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ANIMAL GENETICS

A New Case of Recombination between Nuclear and Mitochondrial Genomes in the Genus *Calliope* Gould, 1836 (Muscicapidae, Aves): The Hypothesis of Origin *Calliope pectoralis* Gould, 1837

L. N. Spiridonova[a], *, O. P. Valchuk[a], and Ya. A. Red'kin[b]

[a] *Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 690022 Russia*

[b] *Zoological Museum, Moscow State University, Moscow, 125009 Russia*

**e-mail: spiridonova@biosoil.ru*

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Abstract—For the first time we propose a hypothesis of hybrid origin of *Calliope pectoralis* from two species, *C. calliope* and *C. obscura,* based on the new molecular genetic data and phenotypic characters. We examined 80 samples of *C. calliope* and one sample of *C. pectoralis tschebaiewi*. We discovered that products of the cytochrome *b* gene, as well as three transport RNAs, ND6, and a control region (3.2 kb) were heterogeneous in 22 specimens of *C. calliope*. The result of cloning of these amplicons produced two clone variants: the cytochrome *b* gene of *C. calliope* and the nuclear pseudogene homologous to the cytochrome *b* gene of *C. pectoralis* (96% match). Computer assisted phylogenetic analysis of the connections between the cloned sequences for the mtDNA cytochrome *b* gene and its nuclear copies revealed a distribution into two clades: *C. calliope* and *C. pectoralis*. This can be explained by an intergenomic recombination event, namely, a transfer of *C. calliope*'s nuclear copy of the cytochrome *b* gene into a mitochondrial genome of a hybrid female that later became the founder of the *C. pectoralis* species. According to morphological features, the second species involved in hybridization with *C. calliope* was probably *C. obscura*, since it is the only species of the *Calliope* genus that has a black breast and black outer tail feathers with white bases similar to those of *C. pectoralis*.

Keywords: interspecific hybridization, NUMT, mtDNA, intergenomic recombination, *Calliope calliope*, *C. obscura*, *C. pectoralis*

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INTRODUCTION

The study of molecular markers of mitochondrial DNA for species identification (DNA coding) led to the emergence of a number of "false" taxa and is associated primarily with NUclear copies of MiTochondrial genes (NUMT), which are co-amplified with orthologous mitochondrial DNA genes [1]. NUMTs are considered as fragments of the ancient mtDNA and are often used as mitochondrial markers to define the basal branches of phylogenetic trees in the reconstructions of the ancestral state of the mitochondrial genome [2]. However, in the paper of B.V. Andrianov et al., data were reported indicating a high degree of variability of the pseudogenes of mitochondrial origin after their integration into chromosomes, which contradicts the idea of them as "molecular fossils" [3].

Nuclear copies of mtDNA are most often regarded as interference, which leads to erroneous reconstructions of the taxa phylogenies of different rank [4]. This is applicable to the cases where nuclear copies are amplified in some taxa, and the mitochondrial genes in others, since the sequences compared have different sources of origin [5–7]. The use of mitochondrial genes and their fragments as markers of the evolutionary process has been questioned by some researchers. This is because in many cases full length mitochondrial genes and their genomic fragments show different rates of mutations [8]. In addition, certain violations were detected in respect to maternal inheritance of mitochondria, mtDNA recombinations, and heteroplasmy. High levels of individual haplotype diversity were also reported [9, 10].

Despite the generally accepted negative opinion about the use of nuclear copies of mitochondrial genes for phylogenetic analysis, our data points to the potential importance of using NUMT to clarify phylogenetic constructs, at least, in some cases. Earlier, in the study of subspecies *C. calliope* (Pallas, 1776), we showed the role of mitochondrial pseudogenes as a source of new variants of mtDNA subspecies haplotypes [11]. Analysis of mitochondrial genes and their nuclear copies in individual members of the genus *Calliope* Gould, 1836 revealed new facts confirming the important role of mitochondrial pseudogenes in

