Sex determination in *Turdus amaurochalinus* (Passeriformes: Muscicapidae): morphometrical analysis supported by CHD gene

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Abstract: Sex determination is important for conservation and population studies, particularly for reproduction programs of threatened species and behavioural ecology. *Turdus amaurochalinus*, Creamy-bellied Thrush, only exhibits sexual dimorphism during the breeding season, when males are considered to show intense yellow bills, and females and immature males show dark brown bills. The objectives of this study were: 1) to determine the sex of individuals using genetic techniques, and 2) to test the hypothesis that sex dimorphism can be detected by morphometry. This study was carried out at Parque Nacional da Restinga de Jurubatiba, a preserved area located on the North coast of Rio de Janeiro State. The birds were captured using ornithological nets, singly marked with metal rings, weighed, measured and had blood samples collected before being released. The sex of 42 *T. amaurochalinus* individuals was determined using the CHD gene marker. A total of 20 males and 22 females were identified from June to August, with peak capture frequency in June. *Turdus amaurochalinus* females and males differed significantly in morphometrical measures. The most important traits to distinguish males from females were wing length (Student t-test=4.34, df=40, p=0.0001) and weight (Student t-test=2.08, df=40, p=0.044): females were heavier and had significantly shorter wing length than males. Females and males were correctly classified in 86% and 75% of cases, respectively, using Discriminant Analysis. The molecular analysis was the most secure method for sex determination in the studied species. Rev. Biol. Trop. 59 (2): 789-794. Epub 2011 June 01.

Key words: sex determination, *Turdus amaurochalinus,* morphometrics, CHD gene, Restinga de Jurubatiba.

Many bird species cannot be sexed by any morphological trait, not differing in morphological external characteristics, while others are sexually dimorphic only in the adult phase (Ellegren & Sheldon 1997, Kahn *et al*. 1998). In the case of *Turdus amaurochalinus*, it is difficult to differentiate young males from adult females.

The sexual identification of birds is very important for population and conservation studies, particularly for reproduction programs of threatened species (Ellegren & Sheldon 1997, Miyaki *et al*. 1998), identification of morphological aspects (King & Griffiths 1994, Burns 1998), behavioural ecology and evolutionary biology (Ellegren 1996, Lessells & Mateman 1998).

Sex is one of the most important variables to distinguish individuals in a population. According to Fisher's theory about sexual proportion, if equal conditions were available for male and female production, the sex ratio at the time parental investment ends should stabilize at 1:1 (Fisher 1930). The individuals of many populations diverge from the expected sex ratio of 50% for each sex, not being able to assume

a 1:1 constant ratio (Krebs 1994). However, these differences between species and populations could have importance, if correlated to some particularity of their life cycle (Bull & Charnov 1988, Lens *et al*. 1998).

Techniques for sex determination in birds include laparoscopy, karyotyping (Basrur *et al*. 1998), analysis of the faecal steroid, DNA fingerprinting and molecular techniques based on the chromo-helicase-DNA binding (CHD) gene (Ellegren 1996, Griffiths *et al*. 1998). The CHD gene has been used successfully in many bird species (Griffiths *et al*. 1998, Miyaki *et al*. 1998, Ito *et al*. 2003, Sacchi *et al*. 2004, Lee *et al*. 2007, 2010), since the gene is preserved in most bird species (Griffiths & Tiwari 1995).

In birds, females are the heterogametic sex (ZW) and males are homogametic (ZZ) (Ellegren 1996). The W chromosome is special for the female, and the determination of the sex is made based on the absence or presence of the marker W-linked. Using polymerase chain reaction (PCR) techniques, it is possible to easily identify the females, since they show two bands on agarose gel. The CHD gene was successfully used for sex determination in the White-necked Thrush *Turdus albicollis* (Vieillot, 1818), not apparently sexually dimorphic in the adult phase. For this species, males and females differed in morphometric characteristics such as wing length and body mass, which were significantly bigger in males (Ritter *et al*. 2003). For this reason, we would expect to find distinct morphological characteristics between sexes in the con-generic species, Creamy-bellied Thrush *T. amaurochalinus* Cabanis 1850, found in the forests of Argentina, Bolivia, Brazil, Chile, Paraguay, Peru and Uruguay.

