

# Molecular sexing: an efficient method to identify individual sex and its implication to differentiate Semipalmated Sandpiper *Calidris pusilla* populations

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**RESUMO:** Sexagem molecular: Um eficiente método para identificar o sexo de indivíduos e suas implicações para diferenciar populações do maçariquinho *Calidris pusilla*. Estudos populacionais que utilizam a definição do sexo das aves com base em características morfológicas e de plumagem têm sido utilizados amplamente em espécies dimórficas. Entretanto, em espécies monomórficas como o caso de *Calidris pusilla* pode haver sobreposições de medidas entre sexos e entre populações migratórias obscurecendo os resultados, especialmente em áreas de parada e invernada onde provavelmente as populações se misturam. Quatro sítios na costa amazônica brasileira foram visitados regularmente durante o período migratório e de invernada entre 1997 e 2000 e as aves foram capturadas em redes de neblina com malha de 36 mm. Para a definição do sexo via análise molecular os primers P2 and P8 foram utilizados para a amplificação dos fragmentos dos introns dos genes CHD-Z e CHD-W. O produto dessa amplificação é maior no caso do CHD-Z em comparação com CHD-W, e as fêmeas apresentam dois fragmentos de diferentes pesos moleculares correspondendo à presença dos dois genes. Machos apresentam fragmentos de pesos iguais, derivados dos dois cromossomos Z. A eficiência do método de definição do sexo via análise molecular foi testada em uma amostra de 14 indivíduos capturados durante o estudo. O sexo desses indivíduos (8 machos e 6 fêmeas) de *C. pusilla* foi confirmado através da dissecação e inspeção gonadal. Análises moleculares identificaram corretamente o sexo de todos os 14 indivíduos de sexo conhecido, comprovando a acurácia desse procedimento usando os Primes P2 e P8. Dos 151 indivíduos analisados usando PCR, 92 foram classificados como machos e 59 como fêmeas. Os resultados das análises moleculares com os primers P2 e P8 indicaram que em média, o comprimento de bico das fêmeas é maior que dos machos. As populações invernantes de *C. pusilla* nos sítios estudados na Amazônia brasileira podem representar uma única população reprodutiva devido à falta de diferenças no comprimento do bico entre indivíduos do mesmo sexo.

**PALAVRAS-CHAVE:** Sexagem molecular, maçariquinho, *Calidris pusilla*.

**ABSTRACT:** In population studies of dimorphic bird species, differences in plumage and morphological characteristics facilitate gender identification. By contrast, in monomorphic species such as Semipalmated Sandpiper, the considerable overlap in measurement between sexes and among different migratory populations impedes reliable gender identification, especially in stopover and wintering areas, where populations are likely to mingle. Four sites on the Brazilian Amazonian coast were visited regularly during the migratory and wintering periods between 1997 and 2000, and birds were captured in mist nets with a 36 mm mesh. Gender identification was based on the molecular primers P2 and P8, which were used to amplify introns of the genes CHD-Z and CHD-W. The product of this amplification is larger in CHD-Z in comparison with CHD-W, and females present fragments of different molecular weights, corresponding to the presence of both genes. Males present fragments of equal weight, from each of the two Z chromosomes. The efficiency of the molecular sexing method was tested in a sample of fourteen of the individuals captured during the study. The sex of these individuals (eight males and six females) was confirmed through dissection and gonadal inspection. Molecular analyses identified correctly the sex of all fourteen individuals of known sex, proving the accuracy of this procedure using P2 and P8 primers in Semipalmated Sandpiper. Of the 151 individuals analyzed using PCR, 92 were identified as male, and 59 as female. The results of the molecular analyses with P2 and P8 primers indicate that the bill is significantly longer in females, on average, in comparison with males. The wintering populations of Semipalmated Sandpiper at the study sites on the northern coast of Brazil may represent a single reproductive population, given the lack of differences in mean bill length among sites for individuals of the same sex.

