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Molecular evolution of β-fibrinogen intron 7 applied to the population genetics of the Semipalmated Sandpiper (*Calidris pusilla*) on the northern Coast of Brazil

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RESUMO. **Evolução molecular do íntron 7 do gene β-fibrinogênio aplicada à genética de populações do maçarico-rasteirinho (*Calidris pusilla*) na costa norte do Brasil.** O maçarico-rasteirinho (*Calidris pusilla*) é um dos mais bem estudados Charadriiformes (Aves), embora pouco se conheça acerca de sua variação genética tanto nas áreas de reprodução quanto de invernada. Neste estudo, nós analisamos os padrões de evolução do íntron 7 do gene nuclear β-fibrinogênio para avaliar a organização da diversidade genética dentro e entre três populações de maçaricos-rasteirinhos da costa norte do Brasil. Adicionalmente, nós acessamos a utilidade deste íntron para análises genéticas de populações de aves limícolas migratórias. Os níveis moderadamente elevados de diversidade genética observados para a combinação de todas as populações (π = 0,0048, h = 0,97) sugerem que elas não sofreram recentes eventos de gargalo, tais como aqueles que parecem haver afetado outras espécies de aves limícolas migratórias durante o Pleistoceno. A estimativa global de diferenciação genética (N_{st} = 0,0182) confirmou a existência de maior diversidade dentro de cada população do que entre populações, indicando a panmixia dos maçaricos-rasteirinhos que invernam na costa norte brasileira. Todos os valores dos testes de neutralidade R_2 foram estatisticamente significantes, apontando para uma rápida expansão demográfica nestas populações. Nossos resultados também indicam que este marcador nuclear pode ser no mínimo tão eficiente quanto muitos marcadores mitocondriais para análise de populações de aves limícolas, ou pelo menos para aquelas que não sofreram drásticas perdas de variação genética em suas histórias evolutivas recentes. No caso específico do maçarico-rasteirinho, esta variabilidade genética, em conjunção com a relativa abundância de suas populações, apontam para sua flexibilidade evolutiva e para uma viabilidade no longo-prazo potencialmente razoável.

Palavras-chave: Genética de populações, maçarico-rasteirinho, *Calidris pusilla*, íntron 7 do gene β-fibrinogênio.

ABSTRACT. The Semipalmated Sandpiper (*Calidris pusilla*) is one of the best studied Charadriiforms (Aves), although little is known of the organization of its genetic variation in either breeding or wintering areas. In this study, we analyzed evolutionary patterns in intron 7 of the β-fibrinogen nuclear gene to evaluate the organization of genetic diversity within and among three wintering Semipalmated Sandpiper populations on the northern coast of Brazil. Additionally, we assessed the utility of β-fibrinogen intron 7 for the genetic analysis of shorebird populations. Genetic diversity levels for all populations were moderately high (π = 0.0048, h = 0.97), suggesting the absence of recent bottleneck events, such as those which may have affected other shorebirds species during the Pleistocene. The estimate of global genetic differentiation (N_{st} = 0.0182) confirmed the existence of more diversity within each population than among populations, indicating panmixia in wintering Semipalmated Sandpiper on the northern coast of Brazil. All values of the R_2 neutrality test were statistically significant, which points to a rapid demographic expansion of these populations. The results also indicate that this nuclear marker may be at least as efficient as most mitochondrial markers for the analysis of shorebird populations, at least those that have not suffered drastic losses of genetic variation in their recent history. In the specific case of Semipalmated Sandpiper, this genetic variability, together with its relative abundant populations, point to its evolutionary flexibility, and potentially good long-term viability.

Key words: Population genetics, Semipalmated Sandpiper, *Calidris pusilla*, β-fibrinogen intron 7.

Given the significant demographic decline suffered by most migratory shorebirds in the past few decades (Clark *et al*. 1993, Baker *et al*. 2004), there is an urgent need for a more detailed understanding of their population dynamics and resource requirements – critical migratory sites in particular – to bolster conservation efforts. Effective conservation strategies will also depend on knowledge of population structure and annual migration routes (Haig and Avise 1995, Gauthreaux 1996). Molecular biology has provided valuable tools not only for the differentiation of populations (see Wenink *et al*. 1993, 1994, 1996, Haig *et al*. 1997, Wennerberg 2001), but also for the evaluation of levels of genetic variability (see Baker 1992, Baker *et al*. 1994) and of the evolutionary potential of a given species.

