CONSERVATION
TE PAPA ATAWHAI

CONSERVATION ADVISORY SCIENCE NOTES

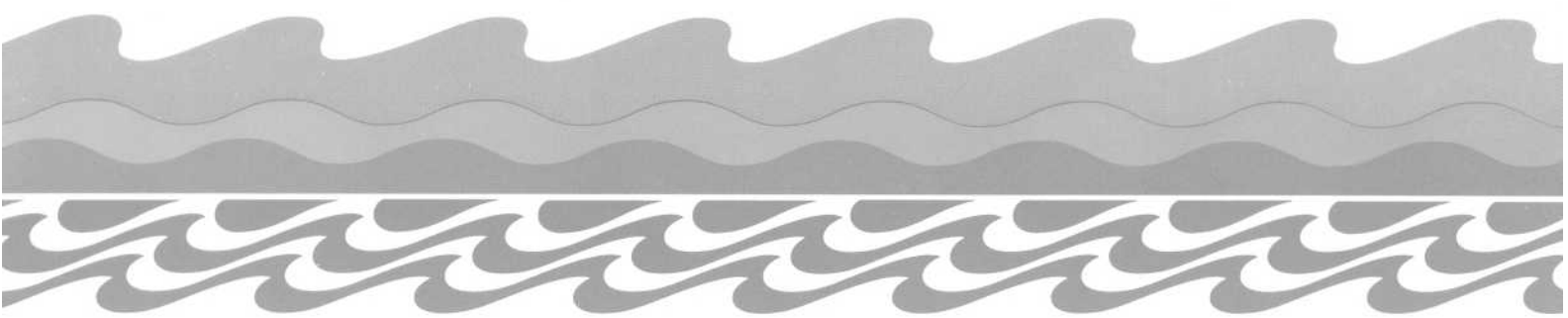
No. 23

DNA FINGERPRINTING STUDIES OF TEALS AND DUCKS

(Short Answers in Conservation Science)

This report is published by Head Office, Department of Conservation, and presents the results of scientific services or advice purchased from a consultant outside the Department or provided by Departmental scientific staff. All enquiries should be addressed to the CAS Coordinator, S&R Division.

Department of Conservation, P O Box 10-420, Wellington, New Zealand



ISSN 1171-9834

© 1993 Department of Conservation

Reference to material in this report should be cited thus:

Lambert, D., 1993.
DNA fingerprinting studies of teals and ducks.
Conservation Advisory Science Notes No. 23, Department of Conservation,
Wellington. 13p

Location: NZMS

Centre for Conservation Biology
(CCB)

DNA Fingerprinting Studies of Teals and Ducks

A report prepared for the Department of Conservation

Dr David Lambert
Centre for Conservation Biology
School of Biological Sciences
University of Auckland

May, 1993

Contents

Executive Summary

- High quality DNA has been extracted from teal blood which has been kept at -80°C. Tissue stored in Tris/NaCI/EDTA buffer also produced DNA of lesser quality. In addition DNA of a poor quality was extracted from tissue such as wing muscle which was preserved 1 to 12 weeks after the death of the animal.

- High quality DNA fingerprints have been produced for Campbell Island teals. The fingerprints of the Auckland Island material were of a lesser quality.

- Minisatellite DNA variation suggests that it may be possible to determine parentage in these species using this technique. The possibility of determining higher order relationships is discussed.

- The hybrid between Auckland and Cambell Island teals is highly distinctive, possessing a large number of restriction fragments not found in the Campbell Island subspecies. This results suggests that there may be a number of minisatellite differences between the two subspecies.

- The polymerase chain reaction (PCR) has been used to amplify annonymous DNA extracted from preserved muscle using DNA primers with a random 10 base sequence. These polymorphic regions could potentially provide appropriate nuclear DNA markers useful in the study of hybridisation between mallard and grey ducks in New Zealand.

