ORNITOLOGÍA NEOTROPICAL
(2018) 29: 225–228
SHORT NOTE

MOLECULAR SEX DETERMINATION IN NEOTROPICAL MONOCHROMATIC HUMMINGBIRDS

Felipe L. S. Shibuya[1] · Flavia Torres Presti[2] · Susana Alexandra Costa Lopes[3] · Paulo Gama Mota[3,4] · James J. Roper[1]

[1] Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal do Paraná, Curitiba, Paraná, Brazil.
[2] Instituto Federal do Paraná, Jacarezinho, Paraná, Brazil.
[3] CIBIO, Research Center for Biodiversity and Genetic Resources, University of Porto, Vairão, Portugal.
[4] Department of Life Sciences, University of Coimbra, Coimbra, Portugal.
E-mail: Felipe L. S. Shibuya · felipe.shibuya@gmail.com

Abstract · Many species of hummingbirds are sexually monochromatic. This is surprising because in all hummingbird species parental care is carried out by females, while males are promiscuous, and this mating system is usually associated with some form of sexual dimorphism. To study sexual selection in monochromatic species, the identification of sex is a prerequisite. Using blood and feathers from live individuals we apply a simple method to identify sex. We amplified fragments of the CHD-1 gene through PCR, using P2 and P8 primers with fluorescence, followed by genotyping in an automatic sequencer. We sexed 110 individuals of five species: Versicolored Emerald (*Amazilia versicolor*), White-throated Hummingbird (*Leucochloris albicollis*), White-vented Violetear (*Colibri serrirostris*), Black Jacobin (*Florisuga fusca*), and Swallow-tailed Hummingbird (*Eupetomena macroura*). We found gene fragments of two sizes: 374 bp in both sexes (Z chromosome) and either 379 or 383 bp in females (W chromosome). Using this method for monochromatic species permits sexing in species with similar fragment sizes between sexes (i.e., hummingbirds). Sexing monochromatic birds will be very useful for field studies that require continuous monitoring of individuals in the wild.

Resumo · Determinação sexual molecular em beija-flores monocromáticos neotropicais
Muitas espécies de beija-flores são sexualmente monocromáticas, o que pode ser surpreendente, já que todas as espécies desta família apresentam cuidado parental realizado pelas fêmeas. Os machos são promíscuos e esse sistema de acasalamento geralmente está associado ao dimorfismo sexual. Para estudar a seleção sexual em espécies monocromáticas, devemos primeiramente identificar os sexos. Aqui, utilizando sangue e penas de indivíduos vivos, aplicamos um método simples para sexagem. Amplificamos os fragmentos do gene CHD-1 através de PCR, utilizando iniciadores P2 e P8 com fluorescência, seguido de genotipagem em sequenciador automático. Foram sexados 110 indivíduos de cinco espécies: *Amazilia versicolor*, *Leucochloris albicollis*, *Colibri serrirostris*, *Florisuga fusca* e *Eupetomena macroura*. Os fragmentos foram identificados em: 374 pb para ambos os sexos (cromossomo Z) e 379 ou 383 pb para fêmeas (cromossomo W). Esta aplicação do método em espécies monocromáticas beneficiará a sexagem em espécies onde há similaridade entre os tamanhos dos fragmentos, como nos beija-flores. Além disso, se tornará uma útil ferramenta para estudos de campo que exijam o monitoramento contínuo de indivíduos na natureza.

Key words: CHD-1 gene · DNA · Molecular sexing · Sex identification · Trochilidae

INTRODUCTION

Hummingbirds (family Trochilidae) are well-known for their typically brightly colored males and their usually plain females that assume all aspects of parental care. Yet several species are sexually monomorphic and monochromatic (Ruschi 1982, Sick 1997, Schuchmann 1999, Fogden et al. 2014, Winkler et al. 2015), which is an enigma because female-only parental care typically goes hand-in-hand with sexual selection of males that result in bright colors or energetic displays (Hurly et al. 2001, Rico-Guevara & Araya-Salas 2015). To understand the lack of sexual dimorphism among hummingbirds we must first identify sex and then test for possible cryptic dimorphism (Miyaki et al. 1998, Bertault et al. 1999, Russello & Amato 2001).

