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Limited geographic genetic structure detected in a widespread Palearctic corvid, *Nucifraga caryocatactes*

The Eurasian or spotted nutcracker (*Nucifraga caryocatactes*) is a widespread resident corvid found throughout the Palearctic from Central Europe to Japan. Characterized by periodic bouts of irruptive dispersal in search of *Pinus* seed crops, this species has potential for high levels of gene flow across its range. Previous analysis of 11 individuals did not find significant range-wide population genetic structure. We investigated population structure using 924 base pairs of mitochondrial DNA control region sequence data from 62 individuals from 12 populations distributed throughout the nutcracker's range. We complemented this analysis by incorporating additional genetic data from previously published sequences. High levels of genetic diversity and limited population genetic structure were detected suggesting that potential barriers to dispersal do not restrict gene flow in nutcrackers.

1 **TITLE:** Limited geographic genetic structure detected in a widespread Palearctic corvid,
2 *Nucifraga caryocatactes*

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9 Introduction

10 In Eurasia, phylogeographic studies of many widespread vertebrate species have revealed a
11 variety of geographical patterns of population structure influenced by current and historical
12 landscapes, with little overall consensus among species. Using mitochondrial DNA, east-west
13 splits have been documented for a variety of vertebrates including bats (Flanders et al. 2009), and
14 several avian species (e.g. Eurasian magpie (*Pica pica*; Kryukov et al. 2004), rook (*Corvus*
15 *frugilegus*; Haring et al. 2007), and red-breasted flycatcher (*Ficedula parva*; Zink et al. 2008)).
16 For other species, multiple splits have occurred (e.g. root vole (*Microtus oeconomus*; Brunhoff et
17 al. 2003) and reed bunting (*Emberiza schoeniclus*; Zink et al. 2008)), or peninsula populations are
18 isolated (e.g. great bustard (*Otis tarda*; Pitra et al. 2000)). In contrast, little population structure
19 has been detected in some widespread species, such as otters (*Lutra lutra*; Ferrando et al. 2004)
20 and several avifauna species (e.g. great spotted woodpecker (*Dendrocopos major*; Zink et al.
21 2002), common sandpiper (*Actitis hypoleucos*; Zink et al. 2008), and Eurasian magpie (*Pica pica*;
22 Zhang et al. 2012)). Some of the observed phylogeographic patterns have been explained by post-
23 glacial colonization from single or multiple refugia, but may also be influenced by barriers to
24 dispersal, such as mountain ranges (e.g. Ural Mountains), large areas of inhospitable habitat (e.g.
25 Tibetan Plateau), or large bodies of water.

26 The Eurasian nutcracker (*Nucifraga caryocatactes*, Linnaeus, 1758) is a corvid with a widespread
27 Palearctic distribution. Although generally classified as a resident species of continental
28 coniferous forests, nutcrackers are known to irruptively disperse to take advantage of mast
29 conifer seed crops (Haring et al. 2007), similar to its North American sister species, Clark's
30 nutcracker (*N. columbiana*; Tomback 1998). Strong geographic genetic structure has not been
31 found in Clark's nutcracker, despite numerous potential physical barriers to dispersal and thus
32 gene flow (Dohms and Burg 2013). A previous study by Haring and colleagues (2007) found no

33 population structure in *N. caryocatactes* throughout Eurasia. However, Haring et al. (2007) only
34 used 11 specimens, thus additional data may shed further light on nutcracker population genetic
35 structure.

36 In this study, we use a highly variable and rapidly evolving mitochondrial DNA marker, the
37 control region, to further investigate population structure of *N. caryocatactes* in Eurasia. Based
38 on the ecology of this species, we predict little range-wide population genetic structure.

39 **Materials & Methods**

40 *Samples*

41 Tissue samples (n = 62) collected throughout the Eurasian nutcracker's range (Fig. 1) were
42 acquired from the Burke Museum of Natural History and Culture at the University of Washington
43 (Supplemental Table S1). Previously published control region (CR) sequences (n = 11) were
44 obtained from GenBank (EU070770 and EU070886-EU070895; Haring et al. 2007).

