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SEX IDENTIFICATION OF PIN-TAILED MANAKINS (*ILICURA MILITARIS*: PIPRIDAE) USING THE POLYMERASE CHAIN REACTION AND ITS APPLICATION TO BEHAVIORAL STUDIES

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Resumo. – Sexagem molecular de Tangarazinhos (*Ilicura militaris*) através da reação de PCR e sua aplicação ao estudo do comportamento. – Na maioria das espécies de tangarás, os machos adultos são reconhecidos por sua plumagem sexualmente dimórfica, mas apresentam atraso de maturação da plumagem. Machos jovens e machos sexualmente maturos apresentam plumagem inicial indistinguível da plumagem das fêmeas, dificultando estudos de comportamento reprodutivo das tais espécies. Testamos dois métodos moleculares para identificação sexual precoce do Tangarazinho utilizando o gene CHD (Helicase dependente de cromo), localizado nos cromossomos sexuais (Z e W) das aves. Os produtos de PCR dos genes CHDZ e CHDW podem ser discriminados pela presença/ausência de sítios de restrição específicos ou pela extensão de seus íntrons. Essa é primeira vez que esses métodos são testados em Passeriformes suboscines. Conduzimos os protocolos em amostras de sangue de 22 Tangarazinhos, capturados em territórios de exibição de machos, em duas florestas do sudeste do Brasil. Os dois métodos, o do polimorfismo de sítio de restrição do CHD e o do dimorfismo intrônico do CHD, identificaram corretamente o sexo de cinco machos e uma fêmea, sexados paralelamente através da observação da plumagem definitiva de machos ou dos ovários na fêmea. 17 indivíduos com plumagem de fêmeas, porém de sexo desconhecido, foram sexados pelos dois métodos. Os resultados revelaram a presença de 12 machos e cinco fêmeas, em uma das áreas de estudo - um desvio significativo da razão sexual ($\chi^2 = 15.12$, $P < 0.0001$) – e um macho na segunda área de estudo. Este resultado indica que a maioria dos indivíduos com plumagem característica de fêmeas, em territórios de exibição de machos, são machos com plumagem pré-definitiva. Esses dados demonstram que a sequência do CHD está conservada em Passeriformes suboscines e enfatizam a importância da aplicação de métodos recentes de sexagem molecular aos estudos comportamentais.

Abstract. – Adult males of most manakin species are recognizable by their distinct sexually dimorphic plumage, but they have delayed plumage maturation. Immature and sexually mature males have an initial plumage indistinguishable from the female plumage, which complicates field studies on their reproductive behavior. We tested two molecular methods for sex identification in the Pin-tailed Manakin (Ilicura militaris) using the CHD (chromo-helicase-DNA-binding) genes, located on Z and W avian sex chromosomes. The CHDZ and CHDW PCR products may be discriminated by absence/presence of a specific restriction site or by intronic length. These methods had never been applied to any suboscine Passeriformes. We conducted both protocols on samples of blood from 22 Pin-tailed Manakins captured at male display sites in

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two forests from southeastern Brazil. The CHD restriction site polymorphism and the intronic CHD size dimorphism methods correctly identified the sex of five males and one female that were sexed by observation of definitive male plumage or ovaries. Both methods were applied to sex 17 female-like plumaged birds of unknown sex from our samples, resulting in the identification of 11 males and five females in one study site, a significant skewed sex ratio toward males ($\chi^2 = 15.12$, $P < 0.0001$), and one male in the second study site. This result indicates that a majority of the female-like plumaged birds at male display sites are pre-definitive plumaged males. Our data show that the CHD sequence is conserved in suboscine Passeriformes and highlights the importance of newly developed molecular sexing techniques in behavioral studies. *Accepted 18 October 2001.*

Key words: Pin-tailed Manakin, *Ilicura militaris*, molecular sexing, CHD, Pipridae, sex-ratio, social organization.

INTRODUCTION

Manakins (Pipridae) are forest frugivorous birds restricted to the Neotropics. They are notable for their elaborate courtship display and polygynous lek breeding system (Sick 1967, Prum 1990). Adult males of most species are recognizable by their distinct sexually dimorphic plumage, but all dimorphic manakin species have delayed male plumage maturation. Immature plumaged and sexually mature males have an initial green plumage that is indistinguishable from female plumage (Snow 1963), and are present during the courtship display performed by adult males (Snow & Snow 1985, Prum 1986, Prum & Johnson 1987, Théry 1992).

