Microsatellite Variability Among Black Skimmer (*Rynchops niger intercedens*) Populations in Southern South America

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Abstract.—*Rynchops niger intercedens*, one of the South American subspecies of the Black Skimmer, is a widely distributed neotropical bird for which many ecological aspects, such as migratory routes and site fidelity, are unknown. Two distinct breeding phenologies have been reported for this subspecies, which may create the genetic isolation of populations. Six microsatellite loci were used to study the genetic structure of the Black Skimmer in southern South America, comparing breeding populations from Brazil and Argentina and individuals from the main non-breeding site at Argentina. A weak genetic differentiation between colonies, although statistically significant, was observed. This low genetic structure (despite different breeding phenologies) could be explained by demographic history of these populations and/or the effect of a very important non-breeding site in southern South America shared by these populations. Further studies applying a broader range of molecular markers plus improvement of extant banding efforts are required to better understand the dispersal mechanisms of this species. *Received 9 August 2013, accepted 10 October 2013.*

Key words.—Black Skimmers, genetic structure, microsatellites, *Rynchops niger intercedens,* South America.

Rynchops niger intercedens, one of the South American subspecies of Black Skimmer, is a neotropical bird widely distributed from northern Brazil to Argentina. Although they are known to breed next to large rivers and continental water bodies, their main non-breeding aggregations are found at coastal areas (Zusi 1996). Differences in breeding locations are also associated with the foraging behavior of these birds. Black Skimmers will feed in freshwater bodies and rivers during breeding but also in brackish water and at sea during winter (Mariano-Jelicich *et al.* 2003, 2008). Black Skimmers have a wide distribution; however, the extant information for this subspecies is scarce and restricted to localized areas (Zusi 1996). The conservation status of Black Skimmers is not of high concern, and the lack of information on this subspecies limits any precise evaluation (International Union for Conservation of Nature 2012). This subspecies shows two distinct breeding phenologies: colonies in spring in Brazil (September to November; Krannitz 1989; Efe *et al.* 2001), and colonies in summer in Argentina (January to March; Klimaitis and Moschione 1984; F. Raffo, pers. commun.). Some of the reported breeding sites are located within two of the main hydrological basins in South America: the Amazonas basin and La Plata basin. The Amazonas basin is characterized by a low-rainfall season in winter and spring months, while the southern part of La Plata basin is characterized by a dry season in summer and autumn months (Robledo and Penalba 2008; Espinoza Villar *et al.* 2009). Given that Black Skimmers depend on the availability of sandbars to breed (Zusi 1996), it is very likely that differences in breeding phenologies could be driven by rainfall and by the flooding regimes that occur in different seasons in both river basins.

Several wintering aggregations of Black Skimmers have been described along the South American Atlantic coast (Favero *et al.* 2001; Branco and Fracasso 2005; Coelho Naves and Vooren 2006; Alfaro and Clara 2007; Barbieri 2007); however, the most important South American wintering site is by far the one located at Mar Chiquita Coastal lagoon in Argentina, with abundances up to 12,000 individuals between late February and May (Ferrero 2001; Mariano-Jelicich *et al.* 2003). The asymmetries in abundance between breeding and non-breeding areas raised the hypothesis of Black Skimmers

migrating from different breeding areas in South America and converging in large non-breeding flocks.

The occurrence of physical barriers disrupting dispersal among populations has been the traditional explanation to the observed genetic structure and speciation of many animal groups. In birds, particularly seabirds, mechanisms of population differentiation seem to be more complex, where several non-physical barriers and behavioral processes might play an important role in promoting genetic differentiation (reviewed by Friesen *et al.* 2007a). Isolation by time, defined as the disruption of gene flow among populations due to heritable reproductive times, has also been described as a key mechanism preventing gene flow in seabirds (Hendry and Day 2005; Friesen *et al.* 2007b).

In this study, samples from five sites (three breeding sites in Brazil, one breeding site in Argentina and the largest wintering aggregation in Argentina) were analyzed using nuclear markers. We tested the hypothesis that significantly different genetic structure has developed between the Brazilian and Argentinean colonies via the influence of distinct breeding phenologies. Moreover, if genetic structure is found in these Black Skimmer populations, then the data could be used to identify the origin of South American Black Skimmers at non-breeding areas.