Abstract

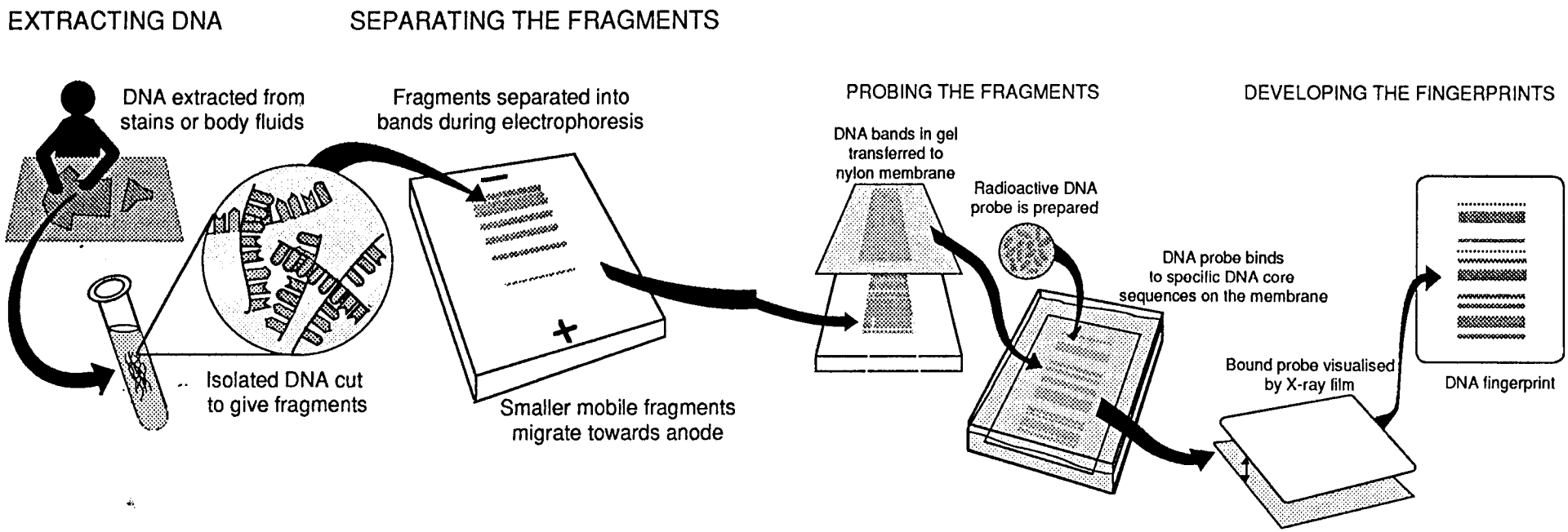
The multilocus DNA fingerprinting technique has been applied to a range of tissue samples from sub-antarctic teals and hyrids between Grey and Mallard ducks. DNA has been extracted from whole blood which was frozen immediately, Tris/NaCI/EDTA preserved tissue and heart and wing muscle which has been preserved in 70% ethanol and later stored in an utlrafreezer. Three multilocus probes, 33.6, 33.15 and pV47-2; were hybridised to genomic DNA from these samples which was digested with the restriction enzyme *Hae* lll. DNA fingerprints of high quality were obtained for the Campbell Island teal material which had been frozen immediately. These membranes were hybridized to three multilocus probes which were originally isolated from human DNA. Approximately 35 restriction fragments were found in the 23 to 2kb size range for the probe 33.15. This is high in comparison to many published studies. The hybrid between the Auckland and Cambell Island populations was highly distinctive. Fingerprints of lesser quality were obtained for the Tris/NaCI/EDTA material of Auckland Island teals, although these were scorable. DNA was able to be extracted from the ethanol-preserved duck material. However, this was not of a sufficient standard to be able to use the fingerprinting technique. Instead, using random 10 base primers, a PCR technique resulted in the amplification of polymorphic annonymous nuclear DNA sequences. A number of fragments in the 5 to 0.3 kb size range were amplified. These appear to be polymorphic in different individuals. This could potentially provide the kinds of nuclear markers needed to investigate the extent of hybridisation between these species.