Whereas mitochondrial DNA sequences have been the principal source of genetic variation in population studies, there have been very few studies based on nuclear sequences. This is due essentially to the much lower rates of evolution of nuclear genes, which is reflected in their reduced polymorphism in comparison with mitochondrial genes. However, the

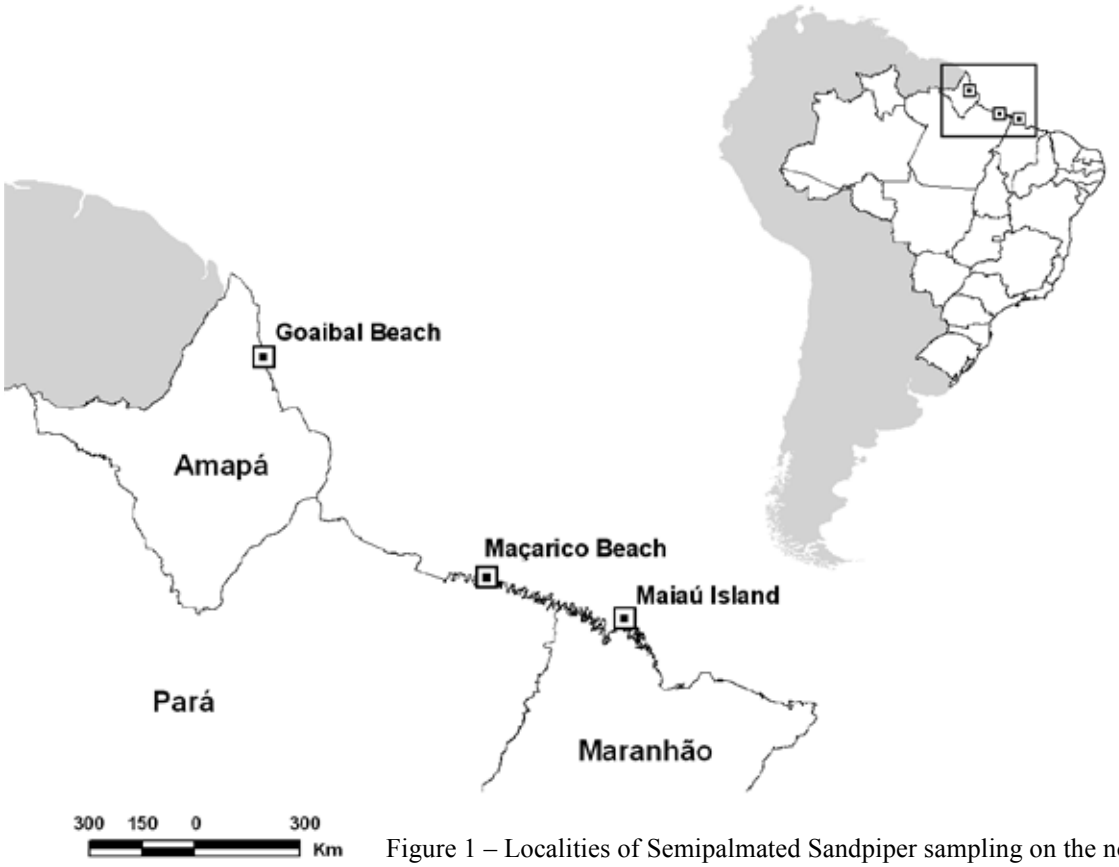

Figure 1 – Localities of Semipalmated Sandpiper sampling on the northern coast of Brazil.

exclusive analysis of mtDNA may provide a relatively simplified and biased view of the phylogenetic history of a species group (Hudson and Coyne 2002, Bensch *et al*. 2006), related primarily to the detection of *numts*, segments of mitochondrial DNA transferred to the nuclear genome (Lopez *et al*. 1994), which mask phylogenetic information. Additionally, in contrast with mitochondrial DNA, which is inherited only through the female lineage, the analysis of nuclear DNA permits evaluation of gene flow mediated by both sexes.

