DNA sexing of brown kiwi (*Apteryx mantelli*) from feather samples

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A B S T R A C T

Raising juvenile kiwi in captivity for later release is a method currently used to help maintain some kiwi populations. Most of these kiwi are of unknown sex at release. If recruitment of captive-raised kiwi into the breeding population is found to be occurring, emphasis could move from monitoring released juveniles to monitoring more breeding males (thus sourcing more eggs/chicks), but an accurate method of sexing juvenile kiwi would be necessary. Feathers were collected from 13 kiwi with radio transmitters in Tongariro Forest Conservation Area during routine handling of the birds between February 1999 and May 2000. Feathers were sent to Massey University, where sex was assigned using a ratite-specific DNA-based sex test test that was being developed. Results from the DNA-based test were in all cases in accordance with sexes assigned by autopsy and behavioural criteria, but there were inconsistencies in the tests for a few individuals. Some potential causes of incorrect sex assignment or an absence of results are discussed: standard procedures for the collection and storage of feather samples for further testing are recommended.

Keywords: sex assignment, juvenile birds, ratites, brown kiwi, *Apteryx mantelli,* non-invasive sampling, polymerase chain reaction, W chromosome, Z chromosome.

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1. Introduction

Traditional methods of assigning sex in brown kiwi involve using behavioural tests (primarily calling) and morphological data (weights and measurements). These methods have proved to be generally reliable with adult kiwi, but it is difficult to apply them to sex immature kiwi.

Female and male kiwi have clearly distinguishable calls, although in the wild at night it can be difficult to verify that the bird heard calling is the individual of interest. Calling has not been known to occur in kiwi juveniles younger than about one year, so young kiwi are unable to be sexed in this way.

Female kiwi are generally larger than males, although weights tend to show some overlap between the largest males and the smallest females. Bill measurements, however, seem to show an almost complete separation between adult males and adult females. For example, Colbourne & Kleinpaste (1983) in a study of 62 adult kiwi at Waitangi State Forest found that tip–cere bill lengths ranged from 90 to 106 mm in males and 117 to 142 mm in females; and P. Miller (unpubl. report 1995) in a study of 146 adult kiwi in four populations near Whangarei found male bills ranged from 86 to 119 mm and females from 120 to 156 mm. Thus in this pooled sample of 103 male and 105 female kiwi, only one male (119 mm) had a longer bill than any female.

There are few data on growth rates in wild juvenile brown kiwi throughout the country, because of the small proportion of chicks that survive longer than a few weeks in the wild (McLennan 1997; McLennan et al. 1996; P. Miller unpubl. report 1995). Juvenile growth rates in captivity often vary considerably, depending on how well each individual copes with the limitations of the captive situation. Thus estimating an expected size/weight range at any stage of growth according to an individual's sex is problematic, making sex assignment of juveniles using morphological data difficult.

1.1 REASON FOR SEXING JUVENILE KIWI

Raising juvenile kiwi in captivity (Operation Nest Egg, ONE) is a method currently used to help maintain some kiwi populations. Kiwi are returned to the wild upon reaching a size of 800–1200 g (a size at which they are believed to be capable of resisting stoats). In the Tongariro/Taupo Conservancy, juveniles have been released from five months of age and most are of unknown sex at release. The Tongariro Forest Kiwi Protection Project states in Objective 1 that the effectiveness of ONE in allowing recruitment of juvenile kiwi into the Tongariro Forest Conservation Area kiwi population should be tested and refined by releasing and monitoring at least 20 juveniles (Martin et al. 1999). An annual survival rate of 75% has been observed to date among the Tongariro released kiwi, though none are known to have yet bred. The target of 20 juveniles released was reached in 2000. After three to four years of monitoring of each of the 20 released kiwi, it should be clear whether captive-raised juveniles are being recruited into the breeding population.