The present study aims to determine the sex proportion of *T. amaurochalinus* using CHD gene marker, and to evaluate if morphological characteristics that may help to identify the sex of an individual in the field.

METHODS

Study sites: The work was carried out in an area of Restinga, which is part of the Atlantic forest biome, in Restinga de Jurubatiba National Park, located at the Eastern coast of Rio de Janeiro State, Brazil (22º00' - 22º23' S, 41º15' - 41º45' W). The region is dominated by a large lake of fresh water (Lagoa Feia) and by a sandy quaternary plain, which stretches towards the continent, advancing from the sea to the interior approximately 2km, where the seasonally flooded forest begins to appear. There is accented seasonality, with maximum rainfall in the summer (approximately 190mm) and minimum in the winter (around 40mm). The mean annual temperature was 22.6°C (Henriques *et al*. 1986).

Data collection at field: Birds were captured twice a month from 2003 to 2004 in the Restinga de Jurubatiba National Park (Alves *et al*. 2004). Each month 10 nets were set up in open *Clusia* scrub and 10 nets in Forest Formation (seasonally flooded forest), with two consecutive days in each area for each excursion (20 nets total per sample period). Nets were exposed for 7 hours of sampling each day, with half of this period in the morning (06:30-10:30h) and half in the afternoon (14:30-17:30h).

Captured birds were placed individually in clean cotton bags, and were marked separately with metal and coloured rings. Shortly after capture, we recorded the following measurements: total length, wing length, length of the tail, tarsus length, exposed culmen, nostril-tip, opening-bill base, bill height in the nostril and in the base, bill width in the nostril and in the base, and length of the head to the bill tip (Sick 1997). The morphometrical data were always taken by the same researcher (one of the authors, M.A.S. Alves) to avoid bias.

We collected approximately 50-150µl of blood from the tarsal vein using a disposable needle 13x4.5mm (26G1/2) and 50μl capillary tubes with heparin. Blood was immediately transferred to 1.5ml plastic tubes with absolute ethanol; all samples were stored at room temperature during field work and at 4°C in the laboratory.

Laboratory analysis: Sex determination was carried out at the Bird Ecology Laboratory, Ecology Department of the Universidade do Estado do Rio de Janeiro (UERJ) using the CHD gene technique developed by Griffiths *et al*. (1998), and modified by Miyaki *et al*. (1998). DNA was extracted using the phenol-chloroform extraction/alcohol precipitation method described in detail by Bruford *et al*. (1992) or by cellular lysis as described by Khatib & Gruenbaum (1996).

The CHD gene was amplified by PCR, using P2 and P8 primers (P2: 5'-TCTGCATCGCTAAATCCTTT-3' and P8: 5'-CTCCCAAGGATGAGRAAYTG-3'; Griffiths *et al*. 1998). The PCRs were carried out in a total volume of 10μl, consisting of 1μl of reaction buffer (10mM KCl, 20mM Tris-HCl pH 8.8, 10mM $(NH_4)_2SO_4$, 0.1% Triton-X-100, 100mg/ml gelatin); 2μM of dNTP mix; 2mM of $MgCl_2$; 100pmoles/μl of each primer; 1-10ng of genomic DNA and 0.05 units of DNA polimerase (Biotools of Brazil). A cycle of 95°C for thirty seconds, 45°C for thirty seconds, 72°C for thirty seconds, preceded by a step of 95°C for five minutes, was repeated 40 times, followed by a step of 72°C for five minutes. The products were separated in 2% agarose gels.

Initially, the CHD gene sex determination technique was tested for several bird species, including *T. amaurochalinus*. Some individuals of *T. albicollis* were used as positive controls for the method (Ritter *et al*. 2003) and *Ramphocelus bresilius* (Linnaeus, 1766) as positive controls for sex differentiation. This last species presents obvious sexual dimorphism of plumage and iris colour: males, including the young males, present red iris colour, and females, chestnut iris colour (Nogueira & Alves 2008). The result proved that the protocol developed for this technique is secure, since *R. bresilius* males presented one band, while females presented two bands.

