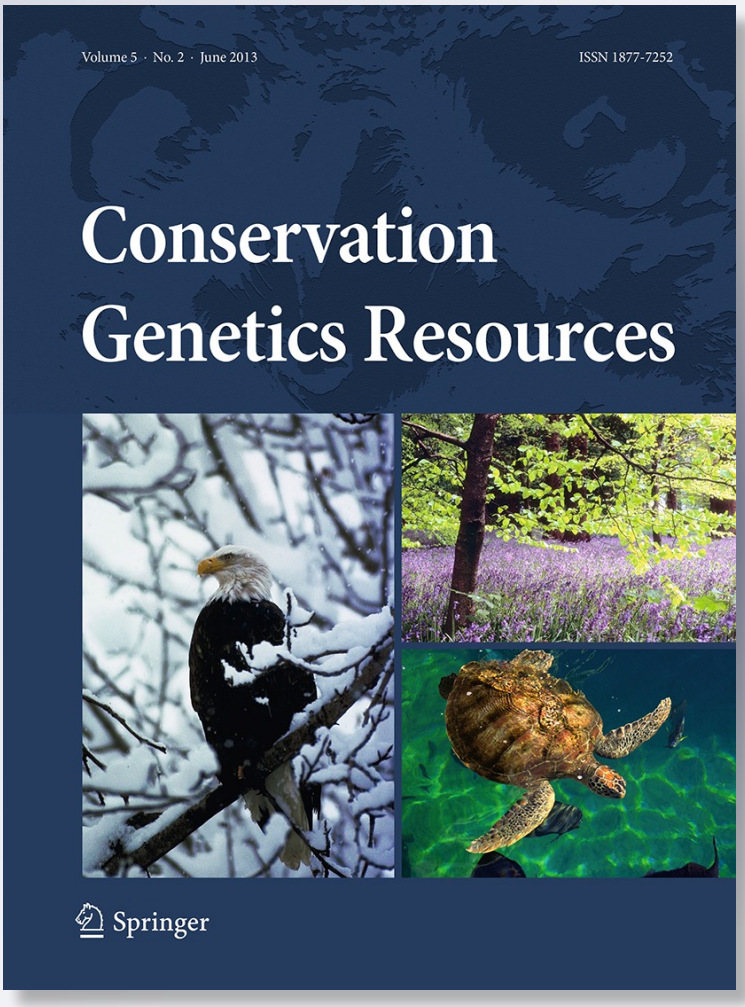
*Development and characterization of thirteen microsatellite loci in Clark's nutcracker (*Nucifraga columbiana*)*

Sara J. Oyler-McCance, Jennifer A. Fike, Todd A. Castoe, Diana F. Tomback, Michael B. Wunder & Taza D. Schaming

Conservation Genetics Resources

ISSN 1877-7252
Volume 5
Number 2

Conservation Genet Resour (2013)
5:303-305
DOI 10.1007/s12686-012-9789-0



Springer

Conservation Genet Resour (2013) 5:303–305
DOI 10.1007/s12686-012-9789-0

TECHNICAL NOTE

Development and characterization of thirteen microsatellite loci in Clark's nutcracker (*Nucifraga columbiana*)

Sara J. Oyler-McCance · Jennifer A. Fike · Todd A. Castoe · Diana F. Tomback · Michael B. Wunder · Taza D. Schaming

Received: 14 September 2012 / Accepted: 28 September 2012 / Published online: 9 October 2012

Abstract Clark's nutcrackers are important seed dispersers for two widely-distributed western North American conifers, whitebark pine and limber pine, which are declining due to outbreaks of mountain pine beetle and white pine blister rust. Because nutcracker seed dispersal services are key to maintaining viable populations of these imperiled pines, knowledge of movement patterns of Clark's nutcrackers helps managers understand local extinction risks for these trees. To investigate population structure within Clark's nutcracker, we developed primers for and characterized 13 polymorphic microsatellite loci. In a screen of 22 individuals from one population, levels of variability ranged from 6 to 15 alleles. No loci were found to be linked, although 4 loci revealed significant departures from Hardy–Weinberg equilibrium and evidence of null alleles. These microsatellite loci will enable population genetic analyses of Clark's nutcrackers, which could provide insights into the spatial relationships between nutcrackers and the trees they help disperse.