45 *DNA extraction, PCR amplification, and sequencing*

46 DNA from muscle samples stored in ethanol or lysis buffer was extracted using a modified
47 Chelex extraction protocol (Walsh et al. 1991; Burg & Croxall, 2001). A 924 bp fragment
48 starting at position 46 of the control region (CR; Saunders and Edwards 2000) was amplified
49 using two primers: L46 SJ (5'-TTT GGC TAT GTA TTT CTT TGC-3'; developed for Steller's
50 jay (*Cyanocitta stelleri*; Birt & Lemmen, unpublished)) and H1030 JCR 18 (5'-TAA ATG ATT
51 TGG ACA ATC TAG G-3'; developed for *Aphelocoma* jays (Saunders and Edwards 2000)). DNA
52 was amplified in a Master gradient thermocycler (Eppendorf) in 25 μ L reactions with 1x goTaq
53 Flexi buffer (Promega), 2.5 mM MgCl₂, 200 μ M dNTP, 0.4 μ M of each primer, and 1 unit goTaq

54 Flexi taq polymerase (Promega). DNA sequencing was performed on an ABI 3730xl DNA
55 Analyzer at McGill University and Génome Québec Innovation Centre.

56 *Alignment and analysis*

57 We edited and aligned sequences from chromatograms and an overlapping subset of 305 bp from
58 previously published CR sequences from GenBank (Haring et al. 2007) using MEGA v5.0
59 (Tamura et al. 2007). Two unrooted statistical parsimony networks (95% probability) were
60 constructed with TCS v1.21 (Clement et al. 2000): one for the samples sequenced as part of this
61 study (924 bp) and a second network that for the 305 bp common fragment (this study; Haring et
62 al. 2007). We calculated the number of haplotypes (H_n), haplotype diversity (H_d), and nucleotide
63 diversity (π) for museum samples using DnaSP v5.10 (Rozas et al. 2003).

64 **Results**

65 *Genetic analyses*

66 We sequenced and aligned the 924 bp control region (CR) sequences for 62 individuals from 12
67 populations (Table 1; GenBank accession nos. XXXX-XXXX). We aligned 11 additional
68 GenBank sequences (Haring et al. 2007) with sequences from our samples and obtained a 305 bp
69 area of overlap. Statistical parsimony networks did not suggest strong geographic structure for
70 the 924 bp sequence (Fig. 2), nor for the larger dataset using the overlapping 305 bp fragment for
71 all 73 individuals (Fig. 3). Ncarcar5 could not be connected to the network in the larger dataset,
72 which was also found by Haring et al. (2007).

73 For the 62 individuals we sequenced, we found 45 unique haplotypes and high levels of genetic
74 diversity in most populations (Table 1). We found 57 polymorphic sites within the 924 bp
75 sequence and 22 within the 305 bp sequence. Haplotype diversity for the 924 bp sequence varied

76 from 0.000 (Ola River headwaters (OR)) to 1.000 (five populations), and all but the OR
77 population had a haplotype diversity equal to or greater than 0.800 (Table 1). Nucleotide diversity
78 ranged from 0.00000 (OR) to 0.00993 (PK; Table 1). Overall haplotype diversity (H_d) 0.967 and
79 nucleotide diversity (π) was 0.00537.

80 Discussion

81 As predicted, analyses of Eurasian nutcracker mitochondrial DNA control region sequences did
82 not detect significant population genetic structure. All populations except nutcrackers from the
83 Ola River headwaters (OR; $n = 2$) exhibited high haplotype diversity and relatively high
84 nucleotide diversity. No geographic clustering was observed in statistical parsimony networks,
85 even when integrating samples from the western part of the range. Despite potential barriers to
86 dispersal for this species, such as isolation on an island (e.g. Sakhalin Oblast (SO)) or peninsula
87 (e.g. Kamtchatka (KA)), most populations of *N. caryocatactes* do not appear to be geographically
88 differentiated from each other, likely due to gene flow during irruptive dispersal in search of mast
89 pine seed crops. Overall, our work supports that done by Haring et al. (2007) where no significant
90 split was seen between the east and west for nutcrackers and is similar to the pattern found in *N.*
91 *caryocatactes* sister species, *N. columbiana* (Dohms and Burg 2013).

92 Compared to Haring et al. (2007), our study found a higher level of haplotype diversity ($H_d =$
93 0.967 vs 0.844 and $\pi = 0.00537$ vs 0.00279). This may be due to the portion of control region
94 sequenced and the larger sample sizes used in this study. The sequences obtained from our
95 samples are predominantly composed of domains I and II of the mtDNA control region (Saunders
96 and Edwards 2000), whereas Haring et al. (2007) sequenced primarily domain II, which is
97 considered less variable (Ruokonen and Kvist 2002).

