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Bird faeces for sex identification and microsatellite analysis

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Abstract: SEGELBACHER, G., & G. STEINBRÜCK (2001): Bird faeces for sex identification and microsatellite analysis. Vogelwarte 41: 139–142.

It is often difficult to obtain blood or tissue samples for population genetic studies of birds without disturbing the animals. We therefore developed a method for extracting DNA from small amounts of bird faeces, and describe the use of this method for sexing birds and for microsatellite analysis. Results obtained from faecal samples were identical to those obtained from tissue or feather samples. With non-invasive sampling, it is possible to gain information about free ranging animals with a minimum of disturbance to them, which is especially important when studying endangered species.

Keywords: Birds, faeces, DNA, sex identification, microsatellite.

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1. Introduction

Blood or tissue samples for population genetic studies of birds are often difficult or impossible to obtain. Particularly in studies of threatened species, non-invasive sampling is highly desirable. In many bird species, faeces can be sampled easily and are an accessible source of DNA (e.g. NOTA & TAKENAKA 1999), because PCR-based methods of genetic analysis require only minute amounts of template DNA from which to amplify additional copies. We describe a method of DNA extraction from small amounts of bird faeces sufficient for identification of the sex of an individual and its genotype at microsatellite loci. Sex determination using molecular methods and analysis of microsatellite variation have proven to be valuable tools in wildlife conservation and for studies of sex allocation and the behavioural ecology in birds (e.g. LENS et al. 1998, SHELDON 1998, GALBUSERA et al. 2001, LEE et al. 2001).

2. Material and Methods

DNA was isolated from fresh (less than one day old) and old (more than one week old) excrement of white-rumped shamas (*Copsychus malabaricus*) (2 females, 2 males), thrush nightingales (*Luscinia luscinia*) (2 females, 2 males), house sparrows (*Passer domesticus*) (2 females, 2 males) and capercaillies (*Tetrao urogallus*) (4 females, 5 males). Samples of white-rumped shamas and thrush nightingales were collected from the cage bottom of caged birds. Samples of house sparrow and capercaillie were sampled at natural roosting sites, both in southern Germany. Droppings were put into paper envelopes and stored dry until analysis. Alternatively a tube containing desiccating silica gel beads was used for large and humid samples.

For sex identification, we used a method which exploits the difference in length between introns in the CHD-Z and CHD-W genes (GRIFFITHS et al. 1998, KAHN et al. 1998) that are located on the avian sex chromosomes of all non-ratite birds. Males of non-ratite birds have two identical sex chromosomes (WW) and therefore two identical CHD-W genes. A PCR reaction using DNA of males and suitable „sex-primers" (GRIFFITHS et al. 1998, FRIDOLFSSON & ELLEGREN 1999) which bind to the W chromosomes gives a single PCR product which can be detected by gel electrophoresis. On the other hand, female birds have different sex chromosomes (WZ) and are therefore heterozygous with respect of the CHD gene (CHD-W and CHD-Z). Due to length differences of introns in the CHD-W and CHD-Z gene copies, a PCR reaction with „sex-primers" results in PCR products which give two bands on a gel. For our analysis, we used the „sex primers" P 2 and P 8 (GRIFFITHS et al. 1998). For microsatellite analysis, we used the primer sets (TUT 1, TUT 4) specially developed for capercaillie (SEGELBACHER et al. 2000).

To test the reliability of faecal typing, we analysed DNA from freshly moulted or plucked feathers of each of the caged individuals from which we had collected faeces. For sex identification, we used samples of 2 females and 2 males of each species. For microsatellite analysis, we analysed droppings and feathers of 9 capercaillie (4 males, 5 females) with primers TUT 1 and TUT 4. As a positive control, we used DNA extracted from muscle tissue of one female and one male capercaillie. To avoid false genotyping of all samples due to low DNA content, three PCR reactions were amplified for each DNA sample (multiple-tube approach).