The statistical analyses of data followed Zar (1984) and were carried out in SYSTAT software (version 10.2). Only data collected from *T. amaurochalinus* adults, selected by plumage, tarsus and bill commissure characters, were used. Discriminant Analysis includes only the variables with observations for all forty individuals: body mass, total length, wing length, length of the tail, tarsus length, exposed culmen and length of the head to the bill tip.

RESULTS

Sexual reason and morphometric analyses: We categorized 42 (20 males and 22 females) *T. amaurochalinus*: males and females differed significantly (Test-t, $p<0.05$; Table 1) on two of 11 analyzed variables. Males presented longer wing length than females, while females appeared heavier than males (Fig. 1).

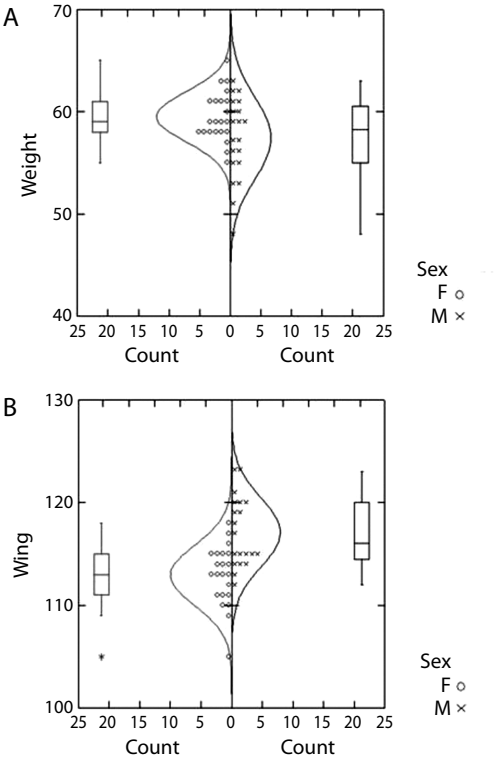

Fig. 1. Student's t-test analysis for weight **(A)** and wing length **(B)** variables in females **(F)** and males **(M)** of *Turdus amaurochalinus* captured. Sex was determined using CHD gene molecular technique.

TABLE 1
Turdus amaurochalinus females and males body mass and morphometrical variables taken in the Restinga de Jurubatiba National Park, Rio de Janeiro, Brazil

Sex	Females		Males				
Body mass (g) and Morphometrical Variables (mm)	n	x ± sd	n	x ± sd	t	df	p
Body mass	22	59.5 ± 2.4	20	57.4 ± 4.0	2.081	40	**0.044**
Total length	22	219.1 ± 9.2	20	217.0 ± 9.5	0.707	40	0.483
Wing length	22	112.9 ± 2.9	20	117.1 ± 3.3	4.335	40	**0.0001**
Length of the tail	22	91.1 ± 4.2	20	93.9 ± 5.9	1.809	40	0.078
Tarsus length	22	32.8 ± 1.1	20	32.1 ± 3.1	0.977	40	0.334
Exposed culmen	22	19.0 ± 1.2	20	18.9 ± 1.4	0.203	40	0.840
Nostril-tip	14	12.5 ± 0.7	9	12.0 ± 0.7	1.601	21	0.124
Bill height in the base	15	6.8 ± 0.3	8	6.6 ± 0.4	1.422	21	0.170
Bill width in the base	15	12.8 ± 0.9	9	12.3 ± 1.0	1.267	22	0.218
Length of the head to the tip of the bill	22	47.2 ± 1.2	20	47.0 ± 1.1	0.441	40	0.661
Opening-bill base	16	11.7 ± 1.5	12	11.7 ± 1.1	0.097	26	0.923

The sex was determined using CHD gene molecular technique. Variables were analysed through Student's t-test (t): n=sample number, x=average, sd=standard deviation, df=degrees of freedom and p=probability.