Haring et al. (2007) state that low genetic diversity may suggest a bottleneck in this species and a single glacial refugium. With high levels of nucleotide and haplotype diversity, our findings do not support a historical bottleneck but rather point toward two possible scenarios: multiple refugia with gene flow or a single refugium with large population size during expansion. Expansion from multiple refugia with gene flow after colonization can produce similar genetic patterns to those in species that expanded slowly from a single refugium with a large population size, retaining high genetic diversity and limited geographic structuring of populations (Hewitt 2004). Given the dispersal potential of nutcrackers, it is possible that a large population expanded out of a single refugium, but it is equally plausible that expansion occurred out of multiple refugia with subsequent gene flow between geographically distinct populations due to irruptive dispersal events. The multiple refugia scenario could have produced the large number of haplotypes, often with high levels of sequence divergence pattern seen here. For example, individuals from Primorsky Krai (PK) are found scattered throughout the parsimony network (Fig. 2), in some cases with a large number of mutations between PK individuals and other haplotypes, yet found clustered with geographically distant individuals from Irkutsk Oblast (KD) and KA. This level of divergence is often associated with isolation in and subsequent colonization from multiple refugia (Hewitt 2004). With the high haplotype diversity across all populations, it is not possible to determine which population(s), if any sampled here, may be in the location of the original refugium or refugia. Without additional present day samples from the Alps and Himalaya Mountain ranges, it is difficult to tell using genetic signatures if these areas served as refugia for nutcrackers during the LGM.

Our findings do not support a single refugium in the Altai Mountains of southern Mongolia, as postulated by Haring et al. (2007). Rather, our data show highly divergent haplotypes which could be the result of prolonged isolation in multiple refugia. Scots pine (*Pinus sylvestris*), an

123 important source of food for nutcrackers, is thought to have survived in refugia near the Alps
124 (Naydenov et al. 2007) and in the east, unglaciated portions of the Himalayas could have served
125 as a refugium for high latitude species (Zhuo et al. 1998; Owen et al. 2002). Alternatively, high
126 levels of plant endemism have been found in the mountains of southern and eastern China,
127 suggestive of long-term suitable habitats (Zhuo et al. 1998). Nutcrackers may have survived in
128 these bands of suitable habitat in the southwest and southeast areas of Eurasia and expanded
129 northward from multiple refugia as glaciers retreated.

130 **Conclusions**

131 Overall, Eurasian nutcrackers exhibit limited geographic genetic structure throughout their range,
132 as might be expected from a resident bird with irruptive dispersal patterns. Our study found high
133 genetic diversity, which suggests that a population bottleneck has not occurred in this species as
134 previously hypothesized. A more detailed phylogeographical study could include additional
135 genetic sampling from northern and southern parts of *N. caryocatactes*' range to further
136 investigate structure across the range of this species.

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139 History and Culture – University of Washington; without tissue samples from their collection,
140 this study would not have been possible.

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Figure 1

Nutcracker tissue sample locations throughout Eurasia

Black squares denote locations; corresponding abbreviations are labelled beside squares. Refer to Table 1 and Supplementary Table S1 for further location information. Black diamonds denote locations of previously published partial control region sequences obtained from GenBank with corresponding sample codes from Haring et al. (2007).

