USGS
science for a changing world

Red Knot (*Calidris canutus*) Research—Preliminary Results and Future Opportunities

Open-File Report 2020–1050

U.S. Department of the Interior
U.S. Geological Survey

Red Knot (*Calidris canutus*) Research—Preliminary Results and Future Opportunities

By David Kazyak, Aaron Aunins, and Robin Johnson

Open-File Report 2020–1050

U.S. Department of the Interior
U.S. Geological Survey

U.S. Department of the Interior
DAVID BERNHARDT, Secretary

U.S. Geological Survey
James F. Reilly II, Director

U.S. Geological Survey, Reston, Virginia: 2020

For more information on the USGS—the Federal source for science about the Earth, its natural and living resources, natural hazards, and the environment—visit https://www.usgs.gov or call 1–888–ASK–USGS.

For an overview of USGS information products, including maps, imagery, and publications, visit https://store.usgs.gov/.

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Although this information product, for the most part, is in the public domain, it also may contain copyrighted materials as noted in the text. Permission to reproduce copyrighted items must be secured from the copyright owner.

Suggested citation:
Kazyak, D., Aunins, A., and Johnson, R., 2020, Red Knot (*Calidris canutus*) research—Preliminary results and future opportunities: U.S. Geological Survey Open-File Report 2020–1050, 6 p., https://doi.org/10.3133/ofr20201050.

ISSN 2331-1258 (online)

Acknowledgments

Dr. Tim King (Leetown Science Center King Conservation Genetics Laboratory) initiated this study, but unfortunately passed away prior to its completion. This report represents an effort to publicly release key findings from his work. We thank the U.S. Fish and Wildlife Service for providing funding to support this research, and two anonymous reviewers for providing helpful feedback.

Contents

Acknowledgments iii
Abstract 1
Project Background 1
Methods 1
Preliminary Results 2
Ideas for Additional Red Knot Genetic Research 3
Summary 5
References Cited 6

Figures

1. Graph showing principal coordinates analysis plot of microsatellite genotypes at 24 loci of *C.c. roselaari* and *C.c. rufa* collections from Massachusetts, Virginia, Alaska, and Quebec 3
2. Graph showing Bayesian clustering results from the program STRUCTURE based on two assumed clusters 4

Table

1. Samples of 13 *C.c. islandica*, 508 *C.c. rufa*, and 37 *C.c. roselaari* received by the Leetown Science Center King Conservation Genetics Laboratory between 2009 and 2016 for genetic analyses 2

Abbreviations

AFLP	amplified fragment length polymorphism
DNA	deoxyribonucleic acid
ETOH	ethanol
GBS	genotyping by sequencing
km	kilometer
LSC-KCGL	Leetown Science Center King Conservation Genetics Laboratory
mtDNA	mitochondrial DNA
PCR	polymerase chain reaction
RAD-Seq	restriction site associated sequencing
RNA	ribonucleic acid
USFWS	U.S. Fish and Wildlife Service
USGS	U.S. Geological Survey

Red Knot (*Calidris canutus*) Research—Preliminary Results and Future Opportunities

By David Kazyak, Aaron Aunins, and Robin Johnson

Abstract

The Red Knot, *Calidris canutus*, is a highly migratory shorebird with a cosmopolitan distribution. Six subspecies have been identified, two of which occur regularly in North America (*C.c. rufa* and *C.c. roselaari*). Given their long-distance migrations through many jurisdictions and conservation status, tools are needed to reliably distinguish the subspecies when captured away from their breeding areas and to examine potential population substructure within each taxa. We used a suite of molecular approaches to develop tools to support Red Knot research and management. Although our microsatellite markers were not able to reliably distinguish *C.c. rufa* and *C.c. roselaari*, we did find evidence of population substructure within *C.c. rufa*.