Keywords Clark's nutcracker · Microsatellites · *Nucifraga columbiana* · *Pinus albicaulis* · Population genetics · Whitebark pine

S. J. Oyler-McCance (✉) · J. A. Fike
U. S. Geological Survey, Fort Collins Science Center, 2150 Centre Ave, Building C, Fort Collins, CO 80526, USA
e-mail: sara_oyler-mccance@usgs.gov

T. A. Castoe
Department of Biology, University of Texas Arlington, Box 19498, Arlington, TX 76019-0498, USA

D. F. Tomback · M. B. Wunder
Department of Integrative Biology, CB 171, University of Colorado Denver, P.O. Box 173364, Denver, CO 80217, USA

T. D. Schaming
Department of Natural Resources, Cornell University, 122 Bruckner Hall, Ithaca, NY 14853, USA

Clark's nutcrackers (*Nucifraga columbiana*) are food and foraging specialists, depending largely on fresh and stored conifer seeds (Tomback 1978, 1998). They forage preferentially on the seeds of whitebark pine (*Pinus albicaulis*) and limber pine (*P. flexilis*), two tree species adapted to avian seed dispersal but affected by unprecedented outbreaks of mountain pine beetle (*Dendroctonus ponderosae*), the invasive pathogen blister rust (*Cronartium ribicola*), and decades of fire-suppression (Tomback et al. 2001; Tomback and Achuff 2010). Nutcracker population numbers and movements appear to be sensitive to the growing losses of high-elevation whitebark pine forest communities, which occur widely throughout the western U.S. and Canada (McKinney et al. 2009; Barringer et al. 2012).

Clark's nutcrackers are obligate seed dispersal mutualists of whitebark pine and thus are essential to the long-term persistence of this tree species (Tomback 1978; Tomback and Linhart 1990). The U.S. Fish and Wildlife Service determined that whitebark pine warrants listing under the Endangered Species Act (USFWS 2011), and whitebark pine was recently listed as Endangered under the Species at Risk Act in Canada. As whitebark pine cone production declines, the probability of nutcrackers visiting a stand and harvesting seeds also declines (McKinney et al. 2009; Barringer et al. 2012). Therefore, as whitebark seed availability declines across the landscape, nutcrackers may emigrate to other regions, potentially resulting in local extirpation of whitebark pine.

Understanding the spatio-temporal dynamics of Clark's nutcracker populations is now essential for conservation of

Table 1 Characterization of 13 polymorphic microsatellite loci developed for the Clark's nutcracker