Minisatellite DNA variation

A 'minisatellite' (also known as a hypervariable region, HVR, or a variable number tandem repeat locus, VNTR) is a DNA sequence, usually less than 20,000 base pairs, comprising multiple copies of a short sequence ('tandem repeat unit') of typically less than 65 base pairs. A minisatellite's organisation is therefore similar to that of 'satellite' DNA, which also consists of multiple repeat sequences, but on a much more extensive scale. Satellite DNAs are non-coding and so, presumably, are most minisatelites, though two are known to form parts of coding sequences.

The key advance that led to the development of DNA fingerprinting came when, in the course of an analysis of the human myoglobin gene, Jeffreys and his colleagues discovered a new family of minisatellite sequences that had in common a 'core' sequence of about 12 nucleotides. They showed that those minisatellites that consisted of multiple repeats of this core sequence could be used as probes to detect simultaneously the hypervariable minisatellites at many separate loci. The theoretical probablility of the same set of DNA fragments being detected in two humans is so small that every human except identical twins is expected to have a unique pattern and the pattern of bands obtained on an autoradiograph is therefore described by analogy as a DNA 'fingerprint'. Two slightly different poly-core probes 33.15 and 33.6 were found which could each be used to obtain distinct fingerprints.

These probes have been shown to exhibit a high degree of homology to DNA sequences from a broad array of species. In particular, avian species have been shown to exhibit variable DNA profiles when digested DNA is hybridised to the 'Jeffreys probes.


EXTRACTING DNA
SEPARATING THE FRAGMENTS
DNA extracted from stains or body fluids
Fragments separated into bands during electrophoresis
PROBING THE FRAGMENTS
DEVELOPING THE FINGERPRINTS
DNA bands in gel transferred to nylon membrane
Radioactive DNA probe is prepared
DNA probe binds to specific DNA core sequences on the membrane
Bound probe visualised by X-ray film
DNA fingerprint
Isolated DNA cut to give fragments
Smaller mobile fragments migrate towards anode

Polymerase Chain Reaction

The PCR technique is a method which provides microgram quantities of DNA copies of either DNA or RNA segments which are themselves present in amounts as small as a single molecule in a nucleic acid preparation. The PCR technique is carried out *in vitro* using DNA polymerase and oliognucleotide primers that permit rapid and inexpensive synthesise of oliognucleotides.

To amplify a DNA segment, two oligonucleotide primers that are each complementary to one of the two 3' borders of the duplex segment to be amplified are synthesised. The PCR technique will act to amplify the sequence between the sites at which the oligonucleotide primers anneal. After annealing the primers to a denatured DNA containing the segment to be amplified, the primers are extended using DNA polymerase and the four deoxynucleotide triphosphates. Each primer is extended towards the other. The resulting duplex DNA's are then denatured and annealed again with the primers, and the DNA polymerase reaction is repeated. This cycle of steps (denaturation, annealing and synthesis) may be repeated as many as 60 times. At each cycle, the amount of duplex DNA segment doubles because both new and old DNA molecules anneal to the primers and are copied. In principle, and virtually in practice 2^n copies of the duplex segment bordered by the primers are produced, where n is the number of cycles.

Using a heat stable DNA polymerase isolated from the thermophilic bacteria *Thermus aquaticus* allows multiple cycles to be carried out after a single addition of the enzyme. The DNA, an excess of primer molecules, the deoxynucleoside triphosphates, and the polymerase are mixed together at the start. Cycle 1 is initiated by heating to a temperature that assures DNA denaturation, followed by cooling to a temperature appropriate for primer annealing. Therefore, the temperature is adjusted so that DNA synthesis can occur. The second and subsequent cycles are initiated by again heating to the denaturation temperature. Thus, cycling can be automated by using a computer-controlled variable temperature heating block. The whole process takes only a few hours.