METHODS

Samples were obtained at four breeding colonies and one non-breeding aggregation in the Amazonas and La Plata basins (Table 1; Fig. 1). Samples were collected between 2003 and 2005 except for 10 individuals sampled on Entre Rios in 2009. Blood samples at breeding colonies were collected from adults and chicks captured with hand nets. To minimize the possibility of sampling closely related individuals, samples were taken either from one chick per nest or from adults caught on the nest, but not from both age groups at the same nest. Non-breeding adults at wintering sites were captured with mist nets and banded to avoid re-sampling. Blood samples (100-200 µl) were taken from the brachial vein and preserved in absolute ethanol until analysis. DNA was extracted from blood samples using a Wizard SV Genomic purification system. All samples were genotyped at six microsatellites that were previously reported to be polymorphic for Black Skimmers (Faria *et al.* 2007). These included three dinucleotide loci isolated from the Red-billed Gull (*Larus novaehollandiae scopulinus*)

(RBG18, RBG27 and RBG28; Given *et al.* 2002); two dinucleotide loci isolated from the Black-legged Kittiwake (*Rissa tridactyla*) (K6 and K16; Tirard *et al.* 2002); and one trinucleotide loci isolated from the Roseate Tern (*Sterna dougallii*) (Sdaat27; Szczys *et al.* 2005).

Microsatellite amplifications were performed in a Biometra thermocycler using a modified primer with a fluorescent tail (HEX or 6-FAM) attached to the 5' end of the oligonucleotide primer. Polymerase Chain Reaction (PCR) amplifications were performed in a reaction volume of 25 µl containing 1 unit of Taq Polymerase (Pb-L, Argentina), 20-40 ng of DNA, 1.5 mM MgCl2, 0.6 µM each primer, and 0.2 mM dNTPs. The thermocycling profile included an initial denaturing at 94 °C (4 min), followed by 30-34 cycles of denaturing at 94 °C (30 sec), an annealing step at 52 °C for RBG18, RBG27, RBG28 and 56 °C for K6 and K16 (45 sec), extension at 72 °C (45 sec), and a final extension at 72 °C (4 min). Relative concentrations of primers were adjusted by trial and error to create multiplexes. Final PCR fluorescently labeled product were visualized and sized in an automated DNA sequencer ABI3730XL. Fragments were scored with software Peak-Scanner (Applied Biosystems 2006).

Arlequin (Excoffier and Lischer 2010) was used to quantify genetic variation in each sampled area using mean number of alleles (A), allele frequencies, observed (H_O), and expected (H_E) heterozygosity for each locus and over all loci. The software MICROCHECKER (Van Oosterhout *et al.* 2004) was used to evaluate the presence of null alleles within each sampling site, and corrected FST values were obtained using the software FreeNa (Chapuis and Estoup 2007). All microsatellite loci were tested for departures from Hardy-Weinberg (HW) equilibrium and linkage disequilibrium, which test for the independence of the loci, using an extension of Fisher exact probability test as performed in Arlequin (Excoffier and Lischer 2010). Significance levels were adjusted for multiple comparisons using Benjamini-Yekutieli corrections (Benjamini and Yekutieli 2001). Allelic frequencies were calculated using FSTAT (Goudet 2002). Allelic richness and private allelic richness were calculated using HP-RARE (Kalinowski 2005). Means ± SD are reported unless otherwise stated. To determine the degree of contemporary relationships among groups, we used global and pairwise FST (Weir and Cockerham 1984) performing a hierarchical analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) as implemented in ARLEQUIN (Excoffier and Lischer 2010). This analysis provides estimates of the percentage of total variance accounted for within and between populations. Analysis variants were performed where sampled sites were placed in separate Amazonas and La Plata groups and where each sampled site were treated as a separate "population" for the analysis. Significance was obtained after 10,000 permutations to determine the probability of a random FST value being greater than or equal to the observed value (Excoffier and Lischer 2010). The Mantel test (Mantel 1967) with 10,000 permutations was performed to assess the relationship between geographic (Euclidean distances between colonies) and genetic distances (FST) using the software FSTAT (Goudet 2002). The software STRUCTURE (Pritchard *et al.* 2010) was

Table 1. Allele frequencies observed (Ho) and expected (He) heterozygosity for each sampled Black Skimmer group at the five microsatellite loci used for analyses. * indicates a significant departure from Hardy-Weinberg equilibrium at $P < 0.05$.