Locus	GenBank accession no.	Primer sequence	T_A (°C)	Motif	A	N	Size range	H_E	H_O
CN21	JX843259	F: M13-AGCAGAAAATGTCTGATTGATATGG R: GGAGGAGTCCCTGTCTCCC	60	$ATCT_{(13)}$	6	22	179–203	0.651	0.636
CN22	JX843260	F: M13-CCGTGTTTCTGATTGACAGAGG R: TATCCTGGCTCCCCTCCC	60	$AC_{(21)}$	15	22	125–161	0.929	0.727[a,*]
CN23	JX843261	F: M13-TGTAAATGGCTCCTGTGTGTTAGC R: CTCCTCCATGCCAGAAAACC	60	$ATC_{(14)}$	6	22	148–166	0.538	0.636
CN24	JX843262	F: M13-TGTCTGTAGGGTTGTTTGGGG R: CAGCCCCTCACCAACAGG	60	$ATC_{(16)}$	8	21	106–130	0.782	0.714
CN25	JX843263	F: M13-CCAAAGAAGTAAAATACACTAAGCCG R: AGTGTATTGAGAACATCTGAGAGGC	60	$AC_{(22)}$	8	22	172–190	0.831	0.409[a,*]
CN26	JX843264	F: M13-TTGTTTAAAGGCCTGGTTTGG R: AAACAAGAGAAGCAAGGCTGC	60	$ATGG_{(14)}$	8	22	166–194	0.863	0.864
CN30	JX843265	F: M13-TAGGTCAAAGGGATGGATGG R: TCTGTTCTTTAATCTCCATAGTATATCAGG	60	$ATGG_{(14)}$	9	22	106–138	0.843	0.864
CN31	JX843266	F: M13-AGAACAATTAAGTAAAATCACTTGCC R: CCGTAACTTACCAACTCAATGG	60	$AGT_{(14)}$	8	22	146–170	0.838	0.364[a,*]
CN33	JX843267	F: M13-CAGAAAAGTACAGAGTCACAGACTGC R: CCCCTTATACAAGAAAGTGTATTTAGGC	60	$ATC_{(14)}$	11	22	139–176	0.887	0.409[a,*]
CN35	JX843268	F: M13-GAAATAACCAAACTATGATTTTAGAACCC R: TTTCTCGCGATACAAACATGC	60	$AC_{(20)}$	11	22	119–141	0.844	0.818
CN36	JX843269	F: M13-CCCAAGGTCGGGTTTCCC R: TTCCAGACCTTCCCAGGGC	60	$TCC_{(12)}$	7	21	154–172	0.734	0.762
CN38	JX843270	F: M13-CGGAAAACAAATTTACCCCG R: AGCCTTCCCTTTTCCATGC	60	$ATGG_{(17)}$	12	21	137–181	0.893	0.905
CN39	JX843271	F: M13-GCATAAGAAGGATTTGCACCTGG R: GTTTCGTGCATGCGTGTGG	60	$AC_{(22)}$	10	22	120–144	0.883	0.864

Annealing temperature (T_A), number of alleles (A), number of individuals screened (N), expected (H_E) and observed (H_O) heterozygosities are reported

* Significant deviation from Hardy–Weinberg equilibrium ($\alpha = 0.05$)

[a] Frequency of null alleles >0.05

whitebark pine communities. Through seed dispersal services, nutcrackers connect populations of high elevation pines, influencing distribution and population structure (Tomback 2005; Tomback and Linhart 1990). The current extent of movement by Clark's nutcrackers within their range is unknown; this information could provide a baseline for detecting future changes in nutcracker movements and distribution resulting from declines in whitebark pine ecosystems. To investigate patterns of individual movements and the genetic structure of Clark's nutcracker populations, we identified and designed primers for 13 polymorphic microsatellite loci.

We extracted genomic DNA from muscle tissue of a previously frozen Clark's nutcracker using the Promega Wizard DNA Purification Kit, following the manufacturer's instructions (Promega Corporation). Microsatellites for nutcrackers were identified from a next-generation sequencing run that provided massive numbers of sequences from which to develop microsatellite markers. Sequencing protocol for the Clark's nutcracker is described elsewhere (Castoe et al. 2012). In brief, a genomic shotgun library from a single individual was sequenced on the Illumina GAIIx platform with 120 bp paired-end reads. The Perl script PAL_FINDER_v0.02.03 (Castoe et al. 2012), which uses Primer 3 (Rozen and Skaletsky 2000) for primer design, was used to identify and design primers for potential microsatellite loci. We chose 20 potential microsatellites (including di, tri, and tetra-nucleotide) with an optimum annealing temperature of 60 °C to facilitate multiplexing PCRs. Of the initial 20 microsatellites, 18 amplified consistently and were used for initial screening.

For population screening, we isolated DNA from blood of 22 Clark's nutcrackers sampled from a local population

in Bridger-Teton National Forest, WY, using an ammonium acetate protocol (modified from the PUREGENE kit; Gentra Systems). Thirteen of those 18 microsatellites were highly polymorphic (>5 alleles), amplified well and were consistent and unambiguous for scoring purposes. Screening PCRs were performed in 10 μL reactions containing 0.2 mM of each dNTP, 1X GoTaq Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.03 μM M13-tailed forward primer (following Boutin-Ganache et al. 2001), 0.5 μM non-tailed reverse primer, 0.5 μM M13 dye-labeled primer with either a 6FAM, VIC, NED, or PET label (Applied Biosystems) and 0.5 U of Taq DNA polymerase (Promega). Amplification conditions were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, then 60 °C for 45 min and a final extension at 72 °C for 10 min. The PCR products were run on an AB3500 Genetic Analyzer (Applied Biosystems). All loci were run with 600LIZ size standard (Applied Biosystems) and analyzed using GeneMapper v4.1 (Applied Biosystems).