Project Background

The Red Knot, *Calidris canutus*, is a highly migratory shorebird with a cosmopolitan distribution. Six subspecies have been identified, two of which occur regularly in North America (*C.c. rufa* and *C.c. roselaari*). The annual migrations of this species may exceed 30,000 kilometers (U.S. Fish and Wildlife Service, 2014). Along their migration routes, these birds pass through many jurisdictions and face a complex suite of threats, including environmental change and reduction of food resources at key stopover sites (Morrison and others, 2004; Atkinson and others, 2007). Consequently, the Red Knot has become the focus of considerable conservation efforts and the *C.c. rufa* subspecies was listed as threatened by the U.S. Fish and Wildlife Service (USFWS). However, tools are needed to reliably distinguish the subspecies when captured away from their breeding areas, and to examine possible population structure within each taxa.

In response to these research and management needs, U.S. Geological Survey (USGS) initiated a study with USFWS in 2009 to develop molecular tools to support Red Knot conservation. This report is intended to summarize the preliminary findings from that study and identify potential directions for continued research.

Methods

Between 2009 and 2016, the USGS Leetown Science Center King Conservation Genetics Laboratory (LSC-KCGL) received Red Knot samples from USFWS, including 14 *Calidris canutus islandica*, 452 *Calidris canutus rufa*, and 37 *Calidris canutus roselaari* samples (hereafter referred to as *C.c. islandica*, *C.c. rufa*, and *C.c. roselaari*, respectively) for genetic analyses from various U.S. and international partners (table 1). Blood or buccal swab samples were preserved in RNAlater (Ambion, Austin, Tex.) or on Whatman FTA cards (GE Healthcare, Buckinghamshire, United Kingdom). Additional samples were received in the form of extracted deoxyribonucleic acid (DNA). Several high throughput shotgun genomic sequencing runs (using multiple instruments, including Illumina GAIIX, Ion Torrent PGM, Ion Proton, and Roche 454 Jr.) of a few individuals of each subspecies were performed to obtain sequence data for microsatellite marker development.

Twenty-four microsatellite loci were optimized for population genetic analyses from *C.c. rufa* sequencing data. Genotypes were determined for 72 *C.c. rufa* and 20 *C.c. roselaari* samples at these 24 newly developed microsatellite loci for preliminary population genetic analyses (table 1). We used principal coordinates analysis and a Bayesian clustering program (STRUCTURE; Pritchard and others, 2000) to assess the ability of the microsatellite markers to distinguish between *C.c. rufa* and *C.c. roselaari*, and to examine potential substructure within *C.c. rufa*. For all STRUCTURE runs, we used a burn-in period of 200,000 steps followed by 200,000 iterations for data collection, and admixture was allowed but capture location was not considered as a prior. Structure Harvester (Earl and vonHoldt, 2012) was used to evaluate model results across *k* values using likelihoods and Evanno's Δk methods (Evanno and others, 2005).

Table 1. Samples of 13 *C.c. islandica*, 508 *C.c. rufa*, and 37 *C.c. roselaari* received by the Leetown Science Center King Conservation Genetics Laboratory between 2009 and 2016 for genetic analyses. The Number genotyped column indicates what samples were genotyped at 24 microsatellite loci developed in this study.

[DNA, deoxyribonucleic acid; ETOH, ethanol; Va., Virginia; Mass., Massachusetts; --, not applicable]

