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ANIMAL GENETICS

Genetic Variation of Manchurian Pheasant (*Phasianus colchicus pallasi* Rotshild, 1903) Inferred from Mitochondrial DNA Control Region Sequences

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Abstract—Sequence variation of the mitochondrial DNA control region was studied in Manchurian pheasants (*Phasianus colchicus pallasi* Rotshild, 1903) representing three geographic populations from the southern part of the Russian Far East. Extremely low population genetic differentiation (F_{ST} = 0.0003) pointed to a very high gene exchange between the populations. Combination of such characters as high haplotype diversity (0.884 to 0.913), low nucleotide diversity (0.0016 to 0.0022), low *R*2 values (0.1235 to 0.1337), certain patterns of pairwise-difference distributions, and the absence of phylogenetic structure suggested that the phylogenetic history of *Ph. c. pallasi* included passing through a bottleneck with further expansion in the postglacial period. According to the data obtained, it was suggested that differentiation between the mitochondrial lineages started approximately 100000 years ago.

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INTRODUCTION

Manchurian pheasant, *Phasianus colchicus pallasi* Rotschild, 1903, is one of numerous subspecies (according to the estimates of different researchers, the subspecies number ranges from 31 to 37 [1]) of common pheasant (*Ph. colchicus* Linnaeus, 1758). The subspecies inhabits a rather narrow territory of the Russian Far East (Primorskii krai, Amur oblast, and Jewish Autonomous oblast) and occupies the northernmost part of the species range. Manchurian pheasant differs from other subspecies not only by a number of morphological, but also by some ethological characters, including seasonal migrations along substantial distances (from 200 to 300 km by straight line) [2].

During two recent decades, analysis of mitochondrial DNA (mtDNA) sequences was successfully applied for investigation of genetic divergence in animal populations and the taxa of different ranks. Among the unique features of mtDNA, differing it from nuclear DNA, the most important for phylogenetic reconstructions are high evolutionary rate, maternal inheritance, the absence of recombinations, and different evolutionary rates of the genes belonging to mitochondrial genome [3–6]. MtDNA control region (D-loop) is hypervariable noncoding part of mitochondrial genome, which is considered to be the most suitable for population analysis due to three- to five-fold higher rate of evolution compared to the rest of mitochondrial genome [7]. Analysis of this region is often used in avian phylogenetic and population studies [8–23]. Depending on the distribution of variable nucleotide positions and nucleotide frequencies, the control region can be divided into three domains. These domains are domain II (central), which is more conserved with the least content of A and highest content of G; domain I (left), and domain III (right). The latter domains are more variable and characterized by highest content of A along with the least content of G, as well as by the presence of long repeats [7, 24–27]. In most of avian species, mtDNA control region is located between the genes for tRNAglu and tRNAphe, while in some species of Picidae, Passeriformes, Falconiformes, and Cuculidae it is located between the genes encoding tRNAthr and tRNApro [24].

The data on the population variation of *Ph. colchicus* and its subspecies are scanty and require further accumulation and classification. Using RAPD markers, a population genetic structure of *Ph. colchicus* from Iowa (United States) was investigated, and high genetic subdivision of the populations was demonstrated [28]. Investigation of a local Far Eastern population of *Ph. c. pallasi* with the help of the same technique [29] demonstrated the high level of within-population variation. Variation of the 464-bp fragment from the 5' end of the mtDNA control region was analyzed in 80 representatives of natural populations of *Ph. colchicus* from Taiwan, and low genetic differences along with high gene flow between the populations was revealed [19].

The present study is the first investigation of variability of the mitochondrial control region performed in the representatives of three natural populations of Manchurian pheasant from the south of the Russian Far

East. The purpose of the study was acquisition of the data on genetic diversity, phylogeographic structure, and evolutionary history of the subspecies of interest.