Besides permitting automation, use of the *T. aquaticus* DNA polymerase has another advantage. This enzyme is most active between 70^{o} and 75^{o} centigrade. At this temperature, base pairing between the oligonucleotide primers (often about 20 residues long) and the DNA is mor specific than at 37^{o}, the optimal temperature for *E. coli* DNA polymerase. Consequently, primers are less likely to anneal to imperfectly matched DNA segments, thereby minimizing amplification of unwanted DNA, especially when an entire genome's worth of DNA is present. Correct annealing of primers is also selecting appropriate conditions of temperature and ionic strength for primer length and base composition. Specificity can be stringent enough to permit simultaneous amplification of two different genomic segments in th same DNA sample, in the presence of two pairs of primers.

Primers can be designed from amino acid sequence data for particular genes. When there is degeneracy in the genetic code at particular sites, a mixture of primers with alternative bases can be used. After PCR the amplified segment can be purified by gel electorphoresis or cloning and subsequently used as a probe.

Nuclear DNA can also be amplified by the use of random primers, as in this work. Such RADPs (randomly amplified polymorphic DNA) represent annonymous regions i.e. those whose function is largely or entirely unknow. It is clear that there are large amounts of detectable variation using this technique, in some species at least. This approach has been used to identify cryptic species in nature and in mapping regions of the genome of a number of species.

Methods

DNA extraction and electrophoresis

High molecular weight DNA was extracted by either of two techniques. In some cases 50ml of blood was resuspended in 5mls of SET buffer (0.1 M Tris HCl pH 8.0, 0.01 M NaCl 1.0 mM EDTA) to which SDS was added to a final concentration of 0.5% and incubated with 5 units of protinase K at 65°C overnight. DNA was extracted three times with phenol and once with chloroform. Alternatively, approximately 60ml of blood cells were lysed by resuspended in 1 ml of lysing buffer (144mM NH4C1; 10MM NH_4HCO_3). This was centrifuged at 7500 rpm for approximately 15mins and the supernatant discarded. The pellet was then resuspended in 1 ml of lysing solution and the centrifugation repeated. The pellet was resuspended in 1 ml of SET buffer, 5 units of protinase K and 0.5% SDS and incubated overnight overnight at 65°C. The remaining procedure was as above. Precipitation was performed according to Sambrook et al.(1989).

All samples were subsequently digested with the restriction enzyme *Hae* III initially overnight with 30 units of enzyme in the presence of 4mM spermadine trichloride and recovered by ethanol precipitation. Digested DNA was separated on a 0.8% agarose gel in TAE running buffer (2mM Tris, 1 mM acetate, 10mM EDTA pH 8.3) for approximately 48hrs at 2V/cm. All gels were run with molecular weight markers which allowed each gel to be standardized with respect to the distance run. The DNA was transferred by Southern blotting to a nylon membrane (Hybond-N, Amersham) by successively soaking in 0.25M HCI for 15 mins, 0.5M NaOH, 1.5M NaCl for 45 mins and 1.5M NaCl, 0.5M Tris pH 7.2 and 1 mM EDTA twice for 15mins. The gels were then capillary blotted overnight in 6 X SSC. After blotting the membranes were washed briefly in 3 X SSC before fixing the transfered DNA by baking at 80°C for 2 hours. ^{32}P probes pV47-2 (Longmire, 1990), the Jeffreys probes 33.15 and 33.6, and 3'HVR were obtained by random priming according to suppliers instructions (Amersham). Filters were prehybridized at either 50°C or 61°C in 0.5M Na phosphate, 1 M EDTA, 7% SDS pH 7.2, then hybridized in the same solution at either 50°C or 61°C in a hybrization chamber. Hybridized filters were washed twice for 30 mins at 55°C in 5 x SSC, 0.1 %SDS and exposed with X-ray film at -80°C for 1-8 days in a casettes with an intensifying screen.

PCR amplification of annonymous regions

A Techne PHC 2 thermal cycler was used to amplify genomic DNA extracted, as above, from ethanol preserved wing and heart muscle. Random 10 base primers were used with the following sequence: **GTTGCGATCC**. The following temperature profiles were used: the DNA was denatured at 94°C for 1:45 mins., primers were annealed at 50°C for 1:30 the extension phase was carried out at 72°C for 1:30 mins. Forty cycles of this profile were run, with a final extension phase at 72°C for 5 mins. The following PCR protocol was used per 25µl reaction: 2.5µl 10XTaq buffer, 10µl 10mM dNTP mix (2.5mM each), 6µl dH20, 5µl 10µM primer, 0.4µl Taq polymerase and 1µl DNA at a concentration of 0.5ng/ml.

