Ornithological Applications

AmericanOrnithology.org

Volume 124, 2022, pp. 1–11
https://doi.org/10.1093/ornithapp/duab053

RESEARCH ARTICLE

Genetic structure in the nonbreeding range of *rufa* Red Knots suggests distinct Arctic breeding populations

Yvonne I. Verkuil,[1,*] Erika Tavares,[2] Patricia M. González,[3] Kristen Choffe,[2] Oliver Haddrath,[2] Mark Peck,[2] Lawrence J. Niles,[4,*] Allan J. Baker,[2†] Theunis Piersma,[1,5,*] and Jesse R. Conklin[1,*]

[1] Conservation Ecology Group, Groningen Institute for Evolutionary Life Sciences (GELIFES), University of Groningen, Groningen, The Netherlands
[2] Department of Natural History, Royal Ontario Museum, Toronto, Ontario, Canada
[3] Fundación Inalafquen & International Conservation Fund of Canada, San Antonio Oeste, Río Negro, Argentina
[4] Wildlife Restoration Partnerships LLC, New Jersey, USA
[5] Department of Coastal Systems, NIOZ Royal Netherlands Institute for Sea Research, Den Burg, Texel, The Netherlands
*Corresponding authors: Yvonne Verkuil, y.i.verkuil@rug.nl; Erika Tavares, erika.tavares@sickkids.ca
[†] This author is deceased.

Submission Date: June 24, 2021; Editorial Acceptance Date: September 28, 2021; Published November 20, 2021

ABSTRACT

An understanding of the migratory connectivity between breeding and nonbreeding areas is fundamental to the management of long-distance migrants under pressure from habitat change along their flyways. Here we describe evidence for genetic structure within the nonbreeding range of the endangered Arctic-Canadian *rufa* subspecies of Red Knots (*Calidris canutus*). Using blood and tissue samples from the major nonbreeding regions in Argentina (Tierra del Fuego and Río Negro), northern Brazil (Maranhão), and southeastern USA (Florida), we estimated genetic structure in 514 amplified fragment length polymorphism (AFLP) loci, applying cluster assignment analyses in DAPC, *assignPOP*, and STRUCTURE. Using a priori location information, individuals could be correctly re-assigned to their nonbreeding regions, which validated that the assignment accuracy of the data was sufficient. Without using a priori location information, we detected 3–5 genotype clusters, and posterior assignment probabilities of samples to these genotype clusters varied among the three regions. Lastly a chi-square test confirmed that allele frequencies varied significantly among nonbreeding regions, rejecting the hypothesis that samples were drawn from a single gene pool. Our findings hint at undescribed structure within the Red Knot *rufa* breeding range in the Canadian Arctic and indicate that each *rufa* nonbreeding area in this study hosts a different subsample of these breeding populations. The observation that nonbreeding sites of *rufa* Red Knots contain different genetic pools argues for separate conservation management of these sites.

Keywords: conservation management, migratory connectivity, population genetics

LAY SUMMARY

- Red Knots of the *rufa* subspecies are famous for their cross-continental migrations, connecting people and cultures in South and North America, where they act as ambassadors for coastal habitat protection.
- Through ongoing habitat loss and degradation along their flyway, *rufa* Red Knots are currently listed as "Threatened" or "Endangered" in the USA, Argentina and other countries in the flyway.
- The internationally collaborative efforts for the protection of this species depend on recurrent updates of the population status, but also on our understanding of how Red Knots from Arctic Canada distribute themselves over their distant nonbreeding areas.
- Therefore, we set out to test whether *rufa* Red Knots spending the boreal winter in Argentina (Tierra del Fuego and Río Negro), northern Brazil (Maranhão) and southeastern USA (Florida) can be considered to come from one interbreeding population, by genotyping 150 Red Knot samples with population genetic markers.
- We detected genetic differences that warrant the recognition of three nonbreeding regions in Argentina, northern Brazil and southeastern USA (each hosting 3,600–13,000 Red Knots) as distinct units, even though the breeding origin of the birds remain unknown.
- The unique assortments of genotypes suggest that these regions receive birds from different areas within the Arctic, and therefore a detailed study of interbreeding across the breeding range is justified, once more samples become available.

La estructura genética en las áreas de estadía no reproductiva de *Calidris canutus rufa* sugiere distintas poblaciones reproductoras del Ártico