MATERIALS AND METHODS

Subject of investigation. The study was performed using the material collected in 2003 through 2005, and containing *Phasianus colchicus pallasi* individuals of different sexes and age from three geographical regions in the southern part of the Far East. These regions were: (1) Jewish Autonomous oblast, floodplain of the Amur river, 22 birds (population BIR): outskirts of the settlements Novoe (115 km to the SSW from the city of Biribidzhan) and Kalinino (86 km to the SSW the city of Biribidzhan); (2) farthest south of Primorskii krai, 6 to 12 km to the NNE from the settlement of Khasan, 26 birds (population KHAS): Ptich'e Lake and Karasik River; (3) Primorskii krai, western bank of the Khanka Lake, 23 birds (population PLAT): outskirts of the settlements of Platonovka (10 to 13 km to the W from the settlement of Novokachalinsk) and Pervomaiskoe (5 to 6 km to the SW from the settlement of Novokachalinsk).

Liver samples were fixed in 96% ethanol in the day of catching. Fixed tissues were kept in a refrigerator at –20°C.

Isolation and analysis of mtDNA. Total genomic DNA was extracted from the 200 mg of tissue samples using standard phenol–chloroform procedure [30]. DNA concentration in the sample was estimated by comparison with the samples containing known concentrations of bacteriophage lambda DNA in electrophoresis in 1.4% agarose gel in Tris–EDTA–borate buffer.

A fragment of pheasant mitochondrial genome containing control region was amplified using polymerase chain reaction (PCR) with forward H19 and reverse L1097 primers in the UNO II 48 (Biometra, Germany) thermocycler (Table 1). Amplification was carried out in 25 μl of the reaction mixture containing 30 to 50 ng of genomic DNA; 0.2 μM of each primer (Sintol, Moscow); 1× buffer; 1 unit of *Taq* polymerase; and 200 μl dNTPs (QIAGEN, Netherlands). The reaction condition included initial denaturation at 94°C for 7 min, followed by 44 cycles of: denaturation at 94°C for 20 s; primer annealing at 52°C for 20 s; and extension at 72°C for 1 min. Final extension was done at 72°C for 7 min. Control sample contained complete amplification mixture without addition of DNA template. The amount of PCR product was estimated by comparison with the samples with known concentrations of bacteriophage lambda DNA, and the lengths of the fragments were determined using lambda DNA/*Eco*RI+*Hind*III markers (Fermentas, Lithuania).

After amplification, the products were subjected to cyclic sequencing using the ABI PRISM BigDye Terminator v. 3.1 reagent kit in one reaction with each of five primers, including two primers (H19 and L1097) used earlier for production of the mtDNA fragment, and three additional internal primers (H636, L612, and L708) (Table 1). Temperature conditions of cyclic sequencing were as follows: initial denaturation 96°C for 1 min, followed by 25 cycles of: denaturation at 96°C for 30 s; annealing at 55°C for 10 s; and extension at 60°C for 4 min. Sequencing of both (forward and reverse) DNA strands was performed using ABI PRISM 310 (Applied Biosystems, United States) DNA analyzer. Using the techniques described, the mtDNA control region was sequenced in 71 Manchurian pheasants.

Table 1. PCR and sequencing primers used in the study

Primer and localization in the D-loop	Nucleotide sequence (5' ⟶ 3')
H19	AACTCCCCTATTGAATGTACCC
H636	TGCGGAGTGCTATTCAAGTG
L612	TGAAGAAGCCCCAGAGAAAA
L708	CGAGGGACACGAGAGGACTA
L1097	AATTTGTGGGGTTTTGTCCT

Analysis of nucleotide sequences and phylogenetic reconstructions. Different manipulations preparing the sequences for further analysis, including sequence alignment, were performed using the Staden 1.53 software package [31] and the SEAVIEW [32] software program [32]. Hierarchical analysis of molecular variance (AMOVA), as well as the computation of such measures as haplotype diversity *h*, nucleotide diversity π, conditional size of ancestral haploid population θ_0, conditional size of contemporary haploid population θ_1, and the age of contemporary population τ were performed using the ARLEQUIN ver. 3.01 software program [33]. The tests for population stability (growth or decline of the population number): Tajima's *D* [34], Fu and Li's *F* [35], Fu's F_S [36], and Ramos-Onsins and Rozas *R2* [37], as well as the analysis of mismatch distributions, which makes it possible to estimate the frequencies of pairwise replacement differences between the individuals and provides information on the sequence demographic histories [38], were performed with the help of the DnaSP 4.10.9 software program [39]. The divergence values between nucleotide sequences (*p* distances) were obtained with the help of MEGA3 software package [40]. Visualization of possible mutational transitions between mtDNA haplotypes was performed using the TCS 1.21 software program [41].