Amplified fragments were separated on a 1 % agarose gel. They were subsequently stained with ethidium bromide and visualised under UV light.

Results

Minisatellite DNA variation in teals

- High resolution DNA fingerprints have been obtained for Campbell Island teals. The mean number of restriction fragments present in the four individual, were 35.2 (probe 33.15), 32.0 (33.6) and 32.6 (PV47-2). This is in the upper range for avian species (see the attached table).

- Given the degree of variability of minisatellite regions in Campbell and Auckland Island teals, it is expected that the technique will be useful in the determination of parentage in these species.

- It is not possible, given the samples available, to use DNA profiles of this type to determine higher order family relationships. This would require a range of individuals of known relationships in order to calibrate the level of band sharing to levels of relatedness. If this were possible then the technique could be used in this way.

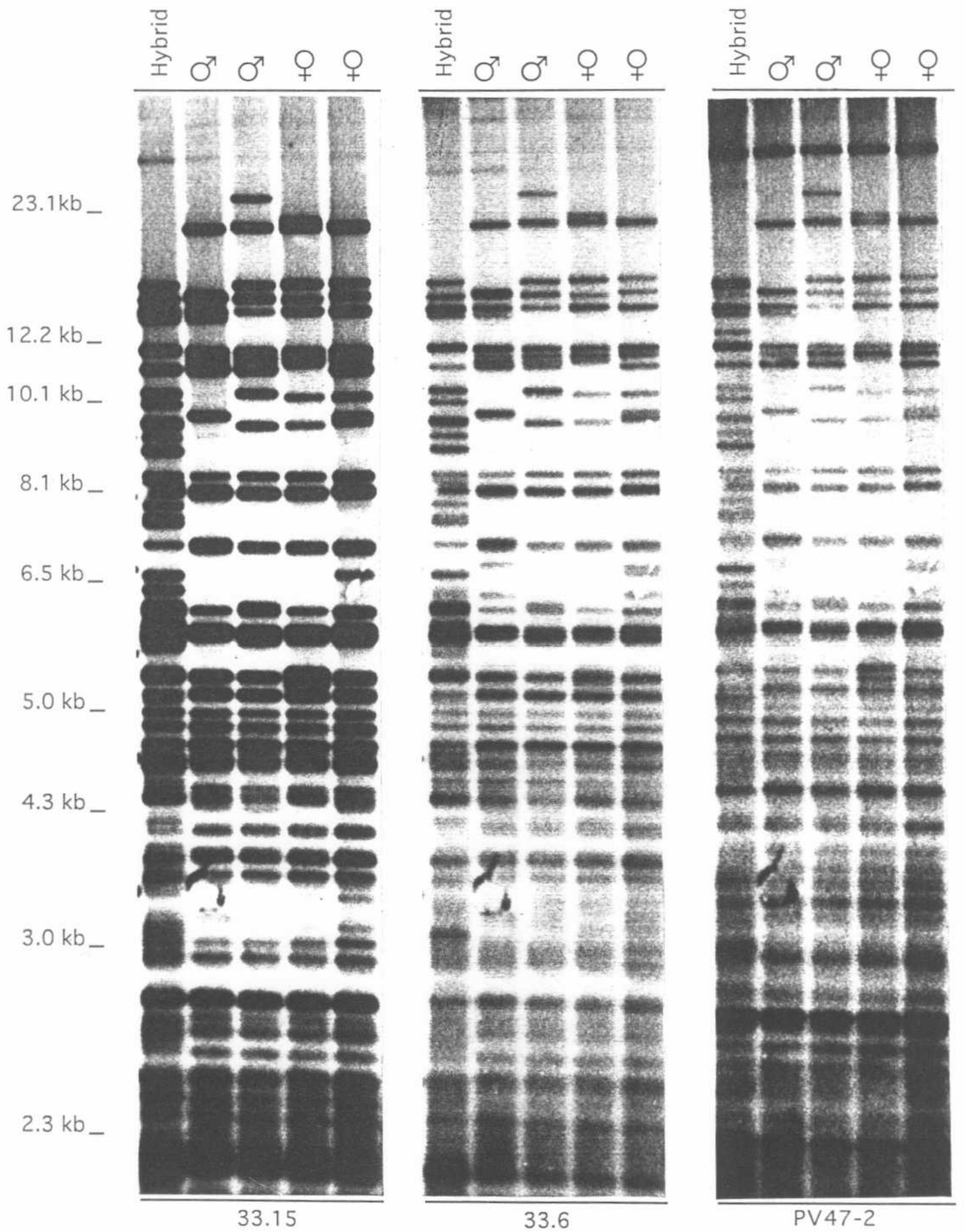
- The one hybrid individual exhibited a large number of restriction fragments not present in the individual Campbell Island teals examined in this study. It is suspected the the extra fragments are derived from the Auckland Island subspecies, although this is difficult to precisely determine since, owing to the very different qualities of the DNA, samples from the two subspecies were not run together on the same gel. This is because the mobility of fragments is affected by DNA quality and hence homologies are not reliable. Nevertheless it appears that there are minisatellite DNA differences between these two subspecies.