A silica-based column method was used for DNA extraction from faeces that was originally developed for the isolation of DNA from human faeces (QIAamp DNA stool kit, Qiagen). DNA from feathers and muscle tissue was extracted as described in SEGELBACHER et al. (2000). A sample of 100 to 300 mg of faeces was put into a 15 ml tube and gently washed with 1 – 3 ml buffer ASL (supplied by the manufacturer) or phospate-buffered saline buffer (PBS) pH 7.4. As bird faeces contains a high amount of uric acid which could interfere with the extraction procedure, only the dark parts of the droppings were chosen. Washing was performed for 20 – 60 minutes on a rotating wheel to release shed epithelial cells of the intestinal lining from the surface of the excrement. We avoided disintegration of the dropping to minimize the amount of PCR-inhibiting substances. This supernatant was then transferred to a 2 ml microcentrifuge tube and centrifuged briefly to pellet particles. Then 1.4 ml of the supernatant was transferred to a new 2 ml microcentrifuge tube and an provided Inhibit EX tablet (Qiagen) added. Additional steps were performed according to the manufacturers protocol. The extracted DNA was then eluted in 50 µl of H_2O. PCR reactions were performed in 10 µl volumes with an Eppendorf Gradient Thermal Cycler. Individual mixes contained 1 µl of the eluted DNA, 0.2 mM dNTPs, 10 pmoles of each primer, 2.5 mM $MgCl_2$, 200 mM $(NH_4)_2(SO)_4$, 750 mM Tris-HCl pH8.8, 1.5 mg/ml BSA, 100 mM beta-mercaptoethanol and 0.25 units of Platinum Taq DNA polymerase (Invitrogen). PCR profiles consisted of 3 minutes denaturation at 94 °C, 35 cycles of 30 s denaturation at 94 °C, 45 s annealing at 60 °C for the microsatellite primers (TUT 1, TUT 4) and 48.5 °C for the sex primers (P 2, P 8), 45 s extension at 72 °C, with a final extension step of 3 min at 72 °C. The products were run on a 6 % denaturing polyacrylamide sequencing gel and visualised by silver staining (Promega). Scoring of the gel bands was done by a person who did not know the sex and origin of the samples. To avoid contamination, DNA extractions, pre PCR and post PCR pipetting were carried out in different rooms and areosol-resistant filter pipette tips were used throughout.

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3. Results and Discussion

Identification of sex proved to be reliable in all tested bird species when using the multiple-tube approach, as proposed by TABERLET et al.(1999). In this approach each DNA amplification is repeated independently for each locus at least three times with the same DNA extract. Droppings of all species (n = 21) could be sexed correctly, and faecal samples showed the same banding patterns as feather and tissue samples (Fig. 1). In only one case was there a drop-out of one allele in the first PCR reaction; only the shorter allele of the CHD gene could be amplified in a female sample. However, two subsequent PCR´s of the same sample gave the correct pair of products. This finding supports the importance of a multiple-tube approach.

Analyses of the 2 microsatellite loci amplified from capercaillie droppings showed that genotypes were identical to those obtained when DNA was extracted from feathers for the four female and five male capercaillies (Fig. 2). Correct genotypes could be obtained from fresh and old droppings, indicating that even faeces, which were collected after some days in the field could be used for genetic analysis. However, amplification success of microsatellite markers was higher in fresh samples than in old samples (SEGELBACHER unpublished data). Effects of sampling and storage of faecal samples should therefore be taken into consideration to optimise genotyping from them.

As bird faeces contains a high amount of uric acid and PCR inhibiting substances, it is difficult to obtain DNA from it that is free of PCR inhibitors using previously published DNA extraction protocols (e.g. standard phenol-chloroform extraction or Chelex methods). We demonstrate that, using the described method, we can use minute amounts of bird faeces for reliable genotyping.