**KEY-WORDS:** Molecular sexing, Semipalmated Sandpiper, *Calidris pusilla*.

The Semipalmated Sandpiper (*Calidris pusilla*) is a small, monogamous and monomorphic in plumage but slightly sexually dimorphic (Cartar 1984, Gratto-Trevor 1992) migratory shorebird. They are abundant on the northern coast of Brazil (Rodrigues 2000), but breed in three distinct populations distributed over a wide area in the low arctic region. These breeding populations present an east-west cline in the length of both the bill and the wing, in both sexes (Manning *et al.* 1956, A.O.U. 1957, Palmer 1967, Harrington and Morrison 1979). However, Prater *et al.* (1977) concluded that only bill length was an adequate criterion for morphometric sexing. Reinforcing the validity of this criterion in Semipalmated Sandpiper, Cartar (1984) found similar differences in the bill lengths observed by Harrington and Morrison (1979) in individuals of the three breeding grounds:  $18.6 \pm 1.36$  mm (range 15.5-23.0 mm,  $n = 147$ ) for males; and  $20.5 \pm 1.38$  mm (range 17.5-23.7 mm,  $n = 107$ ) for females.

Oligonucleotides have been developed for the polymerase chain reaction (PCR) of segments of the CHD-Z and CHD-W genes (chromodomain-helicase-DNA-binding protein) of a large number of bird species (Ellegren 1996, Griffiths *et al.* 1996, Ellegren and Sheldon 1997, Griffiths *et al.* 1998). This has resulted in molecular sexing becoming the standard method for sexually monomorphic birds. An additional advantage of this method is that it avoiding the necessity of sacrificing animals. Moreover, the relatively low cost and simplicity of the method in comparison with traditional procedures, such as endoscopy (Goslowski 1993) and ultra-sonography (Hildebrandt *et al.* 1995), have facilitated its use in testing a number of theories on avian ecology and evolution.

Sexing birds in the field on the basis of morphometry is a practical means of collecting large numbers of records, but it has a major drawback in the overlap of measurements between sexes and/or among populations (e.g. Morrison 1984, Wenink *et al.* 1996), which may obscure possible populations mixing, especially at resting and wintering grounds. Using molecular sexing, the present study evaluated the possible presence of representatives of different breeding populations at wintering grounds on the Brazilian Amazonian coast.

## MATERIAL AND METHODS

Four sites on the Brazilian Amazonian coast were visited regularly during the wintering and migratory periods of the Semipalmated Sandpiper between 1997 and 2000: Goiabá beach ( $02^{\circ}50'N$ ;  $50^{\circ}50'W$ ) – Amapá State; Maçarico/Cuiarana beaches ( $00^{\circ}30'S$ ;  $47^{\circ}20'W$ ) – Pará State and Panaquatira beach ( $02^{\circ}20'S$ ;  $44^{\circ}00'W$ ) – Maranhão State. Approximately 2000 individuals were captured in 36 mm mist nets. Bill was measured to the nearest 0.1 mm from the tip of the upper mandible to

the feather-margin. Mean lengths of the bill were calculated for each site, considering adults only. Differences between means were tested through Student's *t*-test and ANOVA, using BioStat 2.0 (Ayres *et al.* 2000). Following banding, one or two drops of blood were collected from the brachial vein using a heparinized syringe. Total DNA was isolated by enzymatic digestion using K proteinase, extracted with phenol-chloroform and precipitated with ethanol, following standard procedures (Sambrook *et al.* 1989).

For molecular sexing, P2 and P8 primers (Griffiths *et al.* 1998) were used for the amplification of fragments of the introns of the CHD-Z and CHD-W genes. The product of this amplification is larger in the case of CHD-Z in comparison with CHD-W, such that females present two fragments of different molecular weights, corresponding to the presence of the two genes. Males present fragments of equal weight, derived from their two Z chromosomes.

The gene fragments were amplified in a final reaction volume of 25  $\mu$ l, containing 50 ng of genomic DNA, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, 50  $\mu$ M of each dNTP, 0.5  $\mu$ M of each primer, and one unit of Taq DNA polymerase. Cycling was done with: 4 min at 94°C for initial denaturation, followed by 25 denaturation cycles of 45 s at 94°C, annealing for 45 s at 48°C, extension for 45 s at 72°C, and a final run of 5 min at 72°C in order to ensure complete extension of the PCR products. The amplified fragments were separated in 3% agarose gel in 1x TAE buffer and visualized using ethidium bromide under UV illumination.