Phylogenetic reconstructions were made using control region sequences of two pheasant species from the GenBank/NCBI, *Ph. colchicus* (AJ298920 [42], AY874873, and AY874874 [22]) and *Ph. versicolor* (AY376861, AY376862, AY376863, and AY376866 [22]). The sequences obtained in the present study were

Table 2. Control region haplotypes in *Phasianus colchicus pallasi*

Haplotype	Position of nucleotide substitution											Number of certain haplotype samples (relative frequency)		
	1 4 4	1 4 8	1 6 6	1 6 7	1 8 6	1 9 3	2 5 6	2 6 4	3 4 0	8 7 3	9 7 3	BIR, n = 22	KHAS, n = 26	PLAT, n = 23
Ph1	T	C	T	C	T	G	T	C	T	T	C	2 (0.091)	1 (0.038)	–
Ph2	C	·	·	·	·	·	·	·	·	·	·	4 (0.182)	8 (0.308)	3 (0.130)
Ph3	C	·	·	·	C	·	·	·	·	A	·	2 (0.091)	3 (0.115)	2 (0.087)
Ph4	C	·	·	·	C	·	·	·	·	·	·	8 (0.364)	5 (0.192)	4 (0.174)
Ph5	C	·	·	·	·	·	·	·	·	A	·	1 (0.045)	–	1 (0.043)
Ph6	·	·	·	·	·	·	·	·	·	A	·	1 (0.045)*	–	–
Ph7	·	·	·	T	·	A	·	·	·	·	·	1 (0.045)	1 (0.038)	–
Ph8	C	·	·	T	·	A	·	·	·	·	·	1 (0.045)	2 (0.077)	1 (0.043)
Ph9	C	·	·	·	·	·	·	T	·	·	·	1 (0.045)	–	2 (0.087)
Ph10	·	·	·	·	C	·	·	·	·	·	·	1 (0.045)	–	5 (0.217)
Ph11	·	T	·	·	C	·	·	·	·	·	T	–	1 (0.038)*	–
Ph12	C	T	·	·	C	·	·	·	·	·	T	–	3 (0.115)*	–
Ph13	C	·	C	·	C	·	·	·	C	·	·	–	1 (0.038)*	–
Ph14	C	·	·	T	·	A	·	·	·	A	·	–	1 (0.038)*	–
Ph15	·	·	·	·	·	·	·	T	·	·	·	–	–	1 (0.043)*
Ph16	·	·	·	·	C	·	·	·	·	A	·	–	–	2 (0.087)*
Ph17	C	·	·	·	C	·	C	·	·	·	·	–	–	1 (0.043)*
Ph18	C	T	·	T	·	A	·	·	·	·	·	–	–	1 (0.043)*
Haplotypes, total:												10	10	11

Note: Identical nucleotides are designated by dots, unique haplotypes are designated by asterisks, the absence of certain haplotype is designated by dash.

aligned with the GenBank sequence of *Ph. colchicus*, AJ298920, from position 41 to position 1073. Thus, the region of 1033 bp became the subject of comparative analysis. Phylogenetic reconstructions were made with the help of the MEGA3 software package and using the Kimura's two-parametric model of nucleotide substitutions, which takes into consideration substantial prevalence of transitions over transversions in mtDNA evolution, as well as different methods of clustering, including unweighted pair group method with arithmetic average (UPGMA), Neighbor-Joining (NJ), and minimum evolution (ME). Robustness (statistical significance) of phylogenetic trees was evaluated using bootstrap technique [43] with 1000 iterations. The bootstrap values (BP) lower than 50% were not taken into consideration.