A number of studies have used molecular markers for the analysis of shorebird populations (e.g. Wenink *et al*. 1993, 1994, 1996, Wenink and Baker 1996, Baker *et al*. 1994, Haig *et al*. 1997, Wennerberg 2001), but little or no information is currently available for the majority of species. A case in point is the Semipalmated Sandpiper (*Calidris pusilla*), a phylopatric monogamous species which breeds in June and July in the Nearctic region between Alaska and eastern Canada, apparently in three distinct populations – Alaskan, central Canadian Arctic, and eastern Canadian Arctic – based on differences in wing and bill length (Manning *et al*. 1956, A.O.U. 1957, Palmer 1967, Harrington and Morrison 1979). During the other ten months of the year, the species migrates to the northern coast of South America, between Venezuela and Brazil (McNeil 1970, Spaans 1978, Rodrigues 2000), in particular Suriname, and the Brazilian states of Pará and Maranhão (Morrison and Ross 1989, Rodrigues 2000).

As Semipalmated Sandpiper is one of the most common shorebird species, a good deal of information is available on its annual migrations (see Harrington and Morrison 1979, Lank 1983, Morrison 1984, Hiclin 1987, Gratto-Trevor and Dickson 1994, Rodrigues 2000, 2001). Haig *et al*. (1997) found considerable differentiation among five breeding populations, using random amplified polymorphic DNA (RAPD), although they also found ample overlap in the genetic identity of individual birds from different breeding sites. In this context, the degree of differentiation of the three probable breeding populations remains unclear, as does their distribution in the South American wintering areas. Given this, the aims of the present study were to: (i) evaluate the molecular evolution of the nuclear β-fibrinogen intron 7 and its usefulness for the genetic analysis of shorebird populations, and (ii) investigate the pattern of the variation of this intron within and among populations of Semipalmated Sandpiper on the northern coast of Brazil.

METHODS

Sampling. Samples were collected at three sites on the northern coast of Brazil (Figure 1), which were selected according to logistic limitations and the local abundance of the Semipalmated Sandpiper. Sampling was carried out during both spring and autumn migrations, and the wintering season, between 1997 and 2000. Specimen collection was authorized by the Brazilian Environment Institute (license number 088/2002-DIFAS/IBAMA). Two or three drops of blood were taken from the brachial vein of each individual using a heparinized syringe. For this study, genomic DNA of 57 samples was isolated by enzymatic digestion, using K proteinase, extracted with phenol-chloroform and precipitated with ethanol, following standard procedures (Sambrook *et al*. 1989).

Laboratory procedures. Amplification via polymerase chain reaction of β-fibrinogen intron 7 was carried out in 50 µl final volume, containing 5-10 ng of genomic DNA, 50 mM KCL, 2 mM $MgCl_2$, 10 mM Tris-HCL, 50 µM of each DNTP, 0.5 µM of each oligonucleotide (FIB-17L/FIB-17U, Prychitko and Moore 1997), and one unit of Taq DNA polimerase (Invitrogen). The following amplification profile was used: 4 min at 94ºC for initial denaturation; 30 cycles of 1 min at 94ºC, 1 minute at 53ºC, 1 minute at 72ºC; 10 min at 72ºC to insure complete extension of the PCR products.

Amplification products were purified with the Qiaex II gel extraction kit (Qiagen) and cloned in *Escherichia coli* DH5α (Gibco) using the pGEM-T vector System I (Promega).The plasmid DNA of each clone was obtained using the QIAprep Spin plasmid Miniprep Kit (Qiagen) and sequenced automatically in an ALFexpress II (Amersham Biosciences), using the Cy5 thermo sequenase dye terminator kit (Amersham Biosciences), according to the maker's specifications. DNA sequences from both strands were aligned using software Bio-Edit (Hall 1999).