Date received	Location sampled	Sample type	Quantity	Subspecies	Number genotyped
3/26/2009	Europe	Blood in RNAlater[1]	5	*C.c islandica*	--
5/28/2009	Delaware	Blood on FTA card[1]	4	*C.c rufa*	--
5/28/2009	Delaware	Buccal swab in RNAlater	20	*C.c rufa*	--
5/28/2009	New Jersey	Blood on FTA card	5	*C.c rufa*	--
5/28/2009	New Jersey	Buccal swab in RNAlater	19	*C.c rufa*	--
6/4/2009	Hog Island, Va.	Blood on FTA card	2	*C.c rufa*	--
6/2/2010	Florida	DNA	3	*C.c rufa*	--
6/2/2010	Argentina	DNA	3	*C.c rufa*	--
6/2/2010	Brazil	DNA	2	*C.c rufa*	--
6/2/2010	Netherlands	DNA	8	*C.c islandica*	--
6/2/2010	Mexico	DNA	8	*C.c roselaari*	--
4/29/2011	Alaska	Blood on FTA card	29	*C.c roselaari*	20
1/8/2013	New Jersey	Blood on FTA card	67	*C.c rufa*	--
2008–2014	Quebec	Blood on FTA card and blood in ETOH	307	*C.c rufa*	36[2]
6/6/2014	Hog Island, Va.	Blood on FTA card	16	*C.c rufa*	16
12/9/2014	Cape Cod, Mass.	Blood on FTA card	20	*C.c rufa*	20
1/25/2016	Cape Cod, Mass.	Blood on FTA card	41	*C.c rufa*	--

[1]RNAlater and FTA cards are proprietary products.

[2]There were 32 samples genotyped from 2010 and 4 samples genotyped from 2013. Samples from the other years were not genotyped.

Preliminary Results

Although the number of individuals and collections genotyped so far is a small proportion of the total received, we can begin to assess the level of genetic differentiation uncovered by these microsatellite loci between *C.c. rufa* and *C.c. roselaari*. A principal coordinates plot of the genotyped samples indicates some separation of the Alaska *C.c. roselaari* and Quebec *C.c. rufa* samples, but there is still substantial overlap between the two collections, which suggests that the markers we developed cannot be used to reliably distinguish *C.c. rufa* from *C.c. roselaari* with a high level of confidence (fig. 1).

Our microsatellite markers support the presence of at least two populations of *C.c. rufa* with different migratory behaviors (route and [or] phenology). Bayesian clustering analysis using the program STRUCTURE indicated that collections from Massachusetts and Virginia represent a different population than collections from the Mingan Archipelago in Quebec (fig. 2). Although Red Knots routinely migrate long distances, it appears that there are mechanisms that limit gene flow among populations on the breeding grounds, such as geographic isolation or positive assortative mating.

Since the LSC-KCGL study was initiated in 2009, other unpublished genetic results have been presented by various groups investigating population structure within Red Knots. This information was provided by Anne Hecht, U.S. Fish and Wildlife Service (USFWS), and provides important context for our results. Verkuil and others presented results at the Western Hemisphere Shorebird Group meeting in Peru in November 2017, which indicated that they have developed genetic markers that can separate all six subspecies of Red Knots worldwide and have identified Alaskan and Wrangel Island breeding populations of *C.c. roselaari* as genetically distinguishable (G. Morrison, Environment and Climate Change Canada, written commun., April 2018; Verkuil and others presentation is summarized in Tavera and López [2018]). In addition, unpublished work by Baker and others suggests that a panel of 410 amplified fragment length polymorphism (AFLP) loci can unambiguously identify *C.c. rufa* from Tierra del Fuego (Argentina/Chile), Maranhao (Brazil), and Florida (Allan Baker, Royal Ontario Museum, written commun., January 29, 2013). Clearly, multiple research groups continue to pursue genetic investigations of *C.c. rufa* and *C.c. roselaari*, but there is no apparent coordinated collaboration among laboratories to avoid duplication of effort or share results.