RESULTS

Variation and Distribution of mtDNA Haplotypes

Sequence polymorphism in the fragment of the mtDNA control region was examined in Manchurian peasants (*Ph. pallasi*; n = 71) representing three geographical populations from the Far East. Using PCR, a fragment of about 1070 bp, including almost the whole control region, was amplified. The 1033-bp fragment was sequenced. The median nucleotide composition was 32.7% T, 26.2% A, 25.9% C, and 15.2% G. The sequence differences determined were caused by point mutations in 11 (1.1%) sites, of which ten were transitions, and one transversion (Table 2). Among 11 mutations three were single substitutions (positions 166, 256, and 340), and eight mutations were informative (positions 4, 148, 167, 186, 193, 264, 873, and 973). Domain I was shown to be the most variable (eight out of ten mutations), similarly to the most of the representatives of the genera *Alectoris*, *Anser*, *Cepphus*, *Geospiza*, *Cyanoramphus*, and *Grus* [24].

Based on the mtDNA D-loop sequences determined, a total of 18 haplotypes were identified and submitted to the GenBank/NCBI database under accession numbers AM709705 to AM709722. Nine of these haplotypes were unique, with one haplotype observed exclusively in BIR, and by four haplotypes found in KHAS and PLAN. In general, all the population samples examined were similar in terms of the numbers of dif-

ferent haplotypes present in each of them. In BIR and KHAS, 10 haplotypes were identified, while PLAT contained 11 haplotypes (Table 2). Four haplotypes (Ph2, Ph3, Ph4, and Ph8) were found in all populations, and only these haplotypes were shared by KHAS and PLAT. Seven haplotypes were shared by BIR and PLAT, and six haplotypes, by BIR and KHAS (Table 2). The number of polymorphic sites in an individual population constituted six, nine, and eight, for BIS, KHAS, and PLAT, respectively.

Phylogenetic network, constructed using the data on nucleotide polymorphisms between individual haplotypes (Fig. 1) showed the absence of any phylogenetic groups. The topology presented confirmed genetic homogeneity of pheasants within the territory examined. The number of evolutionary events between the nodal mtDNA types constituted one nucleotide substitution. Haplotype Ph4 was most frequent (23.9%). In frequency decreasing order it was followed by haplotypes Ph2 (21.1%), Ph3 (9.8%), Ph10 (8.4%), and Ph8 (5.6%). Then, followed haplotypes Ph1, Ph9, and Ph12, found with similar frequency of 4.2%, and haplotypes Ph5, Ph7, and Ph16 with the frequency of 2.8%. The remaining haplotypes were unique (1.4%).

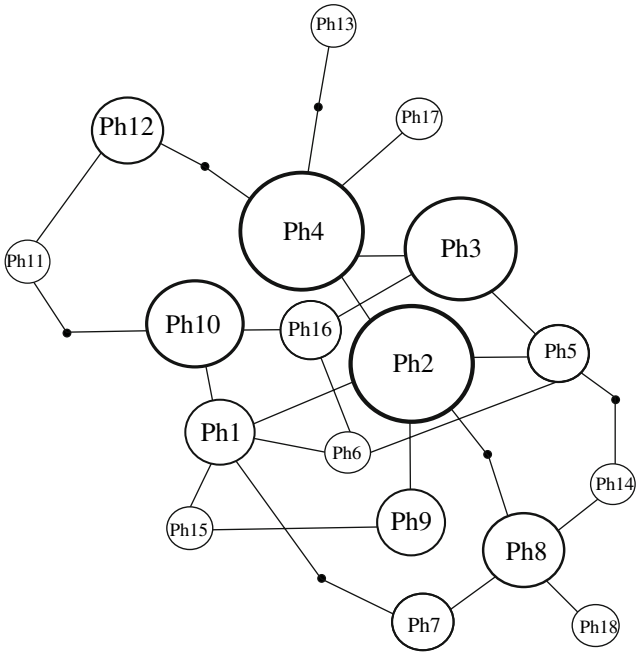

Fig. 1. Phylogenetic network of mtDNA control region haplotypes of *Phasianus colchicus pallasi*. Haplotype numbers are shown at the nodes (see Table 2), the circle sizes reflect approximate haplotype frequencies, and hypothetical haplotypes (black circles).