If recruitment of captive-raised kiwi into the breeding population is found to be occurring, emphasis should move from monitoring released juveniles to monitoring more breeding males. Juvenile females could then be released without transmitters (since monitoring movements of females is not necessary for detection of nesting in North Island brown kiwi), thus freeing up resources and time for monitoring additional breeding males. This would enable a greater number of eggs/chicks to be sourced. An accurate method of sexing juvenile kiwi would be necessary for this management regime to be used.

1.2 DNA SEXING METHODS

As the field of molecular biology advances, more discoveries are being made about the organisation and composition of animal genomes. The resulting DNA sequences can be used to identify species, populations, sexes and individuals. In avian species, the female possesses both a W and Z chromosome (heterogametic), while the male is the homogametic sex, possessing two Z chromosomes (unlike mammals, which show the reverse characteristics, with females being XX and males being XY). It is possible to isolate a piece of DNA within the W chromosome (dotted area on differential region of W chromosome in Fig. 1) that can serve as a marker for sex in bird species. The technique of the polymerase chain reaction (PCR) is used to target and amplify a piece of DNA, allowing it to be stained and visualised using a UV light source. Differences between the DNA amplified from the Z and W chromosomes can then be distinguished and sex readily assigned. Since the PCR only requires nanogramme and sometimes only picogramme amounts of fresh cells, it has made genetic analysis possible from non-invasive sampling such as feather collection (Morin & Woodruff 1996).

The first reported identification of a gene on the avian W chromosome was that encoding a chromo-helicase-DNA binding (CHD) protein. Its high degree of conservation across species meant that it could serve as an almost universal tag

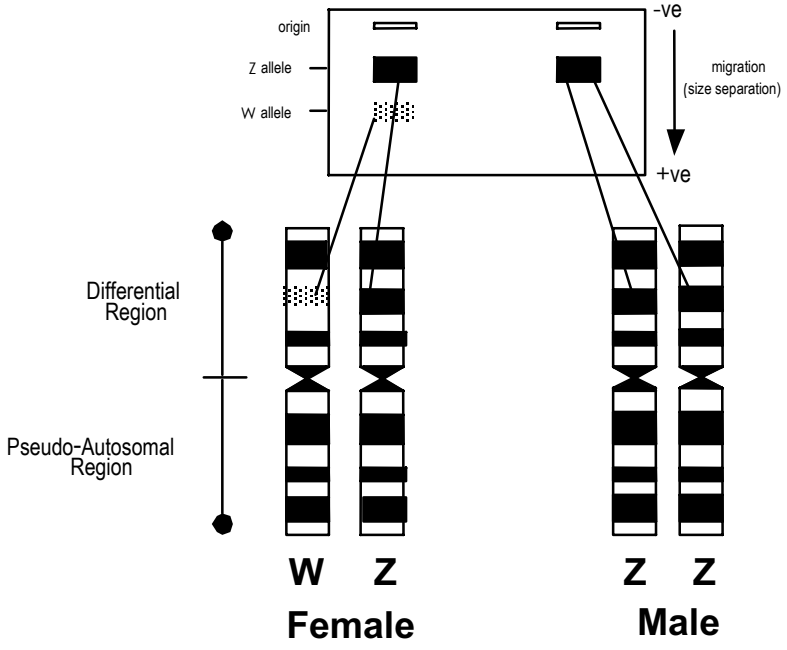

Figure 1. The detection of the ZW and ZZ genotypes during the DNA sexing procedure. The copy of the gene on the W chromosome is shorter than the copy on the Z chromosome. Following amplification of the genes by PCR, they are placed at the negatively charged end of an agarose medium with an electric current running through it. All gene fragments migrate towards the positive terminal. The smaller fragment (from the W chromosome) moves faster than the fragment from the Z chromosome. Thus fragments should be seen in two different positions if the individual is a female. In the male there are two copies of the Z gene which are the same size and therefore overlie each other in the agarose medium.

for sex assignment in birds (Griffiths et al. 1996; Ellegren 1996). The use of the CHD gene to determine the sex of birds is now a patented technique.