*Analyses***.** The program DnaSP 4.10.3 (Rozas *et al*. 2003) was used to calculate levels of polymorphism and nucleotide divergence both within and among populations, and to test the probability of neutral evolution of the sequences. Levels of genetic variability were quantified by the diversity of nucleotides (π) and haplotypes (h). Genetic differentiation was evaluated through estimates of N_{st} (Lynch and Crease 1990), with the Jukes-Cantor (1969) correction. This measure, specific for nucleotide sequences, is an *Fst* (Wright 1978) analog,

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                 1111111112223333333333444444555555566667777788888899  Absolute
              333488144445557066667899999992256333455156734789906667907  Frequency
              189067067890167727499934567894832702320872718570198235414 8  Ap Ma Pa

Hap1     A---AGAATACACCCCCCTGTAATCTAAAAAAGTTCTATGTTATTTTAGCTTCGTTGT  1  -  -
Hap2     .---..--CA.....................A.....................  1  -  -
Hap3     .---.....................TA.............A.....A....  1  -  -
Hap4     .---.....................A.............C.....C..  1  -  -
Hap5     .---.........T.....-----TG...A.....................A....  1  -  -
Hap6     .---.....................A.............A.....A....  1  -  -
Hap7     G---.T.......AT.............T..A....C..........-...TA....  1  -  -
Hap8     .---.....................TA.....................A....  1  4  1
Hap9     .---.....................A.....................A....  1  -  -
Hap10    G---.T...G..A.....................A.....................A....  1  -  -
Hap11    .---.........T.....-----TG...A.C.....................  1  -  -
Hap12    .---.....................TA.....................AA...  1  -  -
Hap13    .---.....................A.....................  1  3  4
Hap14    .---.....................A.....................C.....  -  1  -
Hap15    .---.........T.....-----TG...A.....................G.....A....  -  4  -
Hap16    .---.....................A.....................G.....A....  -  1  -
Hap17    .---..........T.....................G.A.....................  -  1  -
Hap18    .---......TT.....................A..T.....................A....  -  1  -
Hap19    .---.........T...A..-----TG...A.....CC.....................A....  -  1  -
Hap20    .---..........A.....................A.....................  -  1  -
Hap21    .CCA..T.......T...A..-----TG...A......C.....................A....  -  1  -
Hap22    .---.........T...A..-----TG...A......CC.....................A....  -  1  -
Hap23    .---.....................A........C.....................A....  -  1  -
Hap24    G---.....................T..A....C.....GC.......TA....  -  1  -
Hap25    .---.....................A.....................G  -  1  -
Hap26    .---.........T...A..-----TG...A......C.....................  -  1  -
Hap27    .---......TT.......G.....A.........G.....A....  -  1  -
Hap28    .---......TT.....................A.........G.....A....  -  1  -
Hap29    .---.....................A...C.....................  -  1  -
Hap30    .---......TT.......C.....A.....................C..A....  -  -  1
Hap31    .---..........A.....................A.....................A....  -  -  2
Hap32    .---G.........T.....-----TG...A.....................A....  -  -  1
Hap33    .---......TT.....................A.....................A..A....  -  -  1
Hap34    .---.........T...A..-----TG...A......C.....................A....  -  -  1
Hap35    .---......TT.....................A.....................A....  -  -  1
Hap36    .---..........C.....TA.....................A....  -  -  1
Hap37    .---.....................AC.....................  -  -  1
Hap38    .---......T.....................A.....................  -  -  1
Hap39    .---......TT.....................A.....................  -  -  1
Hap40    G---.T.....TT.....................A.....................A....  -  -  1
Hap41    G---.T.......AT.....................TA.....................  -  -  1
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Figure 2 – Alignment of the β-fibrinogen intron 7 haplotypes obtained in this study (GenBank accession numbers EF031925 - EF031965). Only variable positions and indels are shown. Informative sites for parsimony analysis are shaded in grey. Hap = Haplotype, Ap = Amapá, Ma = Maiaú, Pa = Pará.

which represents D_a (the number of net nucleotide substitutions per site between populations, Nei 1987) expressed as the ratio of the total divergence (π average across all within and between populations comparisons) to D_{xy} (the average number of nucleotide substitutions per site between populations, Nei 1987).

Sequence neutrality was tested using the parameters F_s (Fu 1997) and R_2 (Ramos-Onsins and Rozas 2002), which are also powerful tools for the detection of demographic expansion. Ramos-Onsins and Rozas (2002) affirm that R_2 is more effective for samples of small size, and when intragenic recombination is considered, as is the case of the β-fibrinogen gene. Significance was determined based on 1000 coalescent simulations under a model of constant population size using empirical sample sizes, estimates of θ (= 4Neμ) and of the recombination parameter, R (Hudson 1987). These tests were conducted in DnaSP 4.10.3 (Rozas *et al*. 2003).