Population Analysis

In the populations studied, the levels of haplotype (h) and nucleotide (π) diversity varied in the range from 0.844 to 0.913 and from 0.0016 to 0.0022, respectively (Table 3). In the pooled sample, $h = 0.881 \pm 0.022$ and $\pi = 0.0019 \pm 0.0012$. Population BIR was characterized by the lowest levels of haplotype and nucleotide diversity. The highest nucleotide diversity was observed in KHAS, which was geographically southernmost among all populations tested. At the same time, the highest haplotype diversity was observed in PLAT, which occupied geographically central position (Table 3). The mean level of genetic distances between the pairs of populations, as well as between the pairs of individuals constituted 0.002.

Analysis of the distribution of genetic variation AMOVA revealed the high within-population variation, the share of which constituted 99.7%, while the share of between-population variation constituted only 0.03%. These data pointed to the absence of population subdivision (F_{ST} = 0.0003, $P < 0.001$) along with the extremely high level of gene exchange between the populations. The distances between the populations examined (KHAS and PLAT, about 305 km, BIR and PLAT, 300 km by straight line) do not prevent intensive migrations of the bird groups KHAS ⟷ PLAT ⟷ BIR, since it is known [2] that *Ph. c. pallasi* is capable of seasonal migrations along almost 300 km by the straight line.

Demographic Analysis of the MtDNA Data

The Tajima's D values were negative, albeit not significantly different zero for all populations. At the same time, Fu's and Li's F values were relatively low and not statistically significant ($P > 0.10$) (Table 4). Negative statistically significant values of Fu's index F_S were determined for all populations (Table 4); for pooled sample, the value of this index constituted –9.387 (P = 0.00). For all populations, low *R2* values were determined (Table 4). Analysis of mismatch distributions is used as the test for the change of the population size. The histograms show (Fig. 2) a unimodal left-hand peak for the pooled sample, as well as for individual populations. This distribution is supported by the model of sudden population expansion after the pass through bottleneck [38]. Thus, the values of F_S and *R2*, and the distribution of pairwise nucleotide differences obtained for *Ph. c. pallasi* were similar to that for other avian species [10, 15, 16, 23] and were consistent with the hypothesis of population growth.

To test the hypothesis of demographic expansion, which followed from the analyses performed, such

Table 3. Haplotype and nucleotide diversity in populations of *Phasianus colchicus pallasi*

Population	h	π
BIR	0.844 (0.062)	0.0016 (0.0011)
KHAS	0.861 (0.045)	0.0022 (0.0014)
PLAT	0.913 (0.033)	0.0020 (0.0013)

Note: Mean deviation values are given in brackets.

Table 4. Values of the population stability tests in *Phasianus colchicus pallasi*

Population	*D*	*F*	F_S	*R*2
BIR	–0.01*	0.42740*	–5.226***	0.1337
KHAS	–0.16*	0.15050*	–3.078**	0.1235
PLAT	–0.14*	0.08013*	–5.159***	0.1237

Note: * $P > 0.1$; ** $P < 0.05$; *** $P < 0.005$.

Table 5. Population parameters of *Phasianus colchicus pallasi* for the model of sudden dispersal

Parameter	Population		
	BIR	KHAS	PLAT
Conditional size of ancestral population, θ_0	0.000	0.007	0.005
Conditional size of contemporary population, θ_1	∞	12.209	∞
Age of contemporary population, τ	1.701	2.596	2.080

population parameters, as conditional sizes of ancestral and contemporary haploid population, as well as the age of contemporary population, expressed in the mutational time units, were determined (Table 5). Only in case of BIR, the size of ancestral population was determined as zero, while for two other populations the values slightly different from zero were obtained. These findings were consistent with the hypothesized scenario of northwards dispersal of pheasants. In addition, BIR was younger than two other populations. Conditional parameters of contemporary BIR and PLAT were determined as infinite values, while KHAS, compared to them, had very small size.

