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ANIMAL GENETICS

Gene Pool Homogeneity of Western and Eastern Populations of the White-Naped Crane *Antigone vipio* in Different Flyways

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Abstract—The article presents the first data on the genetic structure of the White-naped crane *Antigone vipio*, a rare migratory bird species of Northeast Asia. Based on the analysis of seven polymorphic microsatellite loci and full-lengh mitochondrial DNA Control Region sequencing (1132 bp), the genetic homogeneity of spatially separated western and eastern populations was established. The found high levels of observed (H_O = 0.696 ± 0.033) and expected (H_E = 0.707 ± 0.037) heterozygosity and haplotype diversity (Hd = 0.973) of the White-naped crane were comparable to these parameters in wide-range crane species with a large population sizes. Lack of genetic differentiation by microsatellite loci (F_{ST} = 0.013, P = 0.369), the low level of genetic differences by the Control Region (F_{ST} = 0.041, P = 0.05), and generally low level of intraspecific spatial structuring in the White-naped crane by haplotypes and individual multilocus genotypes may be due to the absence of reproductive isolation between individuals from different populations and changes in the migration routes of immature birds.

Keywords: Gruidae, genetic variation, genetic differentiation, microsatellite loci, Control Region, Russia
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INTRODUCTION

White-naped crane *Antigone vipio* Pallas, 1881 is a globally rare bird species listed as vulnerable on the Red List of the International Union for Conservation of Nature [1]. The breeding part of its range is confined to wetland biotopes of the steppe and forest-steppe zones in the Amur River basin in Russia, Mongolia, and China [2, 3]. In the species structure of the White-naped crane, western and eastern populations are distinguished. They use different breeding and wintering sites and, accordingly, choose different flyways. Birds of the western population breed in Russia (in the southeast of Zabaykalsky krai) and Mongolia and partly in the adjacent territories of China, and winter in China on Lake Poyang. Birds from the eastern population breed in the Russian Far East (in the south of Amur oblast, the Jewish Autonomous Oblast, Khabarovsk krai, and Primorye) and in Northeast China, while winter in Japan and the demilitarized zone of the Korean Peninsula [3] (Fig. 1).

The global abundance of the White-naped crane is estimated at 7000–7800 individuals [3]. The western population is estimated as 500–1000 birds; it has decreased fourfold over the past 20 years, while in the eastern population there are 6200–6500 individuals and its number continues to increase [3, 4]. Such changes can be explained both by a decrease in the level of reproduction of the western population due to long-term drought and by the probable movement of White-naped cranes to the eastern part of the range and from there to the eastern wintering grounds, which have become more attractive owing to the artificial feeding practiced there. Trends within the breeding ranges of the populations in Russia over the past 20–25 years are also multidirectional: the habitats and number of the western population have drastically decreased, while those of the eastern population have increased significantly. The total current abundance of the White-naped crane in Russia is about 610 territorial pairs (90 in the western population, and 520 in the

Fig. 1. Range of the White-naped crane [3] and origin locations of the studied birds: (*1*) western population; (*2*) eastern population (north); (*3*) eastern population (south).

eastern population), and taking into account non-breeding birds, there are about 2200 individuals [5].

Geographical separation of two populations of *A. vipio* can contribute to their genetic differentiation, just as the western grouping decline and the eastern grouping expansion can be reflected in their genetic diversity. Never before have population genetic studies on the species structure of the White-naped crane been carried out in any of the countries that it inhabits. Our study presents the first data on the genetic structure of this species from the territory of Russia.

The aim of this work is to analyze the genetic variability and differentiation of the western and eastern populations of the White-naped crane in Russia on the basis of the data of nuclear microsatellite loci and sequencing of the Control Region of mitochondrial DNA.

