Iris colour as an indicator of age feature in female Brazilian tanagers (Passeriformes: Emberizidae) confirmed by a molecular sexing technique

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Abstract: The Brazilian tanager, *Ramphocelus bresilius* is an endemic species from Brazil that is sexually dimorphic in adult plumage. Young males are similar to adult and young females until their second year. Adults and young females are not distinguishable in plumage. We tested whether iris colour can be used to separate adult females from immature females. We used for the first time the molecular sexing technique based on CHD-genes to confirm the sex of the individuals classified as "female plumage with red iris", and to identify the sex of individuals classified as "female plumage and brown iris". The adult males were used as a positive control. DNA samples from 190 individuals were analysed. The sizes of the PCR products were identified as 350 base pairs (bp) for CHD-Z and 388 bp for CHD-W. We confirmed that adult females have a red iris and the young females a brown iris. We could also separate young males and females which present the same iris colour and plumage. Although there are indications that the iris colour can be used by birds to identify the adults in co-operative breeding species such as the Brazilian tanager, more behavioural data are required to understand the role of iris coloration in this species. Rev. Biol. Trop. 56 (4): 1629-1633. Epub 2008 December 12.

Key words: Molecular sexing, iris colour, CHD-gene, *Ramphocelus bresilius*, Passeriformes.

Adults and particularly juveniles of many bird species are monomorphic, making determination of sex difficult. It is estimated that adult females appear identical to males in over 50% of the world's bird species (Griffiths *et al.* 1998). Besides that, the difference between males and females for some species is only recognisable in adults or subadults. In this case, until the difference in plumage becomes evident, the young are usually similar to adult females. Thus, morphological differences that can be used to easily identify the sex in birds are extremely important to facilitate many studies of behaviour, evolutionary ecology and evolution (Ellegren 1996).

Ramphocelus bresilius, the Brazilian tanager, is an endemic species in Brazil, whose distribution extends from Paraíba state in the northeast to Santa Catarina in the southeast, thus encompassing almost the whole extent of the Atlantic forest. Adult *R. bresilius* exhibit a pronounced sexual dimorphism, with bright blood red plumage in the males, contrasting with black wings, tail and legs. A white callosity at the base of the jaw is emphasized by the dark bill. Adult females are greyish-brown on the back, with scattered reddish feathers dispersed on the rump and upper tail coverts. Immature males and females have similar plumage to adult females and there is no apparent plumage difference between adult and young females (Sick 1997). Adult females and young males can only be distinguished after the first year, when young males start to acquire

the first red feathers. The black and red plumage of the adult male is completed at the second year (Sick 1997).

Based on field observations, Castiglioni (1998) suggests that the iris colour can be used as a feature to separate adult females from young females. According to the author, the young have a brown iris and the adult, a red iris.

Confirmation of the iris as an age related characteristic can help the identification of adult females in the field. This could be particularly important in population genetic studies, since the inclusion of young animals in the analysis could generate too many samples of related individuals (Edwards 1993).

Sex identification at the DNA level is a well-established technique that has proved to be fast and accurate. In the method described by Griffiths *et al.(*1998), homologous copies of the CHD (Chromohelicase-DNA-binding) gene, located on Z and W avian sex chromosomes, are amplified by the polymerase chain reaction (PCR) using a single pair of primers P2 and P8 (Griffiths *et al.* 1996, Griffiths *et al.* 1998). Two different sized PCR products result in females, Z and W, but just one in males, ZZ. This is possible because the primers span differential intronic sequences (Miyaki *et al.* 1998). As the CHD-gene is located on the avian sex chromosomes of all species (except ratites) this technique has been widely used.

In the present study we used the P2 and P8 primers for the first time on *R. bresilius* to confirm if the iris colour is an indicator of age. The molecular sexing technique was also used to identify the gender of the juveniles since there is no difference on iris and plumage colour until the first year.

MATERIALS AND METHODS

The samples were collected from seven sites in Rio de Janeiro state, southeastern Brazil: Ariró (22º54'S, 44º20'W), Marambaia (23º04'S, 43º58'W), Maricá (22º57'S, 42º10'W), Massambaba (22º56'S, 42º12'W) located on the mainland and Abraão (22º51'35''S, 43º49'39''W) Vila Dois Rios (22º50'04''S, 43º51'39"W) and Aventureiro (22º49'28''S, 43º42'32" W) located on the island of Ilha Grande. One hundred and ninety birds were captured with mist nets (12.0 x 2.0 m 36 mm mesh) between October 1999 and February 2001. Individuals were individually marked with numbered aluminium leg bands to identify recaptures. Among the 190 sampled birds, 78 had male plumage and red iris, 45 had female plumage and red iris and 67 had female plumage and brown iris. Based on the iris colour they were classified as: Red iris (Ri) and Brown iris (Bi). The 78 male (M) individuals were included in the analysis as a positive control.

Blood was extracted from each individual captured by puncturing the tarsal vein with a disposable needle 13 x 4.5 mm (26G1/2) and collecting blood in a capillary tube. Approximately 50-150µl of blood was extracted and was immediately placed in a 1.5 ml eppendorf tube containing absolute ethanol. Samples were stored at room temperature during field work and at 4ºC in the laboratory.

DNA was extracted by using a salting-out method (FitzSimmons *et al.* 1995). All individuals were sexed by examining the highly conserved W and Z chromosome linked gene, CHD-W and CHD-Z (Griffiths *et al.* 1998).