The number of pheasants is greatly variable depending on climatic and anthropogenic factors. E.N. Panov in his book *The Birds of Southern Primorye* (1973), analyzing literature and personal data, suggested that temporal decline of the pheasants number was associated with some adverse climatic factors (usually these were summer rains and abundant snow cover in winter), as well as some anthropogenic factors (the most harmful were spring firings) [44]. In addition, population number of *Ph. c. pallasi*, the range of which extends to the territory of China is affected by uncontrolled extermination of this valuable game subspecies. In favorable periods, at absence of the reasons mentioned, population number of pheasants reaches extremely high values, and they can live in conditions of high population density [1, 2, 44].

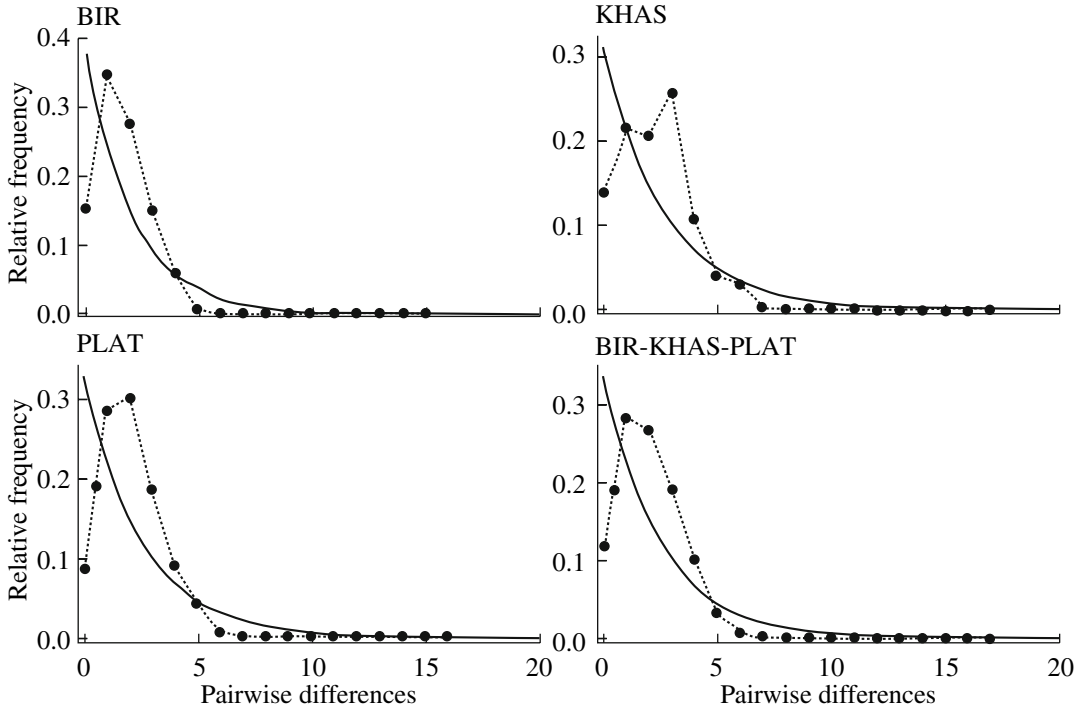

Fig. 2. Distribution of pairwise nucleotide differences in populations of *Phasianus colchicus pallasi* upon the use of the model of constant population size. Expected frequency of pairwise differences between the haplotypes is designated by continuous line, the observed frequency of pairwise differences between the haplotypes is designated by dotted line.

Phylogenetic Relationships of MtDNA Haplotypes

Comparison of the Manchurian pheasant D-loop sequences obtained with those from the GenBank showed that the differences were solely represented by single nucleotide substitutions. Analysis of the data matrix revealed that two GenBank sequences (AJ298920 and AY376866), belonging to different species, the first one, to *Ph. colchicus*, and the second one, to *Ph. versicolor*, were completely identical. To avoid any misunderstanding, these sequences were excluded from further analysis. Analysis of the data matrix with the help of UPGMA, NJ, and ME produced similar phylogenetic trees, which were slightly different only in the positions and statistical significance of some brunches. For these reasons, only NJ tree is presented in the study. As follows from the data of NJ analysis (Fig. 3), the taxa included into analysis, fall into two groups. One group is composed by the three samples of *Ph. versicolor*, while another group with high bootstrap support (BI 99%) unites the sequences of *Ph. colchicus* and its subspecies, *Ph. c. pallasi*. All Manchurian pheasant samples clustered together with moderate support (BI 70%). However, the distribution of the haplotypes examined on the phylogenetic tree was not associated with the attribution to one or another population, supporting genetic homogeneity of the whole group.