Griffith *et al.* (1996, 1998) have described two methods of identifying the sex of birds using the homologous copies of the CHD (chromo-helicase-DNA-binding) gene, located on Z and W avian sex chromosomes (Griffiths & Korn 1997). The CHDZ copy differs from the CHDW copy in presence of a specific restriction site (Hae III) in all 16 studied avian species and in the length of an intronic region in the 27 species analyzed. In species where the CHD gene sequence is conserved, females can be discriminated from males because of the PCR products showing absence/presence of a specific restriction site or differences in the intronic lengths. Males are characterized by absence of the restriction site and monomorphic CHD intronic length, whereas females have the restriction site and dimorphic CHD intronic length. Sex diagnosis is obtained after amplification and electrophoresis in agarose gels. Here we tested for the first time the applicability of both methods for sex identification in a suboscine Passerifomes, the Pin-tailed Manakin (*Ilicura militaris*), in order to establish a standard sexing technique for the species to complement data from behavioral studies in the wild.

METHODS

Samples. Blood samples were obtained from 39 Pin-tailed Manakins mist netted during the beginning of the breeding season, May to October of 1999, at Barreiro Reserve, Belo Horizonte (20°00'S, 44°00'W) and Serra do Brigadeiro State Park, Ervália (20°00'S, 42°40'W); both located in Minas Gerais State, southeastern Brazil. From 39 individuals captured, 22 were morphologically identified as definitive plumaged adult males through plumage inspection, and 17 birds were female-like plumaged. Necropsy or observation of definitive male plumage were performed in one female and five males whose blood samples were analyzed.

Blood samples were collected using heparin as anticoagulant and glass capillary tubes.

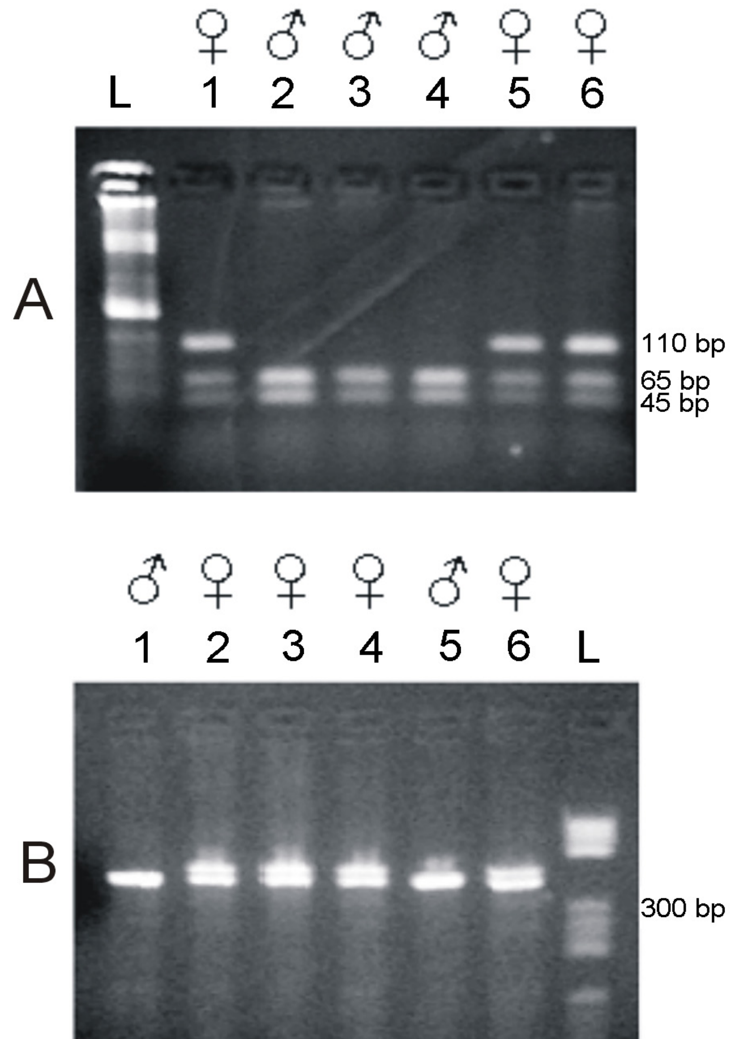

FIG 1. Male and female patterns in both sexing CHD methods: A) PCR products for copies of CHD gene from Z and W chromosomes after digestion with Hae III show two bands for males (samples 2, 3 and 4) and three bands for females (samples 1, 5 and 6); and B) PCR products for intronic copies of CHDZ and CHDW show one band for males (samples 1 and 5) and two bands for females (samples 2, 3, 4 and 6). L = DNA marker pBR 322 Hae III digest (Sigma).