MATERIALS AND METHODS

Biological Material

Individual samples from 51 White-naped cranes from the breeding sites of the western (20 individuals)

Table 1. Characterization of microsatellite loci in the White-naped crane

Locus	Motif	*A*	Alleles	H_O	H_E	Hardy–Weinberg equilibrium
Gram22	$(AAAC)_9$	6	160, 164, 168, 172, 176, 180	0.627	0.753	ns
Gram30	$(AAGG)_7$	10	158, 162, 166, 170, 174, 178, 182, 186, 190, 194	0.745	0.796	ns
Gpa12	$(GATA)_{11}$	8	202, 210, 214, 218, 222, 226, 230, 234	0.843	0.841	ns
Gpa38	$(CTAT)_{13}$	5	186, 190, 194, 198, 202	0.706	0.736	ns
Gpa39	$(GA)_2$ $(GATA)_{13}$	7	100, 108, 112, 116, 120, 124, 128	0.804	0.741	ns
Gj4066	$(ATAG)_9$	5	141, 145, 149, 153, 157	0.608	0.736	*(0.026)
Gj2298	$(CTG)_{13}$	2	142, 145	0.510	0.398	*(0.046)

A is the number of alleles; H_O is the observed heterozygosity; H_E is the expected heterozygosity; 'ns' stands for non-significant deviation from the Hardy–Weinberg equilibrium. * Reliability at $P < 0.05$.

and eastern (31 individuals) populations were used as biological material (Fig. 1). In the western population, samples (plucked cover feathers from the plumage of the breast) were obtained from chicks 1–1.5 months of age during their banding with rings and transmitters in Onon, Aginsky, and Borzinsky districts of Transbaikalia (permits of Rosprirodnadzor no. 50 dated June 30, 2015; no. 44 dated April 27, 2016; and no. 7 dated February 7, 2019), as well as from adult birds originating from the nature of the southeast of Transbaikalia and kept in Oka Crane Breeding Center of Oka State Nature Biosphere Reserve (blood taken in course of scheduled medical examinations). We also used molting feathers from the breeding grounds of the White-naped crane in Transbaikalia. In the eastern population, molting feathers and egg-shell membranes (allantois) from breeding grounds of *A. vipio* in Tambov district of Amur oblast served as a source for DNA isolation as well as confiscated poached specimens from Spassky district of the Primorsky krai (muscle tissue) and blood obtained during planned medical examinations of birds from the wild (Arkharinsky district of Amur oblast) kept at Reintroduction Station of Rare Birds of Khingan State Nature Reserve and Oka Crane Breeding Center.

DNA Isolation

Extraction of DNA from molting feathers was carried out using the innuPREP Forensic Kit (Analytik Jena, Germany) and from plucked feathers and blood using the DIAtom™ DNAPrep100 Kit (Isogen Laboratory Ltd., Russia) according to the protocols of the manufacturers. The total genomic DNA was diluted to a concentration of 50–100 ng/μL (Qubit 2.0, Thermo Fisher Scientific, United States) and used for subsequent amplification of microsatellite fragments of nuclear DNA and the Control Region of mitochondrial DNA (mtDNA) by PCR.

Microsatellite Analysis

Since no specific nuclear microsatellite loci have been developed for the White-naped crane, we tested more than 20 primer pairs for microsatellites characterized in other crane species. As a result, we selected seven loci suitable for our purposes and polymorphic in *A. vipio*: *Gram22*, *Gram30* [6], *Gpa12*, *Gpa38*, *Gpa39* [7], and *Gj4066*, *Gj2298* [8] (Table 1). PCR was performed using the GenPak PCR Core reagent kit (Isogen Laboratory Ltd.) according to the manufacturer's recommendations for primer annealing modes from original sources [6–8] on a Dyad Peltier Thermal Cycler (Bio-Rad, United States). To determine the lengths of the amplified fragments, vertical electrophoresis was performed in a 6% polyacrylamide gel in a Tris-EDTA-borate buffer system using VE-20 chambers (Helicon Company Ltd., Russia). PCR products were stained with ethidium bromide on gel and visualized with UV light in a Kodak Edas 290 (Eastman Kodak Company, United States) gel registration system. The sizes of the obtained DNA fragments (alleles of microsatellite loci) were determined using the GelAnalyzer 19.1 software [9].