RESUMEN

Comprender la conectividad entre las áreas reproductivas y no reproductivas es fundamental para el manejo de las aves migratorias de larga distancia que se encuentran bajo la presión de los cambios de hábitat a lo largo de sus rutas migratorias. En esta publicación describimos la estructura genética de las poblaciones de las áreas de estadía no reproductiva de la subespecie *rufa* del Playero Rojizo *Calidris canutus* del Artico Canadiense que se encuentra en peligro de extinción. Utilizando muestras de sangre y tejidos de sus principales poblaciones de estadía no reproductiva en Argentina (Tierra del Fuego y Río Negro), en el norte de Brasil (Maranhão) y en el sureste de Estados Unidos (Florida), estimamos la estructura genética en 514 loci de polimorfismos en la longitud de fragmentos amplificados (AFLP), aplicando análisis de clúster en DAPC, assignPOP y STRUCTURE. Utilizando información a priori de su ubicación, los individuos podrían reasignarse correctamente a su región no reproductiva, lo que validó que la precisión de la asignación de los datos era suficiente. No utilizando información a priori de su ubicación, detectamos 3 –5 clústeres y la asignación posterior de muestras a estos clústeres, reveló diferencias de frecuencia de genotipos distintas entre las tres regiones. Además, las frecuencias alélicas difirieron significativamente entre cada par de regiones no reproductivas, rechazando la hipótesis de que las muestras se extrajeron de un único acervo genético. Nuestros hallazgos implican una estructura no descrita dentro del rango de Playeros Rojizos en el Ártico Canadiense y sugieren que cada área de estadía no reproductiva de *rufa* en este estudio alberga en promedio una muestra diferente de estas poblaciones reproductoras. La observación de que las regiones no reproductivas de Playero Rojizo muestreadas no son genéticamente idénticas argumenta a favor de un manejo de conservación separado.

Palabras claves: genética de poblaciones, conectividad migratoria, manejo de conservación

INTRODUCTION

Red Knots (*Calidris canutus*) are long-distance migratory shorebirds with six subspecies distributed globally over four flyways, connecting Arctic breeding areas with nonbreeding sites on all continents and as far south as New Zealand, South Africa, and Tierra del Fuego (Piersma and Davidson 1992, Piersma 2007). Just recently, knowledge on Red Knots contributed to the nomination of the wetlands along the Chinese coast of the Yellow Sea as a World Heritage Site (reviewed by Crockford 2019). This well-studied species has often spawned international collaborative conservation efforts and it played a key role in the development of the shorebird "flyway" concept (Western Hemisphere Shorebird Reserve Network 1986, González et al. 1995, Davidson et al. 1998, Boere and Piersma 2012, Atlantic Flyway Shorebird Initiative 2015, Senner et al. 2017). Protection of the shoreline habitats of Delaware Bay in the USA is the best-known case in the Americas where studies on a declining population of Red Knots have highlighted threats and promoted nature conservation actions, thereby preserving the trophic connection between the Atlantic horseshoe crab (*Limulus polyphenus*) and shorebirds (Baker et al. 2004, Morrison et al. 2004, González et al. 2006, Niles et al. 2008).

Although the numbers of Red Knots using Delaware Bay during migration partly recovered after measures to restore spawning horseshoe crab populations (Niles et al. 2009), in the first decade of the 21st Century, the Red Knot subspecies *Calidris canutus rufa* numbered only 42,000 individuals and was still declining (Andres et al. 2012, Hurdle 2021). The *rufa* subspecies breeds in Arctic Canada and has a large range during the boreal winter, stretching all the way from the southeastern USA to Tierra de Fuego in Chile and Argentina. In Canada, *rufa* Red Knots are listed as endangered under Canada's Species at Risk Act, but the threats are considered to occur mostly on the nonbreeding grounds (González et al. 2006, Aldabe et al. 2015, Hope et al. 2019). The subspecies is listed as threatened or endangered in the USA, Argentina, and other countries in the flyway as well, and is included in Appendix I of the Bonn Convention of Migratory Species (CMS) (Aldabe et al. 2015). The continuing declines have raised concerns that the current management of Red Knots may not address the entire *rufa* breeding population, as it disperses across distinct nonbreeding areas using various migratory routes, with one-way distances ranging from 5,000 to 15,000 km. A greater understanding of demographic differences among birds occupying different parts of the nonbreeding range could guide better-targeted conservation action.

The major nonbreeding concentrations of *rufa* Red Knots in North and South America, with 3,600–13,000 birds each, are in the southeastern USA (Gulf of Mexico: Florida and Texas), north-central Brazil, and Tierra del Fuego (Harrington et al. 1988, Morrison and Harrington 1992, Baker et al. 2005a, b; Dey et al. 2011, Andres et al. 2012; Figure 1). Before the population decline, the Patagonian Atlantic coast was also important (Morrison et al. 2004). From South America, *rufa* Red Knots may migrate along the Atlantic Coast, but also often take nonstop inland routes, both through South America (Niles et al. 2010; P. M. González, personal observation) and North America (Newstead et al. 2013). In North America, Delaware Bay is a main staging site (Baker et al. 2004, Atkinson et al. 2005, González et al. 2006, Gillings et al. 2009, Niles et al. 2010). Although stable isotope profiles

of feathers demonstrated that *rufa* Red Knots that spend their nonbreeding season along the Gulf coast of Florida migrate through Delaware Bay (Atkinson et al. 2005), tracking of Red Knots from the Texas coast with light-level geolocators confirmed that some birds from the Gulf only use inland routes (Newstead et al. 2013). These inland routes have long been expected (Morrison and Harrington 1992) and take *rufa* through Saskatchewan, Manitoba along the southern Hudson Bay, including James Bay, potentially to the western part of the *rufa* breeding range (Newstead et al. 2013). The substantial variation in migratory behavior of Red Knots from nonbreeding regions throughout South and North America may indicate that these sites comprise individuals from different breeding areas (Harrington et al. 1988).