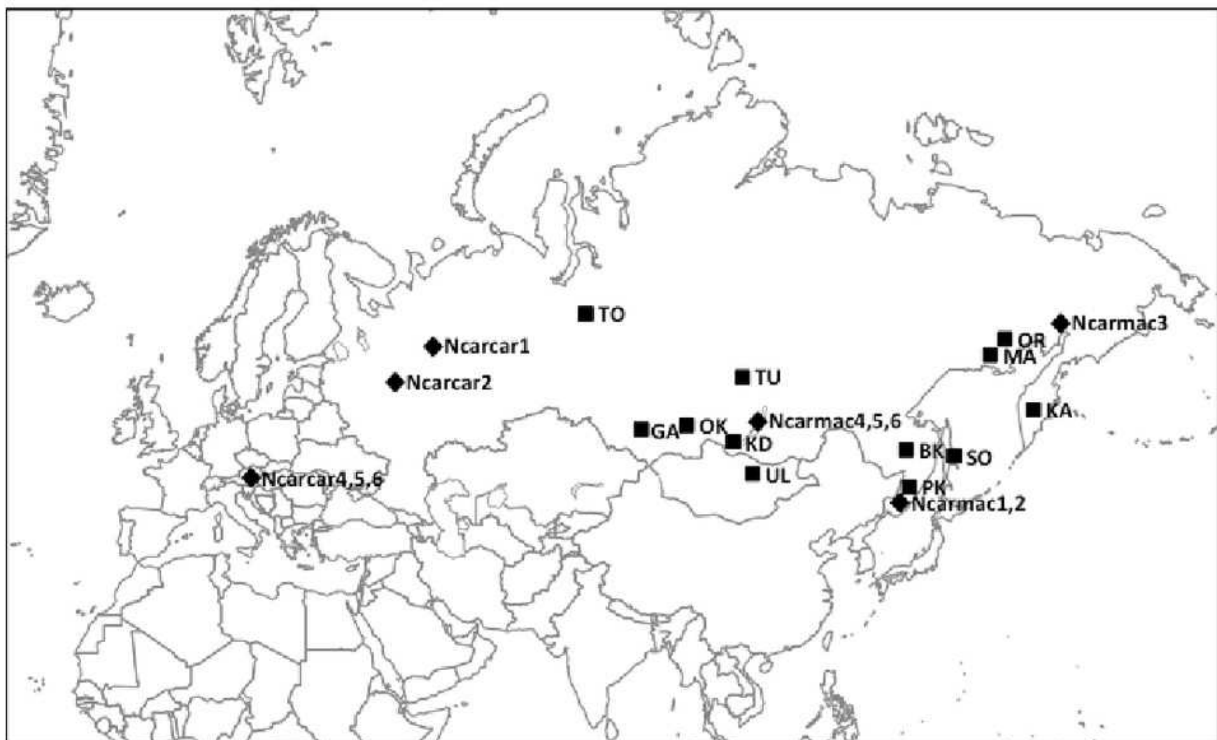


Figure 2

Statistical parsimony network for 924 bp mitochondrial DNA sequence

Statistical parsimony network of *Nucifraga caryocatactes* for 924 bp of the mitochondrial DNA control region sequenced from museum samples (n = 62). Each square represents one individual and colours correspond to author-defined populations, as per figure legend. Circles indicate inferred haplotypes. Refer to Table 1 for population abbreviations.