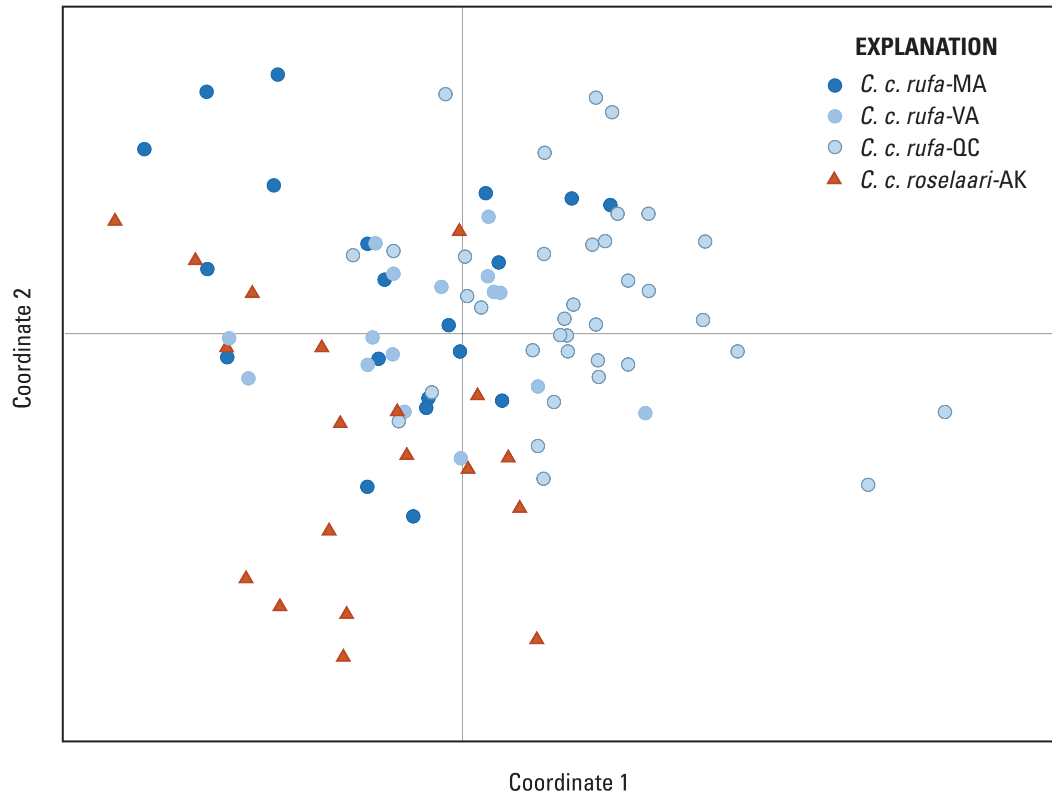

Figure 1. Principal coordinates analysis (PCoA) plot of microsatellite genotypes at 24 loci of *C.c. roselaari* and *C.c. rufa* collections from Massachusetts (MA), Virginia (VA), Alaska (AK), and Quebec (QC) (see table 1).

Ideas for Additional Red Knot Genetic Research

When the USFWS funded the original genetic study at LSC-KCGL in 2009, the agency was interested in three primary objectives, which remain relevant today. Although conference proceedings and unpublished reports suggest there have been significant advancements towards meeting these objectives, these results remain unpublished to date. Here, we discuss each objective individually and offer research recommendations.

1. Determine if there are genetic differences that can be used to reliably distinguish among the *C.c. rufa*, *C.c. roselaari*, and *C.c. islandica* subspecies.

Verkuil and colleagues have apparently developed molecular markers that can separate *C.c. rufa* and *C.c. roselaari* (a presentation by Verkuil and others is summarized in Tavera and López [2018]), whereas our preliminary microsatellite analyses show limited resolution for unambiguous differentiation between collections of *C.c. rufa* and *C.c. roselaari* from Quebec and Alaska, respectively (fig. 1). As the results from Verkuil and others are unpublished, it is currently unknown what molecular markers they are using, and their results cannot readily be tested or confirmed.