- Digested *Hae*III DNA produces an even distribution of restriction fragments.

- There is an apparent high level of band sharing among the four Campbell Island individuals examined and there are no apparent sex-linked fragments in the profiles produced.

- The three probes detect very similar fragments suggesting a single family of minisatellites are being visualised.

Hybridisation of minisatellite DNA probes to *Hae*III digested DNA from Campbell Island Teals



Mean number of bands (*n*), and mean band sharing (*x*) detected in a range of species using various enzyme/probe combinations.

	Restriction Enzyme	Probe	*n*	*x*	Reference
New Zealand Birds					
Kaka	*Hae*III	33.15	51	0.42	Unpublished
(N=6)		pV47-2	33	0.47	
Silvereyes	*Hae*III	33.15	18	0.21	Unpublished
(N=5)		33.6	14	0.22	
Brown Skua	*Hae*III	33.15	14	0.41	Millar et al. 1993
(N=12)		33.6	10	0.28	
Blue Duck	*Hae*III	33.15	22	0.51 (within rivers)	Triggs et al. 1992
(N=58)				0.17 (between islands)	
Other birds					
Tropical wrens	*Hae*III	33.15	24	0.27	Rabenold et al.1990
(N=24)		33.6	30	0.26	
Zebra finches	*Alu*I	33.15	~19	0.17	Birkhead et al.1990
(N=12)		33.6	~30	0.16	
House sparrows	*Alu*I	33.15	15	0.17	Burke and Bruford
(N=2)		33.6	6	0.28	1987
Other taxa					
Pilot whales	*Dde*I	33.15	12	0.6	Amos and Dover
(N=?)					1990
Lions	*Msp*I	FCZ9	23	0.45	Gilbert et al. 1991
(Serengeti population)			(Felid-Specific)		
(N=76)					
Kangaroo rats	*Hae*III	33.6	-	0.30	Keane et al. 1991
(N=20)					
Cats	*Hinf*I	33.15	13	0.47	Jeffreys and Morton
(N=5)		33.6	8		1987
Dogs	*Hinf*I	33.15	19	0.46	Jeffreys and Morton
(N=8)		33.6	16		1987

N=number of individuals
n=mean number of bands scored
x=mean band sharing between unrelated individuals. Band sharing between pairs of individuals is calculated as

$$D=2nAB/(nA + nB)$$

where nA and nB are the numbers of bands scored in individuals A and B respectively, and nAB is the number of shared bands (Wetton et al. 1987).