DISCUSSION

Analysis of the 1033-bp control region fragment of Manchurian pheasant showed that in this subspecies the level of haplotype diversity was two times higher, and the level of nucleotide diversity 2.4 times lower, compared to the values obtained for natural populations of *Ph. colchicus* upon analysis of the 464-bp 5' fragment of the control region (24 variable sites and 13 haplotypes) [19]. It can be suggested in this respect that for *Ph. colchicus*, inhabiting the Taiwan Island which is located in the Pacific Ocean, close to the eastern coast of continental China, the consequences of Pleistocene glaciations were not so dramatic as for its northern subspecies. For instance, paleontological data showed that during the last Glacial Maximum, the temperature in Central Taiwan was 8.0 to 11.0°C lower than at present [45].

The data on haplotype nucleotide diversity are more informative than the data on haplotype diversity, since the range of nucleotide differences reflects the evolutionary distance between the sequences, formed in the course of the mutational process. The level of nucle-

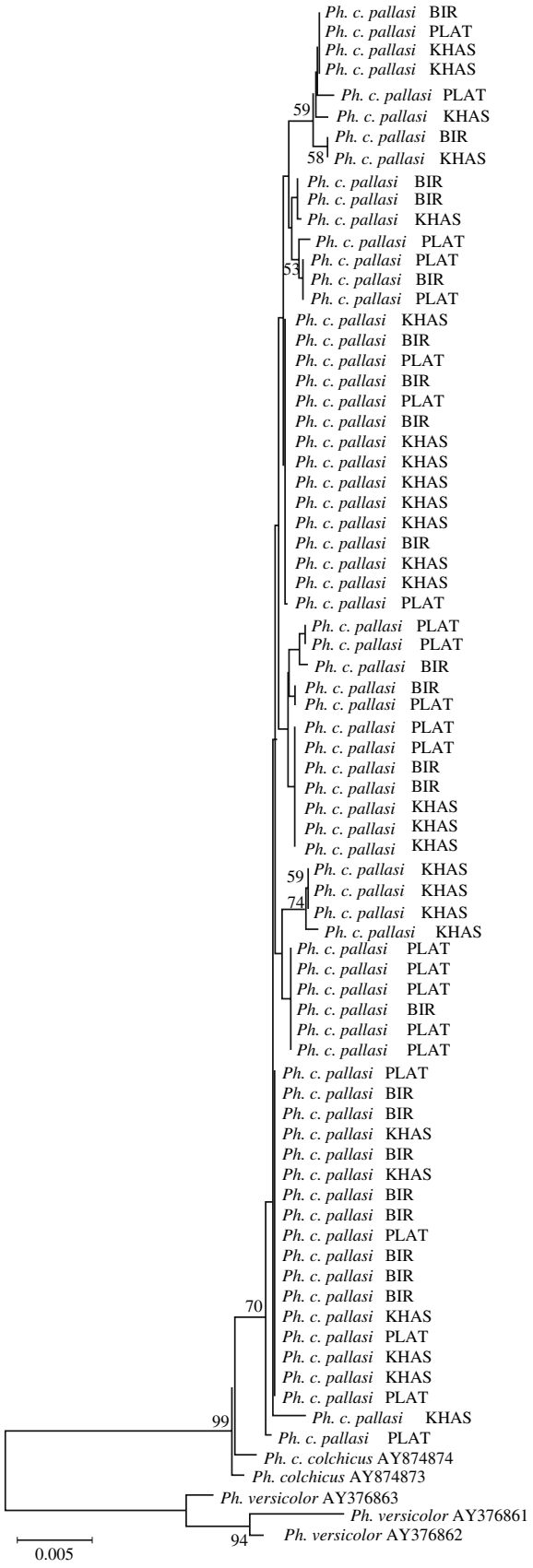

Fig. 3. NJ phylogenetic tree based on comparison of the mtDNA control region sequences from *Phasianus colchicus pallasi* (present study), *Ph. colchicus* (AJ288920, AY874873, and AY874874), and *Ph. versicolor* (AY376861, AY376862, AY376863, and AY376866). Figures at the nodes, bootstrap index values (1000 iterations).