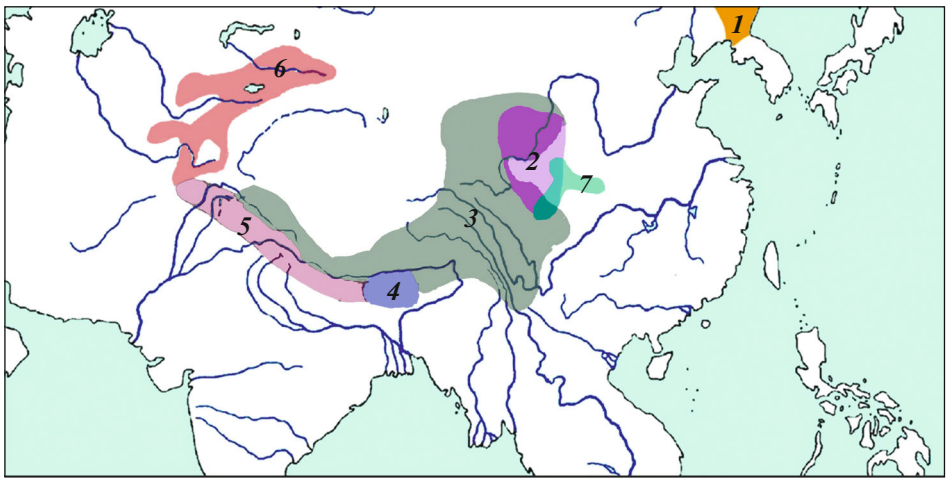

Fig. 1. Areas of species *Calliope* and its subspecies: *C. calliope* (*1*—*C. c. calliope*; *2*—*C. c. beicki*), *C. pectoralis* (*3*—*C. p. tschebaiewi*; *4*—*C. p. confusa*; *5*—*C. p. pectoralis*; *6*—*C. p. ballioni*), *7*—*C. obscura*.

maintaining mtDNA genetic diversity. The subject of this study was the Himalayan rubythroat *C. pectoralis* Gould, 1837, the problem of the origin of which deserves a special commentary.

Split of Siberian rubythroat as an independent *Calliope* genus from a large, morphologically heterogeneous group of "nightingales" *Luscinia* s. l., is based on the results of modern phylogenetic reconstructions [12, 13], has received wide recognition, and has been incorporated in the major modern listings of the birds of the world [14, 15]. This genus includes polytypic species *C. calliope* and *C. pectoralis* and two monotypic species—*C. pectardens* David, 1877 and *C. obscura* (Berezowski et Bianchi, 1891). Most members of the genus *Calliope* inhabit the mountainous areas of Asia (Fig. 1).

The white-tailed rubythroat, represented by a number of subspecies, is distributed from the Tien Shan and Pamir-Alay, across the Himalayas to eastern Qinghai, Gansu, and the western part of Sichuan province [16]. The Siberian rubythroat properly, *C. calliope*, is widespread in most parts of Siberia and the Far East [17]. In Central China, it is represented by an isolated mountain form *C. c. beicki* (Meise, 1937). Monotypic species *C. pectardens* and *C. obscura* inhabit the mountains of southern and central China [15].

C. pectoralis is a high-mountainous species inhabiting alpine and subalpine meadows with shrub vegetation and forms a number of well-separated subspecies [14, 16, 18–20]. *C. p. pectoralis* inhabits the southern slope of the Himalayas from Karakorum to the eastern regions of Nepal; *C. p. confusa* (E. Hartert, 1910) occupies the more eastern regions—the northeastern parts of India and Bhutan; the northernmost form *C. p. ballioni* (Severtsov, 1873) inhabits the mountain systems of Pamir-Alai and Tien Shan. Finally, the range of morphologically isolated form *C. p. tschebaiewi* Przevalski, 1876, extends along the northern slope of the Himalayas from the southern tip of Karakorum to the far north of Myanmar and Yunnan and also covers the western part of Sichuan, Gansu, and East Qinghai (Fig. 1).