The *rufa* breeding range stretches across low-Arctic Canada from Baffin Island and the northern Hudson Bay in the east, to Victoria Island and possibly Banks Island in the west (Morrison and Harrington 1992; Figure 1). The exact distribution and boundaries are not well-described (Lathrop et al. 2018), but it is broken up over several islands or otherwise isolated locations. This extensive and disjunct breeding range could have fostered the evolution of population structure, especially considering the high breeding-site fidelity of Red Knots (Tomkovich and Soloviev 1994).

Together with insights from recent mark-recapture and tracking studies described above (see also Niles et al. 2010, Burger et al. 2012), this implies that *rufa* Red Knots are subdivided into multiple populations with different annual routines that, despite mixing at some sites during migration (Baker et al. 2004, 2020; Lyons et al. 2017), cannot be managed as a single unit. Describing any potential differential distribution of genotypes in the nonbreeding areas is important to assess whether reproductive isolation within *rufa* and migratory connectivity between breeding and nonbreeding areas may exist. Partly because collection of samples of breeding birds was limited and coverage of the range was incomplete, previous population genetic work on the global phylogeography of Red Knots did not address within-subspecies population differentiation (Buehler and Baker 2005, Buehler et al. 2006).

This study aims to improve the understanding of genetic structure within *rufa* by sampling, during the boreal winter, Red Knots from nonbreeding areas in Argentina, northern Brazil (hereafter N Brazil), and southeastern USA (hereafter SE USA). Our main question is whether *rufa* Red Knots breeding in the Canadian Arctic belong to a single panmictic population, freely moving among various nonbreeding regions in North and South America, or if differentiation, as suggested by mark-recapture and tracking studies, indeed exists. We discuss the conservation implications if *rufa* cannot be considered a single distinct unit.

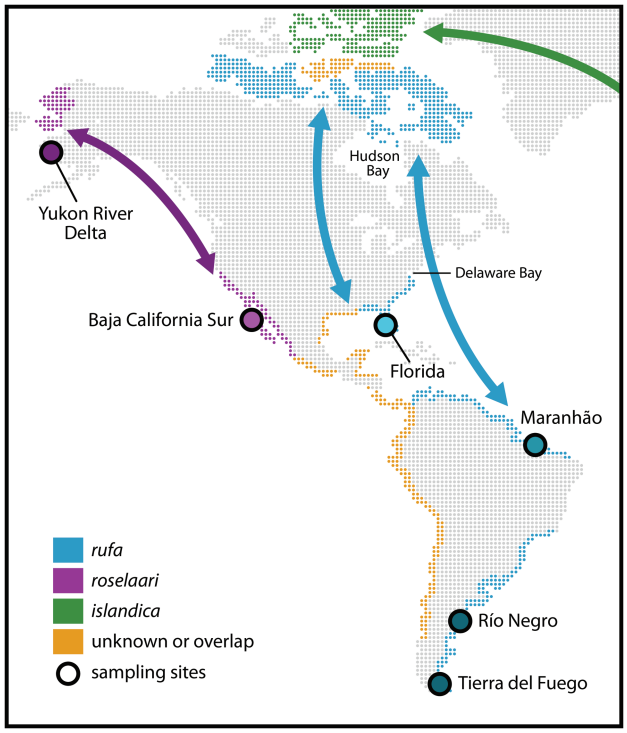

FIGURE 1. Distribution map of the American flyways of Red Knots. (*Blue*) Known distribution of the focal subspecies *Calidris canutus rufa*. (*Purple*) Known range of *C. c. roselaari*, the reference subspecies in this study. (*Green*) Third subspecies breeding in North America, *C. c. islandica* (shown for completeness, not in this study). (*Yellow*) Parts of the range where knowledge of subspecies distribution is incomplete. Arrows are schematic indicators of migration direction from the breeding grounds. Sites mentioned in text are indicated. Sampling sites included in this study are (1) nonbreeding and stopover area in SE USA, Gulf of Mexico (Florida); (2) nonbreeding and stopover area in N Brazil (Maranhão); (3) nonbreeding and stopover areas in Argentina (Río Negro and Tierra del Fuego); (4) stopover area in NW USA (Yukon River Delta); and (5) nonbreeding area in Mexico (Baja California Sur).

METHODS

Sample Collection

For this study, 150 blood and tissue samples of adults of the *C. c. rufa* subspecies of Red Knot were collected; the sample sites represent the major nonbreeding areas in Argentina (n = 72), N Brazil (n = 33), and SE USA (n = 45) (Table 1). Samples in Argentina and SE USA were collected over various years and sites which allows to control for batch effects. Samples were collected during the boreal winter, except for Río Negro, Argentina, which only hosts birds during the migratory season. To understand the relative scale of potential differences and to ensure our sample collection contained *rufa* only, the subspecies *Calidris canutus roselaari* was included (n = 31). For this subspecies, samples were included from a stopover area in

TABLE 1. Information for blood and tissue samples of Red Knots (*Calidris canutus rufa* and *C. c. roselaari*) used in this study. Blood samples were taken from the brachial vein; tissue samples (*) were taken from specimen collected under permits issued to the Royal Ontario Museum, Toronto, Canada. All samples are curated in the Ornithology collection of the Royal Ontario Museum. Locations are shown in Figure 1.