Polymerase chain reaction (PCR) amplification was achieved in a 10 µL reaction volume containing 10ng DNA, 1x buffer (75mM Tris-HCl pH 9.0, 20 mM(NH4)2SO4, 0.01% Tween 20), 0.25 U Taq DNA polymerase (Thermoprime plus, Advanced Biotechnologies), 200 µM dNTPs (Gibco), 2mM de MgCl2 and 1µM of each primer P2 (5'-TCT GCA TCG CTA AAT CCT TT- 3') and P8 (5'-CTC CCA AGG ATG AGR AAY TG-3'). PCR amplifications were performed in a Hybaid Touchdown thermal cycler (Thermo Hybaid, Ashford, Middlesex, UK).

The PCR profile used was an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 45s, 50°C for 45s and 72°C for 45s, with a final extension period of 72°C for 5 min.

The PCR products were analysed on a 6% acrylamide gel using an ABI PRISM™ 377

DNA sequencer and sized with ROX 500 size marker with bands of known size every 50bp. All gels were analysed using GENESCAN TM Analysis 2.0, and GENOTYPER ^{TM} 1.1 software. The primers were labelled with NED dye emitting a yellow fluorescence when read by the laser in the automated sequencer.

RESULTS

The molecular sexing technique was efficient for *R. bresilius*. From 190 DNA samples submitted to the analysis, only seven individuals did not show results. We consider the seven with no results to be related to the quality of the DNA rather than the technique employed. On the other 183 samples, the sex was easily determined by visualizing two yellow bands for females and one band for males on acrylamide gel. By the molecular technique all individuals classified as Red iris (Ri) were females. Among the 67 individuals classified as Brown iris (Bi) with female plumage, 31 were females, 29 were males and seven did not amplify. The sexes of the 78 individuals used as a positive control were confirmed as males. By using a DNA size marker it was possible to identify the size of the PCR products, 350 base pairs (bp) for CHD-Z and 388 bp for CHD-W.

DISCUSSION

The CHD genes are remarkably conservative. Although mammals and birds diverged more than 200 million years ago, a mouse gene that is homologous to the chicken CHD gene is 82.9% identical at the nucleotide level, and 95.6% identical at the amino-acid level (Lessells and Mateman 1996). This is also important as the human CHD-1 gene can be a contaminant in samples (Griffiths *et al.* 1998).

In most cases the female CHD-W gene yields the larger product but in species such as the rock pigeon (*Columba livia*) and European bee-eater (*Merops apiaster*) the reverse is true (Griffiths *et al.* 1998). Product size also differs between species. This is useful since it provides an extra guarantee against cross species contamination (Newton and Graham 1994).

All the individuals sexed in the field as red iris with female plumage were confirmed as female by the molecular sexing technique. Sex was also confirmed in 100% of the adult males used as a positive control, sexed according to plumage and iris colour. The results of the molecular sexing technique confirmed the field observations performed by Castiglioni (1998) according to the relation of the iris color to age in *R. bresilius*.

In all studied species with a coloured iris, the adult iris colour differs from that of juvenile birds. The normal sequence is for juvenile birds to have a dull-coloured iris, which changes to become bright coloured when adults (Sweijd and Craig 1991, Pearson 1966, Ervin 1975, Wilkinson 1988, Wilkinson 1982, Stutterheim 1982, Newton and Marquiss 1982, Picozzi 1981, Peterson 1991, Crook 1964, Craig 1984). Iris colour can thus serve as an indicator of age classes for the human observer (Wood and Wood 1972) and may be used by birds in the same way (Craig and Hulley 2004). There is some evidence in raptors that mate selection may be influenced by age of the partner as indicated by iris colour (Newton and Marquiss 1982, Picozzi 1981).

In the present study, the iris coloration was found to be a reliable morphological feature to identify the adult females of *R. bresilius* in the field, distinguishing them from young birds. Craig and Hulley (2004) noted that many species with a coloured iris are cooperative breeders, and cooperative breeding has been reported in the Brazilian tanager (Sick 1997). Future studies of avian behaviour should examine the role of iris coloration in individual species.

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RESUMEN

El ave *Ramphocelus bresilius* es una especie endémica de Brasil con dimorfismo sexual en el plumaje del adulto. Los machos jóvenes son similares a las hembras adultas y jóvenes hasta el segundo año de vida. Adultos y hembras jóvenes son indistinguibles por el plumaje. Evaluamos si el color del iris puede ser utilizado para distinguir hembras adultas de hembras inmaduras. Utilizamos por primera vez la técnica molecular de identificación de sexos basada en los genes CHD para confirmar el género de individuos clasificados como plumaje femenino con iris rojo, y para identificar el sexo de los individuos clasificados como plumaje femenino e iris marrón. Usamos machos adultos como control. Analizamos muestras de DNA de 190 individuos. Los tamaños de los productos del PCR fueron identificados como 350 pares de bases (pb) para CHD-Z y 388 pb para CHD-W. Pudimos confirmar que las hembras adultas presentan iris rojo y las hembras jóvenes iris marrón. También pudimos distinguir machos jóvenes de hembras, que presentan el mismo color de iris y plumaje.

Palabras clave: identificación de sexos, color del iris, genes CHD, *Ramphocelus bresilius*, Passeriformes.

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