According to many researchers, the taxonomic status *C. p. tschebaiewi* needs a revaluation. Earlier, Ch. Vaurie [18] indicated that *pectoralis* and *tschebaiewi* may be different species. Exploring variations in size and color of *C. pectoralis* subspecies, V.M. Loskot and K.K. Daletskaya [16] came to the conclusion of the greatest divergence of *C. p. tschebaiewi* from other subspecies, possibly, corresponding to a species status. Considering the fact that there were no individuals found in collections with traits strictly intermediate between *tschebaiewi* and other subspecies, the authors proposed to consider this form as mega-subspecies [16]. Later P. Rasmussen and J. Anderton [20] expressed an opinion on the intermediate position of the *C. p. tschebaiewi* form between species *C. calliope* and *C. pectoralis* based on the morphological features. According to the same authors, since vocalization of all three taxa (*calliope*, *pectoralis*, and *tschebaiewi*) is very similar, henceforth, they can be considered as either part of a single polytypic species or as three separate species. Finally, J. Liu et al. [21] proposed to consider the form *tschebaiewi* (Chinese rubythroat) as a separate monotypic species on the basis of the data of multilocus analysis of two mitochondrial and two nuclear genes, as well as comparison of vocalization and external morphological differences. This point of view is also reflected in the last list of sparrow-like birds of the world [15].

The hypothesis of hybrid genesis of the Himalayan rubythroat *C. pectoralis* s. l. was not expressed previously. However, some researchers pointed to the intermediate character of *C. p. tschebaiewi* [20]. In this study, we tested the hypothesis of the hybrid origin *C. pectoralis* from the *C. calliope* and *C. obscura* species

on the basis of the molecular data and morphological appearance of these taxa. In addition, we uncovered a case of a past recombination event between homologous sequences of the nuclear and mitochondrial genomes, which resulted in the emergence of the particular mitochondrial haplotype of *C. pectoralis*.

MATERIALS AND METHODS

DNA samples and amplification of mtDNA cytochrome b gene. We tested 80 samples of *C. calliope* caught in the vicinity of the ringing station of the Amur-Ussuri Center for Biodiversity of Birds (Primorsky krai, valley of the Litovka River, 42.962° N, 132.80° E) during the fall migration of 2011 and one sample of *C. pectoralis tschebaiewi* collected in the northern Myanmar state of Kachin (27.489° N, 97.191° E) (collection of V.N. Sotnikov). DNA was isolated from blood samples fixed in 96% ethanol using the QIAgen DNeasy® Tissue Kit (Qiagen, Inc.). We used gene sequences *cytb* from NCBI GenBank: *C. p. pectoralis* (Himalayas, KJ456329), *C. p. ballioni* (Kazakhstan, HM633321). It should be noted that molecular analysis of migrant *C. calliope* was conducted separately from samples from *C. pectoralis.* Amplification of a mitochondrial DNA fragment (mtDNA) comprising the *cytb* gene, three tRNA, *ND6* and *CR* and sequencing of the *cytb* gene sequence on their basis was described in the previous study [11].

Fragment cloning. Heterogeneous fragments from eight individuals were cloned using the InsTAclone™ PCR cloning Kit (Fermentas, Lituania) according to the manufacturer's instructions and ligated into a pTZ57R/T vector. Transformation was carried out in competent cells of *Escherichia coli* strain XL1-Blue. Testing clones for the presence of the insert was performed with primers M13/pUC$_{fw}$ (5'-GCCAGGGTTTTCCCAGTCACGA-3') and M13/pUC$_{rv}$ (5'-GAGCGGATAACAATTTCACACAGG-3') under the following conditions: predenaturation—2 min/94°C; followed by 35 cycles: denaturation—30 s/94°C, annealing—30 s/55°C, synthesis—1 min/72°C; final synthesis—5 min/72°C. All stages of the experiment were repeated twice to confirm the reproducibility of the results. Identified gene sequences of *cytb* were submitted to GenBank ENA/EMBL under accession numbers LT991756–LT991862.