Sequencing of the mtDNA Control Region

Amplification of the full-lengh 1132 bp Control Region was performed using forward LC16575 (5'-ACA AAA GAA ACC CCC AAA CTC A-3') and reverse HC01342 (5'-AAG AAT TCT GCG GAT ACT TGC ATG T-3') [10] primers according to the following protocol: primary denaturation for 3 min at 94°C; then 29 amplification cycles, including denaturation at 94°C for 45 s, primer annealing at 58°C for 1 min, and elongation at 72°C for 1 min; and final elongation 10 min at 72°C. As in the case of microsatellite loci, PCR of the control region was performed using GenPak PCR Core reagents. Amplification fragments were checked by electrophoresis in 1.5% agarose gel

Table 2. Genetic diversity of the western and eastern populations of the White-naped crane by microsatellite loci

Population	N_A	H_O	H_E	uH_E	F_{IS}	F_{ST}
Western	5.4	0.714 ± 0.030	0.710 ± 0.044	0.728 ± 0.045	–0.029	
Eastern	6.1	0.677 ± 0.010	0.705 ± 0.064	0.716 ± 0.065	0.017	
Average	5.8	0.696 ± 0.033	0.707 ± 0.037	0.722 ± 0.030	–0.006	0.013 ± 0.004

N_A is the number of alleles per locus; H_O is the observed heterozygosity; H_E is the expected heterozygosity; uH_E is the unbiased expected heterozygosity; F_{IS} is the intrapopulation coefficient of inbreeding; F_{ST} is the interpopulation coefficient of inbreeding.

and sequenced in the forward and reverse directions on an ABI 3130 genetic analyzer (Applied Biosystems, United States) at Evrogen Joint Stock Company (Moscow, Russia). The nucleotide sequences of the identified haplotypes were submitted to GenBank under the accession numbers MN929113–MN929130.

Statistical Analysis

Calculations of parameters of intrapopulation diversity, correspondence of the distribution of genotypes of microsatellite loci according to the Hardy–Weinberg equilibrium, *F*-statistics of interpopulation genetic differentiation, gene flow (N_m) and analysis of molecular variance (AMOVA) were performed using the MS Excel–GenAlEx 6.5 add-in [11]. To identify the population structure, we used the Bayesian clustering algorithm in STRUCTURE 2.3.4 software [12]. An estimate of the probable number of genetic clusters *K* according to Evanno method, and structuring and visualization of the results were carried out in CLUMPAK pipeline [13]. For each expected *K* value (ranging from 2 to 4), we performed five independent iterations with simulation length 100000 and a burn-in 10000. We used the options of population data setting LOCPRIOR = 1, an admixture model, and independent allele frequencies between samples. For clustering of individual multilocus genotypes by Principal Component Analysis (PCA), the adegenet [14] and ggplot2 [15] packages in the RStudio 2021.09.1.372 [16] based on R 4.1.2 environment [17] were used. Alignment, editing, and assembly of the sequences after forward and reverse readings were performed against each other and a single reference available from the GenBank (FJ769852) using the MAFFT algorithm [18] in Geneious 9.1.8 [19]. Haplotype and nucleotide diversity, pairwise comparisons of nucleotide differences, Tajima's and Fu and Li's selective neutrality tests, number of migrants per generation (N_m), and coefficient of genetic differentiation F_{ST} were calculated using the DnaSP 6.11.01 software [20]. The haplotype network was constructed using the TCS algorithm [21] in PopART 1.7 [22].