The blood was transferred to vial and maintained in absolute ethanol.

Molecular sexing. Blood cells were disrupted with 0.05M NaOH and boiled at 100°C for ten minutes. The mixture was neutralized with 1.0 M Tris-HCl, pH 8.3 (Khatib & Gruenbaum 1996). One aliquot of 2–8 µl (0.125–0.500 µg DNA) was amplified in 25 µl of PCR reaction mixture. Each amplifica-

TABLE 1. Number of male and female Pin-tailed Manakins (*Ilicura militaris*) in each area. Barreiro Reserve male-females: $\chi^2 = 15.12$, $P < 0.0001$.

Individuals	Barreiro Reserve		Serra do Brigadeiro State Park	
	Number of individuals	Freq. (%)	Number of individuals	Freg. (%)
All males	27	75	7	71
Females	5	14	0	0
Adult males	16	30	6	57
Immature males	11	44	1	14
Unknown	4	11	0	0
Number of individuals	36		7	

tion mixture contained 10mM, pH 8.0, 50mM KCL, 2.5 mM $MgCl_2$, 200 μM dNTP, 1 unit of Taq polimerase (Promega), and 0.5 μM of each primer.

Primers P2 and P3 were used according to Griffiths *et al.* (1996) and each reaction was preheated at 94°C for 2 min. The amplification reaction was developed with 5 cycles using the following thermal profile: desnaturation, 94°C for 30 s; annealing, 53°C for 30 s; and extension 72°C for 30 s, followed by 35 cycles of 94°C for 30 s, 49°C for 20 s; and 72°C for 20 s. The amplified fragment was digested with 5 U of restriction enzyme Hae III at 37°C for 3 h. The digested fragments were separated after electrophoresis in 3% agarose gel with TBE. The gel was stained with ethidium bromide and photographed.

The same composition of the PCR reaction was maintained in the simplified protocol of Griffiths *et al.* (1998), with exception of the primers (P2 and P8). The amplified fragments were resolved in 3% agarose gel.

Control were obtained from five males and one female that were correctly identified by both methods and were previously sexed by observation of definitive male plumage or ovaries.

RESULTS

The CHD restriction site polymorphism method using Hae III restriction enzyme and the intronic CHD size dimorphism method were congruent in identifying the sex in all samples. Figure 1 shows male and female patterns by both CHD sexing methods based on PCR products obtained with DNA samples from the Pin-tailed Manakin. Two bands were detected in males and three bands were detected in females in the method using Hae III restriction enzyme, whereas in the intronic size dimorphism method males presented one band and females two bands. These results presented the same pattern as described respectively by Griffiths *et al.* (1996, 1998).

Eleven males and 5 females were identified in the Barreiro Reserve sample of female-like plumaged birds, demonstrating a significant skew in sex ratio toward males when all individuals captured are considered. No test of sex ratio deviation was performed for the individuals from the Serra do Brigadeiro State Park due to low sample sizes (Table 1 and Fig. 2). Additional analysis of the capture data indicated higher recapture rates for pre-definitive plumaged males (n = 3) and definitive plumaged males (n = 6) than females (n = 1) in a total of 32 individuals of known sex captured in the Barreiro Reserve.