Sequencing and computer analysis. Amplification products were used for cyclic sequencing with the reagent kit ABI PRISM®BigDye™ Terminator v. 3.1. Labeling reactions were performed with same primers. Sequencing was carried out on an ABI PRISM 3130 automatic laser sequencer (Applied Biosystems, USA/Hitachi, Japan).

Forward and reverse sequences were collected using the software package Staden 1.53 [22] and aligned with the program Clustal W provided within the software environment MEGA ver. 6 [23]. We used DnaSP ver. 5.0 to analyze nucleotide diversity of clones and construct a matrix of polymorphic sites [24]. Reconstruction of phylogenetic relationships between haplotypes using the maximum likelihood method was also carried out using MEGA.

Sequencing of the mitochondrial fragment (3.2 kb) from individual migrant no. 16 was performed by pyrosequencing using the Roche GS Junior kit. Preparation of a fast library with ligated adapters, emulsion PCR, and sequencing with GS Junior Titanium Series reagents were performed according to the instructions of the manufacturer of the device and reagents (Roche, United States). The work was performed on the equipment of the Center for Biological Processes, Biotechnology, and Genetic Engineering of the Federal Science Center of Biodiversity of the Far Eastern Branch of the Russian Academy of Sciences. Nucleotide sequences were assembled using the GS De Novo Assembler program (http://454.com/products/analysis-software/index.asp). Sequencing of fragments proceeded with the average coverage 100–300×. To confirm the recombination event between the nuclear and mitochondrial genomes, we tested the nuclear pseudogene and the corresponding mtDNA fragments from *C. calliope*, *C. pectoralis*, *Luscinia svecica* (Linnaeus, 1758), *Larvivora akahige* (Temminck, 1835), and *Tarsiger cyanurus* (Pallas, 1773) using the progam RDP ver. 4 [25].

RESULTS

The reason for conducting this study emerged during a routine selection of amplification conditions for long DNA fragments using a new generation sequencer Roche 454. Analysis of a fragment of about 3.2 kb in length that included the *cytb* gene, three tRNAs, *ND6*, and *CR* (Fig. 2) identified double peaks according to the type of heteroplasmy in sequences from 22 out of 80 *C. calliope* individuals studied. As the result of cloning of amplicons from eight individuals, 162 clones of the *cytb* gene were obtained, on average, 20 clones from each fragment.

Sequences of clones were compared with previously identified haplotypes of *C. calliope* subspecies and with sequences stored in the NCBI GenBank database. Our study revealed a pattern of distribution of heteroplasmic sites that differed from previous analyses [11]. One variant of clones in all birds turned out to correspond to the *cytb* mtDNA gene *of C. calliope*. The second clone showed 96% similarity to the *cytb* gene of *C. pectoralis* on the basis of the distribution of known mutations.

Some of the identified sequences contained the mitochondrial stop codon (TAA), single deletions leading to a shift in the reading frame, and 11 termination TGA codons ("opal" or "umber"). Analysis of cloned sequences revealed a high level of variability

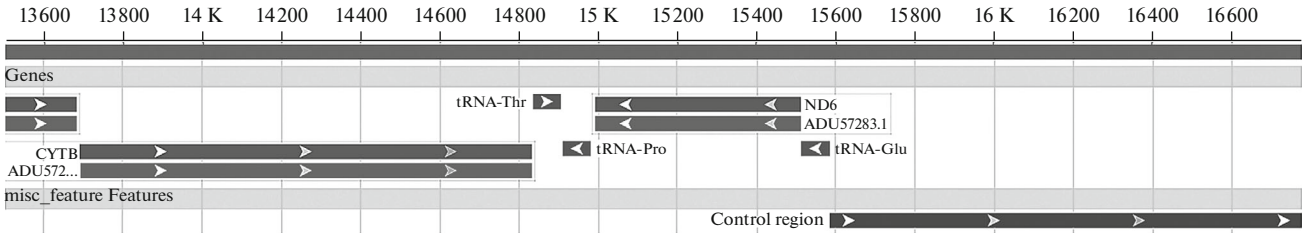

Fig. 2. Scheme of the amplified mitochondrial DNA fragment including the *cytb* gene, tRNA, *ND6*, and the control region (D-loop) (3.2 kb), from which *cytb* gene was sequenced (1141 bp).