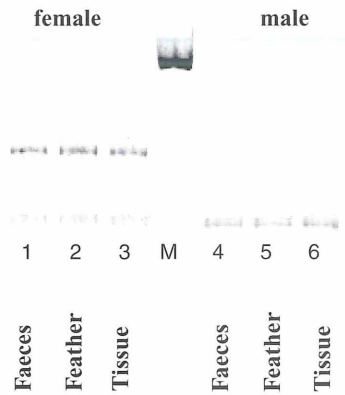

Fig. 1: Sex identification using PCR with template DNA isolated from faeces, feathers or tissue. Lanes 1-3 show the PCR products obtained from a capercaillie female (two alleles amplified) , 4–6 from a capercaillie male (one allele amplified). Lane M shows a 400 basepair molecular weight marker band.

Abb. 1: Geschlechtsbestimmung beim Auerhuhn mittels PCR aus DNA von Kot, Federn und Gewebe. Proben 1–3 zeigen die PCR-Produkte einer Auerhenne (zwei Allele), Proben 4–6 eines Auerhahnes (ein Allel). M zeigt einen 400 bp Längenstandard.

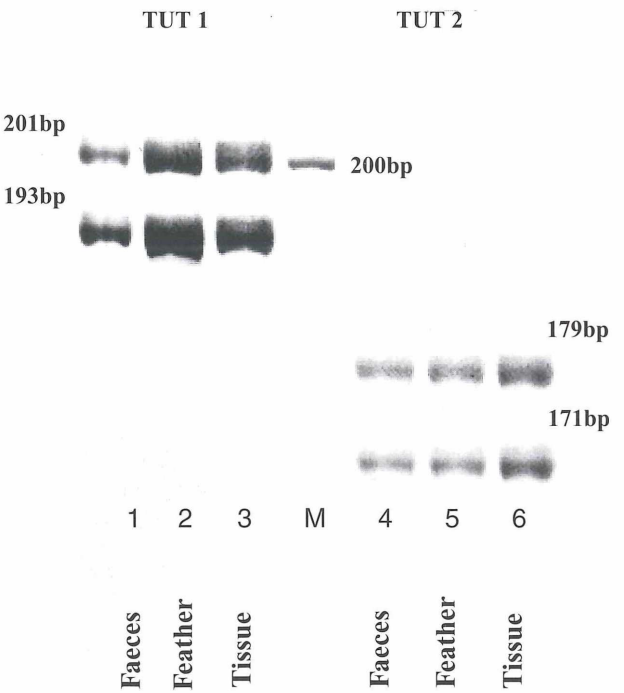

Fig. 2: Banding patterns of two microsatellite loci amplified using DNA isolated from faeces, feather or tissue of capercaillie. Lanes 1–3 show the amplification product of locus TUT 1, lanes 4–6 locus TUT 4. Lane M shows a 200 basepair molecular marker band. The microsatellite banding patterns obtained from the three different sources are identical.

Abb. 2: Bandenmuster zweier Mikrosatellitenloci aus DNA verschiedener Herkunft. Die Proben 1–3 zeigen den Locus TUT 1, 4–6 den Locus TUT 4. M stellt einen 200 bp Größenstandard dar.

Although DNA of droppings, which have been sampled in the field may be highly degraded, we can avoid typing errors that arise to the low amount of template DNA by using multiple, independent PCR reactions. We could also minimise errors in microsatellite genotyping using short (< 200 bp) microsatellite marker fragments.

This approach can be conducted in every molecular biology laboratory performing microsatellite genotyping or sexing of birds without any further need of equipment. Although the basic costs of this approach are similar to those when genotyping blood or tissue samples, one should be aware that multiple extractions and genotyping will be more expensive. As each study is unique and thus

results cannot be transferred to another species or even other populations of the same species, a pilot study to assess the genotyping error rate and the laboratory effort and necessary costs is strongly recommended.