Additional data from more nuclear and mitochondrial markers would help assess the level of genetic divergence between *C.c. rufa* and *C.c. roselaari*. We have mitochondrial deoxyribonucleic acid (mtDNA) sequence data from each of these subspecies yet to be analyzed, but only for a limited number of individuals. New genomic techniques such as reduced representation sequencing (for example, genotyping by sequencing [GBS] or restriction site associated sequencing [RAD-Seq]) have become commonly used molecular tools in the last few years and enable the genotyping of thousands of single nucleotide polymorphisms from throughout the entire genome for multiple individuals. This approach has been used successfully to delineate subspecies in many taxa and would be applicable to addressing the subspecific status of *C.c. rufa* and *C.c. roselaari* (Dierickx and others, 2015; Harvey and Brumfield, 2015; Lim and others, 2017). If the DNA of existing samples is of sufficient quantity and quality, GBS libraries of *C.c. rufa* and *C.c. roselaari* could be developed from the current Alaska and Quebec collections to complement the existing microsatellite dataset. This type of genome-wide approach should have the highest resolution among contemporary genomic methods (besides complete genome sequencing) to determine the extent of differentiation between *C.c. rufa* and *C.c. roselaari*.

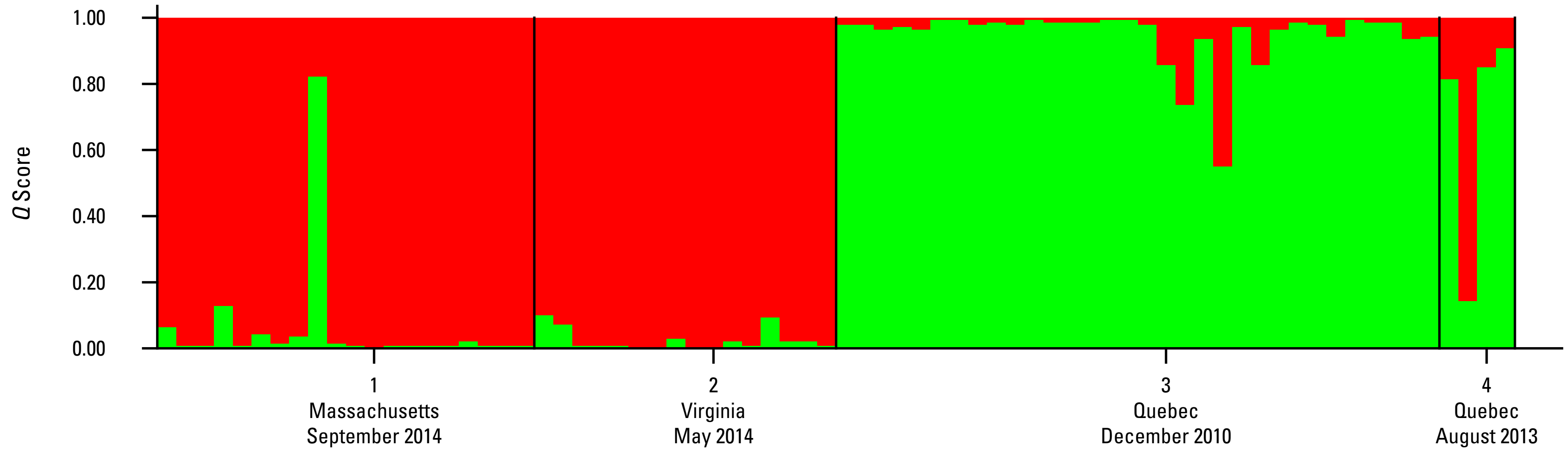

Figure 2. Bayesian clustering results from the program STRUCTURE based on two assumed clusters (k=2). Model runs used 200,000 burn-in iterations followed by 200,000 repetitions, allowed for admixture, and did not incorporate collection location as prior information. Structure Harvester (Earl and vonHoldt, 2012) supported k=2 based on likelihoods and Evanno's Δk methods (Evanno and others, 2005). The colors reflect the admixture proportions for each individual to the two inferred clusters.

2. Characterize the nonbreeding distributions of the *C.c. rufa* and *C.c. roselaari* subspecies.

The sample sizes that were available for *C.c. rufa* and *C.c. roselaari* at wintering areas were exceedingly small (table 1), and more samples would be needed for additional analyses. Microsatellite-based analyses generally benefit from sample sizes of at least 20–30 per population so that allele frequency distributions can be accurately characterized.