Locus	Allele	Mar Chiquita	Entre Rios	Pantanal	Guapore	Mamiraua
K16		20	10	10	9	8
	145	0.025	0	0.200	0.056	0.143
	157	0.075	0	0	0	0
	161	0.025	0.100	0.050	0.167	0.071
	163	0.550	0.500	0.600	0.278	0.286
	165	0.100	0	0.150	0.389	0.500
	171	0	0.050	0	0.056	0
	173	0	0.050	0	0	0
	175	0.225	0.200	0	0.056	0
	177	0	0.100	0	0	0
	Ho	0.650	0.800	0.500	0.670	0.850
	He	0.640	0.720	0.600	0.780	0.690
RBG27		19	10	10	9	8
	185	0.420	0.350	0.400	0.330	0.210
	187	0.550	0.650	0.600	0.670	0.780
	189	0.026	0	0	0	0
	Ho	0.470	0.500	0.400	0.440	0.430
	He	0.530	0.480	0.500	0.470	0.360
RBG18		20	10	10	9	8
	172	0.375	0.700	0.500	0.278	0.214
	174	0.075	0.100	0.050	0	0.071
	176	0.125	0	0	0	0.214
	178	0.100	0.050	0	0	0
	180	0.100	0	0.050	0.278	0.071
	182	0.050	0	0.150	0.110	0.143
	184	0.175	0.100	0.200	0.110	0.214
	186	0	0	0.050	0.056	0.071
	188	0	0.050	0	0.167	0
	Ho	0.800	0.400	0.700	0.780	1.000
	He	0.800	0.510	0.710	0.840	0.890
Sdaat27		18	10	10	9	8
	235	0.056	0	0	0	0.286
	236	0.222	0	0.450	0	0.357
	237	0.139	0	0.400	0.056	0.357
	238	0.028	0.100	0	0.056	0
	239	0.250	0.500	0.150	0.778	0
	240	0.306	0.150	0	0.111	0
	241	0	0.250	0	0	0
	Ho	0.720*	0.600	0.400	0.220	0.570
	He	0.790	0.690	0.640	0.390	0.710
RBG28		17	8	10	9	8
	170	0	0	0	0.167	0
	172	0.618	0.562	0.550	0.667	0.500
	174	0.265	0.312	0.150	0.056	0.167
	176	0	0	0.100	0	0.167
	178	0	0	0.100	0	0.083
	180	0	0	0.100	0	0.083
	182	0.029	0	0	0	0
	Ho	0.410	0.620	0.500	0.440	0.670
	He	0.560*	0.600	0.680*	0.540	0.740

Figure 1. Locations of surveyed breeding and non-breeding sites of Black Skimmers shown by black and white dots, respectively.

used to infer genetic structure. We let K (number of clusters) vary between one and five and used the admixture model (which allows for the possibility that individuals may have mixed ancestry in more than one of the K populations) with correlated allele frequencies, as suggested for closely related populations (Pritchard *et al.* 2000). We conducted six independent runs for each K value. Preliminary runs showed that convergence was achieved after 5,000,000 iterations, and we based the estimations on 5,000,000 additional iterations. We used $Q = 0.2$ as threshold for assigning individuals to populations (Vähä and Primmer 2006; Genovart *et al.* 2012).

The program GENECLASS2 (Piry *et al.* 2004) was used to assign individuals to their origin colonies (Rannala and Mountain 1997).

RESULTS

A total of 56 Black Skimmers from five different sites were genotyped at six microsatellite loci. Due to interruptions in the microsatellite repeats at loci Sdaat27, alleles commonly occurred at one nucleotide apart. Chromatograms for Sdaat27 allowed alleles to be scored consistently. There was not strong evidence of linkage disequilibrium between any pair of loci at any of the sampled sites, or departures from Hardy-Weinberg equilibrium after applying the Benjamini-Yekutieli correction for multiple tests. These results suggest that loci are independent.