The PAUP 4.0 b10 program (Swofford 2003) was used to evaluate the molecular evolution of the intron 7 sequences, and to reconstruct the phylogenetic relationships among the different haplotypes recorded. Molecular evolution was assessed using estimates of the frequencies of nucleotide bases, substitution rates, and uncorrected distances between pairs of sequences. Phylogenetic relationships were obtained from the construction of an unrooted maximum parsimony tree using a heuristic search algorithm with 50 random additional sequence replicates and tree bisection and reconnection branch-swapping. Transitions and transversions were given equal weight, whereas insertions and deletions (indels) were treated as missing data. Support for branching topologies was assessed by 1000 bootstrap (Felsenstein 1985) replicates.

RESULTS

Molecular evolution and phylogenetic relationships. Alignment of the 57 sequences resulted in fragments of up to 993 nucleotide sites, of which 11 were indels and 47 were substitutions (19 of which were informative for parsimony analysis), distributed among 41 distinct haplotypes (Figure 2). There was a bias of approximately 2:1 (transitions:transversions) in nucleotide substitutions. Base frequencies – 30.47% for adenines, 31.80% for tymines, 17.45% for guanines and 20.28% for cytosines – varied little among haplotypes ($\chi^2 = 0.873$, FD = 120, $P = 1.000$). Mean sequence divergence (uncorrected P distance) among the β-fibrinogen intron 7 Semipalmated Sandpiper haplotypes recorded here was 0.0056 (± 0.0026), with a range of 0.0010 to 0.0132.

Maximum parsimony analysis produced a single most parsimonious tree (length = 88, CI = 0.56, RI = 0.52), which distributed the 41 haplotypes in just two, not well-defined clades (77% support from bootstrap values; Figure 3). Clade A included the majority (78%) of haplotypes, and were differentiated from those of clade B by the substitutions C → T, A → T, and A → G at sites 207, 398, and 399, respectively. The deletions at sites 393 and 397 (Figure 2) were also synapomorphies for clade B. It is interesting to note that a restriction site for the EcoRV enzyme, located between positions 393 and 394 in the sequences containing this deletion allowed the haplotypes of the two clades to be differentiated, there being 24 individuals type AB, 28 type AA, and five type BB, indicating that these two groups do not represent evolutionarily