Subspecies	Country	Sampling location	Sampling date	*n*
C. c. rufa (*n* = 150)	Argentina	Tierra de Fuego—Río Grande	8 Nov 2007	40
		Río Negro—Area Natural Protegida Bahía de San Antonio	14 Mar 1997	6
			20 Mar 2003	26
	N Brazil	Maranhão	Feb 2003	33
	SE USA	Florida—Manatee County	31 Dec 2005	20
		Florida—Lee County	2 Jan 2006	8
		Florida—Collier County	30 Jan 1986	16*
		Florida—unknown	unknown	1
C. c. roselaari (*n* = 31)	NW USA	Alaska—Yukon River Delta	19 May 1993	9*
	Mexico	Baja California Sur	31 Oct 2007	22

NW USA (Alaska) (*n* = 9) and from a nonbreeding area in Mexico (*n* = 22). Note that *roselaari* breeds both in Alaska, USA, and on Wrangel Island, Russia and migrates along the Pacific coast of North America to Mexico and Texas, where it has some spatial overlap with *rufa* (Carmona et al. 2013, D.J. Newstead, personal communication; Figure 1).

Marker of Choice: Amplified Fragment Length Polymorphism (AFLPs)

For this study, we apply a few hundred amplified fragment length polymorphism (AFLP) markers. Compared to a single or limited number of loci, a large set of genome-wide nuclear markers is more likely to detect population differentiation (Felsenstein 2006) and AFLPs are generally considered suitable for initial exploration of patterns of genetic diversity (Bensch and Åkesson 2005, Wink 2006, Meudt and Clarke 2007, Rodríguez-Clark et al. 2018). AFLPs are commonly used in polyploid species because they are highly repeatable and homologous across species (e.g., Bryan et al. 2017), and in Red Knots the precursor of AFLP, random amplified polymorphic DNA (RAPD), could detect differences between Florida birds and breeding birds from Ellesmere Island (presumed *Calidris canutus islandica*) (Baker et al. 1994). To avoid batch effects creating spurious structure, we used multiple batches for each region (Table 1; this was not possible for N Brazil), and samples were randomized and processed blindly in the laboratory.

Genotyping Methods

DNA extraction. DNA was extracted in a solution containing 0.1% SDS, 100 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA, and 10 mg mL^{-1} proteinase K (Invitrogen, ThermoFisher Scientific, USA) and incubated overnight at 55°C. DNA purification was performed by a standard phenol-chloroform-isoamyl alcohol method followed by ethanol precipitation (Sambrook and Russell 2001). DNA quality was verified on a 1.5% agarose gel, and DNA concentration was measured with a NanoDrop spectrophotometer (ThermoFisher Scientific, USA). Samples with degraded DNA or with <500 ng total DNA were excluded.

Amplification and processing of AFLPs. Total DNA from each selected individual was normalized to 500 ng in a volume of 10 µL and then digested in 30 µL containing 12U of *EcoRI* and 8U of *MseI I* in 1x buffer R/L (10 mMTris-HCl pH7.5, 10 mM MgAc, 50 mM KAc, 5mM DTT, 50 ng μL^{-1} BSA) by incubation at 37°C for 3 hr. The digestions were verified by running 3 µL on a 1.5% agarose gel. Only samples with a similar smear pattern and intensity were used.

Adapters for *EcoRI* (5'-CTC GTA GAC TGC GTA CC AAT T-3') and *MseI* (5'-GAC GAT GAG TCC TGA GTA-3) were prepared as follows to obtain 5 pmol μL^{-1} *EcoRI* adapter and 5 pmol μL^{-1} *MseI*: mix of the top and bottom strand was heated for 3 min at 90°C, cooled to room temperature on the benchtop, and stored at –20°C. Sticky ends of the DNA fragments were ligated in a 30 µL reaction containing 0.5 mM of each adapter, 1 mM ATP, 1 x R/L buffer, 0.5 U T4 ligase (Invitrogen, ThermoFisher Scientific, USA), and 20 µL of the digestion.

To subdivide the pooled DNA fragment sizes digested with both enzymes, and thus have sufficient size spacing among the PCR products, selective primers recognizing the adapters were attached to the ends of the fragments plus an additional triplet of nucleotides inside the restriction site sequence. Seven triplet extensions linked at the 3' end of the above core sequences were used to generate AFLP profiles: CAT, CCA, CCC, CCG, CGA, CGT, and CTT. This generated 7 independent datasets. The pre-selective PCR was performed in 25 µL of reaction containing Buffer E (10 mM Tris-HCl pH 9, 50 mM KCl, and 1 mM $MgCl_2$), 0.4 mM dNTPs, 0.2 µM of one kind of E-primer (GACTGCGTACCAATTC), 0.2 µM of the corresponding M-primer (GATGAGTCCTGAGTAAA) (Vos et al. 1995), 0.5 U *Taq Polymerase* (Invitrogen,