Differences in sex chromosome morphology are known to exist between ratites and all other avian taxa (non-ratites have heteromorphic while ratites have homomorphic sex chromosomes) (Takagi et al. 1972; de Boer 1980) and it was found that the CHD test failed to distinguish between the sexes in ratites. Therefore in order to sex ratites from DNA, a test specific to ratites needed to be developed. Researchers in Lambert's group at Massey University are working on developing DNA-based sex tests for birds. This group has isolated a W-chromosome-linked DNA sequence in ratite species, using the PCR.

Kiwi from Tongariro Forest were sexed using this sequence as a marker, as part of the process in developing a PCR-based ratite sex test. Results of the Tongariro Forest kiwi sexing are reported here.

2. Methods

Feathers were collected from 13 kiwi with radio transmitters in Tongariro Forest Conservation Area during routine handling of the birds for weighing or transmitter changes between February 1999 and May 2000. Ten were captive-reared (ONE) birds ranging in age from six months to four years, none of which were known to have bred at that time. Three were wild-caught adults, two of which are known to have bred several times.

Feathers which were seen to have been shed from the bird during handling were collected and placed in a plastic bag which was labelled with a number code specific to that individual. Replicate samples from the same individual were collected at subsequent handlings of the bird and labelled with another number also specific to that individual, in order to check for consistency of results[1].

This report was part of a pilot study with David Lambert[2] at Massey University. Feather samples were sent by post to Massey University and the results (Male or Female) were provided in writing.

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[1] The sex testing laboratory procedures were being developed and therefore changed during the testing for this report. Therefore not all of the results from the same individual represent a true replication of the whole sampling procedure.

[2] For further information regarding details of ratite sexing contact D.M. Lambert. e-mail: D.M.Lambert@massey.ac.nz

3. Results

Results from the PCR-based sex test have been obtained for 13 Tongariro Forest kiwi to date (Table 1). At least one independent replicate (see Footnote 1) of the initial test was performed on samples from seven of these 13 kiwi. Of the remaining six kiwi, four (Doug, Te Aukaha, Pihanga and Tahi) are adults whose sex may be assigned by both behavioural and morphological criteria, one (Waitangi) was predated before sex was assigned by morphological/behavioural criteria and had insufficient remains for sexing by autopsy, and one (Anaru – also unsexed by behavioural/morphological criteria) had dropped its transmitter so its whereabouts was unknown. Additional adult birds with transmitters (including some caught since this paper was first submitted that do not appear in Table 1) whose sex has been assigned by behavioural and morphological criteria, have not been sexed by the PCR-based test.

TABLE 1. SEXES ASSIGNED TO 17 TONGARIRO FOREST KIWI USING UP TO FOUR METHODS.

BAND	NAME	DNA					MORPH	BEHAV	AUTOPS	BEST
		1	2	3	4	5				
R-35992	Awhi	F	F	F			F		F	F
R-35994	Waitangi	F								?
R-35995	Moenui	M	M						M	M
R-35999	Hot Chick	M	M							M*
R-36073	Wahanui	M	M	F			F			F?
R-36074	Kahuma	M	F	M	M	F	F			F?
R-36075	Koha	M	M	F	F		F			F?
R-36076	Anaru	M								?
R-36079	Doug	M					M	M		M
R-36080	Te Aukaha	M					M	M		M*
R-36090	Pihanga	F					F	F		F*
R-46251	Tahi	M					M	M		M
R-36081	Titch	F	F				F	F		F*
R-35071	Trevena						F	F		F
R-36072	Harry						M	M		M
R-36077	Lass						F	F		F
R-55401	Mani						M	M		M