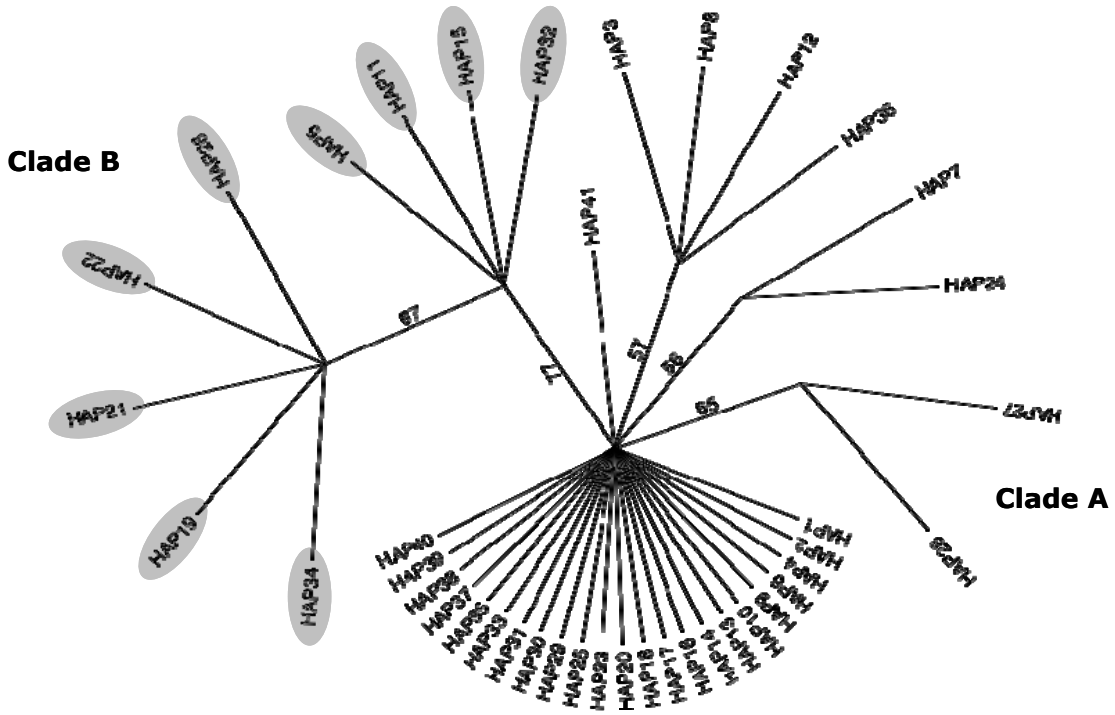

Figure 3 – Unrooted maximum parsimony tree (length = 88, RC = 0.56, RI = 0.52) for the 41 haplotypes of the β-fibrinogen intron 7 of Semipalmated Sandpiper from three wintering populations on the northern coast of Brazil. Clade A include all haplotypes unmarked, haplotypes in grey belongs to clade B.

Table 1 – Genetic diversity (± SD) and neutrality tests for β-fibrinogen intron 7 of three wintering Semipalmated Sandpiper populations on the northern coast of Brazil.

Population	Diversity		Neutrality test	
	π	h	$F_s(P)$	$R_2(P)$
Amapá (n = 13)	0.0048 ± 0.0008	1.00 ± 0.03	- 9.770 (0.016)	0.0835 (0.005)
Maiaú (n = 26)	0.0050 ± 0.0005	0.95 ± 0.03	-8.478 (0.310)	0.0733 (0.013)
Pará (n = 18)	0.0043 ± 0.0006	0.95 ± 0.04	-7.279 (0.256)	0.0793 (0.006)
All (n = 57)	0.0048 ± 0.0004	0.97 ± 0.01	- 41.178 (0.000)	0.0411 (0.000)

P is the probability of an F_s and R_2 value being less than the observed based on 1000 coalescent simulations.

Table 2 – Genetic differentiation estimates between pairs of Semipalmated Sandpiper populations wintering on the northern coast of Brazil based on β-fibrinogen intron 7. Genetic distances – mean number of nucleotide substitutions per site between populations (D_{xy}) and number of net nucleotide substitutions per site between populations (D_a) – were computed with the Jukes-Cantor (1969) correction. All values are percentages.

	N_{st}	D_{xy} (SD)	D_a (SD)
Amapá *vs.* Maiaú	1.702	0.500 (± 0.073)	0.009 (± 0.007)
Amapá *vs.* Pará	1.030	0.460 (± 0.074)	0.005 (± 0.005)
Pará *vs.* Maiaú	2.708	0.476 (± 0.059)	0.013 (± 0.006)

distinct lineages.

Diversity, divergence and demographic changes. The mean pairwise distance (nucleotide diversity, π) between individuals within populations was relatively low (0.48%), whereas the probability that any given pair of birds had different haplotypes (haplotype diversity, *h*) was high, at 96.7% (Table 1). In fact, *h* was 100% for the Amapá population, falling slightly, to 95%, at Maiaú and Pará. Values of π also varied little, between 0.43% (Pará) and 0.50% (Maiaú).

Haplotype 13 was the most common, accounting for 19.5% of individuals, followed by haplotypes 8 (14.6%), 15 (9.8%), and 31 (4.9%). All other haplotypes were recorded only once, and together accounted for just over half (51.2%) of the 57 sequences (Figure 2). As expected for non-isolated populations, the most common haplotypes were recorded at all three collecting sites. This is also consistent with the estimate of global genetic differentiation (N_{st} = 1.82%), which confirms the existence of more diversity within each populations than among populations. Relatively low values of N_{st} were also recorded in the pairwise comparisons between populations, where the largest value was recorded for the comparison between Pará and Maiaú (Table 2).

Significant negative values of F_s were recorded only for Amapá (table 1) and the combined analysis. By contrast, all values of R_2 were statistically significant which points to a rapid demographic expansion of the Semipalmated Sandpiper populations studied here. As in Ramos-Onsis and Rozas (2002), the failure of F_s to detect the demographic expansion in the Maiaú and Pará populations is likely due to a combination of small sample size and intermediate levels of recombination.