Evidence for a low frequency of null alleles was found only at one locus (Sdaat27, $P < 0.15$), while high frequency of null alleles was found at locus K6 at two breeding sites and, therefore, this locus was excluded from subsequent analysis. Total number of alleles per locus ranged from three to nine (mean = 7.2 ± 2.5 over all loci across sampled groups) (Table 1). Levels of genetic diversity as mean expected heterozygosity ranged from 0.60 to 0.68 and similar but low levels of allelic richness (mean ranged from 3.51 to 4.08) were observed (Table 2). Private alleles were found at the Entre Rios breeding colony at locus K16 (allele 173 and allele 177) and locus Sdaat27 (allele 241) and at the Guapore breeding colony at locus RBG28 (allele 170), with three additional private alleles found at the non-breeding aggregation of Mar Chiquita (Locus K16, allele 157; locus RBG27, allele 189; and locus RBG28, allele 182) (Table 2). There was no correlation

Table 2. Sample sites, numbers and genetic diversity descriptors for each sampled Black Skimmer group at five microsatellite loci. Measures of genetic diversity include average allele number per locus (A_N), allelic richness (A_R), number of private alleles (P), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}). * indicates significant values after permutation tests (> 1,000 times).

Sample site	n	A_N	A_R	P	H_O	H_E	F_{IS}
Mar Chiquita	20	5.2	3.89	3	0.61	0.67	—
Entre Rios	10	4.0	3.51	3	0.58	0.60	-0.006
Pantanal	10	4.0	3.55	0	0.50	0.63	0.216*
Guapore	9	4.4	3.84	1	0.51	0.60	0.164*
Mamiraua	9	4.2	4.08	0	0.70	0.68	-0.078

between latitude and allelic richness between breeding colonies (r = -0.908, P = 0.09).

Population Differentiation

Overall FST value was moderate but highly statistically significant (Global FST = 0.09, $P < 0.001$), with pairwise FST ranging from 0.04 to 0.18. All pairwise FST values were significant, with the exception of the pair Mamiraua-Pantanal (Table 3). The estimation of FST per locus using the correction for null alleles (ENA) yielded lower values than the uncorrected FST and showed the same pattern between sites, with the largest pair-wise values observed between the two geographical extreme breeding sites, Entre Rios and Mamiraua, while the lower value occurred between Mamiraua and Pantanal (Table 3). Colonies grouped by hydrological basin in the hierarchical AMOVA revealed that 90.7% of variation was partitioned within populations, and the among-groups component of genetic variation was not significant (FCT = 0.002, P = 0.7). The global Mantel test did not uncover a relationship between genetic and geographic distances between breeding populations (r = 0.20, P = 0.37).

Convergence of the STRUCTURE clustering analysis was confirmed by low variance in $[P(X|K)]$ across replicates of runs and visual inspection of times series plots of likelihood and estimated parameters. The evaluation of $[P(X|K)]$, ΔK and Q for different values of K indicated K = 2 (1n $[P(X|K)]$ = -769.1 ± 1.8) as the best clustering partition. The proportion of membership (average Q) for the studied breeding populations to one cluster ranged from 0.884 to 0.990. One of

Table 3. Microsatellite DNA FST pairwise distance matrices for the Black Skimmer. Values below the diagonal correspond to Weir and Cockerham FST and values above the diagonal correspond to FreeNA FST. Significant values after permutation tests (> 1,000 times) are shown with asterisk * P < 0.05; ** P<0.001.

	Entre Rios	Pantanal	Guapore	Mamiraua
Entre Rios	—	0.07	0.05	0.14
Pantanal	0.10**	—	0.09	0.02
Guapore	0.08*	0.14**	—	0.09
Mamiraua	0.18**	0.04	0.13**	—

the clusters consisted of 100% of individuals belonging to the Entre Rios and Guapore sites. The second cluster comprised 90% of individuals from Pantanal and 100% of individuals from Mamiraua. One individual from Pantanal could neither be assigned to a genetic cluster nor excluded from both of them using the threshold value of Q = 0.2. Correct assignment rates using GeneClass were higher for Entre Rios (80%) and Guapore (67%) compared to Mamiraua (57%) and Pantanal (20%). The assignment of wintering individuals from Mar Chiquita to breeding populations showed a higher assignment of birds to Entre Rios (30%), compared to sites in Brazil (10% for the three sites analyzed).