The **DNA** column shows the sex assigned to individuals by the PCR-based test (1–5 represents replicate samples from the same individual). The **Morph** column shows the sex assigned to individuals using the morphological criterion of bill length. Birds with a tip–cere bill length of over 100 mm were assigned as female and known adults with a bill length under 100 mm were assigned as males by this criterion. Birds still growing (at their last handling), but with a bill length of under 100 mm were not sex-assigned in this column. The **Behav** column shows the sex assigned using behavioural criteria. Individuals were sex-assigned under these criteria in one or a combination of the following situations: Birds found on late incubation eggs were assigned as male; birds heard calling with a characteristic male or female call were assigned accordingly; birds found sharing a roost with a bird who had been assigned as male using one of the other behavioural criteria, were assigned as female. The **Autops** column shows the sexes assigned by autopsy to two individuals who died over the course of the study. The **Best** column gives the best indication of sex (F?/M? indicates the sex assumed, where there are inconsistent indications; F*/M* gives a consistently indicated sex; and M/F gives the sex of known breeders or sex shown by autopsy. ? implies absence of consistent indications or assumptions.)

In total (including replicates), results were received for 27 PCR-based sex tests. Twelve (44.4%) gave a female result and 15 (55.6%) gave a male result.

For three out of the seven individuals for whom replicate PCR tests were performed, conflicting results were obtained—separate tests obtained both male and female results for Wahanui, Kahuma, and Koha.

For all five PCR sex-tested individuals who may also be sex-assigned by both morphological and behavioural criteria, PCR results were in agreement with their behaviourally/morphologically assigned sex. Two individuals were sexed by autopsy and all PCR results were in agreement with the autopsy-assigned sexes of these individuals.

4. Discussion

4.1 CONSISTENCY OF DNA SEXING OF KIWI FEATHERS

Results from the PCR test were in all cases in accordance with sexes assigned by behavioural criteria. Two birds died over the course of the study and were able to be sexed by autopsy. All PCR tests were in accordance with the autopsy-assigned sexes of these birds.

Results were consistent across two or three replications for four out of the seven individuals for whom more than one test was performed. The only inconsistencies seen to date in the results are those for Wahanui, Kahuma, and Koha, for whom both male and female results were obtained in different tests.

Some potential causes of incorrect sex assignment or an absence of results are outlined in Table 2. The first four examples can occur through a lack or absence of DNA, contamination of DNA, or an inability to verify that the feather comes

TABLE 2. POSSIBLE CAUSES OF INCORRECT SEX ASSIGNMENT OR ABSENCE OF RESULTS WITH PCR TECHNIQUE USING DNA EXTRACTED FROM A FEATHER.

POTENTIAL PROBLEMS	RESULT FROM DNA TESTING	EXPLANATION
W allele dropout	F assigned as M	Such small quantities of DNA that the W allele is not detected.
No DNA from feather	No results	DNA degraded due to poor collection or storage procedures.
Contamination	Wrongly assigned as M or F	DNA from an exogenous source enters the DNA sample.
Feather not from named individual	Wrongly assigned as M or F	The feather was collected from around the nest or roost and not directly from the individual.
Unusual genetic sex	Cannot interpret results, or wrongly assigned as F or M	Genetic anomalies[1]: ZZW and ZZZW females, Z0 males, and sex-reversed ZW male and ZZ females.

1. Most of these conditions have been insufficiently characterised in birds, and Z0 males have not yet been described. It is still under debate whether it is the W chromosome or two Z chromosomes that confer sexual differentiation in birds. Many genetic anomalies result in infertility (Clinton 1998).

from the identified individual. Plucking a feather from an individual rather than collecting shed feathers may help to mitigate the problem of a lack or absence of DNA (see Appendix 1) as well as ensuring that the feather is from the named individual. Contamination and degradation of DNA (see Appendix 1) may be avoided by implementing collection and storage procedures which protect and preserve the base of the feather shaft (see Fig. 2).