- The probe 33.15 showns very high homology to the DNA from teals and suggesting that this would be the most appropriate probe to use.

- Although these results are preliminary, it appears that there may be fragments specific to the two subspecies. This would be unusual.

PCR amplification of DNA from ducks

- DNA fingerprinting proved impossible for the samples of Mallard and Grey duck samples available to us. However, the PCR approach described in the methods section yielded amplified fragments which were polymorphic in these species.

- The following figure illustrates amplified products obtained using the polymerase chain reaction. Fragments range in size from 5 to 0.3 kb. Individuals showed between six and nine fragments of high intensity.

- Individual profiles show considerable variation.

- Generally samples amplified well and we are confident that this technique could be employed across a broad range of samples of individuals.

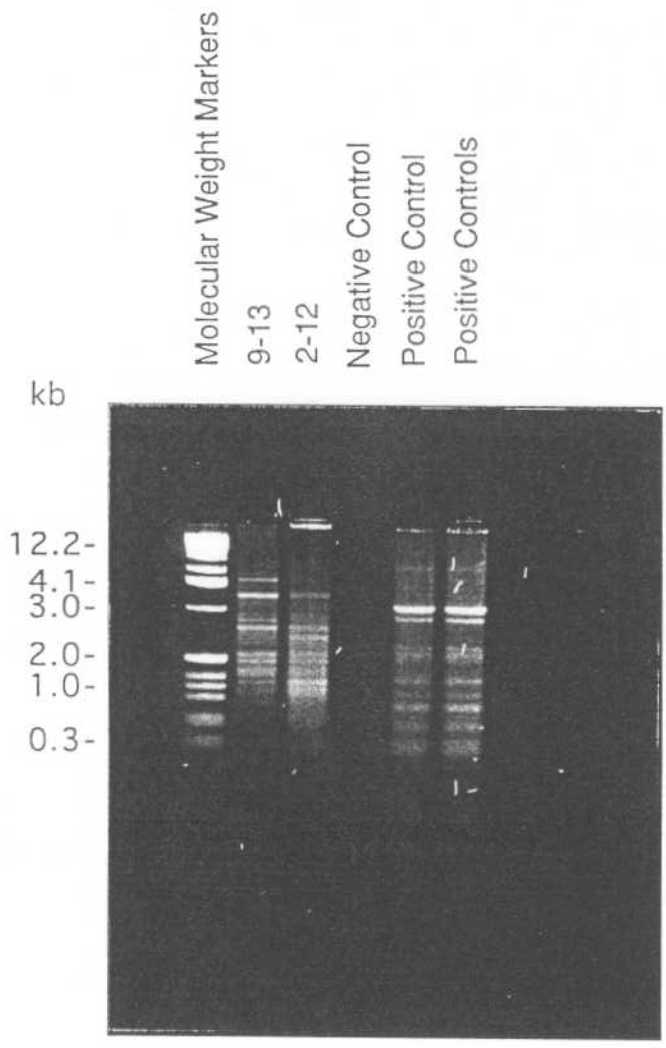
- A large range of 10 base primers are commercially available. Each of these is likely to amplify an independent set of fragments. This suggests that there is a very large set of such RAPDs which could be used in this way.