Several non-invasive sampling studies in mammals demonstrated their potential to identify individuals, to estimate relatedness among individuals, effective population size and level of genetic differentiation between populations (see review in TABERLET et al. 1999). In the case of small endangered populations, species highly sensitive to disturbance, or in behavioural studies where capturing the individual of interest is not feasible, faeces or feathers might be the only accessible source of DNA. Our method offers the possibility to amplify microsatellites and sex markers from the same faecal sample. Therefore one can obtain information about the genotype and sex of the same individual. These non-invasive samples could be also very helpful to gain information on secretive species. As more and more microsatellite markers become available (e.g. SCRIBNER & PEARCE 2000, GALBUSERA et al. 2000), many bird species may become identifiable by their faeces alone. Studies on European grouse species demonstrate the high potential of microsatellite markers to correctly assign any droppings to species (own unpublished data). This information could give additional information when mapping birds distribution and abundance. Analysing faecal samples could even give us the possibility to track endangered populations, to gain information about the number of individuals or their home range size, as has been shown in brown bears (TABLERLET et al. 1997). Using bird faeces for microsatellite analysis and sex identification offers a new perspective in ecological and population studies of birds.

4. Zusammenfassung

Geschlechtsbestimmung und genetische Analysen aus Vogelkot.

Für genetische Untersuchungen an Vögeln ist es oft schwierig, Blut oder Gewebeproben zu erhalten. Daher haben wir eine Methode entwickelt, aus Kotproben DNA zu gewinnen, die für die Geschlechtsbestimmung und Mikrosatellitenanalyse verwendet werden kann. Mittels PCR wurde DNA aus dem Kot verschiedener Vogelarten amplifiziert. Die Ergebnisse stimmten mit denen aus Feder und Gewebeproben gewonnenen überein. Besonders für bedrohte oder störungsempfindliche Arten stellt dies eine Möglichkeit dar, genetische Informationen zu erhalten.

5. References

Fridolfsson, A.K., & H. Ellegren (1999): A simple and universal method for molecular sexing of non-ratite birds. J. Avian Biol. 30: 116–121. ✻ Galbusera, P., S. Van Dongen & E. Matthysen (2000): Cross-species amplification of microsatellite primers in passerine birds. Conservation Genetics 1: 16–168. ✻ Griffiths, R., M.C. Double, K. Orr & R.J.G. Dawson (1998): A DNA test to sex most birds. Mol. Ecol. 7 :1071–1075. ✻ Kahn, N.W., J. St. John & T.W Quinn (1998): Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex identification in birds. Auk 115: 1074–1078. ✻ Lens, L., P. Galbusera, T. Brooks, E. Waiyaki & T Schenck (1998): Highly skewed sex ratios in the critically endangered Taita trush as revealed by CHD genes. Biodiversity and Conservation 7: 869–873. ✻ Nota, Y., & O. Takenaka (1999): DNA extraction from urine and sex identification of birds. Mol. Ecol. 8: 1235–1238. ✻ Scribner, K.T.,g & J.M. Pearce (2000): Microsatellites: evolutionary and methodological background and empirical applications at individual, population and phylogenetic levels. In: Molecular Methods in Ecology (ed. Baker A.), pp 235–271. Blackwell Science Limited, London, England. ✻ Segelbacher, G. (in press): Non-invasive genetic analysis in birds: testing reliability of feather samples. Mol. Ecol. Notes. ✻ Segelbacher, G., R. Paxton, G Steinbrück, P. Trontelj & I. Storch (2000): Characterization of microsatellites in capercaillie (*Tetrao urogallus*). Mol. Ecol. 9: 1934–1935. ✻ Sheldon, B.C. (1998): Recent studies of avian sex ratios. Heredity 80: 397–402. ✻ Taberlet, P., J.J. Cammara & S. Griffin (1997): Non-invasive genetic tracking of the endangered Pyrenean brown bear population: Mol. Ecol. 6: 869–876. ✻ Taberlet, P., L.P. Waits & G. Luikart (1999): Non-invasive genetic sampling: look before you leap. Trends Ecol. Evol. 14: 323–327.