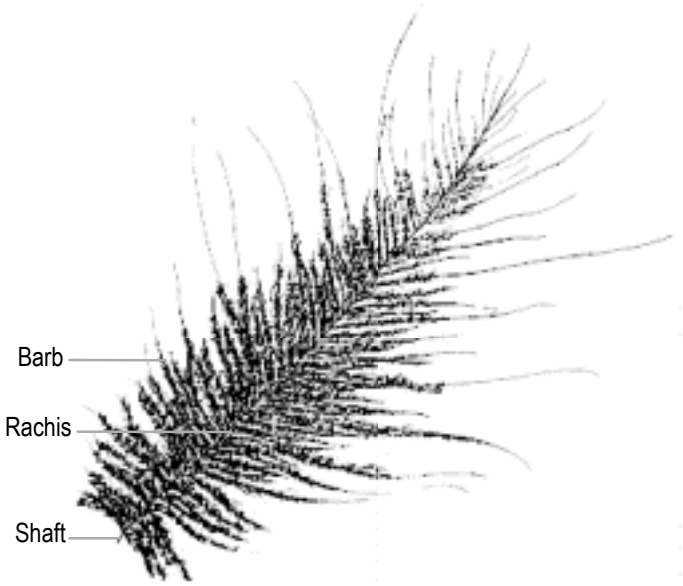

Figure 2. The morphology of a kiwi feather (drawing reproduced courtesy of Tamsin Ward-Smith).

If all three birds for whom inconsistent results were obtained are female (which seems likely according to morphological data), the inconsistencies seen are all of a particular type, namely females are being mis-assigned as males some of the time. Mis-assignment of sex occurring more in one direction than the other may be the result of allelic dropout (see Appendix 1). Allelic dropout can result in an individual from the heterogametic sex (females in birds) being mis-assigned as the homogametic sex, and is most likely to occur when DNA is at a low concentration in the sample from which the DNA is extracted.

4.2 SEXES OF THE KIWI IN TONGARIRO FOREST

The right-hand column of Table 1 gives the best indication of sex for 15 Tongariro Forest kiwi, using up to four methods of sex assignment.

4.3 USE OF DNA FROM KIWI FEATHERS TO IDENTIFY MALES

It may be possible in the future to use DNA sexing from kiwi feathers to identify males prior to release. More sampling of individuals of known sex and replicate sampling of those of unknown sex (using more rigorous collection procedures) needs to be carried out. If it becomes evident that allelic dropout is occurring,

but that all other problems (such as contamination) can be overcome through better collection procedures, the test should be able to identify, prior to release, individuals which are definitely females. This could still be of some use for the purposes of Operation Nest Egg.

If it is found that allelic dropout is not occurring or can be overcome by maximising the DNA in a sample, and that problems such as contamination can be overcome by better collection procedures, males should be able to be identified prior to release using this method.

5. Conclusions

Continuing to collect feather samples for sex testing is recommended, while ensuring that all those involved in collection are aware of and use procedures to avoid contamination, degradation, or mixing of samples. Recommended procedures for collection are:

- Only feathers seen to come from the kiwi being handled should be collected (as only plucked feathers can be guaranteed to have come from the individual, it would be preferable to pluck feathers rather than picking up shed feathers – DOC Animal Ethics Committee approval must be obtained before feathers are plucked).
- Feathers should be picked up before they fall on the ground.
- Feathers picked up by hand should be held by the barb rather than the shaft end and placed into a clean, dry, sealable bag as soon as possible (if feathers are to be stored for some time prior to analysis, they should be placed in a paper bag or envelope, and plastic bags should only be used if the sample is to be stored in a freezer).
- About 4–5 feathers from each individual should be collected, if possible.
- Feathers should be stored in a cool, dry and sterile place.
- The possibility of using the shafts from several feathers in one DNA extraction (in order to maximise the amount of DNA in an extraction) should be explored.

Sampling adults (especially known breeders) is a useful indicator of the reliability of the test and it is recommended that feather samples be collected from those birds of known sex that have not yet been tested.

6. Acknowledgements

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Appendix 1. DNA from feathers and allelic dropout

The main part of a feather is composed of keratin protein which does not contain living cells and therefore does not contain DNA. The lower part of the shaft, which is embedded into the bird's skin, is the only part of the feather that contains cells. Taberlet & Bouvet (1991) estimated a feather with a shaft < 0.2 mm in diameter contains several hundred cells and therefore several hundred copies of a gene. Growing feathers have a higher number of intact cells in the shaft than shed feathers. Many cells in the lower shaft of a shed feather could be dead and the DNA in poor condition.

