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

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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 the areas of study. The results demonstrate that the CHD sequence is conserved in suboscine Passeriformes and emphasize the importance of applying recent methods of molecular sexing to behavioral studies.

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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 Passeriformes, 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, Ervalia (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.
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-
tion mixture contained 10mM, pH 8.0, 50mM KCl, 2.5 mM MgCl₂, 200 µM dNTP, 1 unit of Taq polymerase (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: desnaturación, 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

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Barreiro Reserve</th>
<th>Serra do Brigadeiro State Park</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of individuals</td>
<td>Freq. (%)</td>
<td>Number of individuals</td>
</tr>
<tr>
<td>All males</td>
<td>27</td>
<td>75</td>
</tr>
<tr>
<td>Females</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Adult males</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>Immature males</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>Unknown</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Number of individuals</td>
<td>36</td>
<td>7</td>
</tr>
</tbody>
</table>

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

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 (Ellegren & 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

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