ThermoFisher Scientific, USA), and 3 µL of ligated digestions (Hagelberg 1994). The selective PCR was performed in 10 µL of reaction containing 1x Buffer E, 0.4 mM dNTPs, 0.2 µM primer forward recognizing *EcoRI* adaptor and M13 tail, 0.2 µM of reverse primer recognizing the *MseI* adaptor and M13 tail, 0.2 µM of M13-FAM (blue fluorescence) primer, 0.25 U of *Taq Polymerase* (Invitrogen, ThermoFisher Scientific, USA), and 1 µL of 1:10 dilution of the pre-selected products. The PCR products were run in an ABI 3730 DNA analyzer (Applied Biosystems, USA) and only the variable fragments with well-defined fluorescence peaks were selected for each of the 7 datasets after scoring in GeneMarker HID (Holland and Parson 2011). Sampling location identification tags for the samples were added after scoring, to certify that this step was performed blindly regarding sample origin. To test the error rate associated with not detecting a real peak and/or calling the wrong peak, six samples were randomly processed twice across all primer combinations, starting from the DNA extraction step.

Genotype Cluster Analyses

We conducted Bayesian cluster analyses with and without using sampling region priors. Our first objective was to investigate the maximal genetic diversity among the nonbreeding regions, and the assignment accuracy of the data, and therefore the individuals were labeled with their geographically predefined region: Argentina, N Brazil, or SE USA. For this, we used discriminant analysis of principal components (DAPC) for genetic data, implemented in the *Adegenet* package (Jombart 2008, Jombart et al. 2010) in R v3.5.1. DAPC is a multivariate method that makes no assumptions about the underlying population genetic model. First, genetic data are transformed and subjected to a principal component analysis (PCA). Second, the principal components are subjected to a discriminant analysis that summarizes the genetic differentiation between groups while disregarding within-group variation (Jombart et al. 2010). The 3D plots of the DAPC analysis were obtained in R with the package *rgl* (Adler and Murdoch 2019). We ran a cross-validation analysis with the R package *assignPOP* (Chen et al. 2018) to test the robustness of sampling region assignment by random sampling the dataset.

Next, the probability of K clusters was estimated amongst the pooled genotypes without prior sampling region assignment, by individual-based clustering of genotypes using the program STRUCTURE 2.3.4 (Pritchard et al. 2000). For each K, ranging from 1 to 8, independent tests were run 10 times, with burn-in and iteration values set at 10,000 and 100,000, respectively. Convergence of runs was assessed by the data plots and histograms (deviation from normal distribution) of the summary statistics fixation index (F_{ST}) and alpha. Two different models were run, assuming either symmetric or asymmetric ancestry; the latter allows for a unique ancestry parameter α for each inferred population. In both models, individuals could have mixed ancestry, and allele frequencies could correlate between inferred populations. Model comparisons and the choice of K were executed in CLUMPAK (Kopelman et al. 2015) and Harvester (Earl and vonHoldt 2012). The symmetry model was slightly more stable (major clusters supported by 10/10 runs at K = 3–5) than the asymmetry model (major clusters supported by 8/10 or 9/10 runs), while the posterior assignments were not significantly different from the asymmetry model (P-values of 0.87–0.99 for K = 3–5). We evaluated the best-supported value of K by two methods: by identifying the greatest posterior probability (ln(Pr(X|K); Pritchard et al. 2000) and the greatest increase in log likelihood (Delta K; Evanno et al. 2005).

Allele Frequency Differentiation

To determine whether alleles sampled at each of three *rufa* nonbreeding regions can be considered a different, nonrandom sample from the gene pool, traditional population genetic statistics such as F_{ST} were avoided because (1) at nonbreeding sites the assumption of random mating may not apply, (2) our study design does not allow the inference that detected genotype clusters in *rufa* may represent breeding populations, and (3) the ascertainment bias associated with AFLPs may inflate F_{ST} values. Instead, we tested whether allele frequency differences between regions were large enough to reject homogeneity, using a chi-square (χ^2) approach. In the program CHIFISH (v. 2017, default settings) testing for no differentiation, Pearson's traditional chi-square statistic and its P-value were calculated for each AFLP locus; subsequently, with the sums the chi-squares and associated degrees of freedom, the overall P-value was calculated (Ryman 2006). We also report the number of loci with significantly different allele frequencies (i.e. segregating loci) for each pairwise comparison of nonbreeding regions.

RESULTS

AFLP Markers and Genotyping Success

The dataset of 181 samples (150 *rufa* Red Knots and 31 reference samples of *roselaari*) contained 514 fragment length polymorphisms (AFLP) amongst a total of 665 amplified fragments. The error rate was 13.8%, as estimated for 6 repeated samples for 378 loci. The rates of missing data in sample batches from Argentina, N Brazil, and SE USA were 58.9%, 25.3%, and 23.8%, respectively, and 11.6% in *roselaari*. The reduced dataset, omitting samples with >35% missing data, contained 498 AFLPs, and consisted of 98 samples: 67 *rufa* (Argentina = 15; N Brazil = 21; SE