Baker and colleagues compared the ability of a panel of 10 microsatellites and ~400 AFLP loci to differentiate nonbreeding *C.c. rufa* from Tierra del Fuego (Argentina/Chile), Maranhao (Brazil), and Florida (Allan Baker, Royal Ontario Museum, written commun., January 29, 2013). Although the microsatellite analyses did allow some individuals to be assigned to their collection of origin, the assignments based on the ~400 AFLP loci were completely unambiguous. Although AFLP and GBS sample loci from throughout the genome, GBS has many advantages over AFLP: GBS can score many more loci reliably than AFLP, the loci are easily scored as codominant in GBS, and GBS costs substantially less per sample.

We do not currently possess enough samples of *C.c. rufa* or *C.c. roselaari* from the wintering grounds to effectively analyze with microsatellites, GBS, or any other technique. Given the results of Baker and others (Allan Baker, Royal Ontario Museum, written commun., January 29, 2013), a genomic approach like GBS may have more resolving power to assess differences among collections if samples become available.

3. Determine if *C.c. rufa* Red Knots from different wintering regions segregate on the breeding grounds (in other words, determine whether there are identifiable genetic differences among *C.c. rufa* Red Knots using four different wintering areas: Argentina/Chile, Brazil, southeastern United States/Caribbean, northwest Gulf of Mexico).

Unpublished work by Baker and others found genetic differentiation among Red Knots from different wintering locations (Allan Baker, Royal Ontario Museum, written commun., January 29, 2013). This indicates that there is population structure (in other words, multiple populations) within *C.c. rufa*, with these populations wintering in different areas (or at least occurring in different proportions at different wintering areas). We used our microsatellite markers to examine population structure among *C.c. rufa* stopover locations and found clear genetic differences among collections in Virginia and Massachusetts in comparison to collections from the Mingan Archipelago (Quebec). To maintain the observed population structure, birds from different populations would either need to be spatially isolated in breeding areas or exhibit some form of assortative mating. Given the difficulty of obtaining samples from the breeding grounds, this type of analysis might best be conducted using a combination of genetics and telemetry.

Currently, we have genotyped a relatively modest number of individuals from three stopover areas. Moving forward, microsatellite markers could be applied to a larger number of individuals from more stopover/overwinter locations to attempt to understand when and where each *C.c. rufa* population occurs, and their relative abundance at different locations. If historical samples were available, those samples could be run to see if the relative abundance or migratory corridors of the populations have changed through time.

Summary

Overall, additional genetic work is needed to address the research needs of the U.S. Fish and Wildlife Service regarding *C.c. rufa* and *C.c. roselaari*. The following opportunities may warrant consideration:

1. Additional microsatellite genotyping of existing *C.c. rufa* and *C.c. roselaari* samples, such as the 411 specimens currently at the USGS LSC-KCGL (table 1). Additional samples from the breeding sites or overwintering sites may improve our understanding of population structure within *C.c. rufa* and *C.c. roselaari*. In particular, the migratory pattern and relative abundance of each *C.c. rufa* population could be inferred if enough samples were available.

2. Genotyping by sequencing (GBS) of *C.c. rufa* and *C.c. roselaari* samples could be used to better understand genetic differentiation between *C.c. rufa* and *C.c. roselaari*, as well as population-level structure within each subspecies. Additional samples from the breeding sites or terminal overwintering sites would be provide additional insight if included in a GBS analysis.

To avoid redundant efforts, it would be beneficial to have a discussion among the broader Red Knot genetics research community to update the unpublished results noted and identify the most effective approaches before initiating any new genetic studies.