DISCUSSION

Digestion of CHD products amplified with

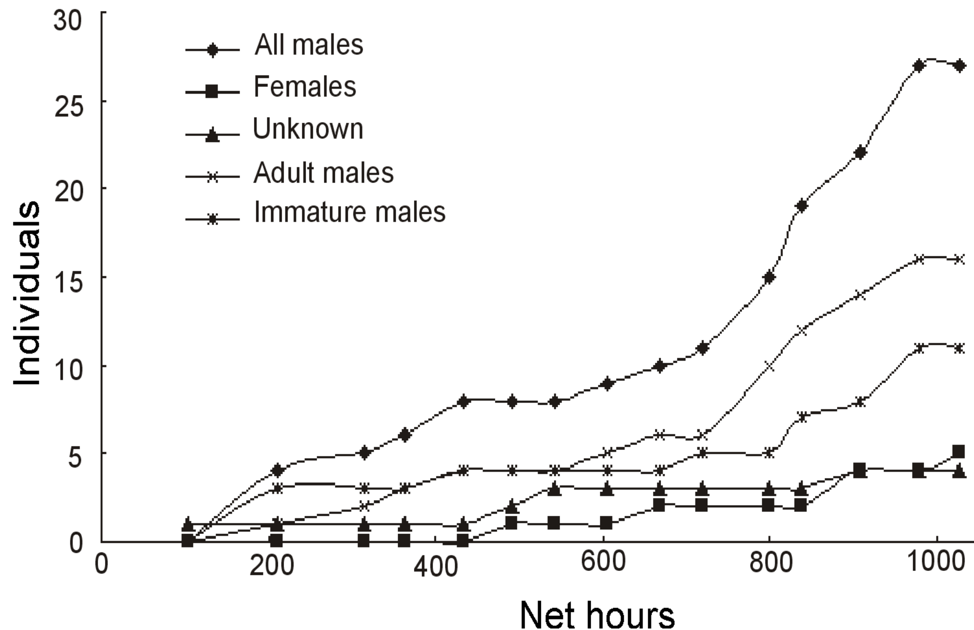

FIG. 2. Accumulation curves of Pin-tailed Manakins in the Barreiro Reserve. Recaptures were not considered.

restriction enzyme differentiates male and females. The Hae III site distinguishes the CHDW from the CHDZ allele, according to patterns obtained by Griffiths *et al.* (1996), who also showed females with three bands and males with two bands. The simultaneous application of two sexing methods insures the correct sex identification and decreases the probability of error due to sample contamination with human DNA. Human CHD gene can be amplified when Griffiths *et al.* (1996) method is used. However, the difference observed between fragment size obtained by Griffiths *et al.* (1998) for human and bird species eliminates this problem of cross contamination. Complete correspondence between results obtained by two molecular sexing methods demonstrates that there is no human DNA contamination.

These protocols have been successfully applied to field studies, revealing differential sex-related selection on Old World flycatchers (Merila *et al.* 1997) and consequences of skewed sex ratios to conservation of small populations of African thrushes (Lens et al. 1998). Patterns obtained by the protocol described by Griffiths *et al.* (1998) showed females with CHDW and CHDZ intronic bands and males with CHDZ band, repeating the findings of the previous study.

The application of DNA-based methods for the identification of sex in birds is profitable for studies of sex allocation and its consequences in birds (Ellegrens & Sheldon 1997). Our results indicate that a majority of the green-plumaged Pin-tailed Manakins observed at male display sites are pre-definitive plumaged males. Field studies considering lekking species should attempt to determine sex ratios in display sites, even in solitary lekking species such as the Pin-tailed Manakin (e.g., Snow & Snow 1985). The

number of sub-ordinate males in a display area was shown to be higher than the number of females attending the lek.

Males also had higher recapture rates, indicating that females may have larger home ranges than pre-definitive and definitive plumaged males in this lekking species. Other studies have shown that males and females show different patterns of space utilization in manakin species (Théry 1992) and that females disperse across male territories (Westcott 1997). Graves *et al.* (1992) found a strong tendency for greater captures of new males than of females on the first 2000 hours of mist netting after removing all captured individuals from the study site. Increasing sampling effort also increased the number of new females captured, indicating that captured males are not replaced, but females are. Extended mark-recapture studies, without removal, will allow a test of whether the results by Graves *et al.* (1992) for increased newly captured females is an effect of removal *per se* or truly a result of their higher vagility.

In our Pin-tailed Manakin study during 1999, the only recaptured female was first captured in 1997, approximately 1000 m away from the recapture site, whereas all recaptured males (from which all three immature males were sexed by the CHD method) were first captured in the same sampling area, including one individual from 1998. These data corroborate the hypothesis that females are more vagile, suggesting that they would be less frequently recaptured in a given male display area.

Our results demonstrate that the CHD system is effective in suboscine Passeriformes and further establish that the CHD sequence is highly conserved among birds. The method using size dimorphism of an intronic region from the CHD gene on the Z and W chromosomes is simpler and less labor-intensive. Our data thus highlight the potential of these newly improved molecular sexing techniques to reveal demographic patterns in behavioral studies.

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