RESULTS

Genetic Variation and Differentiation by Microsatellite Loci

In the total sample (51 individuals) of White-naped cranes at seven polymorphic loci, from two (*Gj2298*) to ten (*Gram-30*) alleles were identified (Table 1). The total number of alleles was 43. At five loci genotype distributions were in an Hardy–Weinberg equilibrium, while deviations were observed for two loci, significant at a probability level of less than 0.05%, toward a deficiency (*Gj4066*) and excess (*Gj2298*) of heterozygotes. The values of observed (H_O) and expected (H_E) heterozygosity ranged from the smallest by locus *Gj2298* ($H_O = 0.510$, $H_E = 0.398$) to the largest by locus *Gpa12* ($H_O = 0.843$, $H_E = 0.841$).

The average number of alleles per locus for both populations was 5.8, and the average levels of heterozygosity were high: $H_O = 0.696 \pm 0.033$, $H_E = 0.707 \pm 0.037$. Unbiased estimates of expected heterozygosity (uH_E) in both populations were similar (Table 2). Out of 43 alleles, 38 turned out to be common for the western and eastern populations; private alleles were found only in the eastern population (*Gram30*^{190}, *Gram30*^{194}, *Gpa12*^{202}, *Gpa39*^{100}, and *Gpa39*^{108}).

The values of intrapopulation inbreeding coefficient F_{IS} indicated a slight excess of heterozygotes in the western population (2.9%) and a slight deficiency of heterozygotes in the eastern population (1.7%); however, in general, this index for the species was close to zero ($F_{IS} = -0.006$). The interpopulation genetic differentiation of the White-naped crane by microsatellite loci was low and did not significantly differ from zero ($F_{ST} = 0.013$, $P = 0.369$; unbiased estimate $G_{ST} = 0.001$, $P = 0.359$) (Table 2). According to AMOVA, 96% of the genetic variability of the species was found within individuals and 4% among them. Gene flow (N_m) between western and eastern populations of *A. vipio* measured at 29.09 migrants per generation.

Structuring of Populations by Microsatellite Loci

Results of Bayesian clustering in STRUCTURE software for the most probable values of genetic clusters *K* ranging from two to four at optimal $K = 3$ revealed no differences in the population structure of

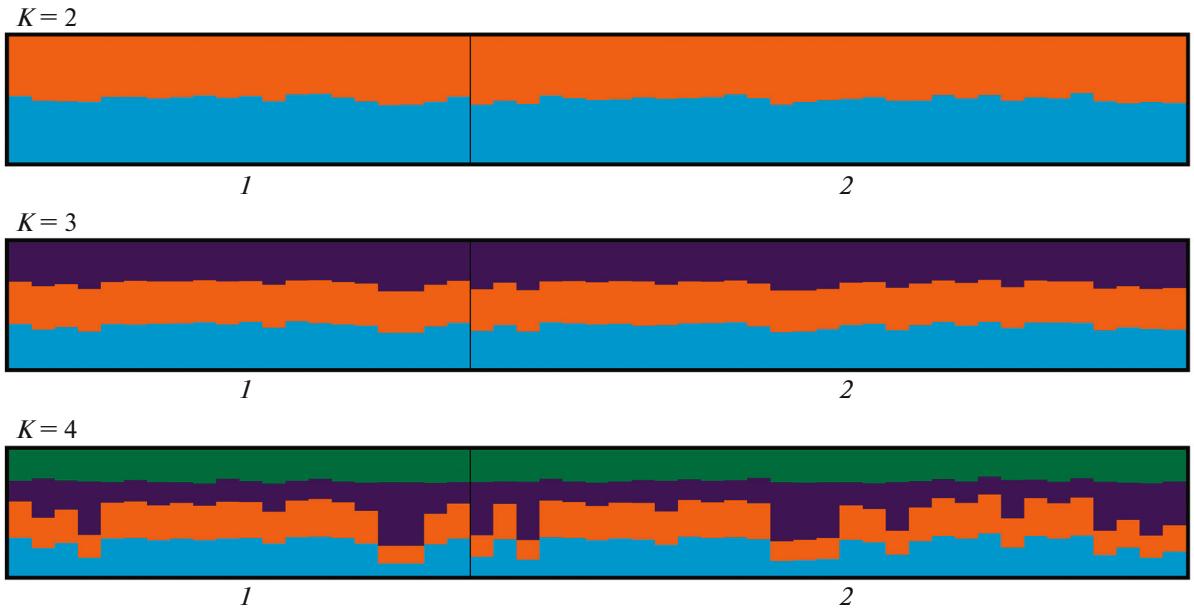

Fig. 2. Clustering of individual haplotypes of the western (*1*) and eastern (*2*) populations of the White-naped crane by microsatellite loci in STRUCTURE for the most probable values of genetic clusters from $K = 2$ to $K = 4$ at optimal $K = 3$.