The efficiency of the molecular sexing method was tested in a sample of fourteen of the individuals captured during the study. The sex of these individuals (eight males and six females) was confirmed through dissection and gonadal inspection.

## RESULTS

Molecular analyses identified correctly the sex of all fourteen individuals of known sex, proving the accuracy of this procedure using P2 and P8 primers in Semipalmated Sandpiper. Of the total of 151 individuals analyzed using PCR, 92 were classified as males, and 59 as females. The largest mean for males was recorded in Maranhão and for females in Pará, but no statistically significant differences were found between sites (Table 1). However, all between-sex comparisons were significant ( $p < 0.05$ ). In this case, all birds were grouped in males and females groups only. The global comparison considering the different sites between males and females with median values equal to 20.00 and 22.1, respectively, showed highly significant statistical differences (ANOVA,  $p < 0.01$ ).

TABLE 1: Sexual dimorphism based on the bill length (mm) of Semipalmated Sandpiper from the northern coast of Brazil sexed through PCR, as described in the text. All differences between the sexes were statistically significant,  $p < 0.05$  (Student's *t*-test).

Sex	Amapá		Pará		Maranhão			
	mean $\pm$ SD n	range	mean $\pm$ SD n	range	Ilha de Maiaú		Panaquatira	
	mean $\pm$ SD	range	mean $\pm$ SD	range	mean $\pm$ SD	range	mean $\pm$ SD	range
Males	19.86 $\pm$ 0.8 n = 8	18.5 – 20.8	19.96 $\pm$ 1.3 n = 46	17.5 – 23.6	20.05 $\pm$ 0.9 n = 29	18.2 – 21.5	20.42 $\pm$ 1.7 n = 9	18.3 – 23.8
Females	21.14 $\pm$ 1.5 n = 5	19.3 – 23.3	22.72 $\pm$ 1.5 n = 18	19.2 – 24.6	21.97 $\pm$ 1.5 n = 31	18.7 – 26.0	21.06 $\pm$ 2.4 n = 5	18.2 – 23.5
M/F ratio	0.94		0.87		0.92		0.94	

Bill length was more variable in females in comparison with males. Coefficients of variation in females were 6.83% (Maiaú), 6.84% (Pará), 11.83% (Panaquatira) and 7.15% (Amapá) in comparison with 4.86%, 6.83%, 8.68% and 4.22%, respectively, in males.

## DISCUSSION

The results of the molecular analyses with P2 and P8 primers in Semipalmated Sandpiper indicate that the bill is significantly longer in females, on average, in comparison with males, corroborating Prater *et al.* (1977) and Cartar (1984). However, as in a similar study of Red Knots, *Calidris canutus* (Baker *et al.* 1999), our data indicates that Semipalmated Sandpiper cannot be sexed reliably on the basis of bill length, given the overlap between sexes (Table 1). Given this, the use of morphometric criteria for sexing individuals should be avoided, especially in wintering grounds, where different breeding populations may be represented, resulting in a high degree of error.

An important application of molecular sexing in the case of Semipalmated Sandpiper is thus the discrimination and inference of the reproductive origins of wintering populations in the southern hemisphere. In fact, our results indicate that the wintering populations of Semipalmated Sandpiper at the study sites on the Brazilian Amazonian coast may represent a single reproductive population, given the lack of differences in mean bill length among sites for individuals of the same sex. A comparison of the data presented here (Table 1) with those of Harrington and Morrison (1979) suggests that these birds originated in the breeding grounds of the eastern Canadian Arctic, specifically western Baffin Island (mean bill length: males = 19.3 mm; females = 21.03 mm) and eastern Hudson Bay/Belcher Island (males = 19.99 mm; females = 21.54 mm).

Molecular sexing, together with other genetic tools should provide important insights into the migratory patterns of Semipalmated Sandpiper populations, how they evolved, and their ecological implications.

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