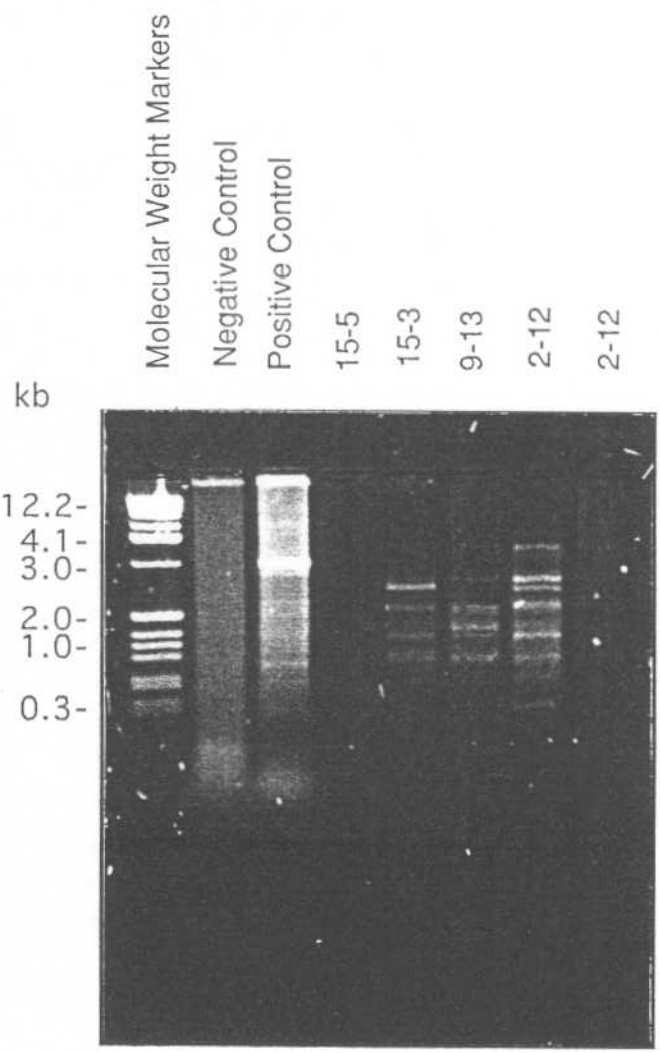
- It is likely that at least the majority of these fragments represent nuclear markers and would consequently complement a study of the mitochondrial DNA of these species.

- RAPD amplification has been shown to be inconsistent under variable experimental conditions (see, for example, samples 9-13 and 1-12). This remains a serious limitation. Single locus DNA probes may provide a more informative and reliable approach to the study of hybridisation between Grey and Mallard ducks.

CCB

PCR amplified DNA fragments of teal and mallard ducks using the RAPD (Randomly Amplified Polymorphic DNA) technique.





References

Birkhead TR, Burke T, Zann R, Hunter FM, and Krupa AP, 1990. Extra-pair paternity and intraspecific brood parasitism in wild zebra finches *Taeniopygia guttata*, revealed by DNA fingerprinting. Behav Ecol Sociobiol 27: 315-324.

Burke T and Bruford MW, 1987. DNA fingerprinting in birds. Nature 327: 149-152.

Gilbert DA, Packer C, Pusey AE, Stephens JC, and O'Brien SJ, 1991. Analytical DNA fingerprinting in lions: parentage, genetic diversity, and kinship. J Hered 82: 378-386.

Jeffreys AJ and Morton DB, 1987. DNA fingerprints of dogs and cats. Anim Genet 18:1-15.

Keane, B., Waser, P.M., Danzl-Tauer, L., and Minchella, D.J. 1991. DNA fingerprinting: estimating background band-sharing in banner-tailed kangaroo rats. Anim. Behav. 42: 141-143.

Longmire JL, Kraemer PM, Brown NC, Hardekopf LC, and Deaven LL, 1990. A new multi-locus DNA fingerprinting probe: pV47-2. Nucleic Acids Res. 18: 1658.

Millar CD, Lambert DM, Bellamy AR, Stapleton PM, and Young EC, 1992. Sex-specific restriction fragments and sex ratios revealed by DNA fingerprinting in the brown skua. The Journal of Heredity 83: 350-355.

Rabenold PP, Rabenold KN, Piper WH, Haydock J, and Zack SW, 1990. Shared paternity revealed by genetic analysis in cooperatively breeding tropical wrens. Nature 348: 538-540.

Sambrook, J, Fritsch, EF and Maniatis, T, 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 3v.

Triggs, S.J., Williams, M.J., Marshall, S.J., and Chambers, G.K. 1992. Genetic strucutre of blue duck (*Hymenolaimus malacorhynchos)* populations revealed by DNA fingerprinting. Auk 109 (1) : 80-89.

Wetton JH, Carter RE, Parkin DT, and Walter D, 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327: 147-149.