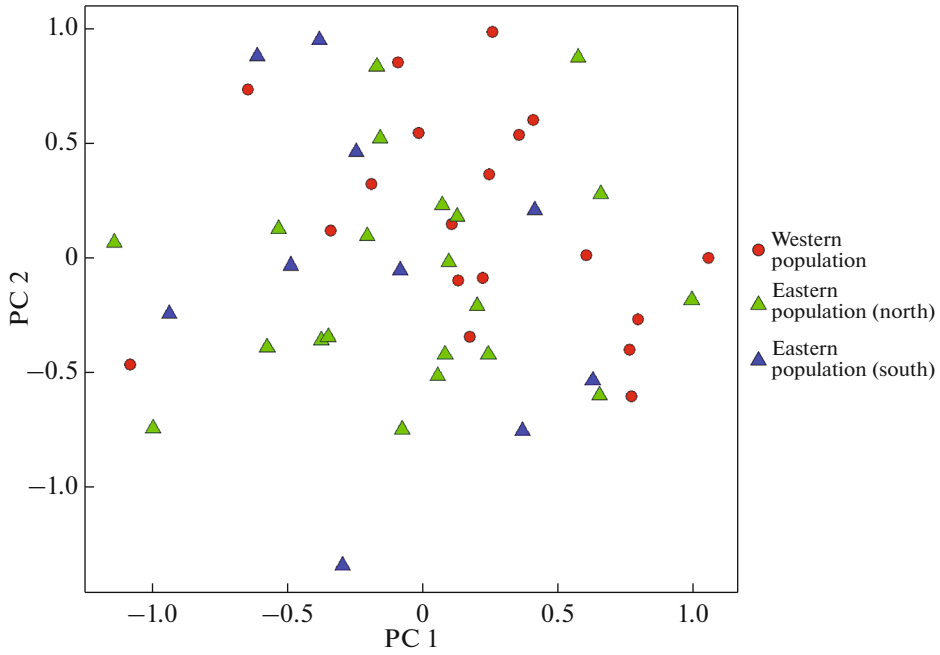

Fig. 3. Distribution of individual multilocus genotypes of the White-naped crane by microsatellite loci from the western population and north and south of the eastern population in the principal component space (PCA).

the western and eastern populations (Fig. 2). In fact, the data of Bayesian analysis showed the existence of a single population of the species on the territory of Russia. The same homogeneous genetic structure of the White-naped crane was revealed by PCA analysis. Since birds from the northern and southern parts of the eastern population used the same flyway, but were geographically separated (Fig. 1), we divided them into separate groups for PCA clustering. However, this also did not affect the spatial distribution of genotypes: the set of points denoting the multilocus individual genotypes of the western, northern, and southern eastern groups of White-naped cranes overlaps on the PC1–PC2 two-dimensional plot (Fig. 3).