After removal from the bird's skin, the semi-exposed cells in the lower shaft become open to contamination by DNA from an exogenous source. In addition, the DNA is continually under attack and can be degraded by moisture, warm temperatures and microbes (Lindahl 1993).

To date there are no comprehensive studies on the reproducibility of genetic results from feather DNA extractions, although studies on DNA extracted from hair samples have been conducted (Gagneux et al. 1997; Taberlet et al. 1997; Goossens et al. 1998). Gagneux et al (1997) quantified the genotyping error rate from an analysis of 791 single shed chimpanzee hairs. Thirty-one percent of these showed DNA of sufficiently low quantity for allelic dropout to occur. When only minute quantities of DNA are present in a sample, allelic dropout can occur (the theoretical probability of allelic dropout occurring at various template DNA quantities is illustrated in Fig. 3) due to the stochastic amplification of one of the two alleles (Taberlet et al. 1996). This assumes that there is no bias in the technique towards amplification of one allele over the other. If allelic dropout occurred during avian sexing, only the Z or only the W allele would be detected. If the individual is a female but only the Z allele is detected, the individual would be assigned as male (see Fig. 1). If the individual is male and only one Z allele is detected, the individual will still be assigned as male; and if the individual is female and only the W allele is detected, the individual can still be assigned as female.

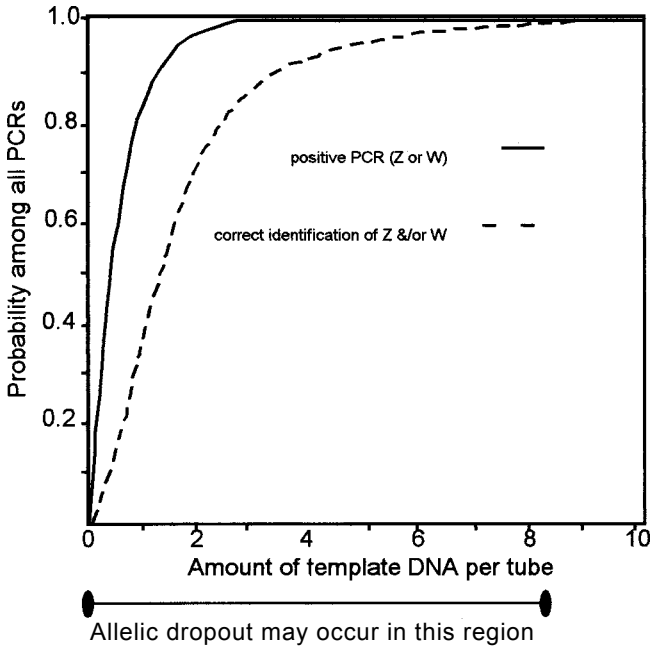

Figure 3. A simulation concerning the genetic typing of an individual bearing a Z and W allele. This shows the probability among all polymerase chain reactions (PCRs) of obtaining a positive PCR (i.e. obtaining a result: either Z, W, or both) and of correctly identifying Z and/or W, according to the amount of template DNA. One unit on the x-axis corresponds to the equivalent of the DNA content of one diploid cell. The greater the difference between the probability of a result and the probability of correct gene identification, the greater the probability of incorrectly assigning sex due to allelic dropout. The line below the x-axis identifies the range of template DNA concentrations within which allelic dropout may occur. The graph has been adapted from Taberlet et al. (1996).

Gagneux et al. (1997) noted that they did not observe allelic dropout in mammalian ear and toe clips in ethanol, dry bird feathers, or reptile blood in ethanol. However, if a feather is in poor condition at the time of collection or the DNA becomes degraded during collection or storage, the DNA concentration may become low enough for allelic dropout to occur.