We wanted to identify sex in an assemblage of species during a study of population dynamics and so needed to minimize stress and handling. Thus, we used molecular analysis of simple tissue samples in which sex identification should be possible through variation in intron lengths of the chromodomain-helicase-DNA-binding (CHD-1) genes on the CHD-Z and CHD-W (on Z and W chromosomes, respectively) that can be compared through electrophoresis (300–400 and 400–700 bp, respectively; Griffiths et al. 1998, Fridolfsson & Ellegren 1999). This inexpensive method can be performed in most genetics laboratories. Differences in size of these genes by sex are often variable (Griffiths et al. 1998, Wang et al. 2007), and so the generality of the method is unclear. In hummingbirds, sex was identified by comparison of the CHD-1 genes using electrophoresis in the Sparkling Violetear (*Colibri coruscans*; Jensen et al. 2003), using P2/ P8 primers (Griffiths et al. 1998) and 1272H/ 1237L primers

Receipt 15 January 2018 · First decision 29 May 2018 · Acceptance 6 September 2018 · Online publication 12 September 2018
Communicated by Kaspar Delhey

(Kahn et al. 1998) with 20 bp of difference between genes on the Z and W chromosomes, but this method was unsuccessful in other species (Presti pers. observ.). When sex identification was not possible, it was due to the very small difference in length of Z-linked and W-linked CHD-1 gene fragments (Kahn et al. 1998). Recently, the sexing technique by Griffiths et al. (1998) was tested in dichromatic North American hummingbirds (Hagadorn et al. 2016). Here, our goal was to demonstrate that this method (employing only blood and feathers as sources of DNA) using a single pair of primers (P2 and P8) can be applied to monochromatic species. We applied this technique to identify sex in five species of hummingbirds in southern Brazil, all of which cannot be sexed in the hand: Versicolored Emerald (*Amazilia versicolor*), White-throated Hummingbird (*Leucochloris albicollis*), White-vented Violetear (*Colibri serrirostris*), Black Jacobin (*Florisuga fusca*), and Swallow-tailed Hummingbird (*Eupetomena macroura*; Figure 1).

METHODS

Study site and samples. Hummingbirds were captured in the Atlantic forest in the municipality of Piraquara, in the state of Paraná in southern Brazil (25.5214°S, 49.0925°W) from August 2014 to March 2015. One contour feather from the pectoral region was taken from each bird to obtain the tissue at the feather base. Feather samples were placed in absolute alcohol and stored at room temperature. In 17 *L. albicollis*, we also sampled a small quantity of blood (< 10 µl) by cutting a nail to the quick and collecting a drop or two of blood on filter paper. All individuals were released immediately after collecting the material.

Polymerase chain reactions of the CHD-1. DNA was isolated using two kits from Qiagen, the DNeasy Blood & Tissue Kit (for blood) and the QIAamp DNA Micro Kit (for feathers), both following the manufacturer's recommended protocol (Bruford et al. 1992). To sex birds, we amplified fragments of the CHD-1 gene through polymerase chain reactions (PCR), using the primers P2 and P8 (Griffiths et al. 1998). P2 primers were labeled with the fluorescent dye FAM. PCR amplification was carried out with Mytaq (Bioline) in a 10 µl reaction mixture following the manufacturer protocol, in a T100 Bio-rad thermal cycler at 95ºC for 5 min, 28 cycles at 94ºC for 30 s, 48ºC for 45 s and 72ºC for 45 s, which was then continued for another 5 min. PCR products were separated by capillary electrophoresis using the automatic sequencer ABI 3130xl Genetic Analyzer. Fragments were scored against GeneScan 500 LIZ size Standard, using GeneMapper (version 4.1, Applied Biosystems). Previous study had shown that amplified fragments of the CHD genes are of different sizes. Males are homogametic (ZZ) and have fragments of only one size, while females are heterogametic (ZW) and have two fragment sizes (Griffiths et al. 1998). Thus, all birds with bands having one gene fragment size were considered male while those with two fragment sizes were assumed to be female.

RESULTS

All individuals had a 374 bp gene fragment on the Z chromosome and females had an additional fragment on the W chromosome, all easily seen using capillary electrophoresis and with DNA extracted from feathers or blood. The additional fragment in females was 379 bp in *A. versicolor*, *L. albicollis*, and *E. macroura*, and 383 bp in *C. serrirostris* and *F. fusca*. With this information, we identified the sex of 110 individuals of the five species, with 33 females and 77 males: *A. versicolor* (4 females, 13 males), *L. albicollis* (5, 30), *C. serrirostris* (2, 20), *F. fusca* (12, 4), and *E. macroura* (10, 10).