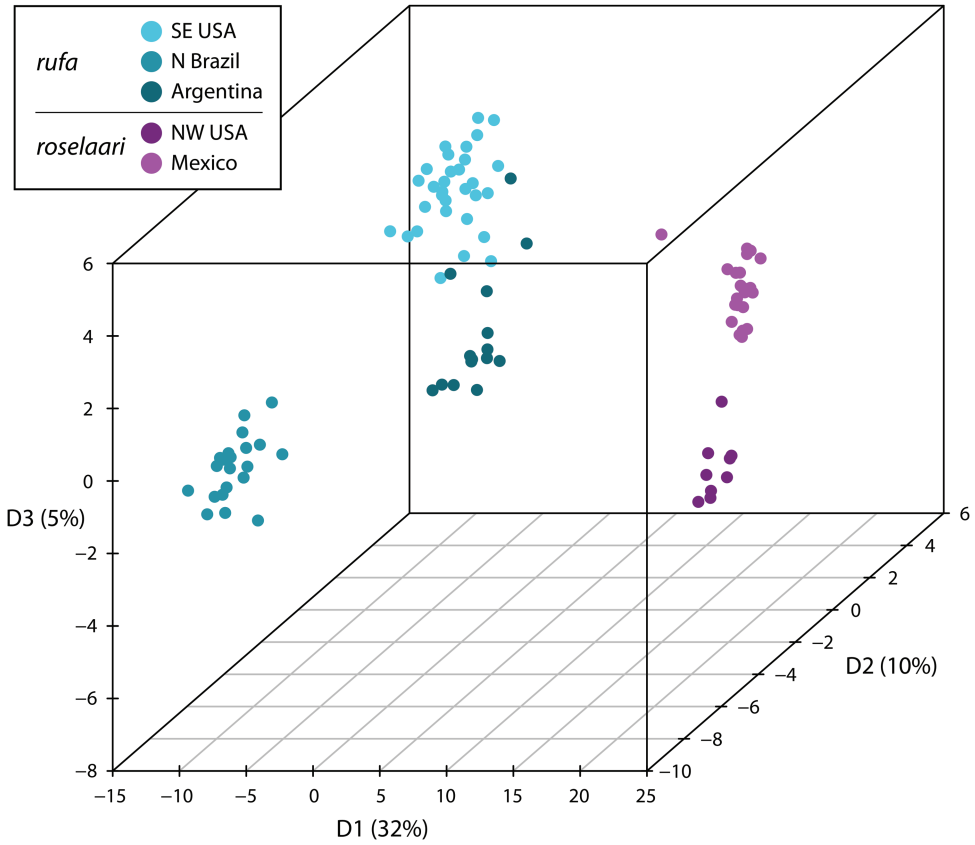

FIGURE 2. Clustering of samples detected with the DAPC Bayesian clustering analyses, using a priori sampling location information and retaining three discriminant functions (see Supplementary Material Figure S1A). Number of PCs retained was 30, which represents ~80% of the variance (Supplementary Material Figure S1B). Shown are the first three principal components (D1 to D3; with the % variance explained). Samples from *Calidris canutus rufa* are from Argentina (Tierra del Fuego/Río Negro Province), N Brazil (Maranhão), and SE USA (Florida coast of Gulf of Mexico). The subspecies *C. c. roselaari* is included for reference, represented by samples from NW USA (Yukon River Delta, Alaska) and Mexico (Baja California Sur).

USA = 31) and 31 *roselaari*. Subsequent analyses were run with the reduced dataset unless stated otherwise.

Genetic Differentiation

Using the sampling regions as a priori defined groups, DAPC analysis of the 498 AFLP markers clearly differentiated the three *rufa* regions from each other, and *rufa* from *roselaari,* while showing some differentiation between the two *roselaari* sampling locations (Figure 2). The cross-validation analysis showed that when >50% of the loci were used, the assignment of the samples to the correct sampling region was robust (Supplementary Material Figure S2).

In the STRUCTURE analysis, conducted without using a priori sampling locations, the model with five genetic clusters ($K = 5$) achieved the highest probability (Supplementary Material Figure S3A), whereas the greatest change in likelihood supported $K = 3$ (Supplementary Material Figure S3B). Therefore, we considered posterior assignments from scenarios of $K = 3$–5. In all models, each *rufa* nonbreeding region had a unique distribution of genotypes, while a single genotype was dominant in *roselaari* (Figure 3). The patterns of substructuring were similar across models and datasets, as depicted for the reduced dataset (Figure 3) and the full dataset (Supplementary Material Figure S4). Generally, the regionally common genotypes were present in each sample batch of that specific region or subspecies. This was true for the sample batches of *roselaari* from the Yukon River Delta and Baja California Sur, the batches from Argentina (Tierra de Fuego 2007 and Río Negro 1997/2003), and for two batches from Florida; however, the Collier County 1986 batch was dominated by a cluster not typically found in the other years and counties in Florida (Supplementary Material Figure S4).