References Cited

Atkinson, P.W., Baker, A.J., Bennett, K.A., Clark, N.A., Clark, J.A., Cole, K.B., Dekinga, A., Dey, A., Gillings, S., Gonzalez, P.M., Kalasz, K., Minton, C.D.T., Newton, J., Niles, L.J., Piersma, T., Robinson, R.A., and Sitters, H.P., 2007, Rates of mass gain and energy deposition in red knot on their final spring staging site is both time- and condition-dependent: Journal of Applied Ecology, v. 44, no. 4, p. 885–895, accessed September 2018 at https://doi.org/10.1111/j.1365-2664.2007.01308.x.

Dierickx, E.G., Shultz, A.J., Sato, F., Hiraoka, T., and Edwards, S.V., 2015, Morphological and genomic comparisons of Hawaiian and Japanese Black-footed Albatrosses (*Phoebastria nigripes*) using double digest RADseq—Implications for conservation: Evolutionary Applications, v. 8, no. 7, p. 662–678, accessed September 2018 at https://doi.org/10.1111/eva.12274.

Earl, D.A., and vonHoldt, B.M., 2012, STRUCTURE HARVESTER—A website and program for visualizing STRUCTURE output and implementing the Evanno method: Conservation Genetics Resources, v. 4, no. 2, p. 359–361, accessed September 2018 at https://doi.org/10.1007/s12686-011-9548-7.

Evanno, G., Regnaut, S., and Goudet, J., 2005, Detecting the number of clusters of individuals using the software STRUCTURE—A simulation study: Molecular Ecology, v. 14, no. 8, p. 2611–2620, accessed April 2020 at https://www.ncbi.nlm.nih.gov/pubmed/15969739.

Harvey, M.G., and Brumfield, R.T., 2015, Genomic variation in a widespread neotropical bird (*Xenops minutus*) reveals divergence, population expansion, and gene flow: Molecular Phylogenetics and Evolution, v. 83, p. 305–316, accessed September 2018 at https://doi.org/10.1016/j.ympev.2014.10.023.

Lim, H.C., Gawin, D.F., Shakya, S.B., Harvey, M.G., and Rahman, M.A., 2017, Sundaland's east-west rain forest population structure—Variable manifestations in four polytypic bird species examined using RAD-Seq and plumage analysis: Journal of Biogeography, v. 44, no. 10, p. 2259–2271, accessed September 2018 at https://doi.org/10.1111/jbi.13031.

Morrison, R.I.G., Ross, R.K., and Niles, L.J., 2004, Declines in wintering populations of red knots in southern South America: The Condor, v. 106, no. 1, p. 60–70, accessed September 2018 at https://doi.org/10.1093/condor/106.1.60.

Pritchard, J.K., Stephens, M., and Donnelly, P., 2000, Inference of population structure using multilocus genotype data: Genetics, v. 155, no. 2, p. 945–959, accessed April 2020 at https://www.genetics.org/content/155/2/945.

Tavera, E.A., and López, E.O., 2018, Seventh meeting of the Western Hemisphere Shorebird Group, 10–14 November 2017, Paracas, Peru: Wader Study, v. 125, no. 1, p. 68–76, accessed November 2019 at https://www.waderstudygroup.org/article/10720/.

U.S. Fish and Wildlife Service, 2014, Rufa Red Knot background information and threats assessment—Supplement to endangered and threatened wildlife and plants; final threatened status for the Rufa Red Knot (*Calidris canutus rufa*): U.S. Fish and Wildlife Service web page, 383 p., accessed April 2020 at https://fws.gov/northeast/red-knot/pdf/20141125_REKN_FL_supplemental_doc_FINAL.pdf.

For more information, contact:
Director, Leetown Science Center
U.S. Geological Survey
11649 Leetown Road
Kearneysville, WV 25430

Publishing support provided by the
West Trenton and Pembroke Publishing Service Centers

USGS

Kazyak and others—**Red Knot (*Calidris canutus*) Research—Preliminary Results and Future Opportunities**—OFR 2020–1050

ISSN 2331-1258 (online)
https://doi.org/10.3133/ofr20201050