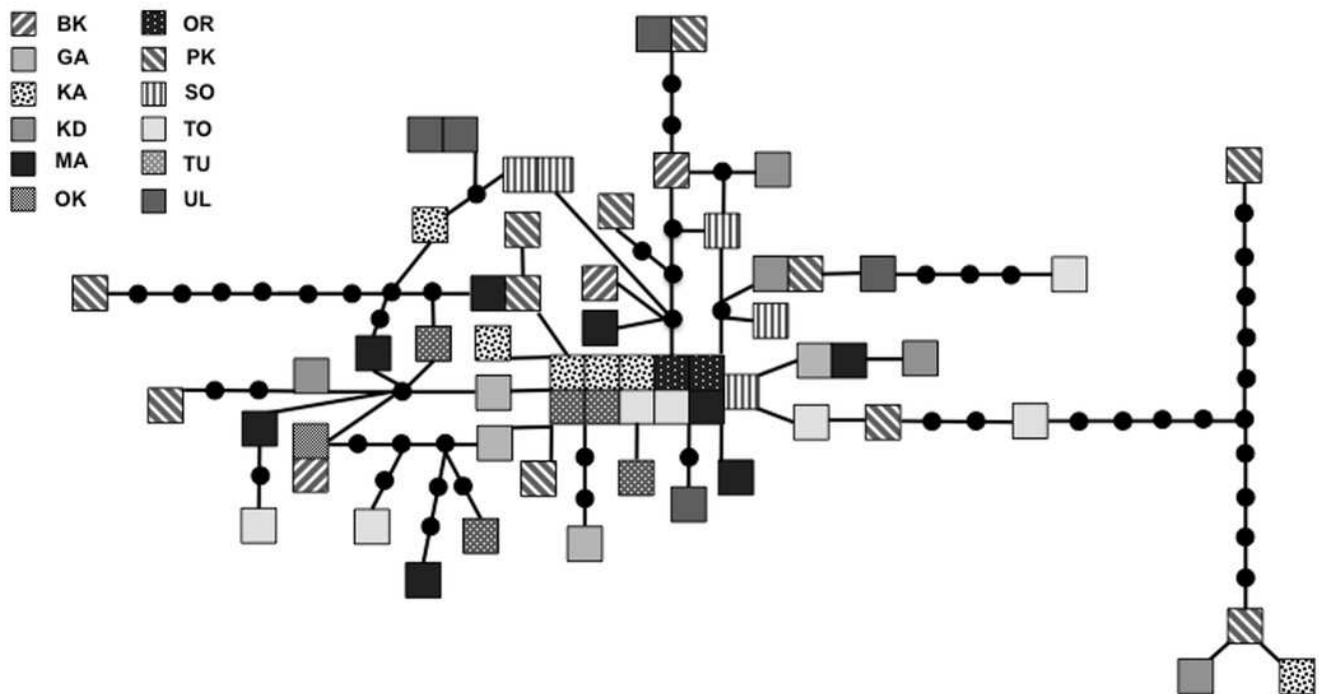


Figure 3

Statistical parsimony network of 305 bp mitochondrial DNA sequence

Statistical parsimony network of *Nucifraga caryocatactes* for overlapping sequences of 305 bp of the mitochondrial control region (Domain II) sequenced from museum samples (n = 62) and GenBank sequences (n = 11; Haring et al., 2007). Each coloured square represents one individual and colours correspond to author-defined populations. Black solid circles indicate inferred haplotypes. Open circles represent haplotypes; text in circles represents GenBank sequences as per Fig. 1 and Haring et al. (2007). Refer to Table 1 for population abbreviations found in the legend.

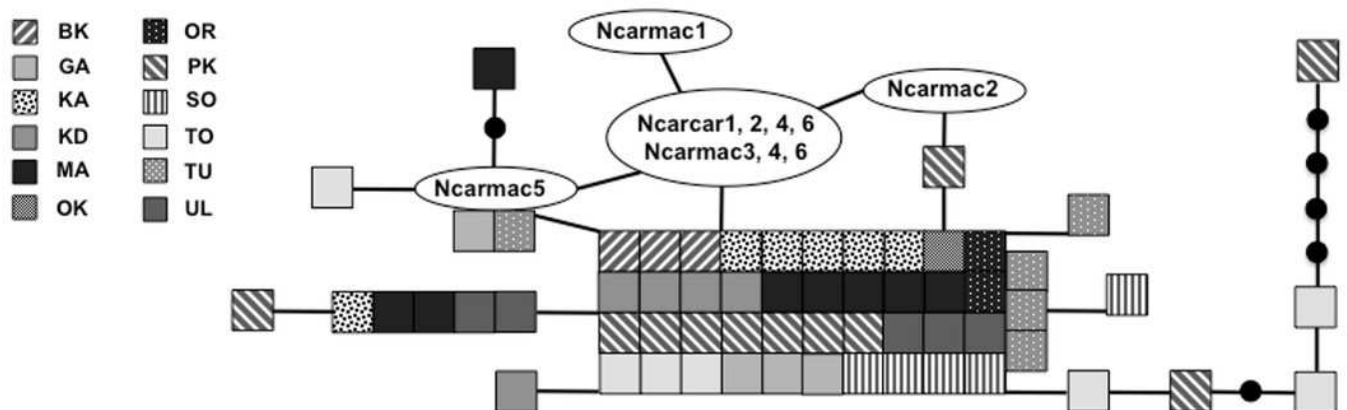


Table 1 (on next page)

Diversity of a 924 bp mitochondrial DNA sequence

Haplotype diversity for a 924 bp fragment of mtDNA control region from 12 populations of *Nucifraga caryocatactes* throughout Eurasia. n = number of individuals in population, H_n = number of haplotypes, H_d = haplotype diversity, and π = nucleotide diversity within the population.

Population	Location	n	H_n	H_d	π
BK	Badzhal'skiy Kreet, Russia	3	3	1.000	0.00361
GA	Gorno-Altaysk, Russia	4	4	1.000	0.00328
KA	Kamchatka, Russia	6	4	0.800	0.00369
KD	Irkutsk Oblast, Russia	5	5	1.000	0.00523
MA	Magadanskaya Oblast, Russia	8	8	1.000	0.00397
OK	Kyzyl, Russia	1	1	-	-
OR	Ola River headwaters, Russia	2	1	0.000	0.00000
PK	Primorsky Kray, Russia	11	11	1.000	0.00993
SO	Sakhalinksya Oblast, Russia	5	4	0.900	0.00195
TO	Tyumenskaya Oblast, Russia	7	6	0.952	0.00622
TU	Irkutsk Oblast, Russia	5	4	0.900	0.00326
UL	Ulaanbaatar, Mongolia	5	4	0.900	0.00611
Overall		62	45	0.967	0.00537