For each polymorphic locus, we calculated observed heterozygosity (H_O), expected heterozygosity (H_E) and null allele frequencies using CERVUS 1.0 (Marshall et al. 1998). GENEPOP version 3.4 (Raymond and Rousset 2000) was used to test for evidence of linkage disequilibrium and deviations from Hardy–Weinberg equilibrium. The number of alleles per locus ranged from six to 15, and single locus heterozygosities ranged from 0.364 to 0.905 (Table 1). Significant deviations from Hardy–Weinberg equilibrium were observed at 4 loci, and high null allele frequencies were detected at those same 4 loci (Table 1). We found no evidence for genotypic linkage disequilibrium between any set of paired loci after a sequential Bonferroni was applied ($P < 0.0001$). These microsatellite loci will enable researchers to investigate movements and population structure of Clark's nutcrackers, which could provide insights into the relationship between nutcrackers and the tree species they disperse, and provide information for managing declining nutcracker-dependent pine populations.

Acknowledgments This work was funded by a grant to DFT and MBW from the College of Arts and Sciences Research Innovation Seed Program (CRISP), University of Colorado Denver. We thank Alan Kamil (University of Nebraska), Russell Balda, and Russell Benford (Northern Arizona University) for the Clark's Nutcracker specimen. The use of any trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

References

Barringer LE, Tomback DF, Wunder MB, McKinney ST (2012) Whitebark pine stand condition, tree abundance, and cone production as predictors of visitation by Clark's nutcracker. PLoS ONE 7(5):e37663. doi:10.1371/journal.pone.0037663

Boutin-Ganache I, Raposo M, Raymond M, Deschepper CF (2001) M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. Biotechniques 31:25–28

Castoe TA, Poole AW, de Koning APJ, Jones KL, Tomback DF, Oyler-McCance SJ, Fike JA, Lance SL, Streicher JW, Smith EN, Pollock DD (2012) Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. PLoS ONE 7(2):e30953. doi:10.1371/journal.pone.0030953

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655

McKinney ST, Fiedler CE, Tomback DF (2009) Invasive pathogen threatens bird-pine mutualism: implications for sustaining a high-elevation ecosystem. Ecol Appl 19:597–607

Raymond M, Rousset F (2000) GENEPOP version 3.4. Available at http://wbiomed.curtin.edu.au/genepop

Rozen S, Skaletsky HJ (2000) PRIMER 3. In: Krawetz S, Misener S (eds). Bioinformatics methods and protocols: methods in molecular biology, pp. 365–368. Humana Press, New Jersey. URL http://www-genome.wi-mit.edu/genome_software/other/primer3.html

Tomback DF (1978) Foraging strategies of Clark's nutcracker. Living Bird 16:123–161

Tomback DF (1998) Clark's nutcracker (*Nucifraga columbiana*). In: Poole A, Gill F (eds). The birds of North America. No. 331, Philadelphia, PA, USA: The Birds of North America, Inc.

Tomback DF (2005) The impact of seed dispersal by Clark's nutcracker on whitebark pine: multi-scale perspective on a high mountain mutualism. In: Broll G, Keplin G (eds) Mountain ecosystems: studies in Treeline ecology. Springer, Berlin, pp 181–201

Tomback DF, Achuff P (2010) Blister rust and western forest biodiversity: ecology, values and outlook for white pines. Forest Pathol 40:186–225

Tomback DF, Linhart YB (1990) The evolution of bird-dispersed pines. Evol Ecol 4:185–219

Tomback DF, Arno SF, Keane RE (2001) The compelling case for management intervention. In: Tomback DF, Arno SF, Keane RE (eds) Whitebark pine communities: ecology and restoration. Island Press, Washington, D.C., pp 3–25

U. S. Fish and Wildlife Service (2011) Endangered and threatened wildlife and plants; 12-month finding on petition to list *Pinus albicaulis* as endangered or threatened with critical habitat. Fed Reg 76:42631–42654