DISCUSSION

We found that sex can be identified clearly using small quantities of blood or feathers that can be collected quickly and easily in the field, and this is the first study using this method in sexually monochromatic hummingbirds. All individuals were successfully and unequivocally sexed because the differences in base-pair lengths are clearly visible by capillary electrophoresis (but not by electrophoresis on agarose gel; Griffiths 2000, Wang et al. 2007, Shibuya 2016). This information is important because it will allow future studies to direct their efforts to quicker, less costly and more efficient methods. Thus, capillary electrophoresis using an automatic sequencer is an efficient combination for sexing these hummingbirds, and perhaps any species with a 1 bp or larger difference between fragment sizes (Lee et al. 2010).

About half of all hummingbird species are sexually monochromatic, most often without size or other morphological differences between the sexes (Schuchmann 1999, Fogden et al. 2014, Shibuya 2016). With this technique, these birds can now be sexed and issues related to sex can now be studied in the field. Also, we demonstrate that a minimal amount of tissue or blood can quickly be taken from the birds, thereby minimizing stress and time in hand, since it could be fatal for small birds like hummingbirds.

Molecular sexing is the most reliable method for identifying sex and is commonly used in birds (Griffiths 2000, Bantock et al. 2008, He et al. 2013). The method described by Griffiths et al. (1998) has proved to be an effective tool (Griffiths 2000, Wang et al. 2007, Lee et al. 2010, Hagadorn et al. 2016), now also in monochromatic hummingbirds. When two fragment sizes are found, it is clear that the individual is a female. Thus, we did not use another independent method of sexing birds. In our case, other methods would have often been fatal or have interfered with the concurrent population dynamics study (i.e., laparoscopy, laparotomy, or tomography; see Prus & Schmutz 1987, Taylor 1994). Moreover, comparisons of morphology between the sexes in all these species demonstrated that there is no sexual dimorphism (Shibuya 2016). Anecdotally, we sexed a color-marked *F. fusca* as female that subsequently was found incubating on a nest, and thus is an independent confirmation of sex. Nonetheless, further tests of this method with known-sex specimens should be carried out, perhaps by extracting DNA from already sexed museum specimens.

Understanding differences in behavior and ecology between the sexes is increasingly important for understanding migration, ecology, and reproduction in fragmented habitats and with climate change (Montalti et al. 2004, Gill & Vonhof 2006, Sekercioglu 2006, Smith 2010). Birds can be marked (several methods are available for marking hummingbirds; see Stiles & Wolf 1973, Kapoor 2012) and then sexed, thereby making it possible to include sex in behavioral and ecological studies, including long-term population studies.

Figure 1. Study species of monochromatic hummingbirds in southern Brazil: a) Versicolored Emerald (*Amazilia versicolor*); b) White-throated Hummingbird (*Leucochloris albicollis*); c) White-vented Violetear (*Colibri serrirostris*); d) Black Jacobin (*Florisuga fusca*); and e) Swallow-tailed Hummingbird (*Eupetomena macroura*). Photographs by Luiz Carlos da Costa Ribenboim.

ACKNOWLEDGMENTS

We thank Aspects Inc. for their donation of HummZinger® hummingbird feeders. We especially thank Jaqueline Evelyn Persegona for her help in the field. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) under grant 99999.010906/2014-02 and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) under grant 306963/2012-4. All birds were captured under permit number 30803-1 of the Ministério do Meio Ambiente of Brazil, and treated following ethics standards of the Universidade Federal do Paraná and Brazilian legislation.

REFERENCES

Bantock, TM, RP Prys-Jones & PLM Lee (2008) New and improved molecular sexing methods for museum bird specimens. *Molecular Ecology Resources* 8: 519–528.

Bertault, G, D Joulia, AR Johnson & M Raymond (1999) Sex determination in Greater Flamingo chicks through DNA analysis. *Waterbirds* 22: 282–284.

Bruford, MW, O Hanotte, JFY Brookfield & T Burke (1992) Single-locus and multilocus DNA fingerprinting. Pp 225–269 *in* Hoelzel, AR (ed). *Molecular genetic analysis of population: a practical approach*. IRL Press, Oxford, UK.

Fogden, M, M Taylor & SL Williamson (2014) *Hummingbirds: a life-size guide to every species*. Ivy Press, Brighton, UK.

Fridolfsson, AK & H Ellegren (1999) A simple and universal method for

molecular sexing of non-ratite birds. *Journal of Avian Biology* 30: 116–121.

Gill, SA & MJ Vonhof (2006) Sexing monochromatic birds in the field: cryptic sexual size dimorphism in Buff-breasted Wrens (*Thryothorus leucotis*). *Ornitología Neotropical* 17: 409–418.