DISCUSSION

We provide initial evidence that at least southern South American populations of the Black Skimmer are not completely panmictic. Three exclusive microsatellites were found in the Entre Ríos (northern Argentina) population and one exclusive microsatellite allele was observed in the Guapore (central Amazon) population (Table 1). Results revealed differences between populations but no structuring among populations located in different hydrological basins and suggested the existence of two genetic groups, one formed by two of the Brazilian populations studied (Mamiraua and Pantanal sites) and another including one of Brazilian sites (Guapore) and an Argentinean site (Entre Ríos). Temporal isolation has been described as an important force preventing gene flow among seabird populations and has been largely described (Friesen *et al.* 2007b). In this case, genetic differentiation between Black Skimmer breeding sites in Argentina and Brazil was low, indicating that despite different breeding phenologies, gene flow is high enough to prevent genetic isolation. This scenario has also been described for the South American Tern (*S. hirundinacea*) populations from Brazil and Argentina where, despite the different breeding phenologies between regions, population structure was very low (Faria *et al.* 2010). Low genetic structure at regional scale has been

described as well for the Roseate Tern (Lashko 2004). The absence of a strong genetic structure has been explained through the occurrence of contemporary gene flow among populations that were either historically isolated or had a strong historical association or both (Faria *et al.* 2010; Bicknell *et al.* 2012). Disentangling historical processes requires, in part, the use of other molecular markers like mitochondrial DNA; however, previous studies highlighted that, despite the selected molecular marker, terns and skimmers show low levels of genetic variation (Lashko 2004; Whittier *et al.* 2006; Faria *et al.* 2007; 2010). Particularly for the Black Skimmer, the occurrence of nuclear copies on the mitochondrial control region and a low genetic variation on several mitochondrial genes limited the usefulness of these markers for genetic studies (Faria *et al.* 2007).

Low genetic diversity was observed in the Entre Ríos and Pantanal breeding populations. A reduction in allele number and/or heterozygosity at variable loci is expected to occur under a scenario of effective population size reduction (Chakraborty and Nei 1977). Moreover, allelic diversity decreases more quickly than heterozygosity due to the loss of rare alleles (Nei *et al.* 1975; Bouzat 2010). This has been supported empirically, where low genetic diversity is attributed to bottlenecks and/or founder events in several species (Bouzat *et al.* 1998; Abbot and Double 2003; Barlow *et al.* 2011). Unfortunately, the lack of historical reports on skimmer abundance and population dynamics in these sites and the limited sample size of the present study preclude any strong conclusion on this aspect.

Higher genetic diversity was observed in the Mar Chiquita winter aggregation, supporting the hypothesis that Black Skimmers converged here from different breeding areas. Wintering areas have been suggested to provide an opportunity for contact between birds from distant colonies and are places where birds can potentially switch future reproduction sites (Friesen *et al.* 2007a). The occurrence of a main non-breeding area for a species rather than multiple non-breeding areas has been related to overall low population genetic structure (Friesen *et al.* 2007a). The large numbers of Black Skimmers observed in Mar Chiquita and records of Black Skimmers banded at Brazilian grounds wintering in this and nearby areas (~250 km) (Mariano-Jelicich 2007) suggest that individuals from different populations may coexist at these grounds. This potential mixture of individuals has also been reported for the Common Tern (*S. hirundo*) (Bugoni and Vooren 2005). In our study, the overall trend of low and, in some cases, lack of genetic structure among the breeding colonies analyzed restrains the usefulness of microsatellites to identify the origin of individuals in non-breeding aggregations.

Further studies are required to understand the origin of the high abundance of Black Skimmers at Mar Chiquita Coastal Lagoon. Particularly, applying a broader range of molecular markers, the improvement of the extant efforts of bird banding and the inclusion of other breeding and non-breeding groups throughout the distribution range of this species is needed to enhance these results and help elucidate dispersal mechanisms of the Black Skimmer in southern South America.

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