Allele frequencies differed significantly among the *rufa* nonbreeding regions: at the 458 polymorphic loci within *rufa*, the null hypothesis of no allele frequency differentiation among the sampling regions was rejected ($\chi^2 = 8331$, df = 918, $P < 0.001$, $n = 150$). In each pairwise comparison of nonbreeding regions, we detected 185–196 segregating

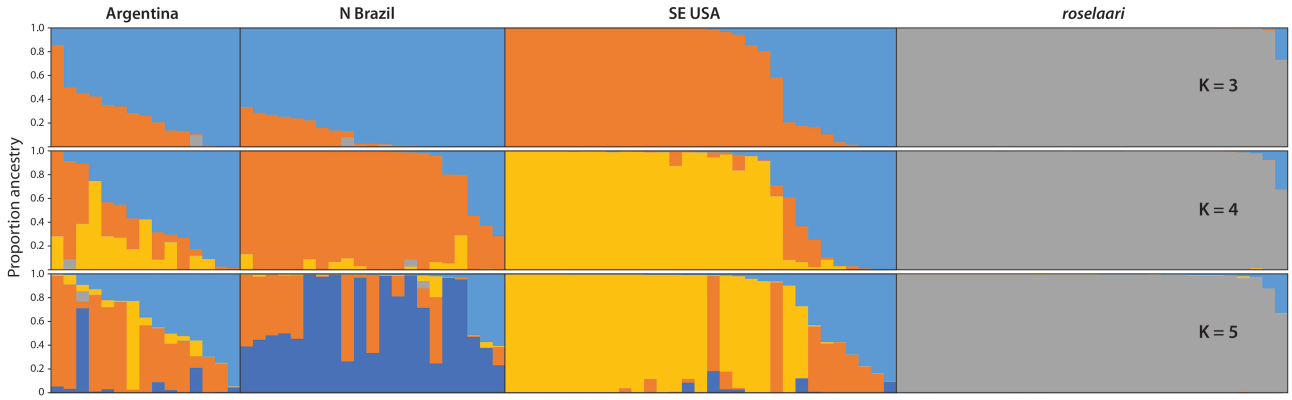

FIGURE 3. Clustering detected with Bayesian clustering analyses without prior population information, depicting the assignment of each individual to one of 3–5 posterior genetic clusters detected in the data. Samples are from *Calidris canutus rufa* from Argentina (Tierra del Fuego/Río Negro Province), N Brazil (Maranhão), and SE USA (Florida coast of Gulf of Mexico), and from *C. c. roselaari*.

loci among 419–428 polymorphic AFLPs included in the comparison (Table 2).

DISCUSSION

Our results demonstrate genetic differentiation among *rufa* Red Knots occurring at the main nonbreeding regions in Argentina, N Brazil, and SE USA. While clearly diverged from the neighboring North American subspecies *C. c. roselaari*, *rufa* Red Knots are not genetically homogenous across their nonbreeding range. The precise interconnections between nonbreeding, staging, and breeding areas cannot be inferred from this dataset and remain to be studied. Nevertheless, the results strongly hint at substructuring within the breeding range of *rufa*, coupled with some degree of migratory connectivity between nonbreeding and breeding sites.

Population structure would be expected within birds with disjunct breeding ranges (e.g., Zink et al. 1995). Indeed, we find a contrast between Red Knots and two congeneric long-distance migratory shorebirds with generally similar breeding ranges across the North American low-Arctic, White-rumped Sandpiper (*Calidris fuscicollis*) and Semipalmated Sandpiper (*C. pusilla*), in which no population genetic structure was detected (Wennerberg et al. 2002, Miller et al. 2013). Although undetected fine-scale structure could yet be revealed in these species using different (e.g., genome-wide) genetic markers, a difference with Red Knots is expected based on ecological differences. The small sandpipers breed abundantly in low-elevation wet meadows available throughout the Arctic, whereas Red Knots occur in lower densities in disjunct regions with barren, rocky slopes (Baker et al. 2020). On migration, the small sandpipers use various inland stopover habitats (e.g., Cheyenne Bottoms in Kansas, USA; Hicklin and Gratto-Trevor 2020, Parmelee 2020) whereas Red Knots mainly make long nonstop flights among discrete coastal habitats. Lastly, in the boreal winter White-rumped and Semipalmated sandpipers are found in a small range of latitudes (respectively, approximately 28–55°S; Parmelee 2020, and approximately 24°N to 25°S; Hicklin and Gratto-Trevor 2020), whereas *rufa* Red Knots occur in a discontinuous winter range spanning approximately 30°N to 55°S. Generally, in coastal obligate shorebird species, population structuring is more prominent than in species with a more inland distribution (Verkuil et al. 2012) and, in comparison with White-rumped and Semipalmated sandpipers, *rufa* Red Knots appear to follow this pattern.

TABLE 2. Chi-square (χ^2) test results rejecting the null hypothesis of no allelic frequency difference among nonbreeding populations of *rufa* Red Knots. Shown are the pairwise comparisons between the nonbreeding sampling regions. Above the diagonal: number of polymorphic loci/number of loci with significantly different allele frequencies (at $P < 0.05$), and sample size of Red Knots included in each pairwise comparison. Below diagonal: sum of chi-squares over all polymorphic loci (summed χ^2), and their *P*-value.