otide variation of the mtDNA control region revealed in *Ph. c. pallasi* was comparable to that in *Columbia inornata* (0.2 to 0.4 [8]), *Parus major major* (0.19 [10] and 0.113 to 0.384 [23]), *Luscinia svecica* (0.02 to 0.3), and *Motacilla flava* (0.25 to 0.47) [16]. At the same time, the value of this index was considerably lower than in *Parus montanus borealis* (0.53 [9]), *Sterna fuscata* (2.9 [14]), *Alectoris graeca* (0.8 [17]), *Gypaetus barbatus* (2.92 [20]), and *Somateria mollissima* (0.4 to 3.13 [21]). Low level of nucleotide diversity is usually associated, first, with the young age of the group, and second, with passing through the so-called bottleneck, i.e., with dramatic reduction of the population number and its recent recovery [8, 10, 16, 23].

The absence of genetic divergence between the populations of Manchurian pheasant (F_{ST} = 0.0003) was not surprising. As early as in 1963, Mayr [46], analyzing large data sets on geographic variation of birds, indicated that application of rather sensitive techniques usually resulted in identification of various features distinguishing individual population from all the others belonging to the same species. The cases of complete absence of geographic variation are extremely rare, while the cases of low geographic variation can be observed somewhat more often. The latter can be explained by a number of reasons or by their combination. For instance, in the case of a small species range, geographic variation is impossible due to homogenous conditions allover the range [46]. The absence of population differentiation in *Ph. c. pallasi* is in agreement with such characteristics of the species and range, as small territory occupied, polygamous relations between the sexes along with the absence irresistible physical barriers within the range [1, 2]. Similar patterns can be followed for many duck species, some passerines, and some other groups.

To date, phylogeographic variation of the mtDNA control region variation in birds has been extensively studied [9–12, 15–17, 47, 48]. Usually widely distributed species are characterized by certain degree of genetic differentiation depending on the geographic scale [47, 48]. However, in some *Parus* species, population differentiation was either absent, or was very low [9–12]. As it was mentioned by the authors of the studies, one of the reasons for the absence of the population structure could be the pass through the "bottleneck" in the history of the species with subsequent recolonization and further population expansion.

Similarly to *Parus major major* [10], northern populations of *P. caeruleus* [11], and many other forest bird species of Northern Eurasia [15], the absence of population differentiation and phylogeographic structure in Manchurian pheasant is thought to be associated with the range extension after the end of the last Ice Age. The values of population parameters obtained in the present study for the model of sudden dispersal, specifically, null size of conditional ancestral population, and the young age of contemporary populations, also confirm the bottleneck hypothesis for the subspecies examined.

Using generally accepted estimate of the mtDNA D-loop divergence rate as equal to 2% per million years [9–12], along with the data on the control region sequence divergence obtained for Manchurian pheasant (0.2%), the divergence time of the populations examined was determined. The calculations showed that differentiation between the mitochondrial lineages started, approximately, 100000 years ago in Upper Pleistocene.

Thus, sequence analysis of the mtDNA control region in Manchurian pheasant provided identification of some specific genetic features of the species, specifically, high haplotype diversity, low nucleotide diversity, certain patterns of pairwise-difference distributions in combination with negative F_S values, low $R2$ values, absence of phylogenetic structure, and young population age. These findings suggest that *Ph. c. pallasi* passed the bottleneck during Pleistocene glaciations, while the modern period is characterized by the range extension in this species coupled with intensive population growth and high gene flow.

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