Sexing Avian Species Using DNA Technology

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Introduction

Many avian species are sexually monomorphic or display sexual dimorphism upon maturity. The main method for the determination of sex in birds utilises laparoscopic procedures (surgical sexing). Such procedures require the bird to be lightly anaesthetised for a period of time and a laparoscope inserted through the body wall to observe the sex organs. Whilst there have been many improvements in the use of anaesthetics and the availability of smaller laparoscopic instruments, surgical sexing remains a risky diagnostic procedure which in young birds may give equivocal results due to the immaturity of the sex organs.

The relatively recent development of molecular biological technology using recombinant DNA sequences provides a new approach to identifing genetically defined characteristics in cluding the sex of an individual. This report describes the nature of the DNA technology that is currently being used to determine the sex of many avian species.

What is DNA?

DNA is an acronym that stands for "DeoxyRiboNucleotide". DNA is composed of four bases denoted "A", "G", "C" and "T". These four bases are arranged such that they form two opposite strands which are ordered into a helical structure such that where an "A" is on one strand a "T" is on the other strand and vice versa, and where a "G" is on one strand a "C" is on the other and vice versa. DNA is a large molecule (approximately 1metre per cell) found in the nuclei of cells where it is tightly folded into discrete bundels called chromosomes. In birds, this means that DNA is found in the red blood cells. DNA can be removed intact from these cells by firstly breaking the cell open to release the DNA followed by the removal of non-DNA components by a series of chemical or chromatographic extraction procedures. Typically, this needs to be done in a laboratory equipped for such procedures. Once the DNA has been isolated it is very stable and can be stored frozen for many years.

How do we analyse the DNA?

In most vertebrates species where the male is characterised by two different sex chromosomes (X and Y chromosomes) and is referred to as the heterogametic sex. However in birds, it is the female which has the two different chromosomes, designated W and Z and is therefore the heterogametic sex. Male birds have two copies of the Z chromosome.

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RFLP (Restriction Fragment Length Polymorphism) analysis is a technique where the DNA is broken down specifically using an enzyme called a restriction endonuclease (RE). These RE's identify specific sequences of bases in DNA (eg GATC - recognised by a restriction endonuclease called *Sau* 3A) and break the two strands apart at these sequences (endo - meaning inside and nuclease meaning breaking DNA bonds). There are other RE's which detect different sequences from that shown above. Different RE's recognise base sequences from 4-8 bases. After digestion of the DNA with a RE, the DNA is now composed of a complex mixture of smaller molecules which vary in length. The length of the DNA is commonly called base pairs (bp) to kilo base pairs (kbp). The DNA can be separated according to size by a technique called agarose gel electrophoresis. Basically, electrophoresis is where the DNA is placed in a gel made of agarose and a charge is applied across the gel. DNA has a negative charge and therefore migrates towards the positive electrode. However, when doing so the DNA is retarded by the agarose matrix and the rate of retardation is primarily dependent upon its size and the agarose concentration. That is, smaller molecules are less retarded and therefore migrate faster than the larger molecules. Once the electrophoresis is completed the DNA is eluted out of the gel and bound to a solid membrane by a process called Southern blotting. The three basic stages of Southern blotting are:

1. denaturing the DNA from double stranded to single stranded using a alkaline solution.

2. transferring the DNA from the gel onto a filter support using continuous flow of buffer through the gel and membrane. This carries the DNA from the gel onto the membrane.

3. fixing the single stranded DNA to the membrane. This is achieved by drying the membrane at 60ºC. Using the Southern blotting technique, an exact replica of the pattern of DNA fragments in the gel is made on one side of the solid membrane and at the same time changes the DNA fragments from being double stranded to single stranded.

DNA is composed of two strands that are paired (A to T and G to C). So if we have a single stranded DNA probe (from a piece of cloned DNA) which has a sequence complementary to that of any strand on the membrane then under certain conditions this can bind and reform a double stranded hybrid. Called a hybrid because it is composed of the native DNA and a genetically engineered piece of DNA. For example if the DNA sequence on the membrane was AATTCCGGATCG and the probe was CGATCCGGAATT then these two sequences will reform the double helix. This is the principle of DNA hybridisation. Furthermore, if the probe DNA was tagged with a radioactive tag then where it formed a hybrid radioactivity would also exist. This radioactivity can be detected by exposing an X-ray film (autoradiograph) to the radioactivity on the membrane.

How is the DNA sexing performed?

Zoogen Inc in California (USA) identified a DNA fragment from a turkey embryo which was found to occur only the W and Z chromosomes (Dvorak *et al*, 1992). They cloned this fragment and applied for a patent for its sequence since detection of this fragment permitted

sex determination in a wide variety of avain species. The basis of this test is that the DNA of male birds will contain DNA fragments from either of the two Z chromosomes defining this sex. In contrast DNA from the female birds will manifest similar fragments to those occuring in the males, but also additional DNA fragments corresponding to the W chromosome characteristic of females. At the present time detection of the Zoogen sex specific DNA fragment must be performed using the hybridisation procedure. An example of these patterns is seen in Figure 1. Thus we are able to determine the genetic sex of the species under investigation. DNA from birds of known sex is also included in each test performed to act as controls for the procedure.