	Argentina	N Brazil	SE USA
Argentina		428/185 *n* = 105	422/189 *n* = 117
N Brazil	4,201 *P* < 0.0001		419/196 *n* = 78
SE USA	3,170 *P* < 0.0001	4,422 *P* < 0.0001	

Limitations to Phylogeographic Inferences I: Choice of Sampling Locations

The large genetic differences between the *rufa* and *roselaari* subspecies excluded the possibility that samples taken in Florida contained *roselaari* (Figure 3). It was previously thought that Red Knots wintering in the SE USA included *roselaari* (e.g., Buehler and Baker 2005). However, it is now clear that Florida-wintering Red Knots migrating

northward along the Atlantic Coast are *rufa* (Atkinson et al. 2005), and no individually marked *roselaari* individuals have been reported east of the Texas coast of the Gulf of Mexico (Carmona et al. 2013, D.J. Newstead, personal communication). Sites in this zone of apparent overlap between the subspecies (Figure 1) were not sampled in this study. The clear genetic differentiation between *roselaari* and *rufa* is consistent with a separate study of genome-wide single nucleotide polymorphisms (SNPs; Conklin et al. 2021).

Importantly, while we demonstrated that the AFLP data were sufficient to assign individuals with confidence to their a priori defined populations (Figure 2), which suggests that migratory connectivity exists, as is expected from the banding and tracking results (see Introduction), these results do not allow inferences about the strength of this connectivity. The analyses without prior sampling information (Figure 3) do show that connectivity is not absolute, because genetic clusters were only partly sorted by sampling region and genotypes were shared. Therefore, although the detected genotype clusters in the *rufa* nonbreeding range suggest that distinct breeding lineages exist, the actual number or spatial organization of subpopulations within *rufa* cannot be inferred without sampling the remote breeding areas. Here we note that instead of visiting the Arctic, DNA samples could be collected from birds that are remotely tracked to breeding areas (e.g., by satellite-telemetry).

We feel that our study is a strong incentive for a comprehensive population genetic study of *rufa* with many genome-wide markers, which would allow detailed demographic inferences, with regard to population sizes, trends, and composition. Additionally, the degree of isolation and potential spatial overlap with both *roselaari* and the high-Arctic subspecies *islandica* (see Figure 1) requires further investigation. To fully understand differentiation within *rufa* there is clearly a need for a reassessment of the phylogeography of Red Knots in a global context.

Limitations to Phylogeographic Inferences II: Marker Choice

Overall, the AFLP markers yielded results that were congruent with earlier studies using nuclear genetic markers and stable isotopes, which revealed similar patterns of divergences in American Red Knots. Stable isotope analyses found Florida samples to be distinct from Argentina and Brazil (Atkinson et al. 2005). Electrophoresis assays of 37 protein-coding loci indicated that individuals spending the nonbreeding season in Florida were differentiated from those from Argentina (Baker 1992). Nevertheless, a concern for this study is that some of the differentiation signals may have been obscured by the error rates and missing data, or by homoplasy (Vekemans et al. 2002, Herrmann et al. 2010), causing individuals to be considered invariable while they are not. Alternatively, the level of differentiation is the result of incomplete lineage sorting, a phenomenon expected to play a role in the relatively recent evolution of flyways of Red Knots (Buehler et al. 2006). AFLP markers cannot distinguish between these two scenarios. A second concern is that variation in quality of sample batches has systematically shifted the presence or absence of alleles, thereby creating an overestimation of the number of populations (Meudt and Clarke 2007). We avoided this pitfall by excluding samples with >35% missing data. After that, we still observed that the samples taken 20 years earlier in Florida, in 1986, were different (Figure 3). This could mean that a few generations ago, a different subset of *rufa* used Florida, or that the older Florida samples suffered from technical error, and hence these historical samples do not contribute to our understanding of the extant genetic structure.

Implications For Flyway Conservation

Our findings of genetic differentiation among the three *rufa* Red Knot nonbreeding regions is remarkable considering that birds from all three regions partly use the same stopovers, such as Delaware Bay during northward migration (Baker et al. 2004, Atkinson et al. 2005, Gillings et al. 2009) and Massachusetts during southward migration (Harrington et al. 2010). Therefore, during migration birds from different nonbreeding regions mix, as was shown for Red Knots from Tierra del Fuego making a stopover in Maranhão or Ceará in N Brazil and for some birds from South America stopping in Florida during northward migration (Baker et al. 2013). Many juveniles, but rarely adults, spend large parts of the nonbreeding season in northern sites along the flyway (Harrington et al. 2010, Baker et al. 2020). Nevertheless, despite the intense re-sighting efforts in Argentina from 1995 onwards, and later in Chile, no Red Knot marked in Florida or northern South America during the boreal winter months was ever seen at the southern tip of the range (apart from one adult marked as juvenile in French Guiana), suggesting that ecological differences, such as migration schedules and molt phenologies, may keep Red Knots from the three nonbreeding regions segregated (Baker et al. 2020).

Little is known about the demography of nonbreeding Red Knots in the Gulf of Mexico, from where birds take inland routes to the Arctic (Newstead et al. 2013). The larger variation of genotypes in Florida than at the other two locations is consistent with the expectation that the Gulf may receive birds from various breeding areas, including those in the far west of the range, such as Victoria Island. These western breeders may occur in small numbers and would have unknown population trends, while facing potentially different threats during the nonbreeding season,