Table 3. Distribution of 18 Control Region haplotypes in populations of the White-naped crane

Haplotype	GenBank accession number	Population		Total individuals
		western	eastern	
h1	MN929113	1		1
h2	MN929114	2		2
h3	MN929115	2		2
h4	MN929116		1	1
h5	MN929117		1	1
h6	MN929118	1	2	3
h7	MN929119		1	1
h8	MN929120		2	2
h9	MN929121		1	1
h10	MN929122		1	1
h11	MN929123	1		1
h12	MN929124	1		1
h13	MN929125		1	1
h14	MN929126		2	2
h15	MN929127		1	1
h16	MN929128	1		1
h17	MN929129	1		1
h18	MN929130		2	2
		10	15	25

Table 4. Estimates of the mtDNA Control Region variability and values of selective neutrality tests in populations of the White-naped crane

Population	*Hd*	π	*k*	*D*	*F*
Western	0.956 ± 0.059	0.00635 ± 0.00065	7.178	–0.36410 ns	–0.46458 ns
Eastern	0.962 ± 0.034	0.00655 ± 0.00659	7.410	–0.30614 ns	–0.00950 ns
Average	0.973 ± 0.018	0.00662 ± 0.00064	7.487	–0.81269 ns	–0.8890 ns

Hd is the haplotype diversity; π is the nucleotide diversity; *k* is the average number of nucleotide differences; Tajima's (*D*) and Fu and Li's (*F*) selective neutrality tests; 'ns' stands for non-significant values.

Genetic Diversity of mtDNA Control Region Haplotypes

The analysis included nucleotide sequences of the full-length Control Region (1132 bp) of 10 and 15 individuals from the western and eastern populations of the White-naped crane, respectively. Among these 25 individuals, 18 haplotypes were identified (Table 3). Only one of them (h6) was shared, while the others were found either in one or in the other population. In total, eight haplotypes belonged to the western population, and 11 belonged to the eastern population.

Both populations were characterized by high haplotype and low nucleotide diversity. The average values of these parameters were, respectively, $Hd = 0.973 \pm 0.018$ and $\pi = 0.00662 \pm 0.00064$ (Table 4), which indicates a high similarity between the haplotypes of the White-naped crane. The average number of nucleotide substitutions in haplotypes (*k*) was equal to 7.487. The distribution of nucleotide differences in pairwise comparisons of haplotypes corresponded to the population expansion model (Fig. 4); however, both Tajima's ($D = -0.81269$) and Fu and Li's ($F = -0.8890$) selective neutrality tests were not significant. The level of genetic differentiation of the eastern and western populations bythe Control Region exceeded that for microsatellite loci and significantly differed from zero ($F_{ST} = 0.041$, $P = 0.05$), and the number of migrants per generation according to mtDNA, respectively, was lower: $N_m = 5.83$. According to the AMOVA data, 97.4% of the genetic variability of the White-naped cranes was found within individuals and 2.6% was among them.

The TCS-haplotype network demonstrated a ring structure of western and eastern variants without the formation of any geographic clusters (Fig. 5). The only