Griffiths, R (2000) Sex identification in birds. *Journal of Exotic Pet Medicine* 9: 14–26.

Griffiths, R, M Double, KCY Orr & RJG Dawson (1998) A DNA test to sex most birds. *Molecular Ecology* 7: 1071–1075.

Hagadorn, KA, LA Tell, TL Drazenovich & HB Ernest (2016) Molecular sex identification markers for five North America hummingbird species. *Conservation Genetics Resources* 8: 427–430.

He, XL, BP Qing, JL Han & CQ Ding (2013) Improved molecular assay for sex identification of the endangered Crested Ibis (*Nipponia nippon*) based on the CHD1 gene and a sex-linked microsatellite locus. *Zoological Science* 30: 742–747.

Hurly, TA, RD Scott & SD Healy (2001) The function of displays of male Rufous Hummingbirds. *The Condor* 103: 647–651.

Jensen, T, FM Pernasetti & B Durrant (2003) Conditions for rapid sex determination in 47 avian species by PCR of genomic DNA from blood, shell-membrane blood vessels, and feathers. *Zoo Biology* 22: 561–571.

Kahn, NW, JS John & TW Quinn (1998) Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex identification in birds. *The Auk* 115: 1074–1078.

Kapoor, JA (2012) Improved methods for color-marking hummingbirds. *Journal of Field Ornithology* 83: 186–191.

Lee, JCI, LC Tsai, PY Hwa, CL Chan, A Huang, SC Chin, LC Wang, JT Lin, A Linacre & HM Hsieh (2010) A novel strategy for avian species and gender identification using the CDH gene. *Molecular and Cellular Probes* 24: 27–31.

Miyaki, CY, R Griffiths, K Orr, LA Nahum, SL Pereira & A Wajntal (1998) Sex identification of parrots, toucans, and curassows by PCR: perspectives for wild and captive population studies. *Zoo Biology* 17: 415–423.

Montalti, D, G Kopji & R Maragliano (2004) Morphometrics and sexual dimorphism of some neotropical passerines. *Ornitología Neotropical* 15: 271–278.

Prus, SE & SM Schmutz (1987) Comparative efficiency and accuracy of surgical and cytogenetic sexing in psittacines. *Avian Diseases* 31: 420–424.

Rico-Guevara, A & M Araya-Salas (2015) Bills as daggers? A test for sexually dimorphic weapons in a lekking hummingbirds. *Behavioral Ecology* 26: 21–29.

Ruschi, A (1982) *Aves do Brasil: beija-flores*. Expressão e Cultura, Rio de Janeiro, Brazil.

Russello, MA & G Amato (2001) Application of a noninvasive, PCR-based test for sex identification in an endangered parrot, *Amazona guildingii. Zoo Biology* 20: 41–45.

Schuchmann, KL (1999) Family Trochilidae (hummingbirds). Pp 468–680 *in* del Hoyo, J, A Elliott & J Sargatal (eds). *Handbook of the birds of the world. Volume 5: Barn-owls to hummingbirds*. Lynx Edicions, Barcelona, Spain.

Sekercioglu, CH (2006) Increasing awareness of avian ecological function. *Trends in Ecology & Evolution* 21: 464–471.

Shibuya, FLS (2016) *Características das espécies que podem influenciar as dinâmicas populacionais de beija-flores na Floresta Atlântica no sul do Brasil.* Ph.D. diss., Univ. Federal do Paraná, Curitiba, Paraná, Brazil.

Sick, H (1997) *Ornitologia brasileira*. Ed. Nova Fronteira, Rio de Janeiro, Brazil.

Smith, CA (2010) Sex determination in birds: a review. *Emu* 110: 364–377.

Stiles, FG & LL Wolf (1973) Techniques for color-marking hummingbirds. *The Condor* 75: 244–245.

Taylor, M (1994) Endoscopic examination and biopsy techniques. Pp 327–354 *in* Ritchie, BW, GJ Harrison & LR Harrison (eds). *Avian medicine: principles and applications*. Wingers Publishing Inc., Lake Worth, Florida, USA.

Wang, LC, CT Chen, HY Lee, SH Li, JT Lir, SC Chin, CE Pu & CH Wang (2007) Sexing a wider range of avian species based on two CHD1 introns with a unified reaction condition. *Zoo Biology* 26: 425–431.

Winkler, DW, SM Billerman & IJ Lovette (2015) *Bird families of the world*. Lynx Edicions, Barcelona, Spain.