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
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. roselari*). 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. roselari*, we did find evidence of population substructure within *C.c. rufa*.

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 roselari* samples (hereafter referred to as *C.c. islandica*, *C.c. rufa*, and *C.c. roselari*, 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. roselari* 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. roselari*, 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.

| 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.
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 non-breeding 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., Dekking, 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.