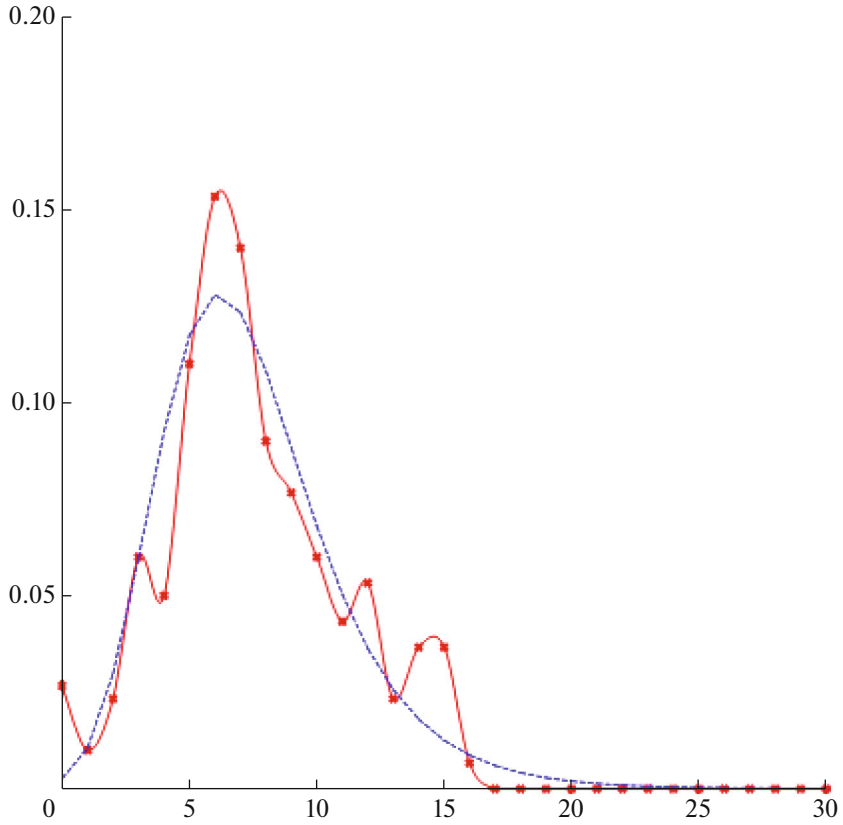

Fig. 4. Mismatch distribution of the White-naped crane populations under the population expansion model. The *x* axis shows the number of nucleotide substitutions in pairwise comparison of haplotypes, and the *y* axis shows the hyplotype frequency. The dotted line corresponds to the expected distribution; the solid line corresponds to the observed one.

northeastern h4 haplotype formed a node associated both with other eastern northern, eastern southern and western variants.

DISCUSSION

Our data on the genetic structure of two populations of the White-naped crane using different breeding and wintering sites and, accordingly, different migration routes, indicated the homogeneity of the gene pool of this rare bird species. Both the declining western and expanding eastern populations were characterized by a high level of genetic diversity by nuclear microsatellite loci and mtDNA Control Region, comparable to that of the wide-range crane species with large population size—the Sandhill crane *Antigone canadensis* [23], the Eurasian crane *Grus grus* [24], and the Demoiselle crane *Anthropoides virgo* [25, 26], but exceeding this value in the rare Whooping *Grus americana* [6, 27] and Red-crowned *G. japonensis* [28] cranes.

Low genetic differentiation, the absence of a clearly defined population structure, and a rather intense gene flow between the western and eastern groupings of the White-naped crane indicated the absence of reproductive isolation between populations using different flyways. The use of remote tracking methods made it possible to identify a case of a change in wintering places by a female of the White-naped crane from the western group: it spent her first winter in the traditional place on Lake Poyang in China (2016) but it wintered in the grounds of the eastern population in Korea and Japan in 2017–2020; however, it did not change the breeding site in Southeastern Transbaikalia from year to year (https://savingcranes.org/the-journey-of-white-naped-crane-borzya). This female formed a mating pair at the wintering grounds of the eastern population; so its partner, who probably belonged to the eastern population, changed the breeding site. There are other reliable cases of the change of wintering grounds and migration routes by the White-naped cranes. Most likely, changes in routes are more appropriate to immature individuals during their first independent migration not related to parents. Thus, the association of birds within common wintering grounds with representatives of different flyway can lead to a change of breeding site.

The state of the western population in arid conditions is greatly influenced by climatic cycles lasting about 30 years with alternating wet and dry periods, during which bird habitat conditions can radically