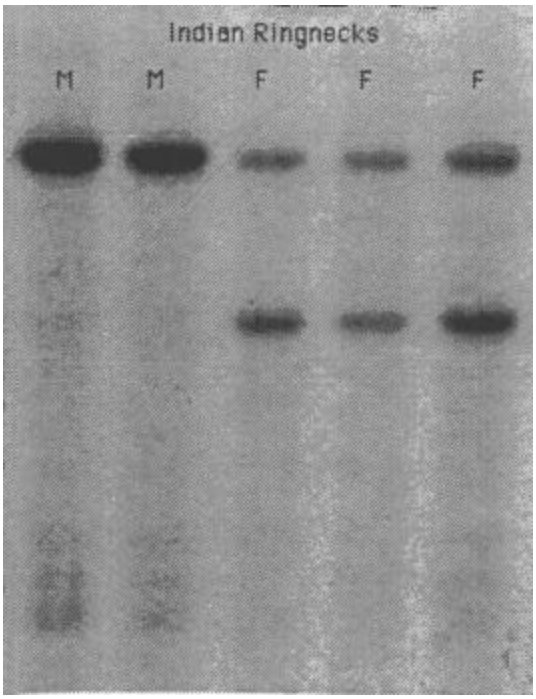

Figure 1 This figure shows results from the RFLP technique. DNA was extracted from five Indian Ringneck Parakettes and probed with the Zoogen sex probe. The male animals are designated M and the female F. It can be clearly seen that male birds display one band and females two with the larger band in the same position as the band seen in males. The smaller band originates from the W chromosome.

For emus and ostriches the RFLP technique has been replaced by an alternative technique called PCR. This alternative technique uses specific sequences flanking the desired sequence to amplify the target such that it can be visualised directly on an agarose gel without resorting to the Southern Blotting procedure. The test detects only the presence of the W chromosome homologue ie animals that are female show the band. An example of this is shown in Figure 2.

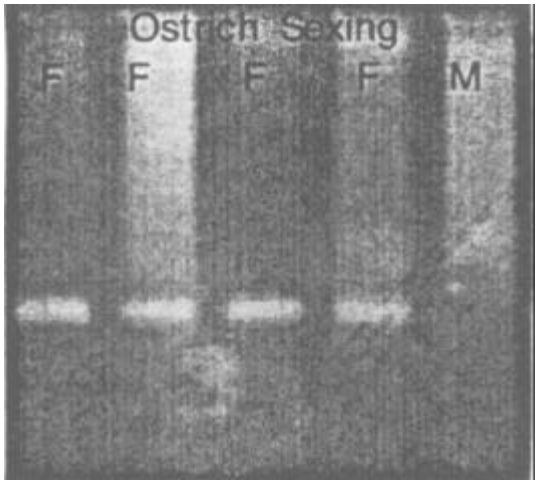

Figure 2 This figure shows results of the sexing by the PCR procedure on five Ostrich samples. Female animals show one PCR band whereas in males it is absent.

What are the advantages?

The DNA sexing procedure can easily be performed in a laboratory equipped for recombinant DNA technology. Such a laboratory is available at Curtin University. Avigen Pty Ltd has successfully negotiated a exclusive licence from Zoogen to exploit the technology in Australasia and in conjunction with Curtin Consultancy Services and Fauna Technologies is offering it to veterinarians and aviculturists. The technique requires the transfer of the very small blood sample to Curtin University's DNA profiling laboratory. Blood can be easily collected from the toe nail vein into special collection tubes containing a preservative which allows the sample to be sent without deterioration (even after weeks of storage) at room temperature. The test takes between 10-15 working days from receipt of the sample. The test cannot be used on bird species which have not been previously validated, aslthough the list of bird species for which the test has been validated is now quite large. For such species blood specimens from known males and females must be tested first to prove the sex specificity of the DNA fragments produced by the test procedure.

A major advantage of DNA sexing is that it has none of the associated potential risks of the anaesthesia and surgery required in the laparoscopic method. The blood sample required is very small, only 30-50ul, and can easily bet collected from young birds for which surgical sexing is not a viable option. Finally, as DNA testing is based upon the genetic makeup of the animal, it is unaffected by age, sexual maturity and breeding cycles.

References

Dvorak, J., Halverson, J.L., Gulick, P., Rauen, K.A., Abbott, U.K., Kelly, B.J. and Shultz, F.T. (1992). cDNA Cloning of a Z- and W-Linked Gene in Gallinaceous Birds. J. Hered. 83: 22-25.