DISCUSSION

Analytic power of the nuclear β-fibrinogen intron 7. A comparison with the variation observed in the mitochondrial DNA of other shorebird species (Table 3) demonstrates clearly the potential of the nuclear β-fibrinogen intron 7 for phylogeographic analyses. The proportion of variable sites observed in the present study was only smaller than that found by Wenink *et al*. (1993, 1996) in the D-loop of the Dunlin (*Calidris alpina*). This difference might be expected, however, given the

Table 3 – Comparison of the variation in the DNA sequences of different shorebird species.

Species	N	Number of variable sites	Sequence size (pbs)	% of variable sites	Molecular marker	Study
Arenaria interpres	75	23	1192	1.93	D-loop	Wenink et al. (1994)
Calidris alpina	73	8	302	2.65	Cytochrome b	Wenink et al. (1993)
Calidris alpina	73	42	608	6.90	D-loop	Wenink et al. (1993)
Calidris alpina	3	32	1168	2.74	D-loop	Wenink et al. (1994)
Calidris alpina	155	43	608	7.07	D-loop	Wenink et al. (1996)
Calidris alpina	52	31	608	5.09	D-loop	Wenink & Baker (1996)
Calidris canutus	25	7	555	1.26	D-loop	Baker et al. (1994)
Calidris ferruginea	70	18	664	2.70	D-loop	Wennerberg (2001)
Calidris fuscicollis	52	18	607	2.80	D-loop	Wennerberg (2001)
Calidris pusilla	57	58	993	5.84	β-fibrinogen intron 7	Present study

fact that Dunlin has five subspecies and a distinct phylogeographic structure, whereas Semipalmated Sandpiper is monotypic (Prater 1977, Haig *et al*. 1997). Sample size may also be a factor here, in fact, the Dunlin study with sample size most similar to that of the present study (Wenink and Baker, 1996) returned a slightly lower proportion of variable sites.

On the other hand, the differences observed in comparison with the mitochondrial markers of other shorebird species may reflect lower levels of genetic variability in these species in comparison with Semipalmated Sandpiper. Overall, the present study supports the use of β-fibrinogen intron 7 as an alternative to mitochondrial DNA for the analysis of shorebird populations, especially those which have not suffered any major loss of genetic variation during their evolutionary history.

Diversity, Genetic Differentiation and Demographic History. Levels of genetic differentiation observed in the present study indicate that the Semipalmated Sandpiper populations on the northern coast of Brazil are panmitic, a conclusion supported by the morphological similarities among them (Rodrigues 2001). A comparison of the data presented by Rodrigues (2001) 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). This analysis also suggests that this eastern Canadian population has recently grown in size. Its relatively high levels of genetic diversity nevertheless indicate that this growth was not the result of a recent bottleneck event, as suggested by Baker *et al*. (1994) for the Red Knot (*Calidris canutus*).

The relatively high genetic variability of the Dunlin, also suggests a lack of bottleneck events during the recent evolutionary history of this species (Wennerberg 2001). As the Semipalmated Sandpiper, like the Dunlin, reproduces in areas south of the Arctic tundra, it probably suffered less from climate changes during the Pleistocene that species that breed further north. This hypothesis is reinforced by the relatively reduced genetic variability of the other shorebirds mentioned here (Table 3), which reproduce in the Arctic tundra.

On the basis of these conclusions, it seems reasonable to expect that the central Canadian and Alaskan breeding populations of Semipalmated Sandpiper are also characterized by at least moderately high levels of genetic diversity. This, together with the relative abundance of this species in the wild (Rodrigues 1993, 2000, 2001) implies that its potential viability over the long term is better than that of other, less abundant and genetically less variable species. Nevertheless, the ongoing decline in shorebird populations worldwide reinforces the need for more detailed studies of intraspecific phylogeography. In the specific case of the Semipalmated Sandpiper, it will be necessary to confirm possible differences in the genetic diversity of different breeding populations, and also whether these populations intermix on the coast of Venezuela and the Guyanas, given that the Brazilian wintering population of Semipalmated Sandpipers appears to correspond to that of a single breeding population. Information of this kind will be fundamental to the development of efficient strategies of conservation and management.

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