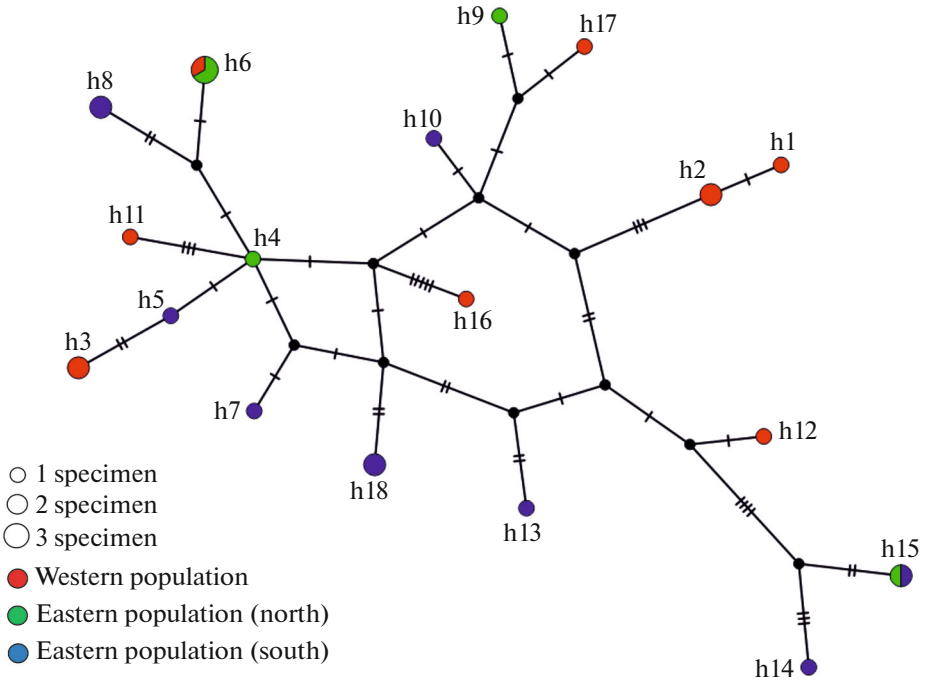

Fig. 5. The network of haplotypes of the mtDNA Control Region of the White-naped crane built using the TCS algorithm [21]. The size of the circles is proportional to the number of individuals, the cross marks on the connecting lines correspond to the number of mutational events between haplotypes; the pie charts represent the frequencies of haplotypes in populations.

change from favorable to extremely unfavorable [29–33]. This causes large-scale movements of individuals and a change in the spatial structure of the population both within the western population of the White-naped crane and, probably, within the species in total [31]. The rapid decline of the western group in 2000–2017 occurred during an unfavorable dry period, when the vast majority of its habitats dried up, which suggests that a part of the western White-naped cranes moved to the breeding sites of the eastern population, where the habitat conditions at that time were relatively favorable, and the species number grew quite quickly [5, 31]. Such movements of individuals, of course, contribute to the homogenization of the genetic structure of the species.

Weak or moderate genetic differences were also shown for other migratory crane species, both widespread and rare. So, for microsatellite loci, the value of F_{ST} among populations of the western subspecies of the Eurasian crane was 0.012 [34] and between the western and eastern subspecies 0.011 [24]. As well, similar low (0.3–1.1%) genetic differentiation was observed between Eurasian crane subspecies by mtDNA Control Region [35]. In the Demoiselle crane, a species with a large but fragmented range in Russia, the differences in the genetic structure for microsatellite loci (F_{ST} = 0.052 [25]) were higher than that of the more numerous and widespread Eurasian crane and, as shown in this study, the rare White-naped crane inhabiting a more compact area. For the Control Region, the level of genetic differentiation of the Demoiselle crane throughout the range (F_{ST} = 0.075 [26]) also exceeded that of the White-naped crane. Genetic differences between wintering populations of the Hooded crane *G. monacha* [36], as well as between the continental migratory and non-migratoryisland populations of the Red-crowned crane [10], according to the distribution of the Control Region haplotypes in median networks and phylogenetic trees, almost absent.

The presented here mismatch distribution of pairwise differences in nucleotides which indicates an increase in species number in the recent past, is not supported by reliable selective neutrality tests. However, these tests are very sensitive to sample sizes, which are not large enough in our study. At the same time, the TCS network of the Control Region haplotypeslacks star-like shapes typical for populations that have passed through a “bottleneck,” which also testifies in favor of premature conclusions about the population expansion of the White-naped crane in the past.

In general, the high rates of genetic variability of the White-naped crane according to the studied molecular genetic markers indicate a stable state of its gene pool on different flyways; however, owing to the decline of the western population and in order to maintain the eastern population, conservation measures for this rare species of cranes are relevant and necessary.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for animal care and use have been followed.

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