The restriction enzyme analyses offer good prospects for the eventual development of robust and efficient genetic assay systems for haplotype diagnosis. It is disappointing that so few NZHS could be examined in the time available. The small sample size (four individuals) precludes drawing any firm conclusions regarding the extent and direction of hybridisation. However, this is being remedied by using the methods developed here to examine DNA extracted from more hybrid birds.

7. Conclusions

- New Zealand black stilt exhibit two similar composite mitochondrial haplotypes, designated BB1 and CB2 which are different from the single AA composite haplotype found in New Zealand pied stilts and Australian black-winged stilts.
- New Zealand black stilts possess a different mitochondrial gene pool to pied stilts. This together with morphological and behavioural differences lead us to consider black stilts as a separate species.
- These haplotypes serve as good species-specific markers for genetically 'pure' New Zealand pied stilts and New Zealand black stilts and can be scored by DNA sequencing or by restriction enzyme digestion of PCR amplified target regions.
- The restriction enzyme assays reported here offer the potential to develop an efficient and relatively inexpensive means of monitoring gene flow in *Himantopus* spp.
- The modern population of New Zealand pied stilt is derived from colonising invasions of at least two maternal lineages of Australian black-winged stilt, and these events took place too recently for their mitochondrial DNA haplotypes to have diverged.
- There are no 'cryptic' hybrids among the 'pure' New Zealand pied stilts and 'pure' black stilts examined.
- Gene flow between New Zealand black stilt and New Zealand pied stilt appears to be occurring in both directions, but analysis of more New Zealand hybrid stilt samples will be required to estimate its extent and the directionality of hybridisation.

8. Recommendations

The data presented here provide good evidence that NZBS is a distinct genetic entity which can be reliably differentiated from NZPS. It is unfortunate that problems encountered with the sample numbering system meant that we were not able to concentrate our efforts on specimens that we originally intended to assay. The result is an unbalanced database in which both ABWS and NZHS are underrepresented. To remedy this, additional samples of hybrid stilts are being tested to increase the number of cytochrome *b* and control region sequences for several representatives from each compound plumage node class.

The recommended additional information on mtDNA haplotypes in ABWS, NZHS and NZBS could be obtained either by DNA sequencing or by restriction enzyme analysis. The former method is very reliable, but may be considered time consuming and expensive. The latter method may eventually represent a cost-effective alternative, but will still require some careful developmental work to meet proper quality assurance standards.

9. Acknowledgements

We thank Murray Williams and Richard Maloney for their advice and help in obtaining tissue samples from Twizel Field Centre. We also thank B.S. Green for her help in supplying information on her numbering system of the frozen samples. We are especially grateful to Rod Hitchmough and Thomas Buckley for their assistance with the phylogenetic analysis and to Rod for his constructive comments on the manuscript.

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