Discriminant Analysis showed that 86% of the females and 75% of the males were categorized correctly (Wilk's lambda=0.549 and p-tail=0.0028). The variables that contributed more to distinguish the sexes were wing length and exposed culmen (Table 2). The males have longer wing length than females, while females presented a bigger value for exposed culmen.

TABLE 2
Canonical Discriminant Functions of the Discriminant Analysis for body variables of *Turdus amaurochalinus* sampled in the Restinga de Jurubatiba National Park, Rio de Janeiro, Brazil

Variables	Canonical Discriminating Functions
Body mass	0.157
Total length	0.028
Wing length	**0.326**
Length of the tail	0.009
Tarsus length	0.070
Exposed culmen	**0.167**
Length of the head to the bill tip	0.131

The sex was determined using CHD gene molecular technique.

We effectively amplified a selected region of the CHD-W gene and its homologous copy CHD-Z from *T. amaurochalinus* with P2/P8 primers (Griffiths *et al*. 1998). PCR products were 380/340 base pairs (bp) in length (CHD-W/CHD-Z, respectively).

DISCUSSION

It is reported that during breeding period, *T. amaurochalinus* males show intense yellow upper bill, and females and immature males show black coloured upper bill (Sick 1997). However, this correlation was not demonstrated in this study, since females were recorded with partially yellow bills.

Just wing length and body mass variables were statistically significant to separate sexes when Student's t-test was applied (Table 1 and Fig. 1). Regarding body mass, this result is already anticipated, since capture was carried out during species' reproduction time, when females are heavier due to eggs production. The fact that males presented larger wing length can be due to their major displacement (i.e. flies longer distances) in living area regarding females.

When Discriminant Analysis was applied to *T. amaurochalinus* morphometrical data, 86% of the females and 75% of the males were classified correctly. Wing length was the most significant variable in sex distinction of this species, with exposed culmen the second most significant variable (Table 2).

Student's t-test and Discriminating Analysis ratified wing length as a very significant variable to sexually separate individuals. Lens *et al*. (1998) and Ritter *et al*. (2003) also concluded that wing length is a relevant factor to separate adult males from females in *Thorichthys helleri* and *Turdus albicollis*, respectively.

CHD-W and CHD-Z gene sizes are compatible with con-generic species: 394/349 bp, respectively, in length for *Turdus merula* (Dybus *et al*. 2009) and 379/341 bp, respectively, in length for *Turdus pallidus* (Lee *et al*. 2010).

Turdus amaurochalinus males presented wing length larger than females, while body mass was larger in females than in males. These characters can be used as indication of sex status in field. However, molecular analysis remains the most accurate method to determine sex for this species.

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RESUMEN

La determinación del sexo es importante para la conservación y los estudios poblacionales. *Turdus amaurochalinus* no presenta aparente dimorfismo sexual. El objetivo de este estudio fue determinar el sexo a través de una técnica genética, mediante el uso del marcador del gen CHD y se puso a prueba la hipótesis de que el dimorfismo sexual puede ser detectado por morfometría. Este estudio se llevó a cabo en el Parque Nacional da Restinga de Jurubatiba, una zona protegida situada en la costa norte de Río de Janeiro. Las aves fueron capturadas con redes de niebla, los individuos se marcaron con anillos de metal, se pesaron, medieron y se les tomó una muestra de sangre antes de ser liberados. Un total de 20 machos y 22 hembras fueron identificados en el área de estudio desde junio hasta agosto, con la frecuencia máxima de captura en junio. La prueba de t-student fue usada para evaluar si hembras y machos se diferencian considerablemente en relación a medidas morfométricas. Los rasgos más importantes para distinguir machos de hembras fueron la longitud del ala y el peso: las hembras eran más pesadas y tenían longitud de ala considerablemente más corta que los machos. Hembras y machos fueron correctamente clasificados en un 86% y 75% de casos respectivamente, donde se usó un análisis discriminante. El análisis molecular es el método más seguro para la determinación sexual en la especie estudiada.

Palabras clave: determinación de sexo, *Turdus amaurochalinus*, morfometría, gen CHD, Restinga de Jurubatiba.

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