caused by randomly distributed single mutations and numerous recombination rearrangements between different variants of haplotypes represented in the nuclear genome of *C. calliope* (Fig. 3). For example, the length of the recombinant sites in the cloned fragments ranged from 85 bp in clone 16.6 (sample no. 16) to 869 bp in clone 6.2 (sample no. 6). Interestingly, insertions of other haplogroup variants were found in the nuclear copies of mitochondrial genes in one individual, which indicates a high rate of variation of pseudogenes owing to recombination processes of the nuclear DNA itself. The pattern of nucleotide distribution of the nuclear pseudogens in C. *calliope* and *cytb* gene in *C. pectoralis* revealed a well-defined homology, supporting their common origin. At the same time, we uncovered a significant divergence between the nuclear pseudogene in *C. calliope* and mitochondrial *cytb* gene in *C. pectoralis*. This can be explained by a prolonged location of the paralog in the nuclear genome after the recombination event.

Figure 4 presents the known relationships between *C. calliope* and *C. pectoralis* species and sequences of *C. calliope* cloned in this study. Reconstruction of mt-haplotypes of the *cytb* gene and its nuclear copies using the maximum likelihood method showed that all clones are distributed in three clades: *C. calliope* (variants I and II) and *C. pectoralis* (variant III). This result suggests a familiar relationship between the nuclear pseudogene of *C. calliope* and mitochondrial gene in *C. pectoralis*. Since the nuclear paralog and the mitochondrial gene are in different genomes, we believe that an intergenomic recombination event took place, as in the case of the origin of *C. calliope* [11, 17].

To confirm the recombination event between the nuclear pseudogene and mtDNA, we tested NUMT and mtDNA fragments from *C. calliope*, *C. pectoralis*, *Luscinia svecica*, *Larvivora akahige*, and *Tarsiger cyanurus* using the program RDP ver. 4. As result of this analysis, we confirmed that the *cytb–CR* fragment of mtDNA in *C. pectoralis* is of recombinant origin with reliable probability values (Chimaera, $P = 1.458 \times 10^{-04}$; MaxChi, $P = 3.322 \times 10^{-05}$; GENECONV, $P = 1.123 \times 10^{-08}$; SiScan, $P = 5.562 \times 10^{-13}$).

DISCUSSION

Population studies of mitochondrial markers in many avian taxa reveal two phylogeographic patterns. One of them is characterized by the absence of a well-defined phylogeographic structure and is described, for example, for *Parus major* s.l., *P. montanus* s.l. [26], *Perisoreus infaustus*, *Nucifraga caryocatactes* [27], *Nucifraga columbiana* [28], and *Luscinia svecica* [29]. The other pattern, on the contrary, has a well-defined phylogeographic structure and is found in *C. calliope* [17], *Motacilla flava* s.l., *M. citreola* s.l. [30], *Ficedula parva* s.l. and *Alauda arvensis* s.l. [31], and *Pinicola enucleator* [32]. The causes of such phenomena are usually explained by the events of the ice age. *C. pectoralis*-complex on the basis of the distribution of the mitochondrial marker (*cytb*) is characterized by a well-defined phylogeographic structure and, according to Y. Liu et al., separated from its closest relative, *C. calliope*, populating the northern regions in the period of the Early Pliocene (3.2–4.8 Ma) [21].

Earlier, using the example of the Siberian rubythroat, we demonstrated alternative ways of forming phylogeographic structure of species with participation of nuclear pseudogenes [17]. Our present data suggest a similar scenario for the formation of the phylogeographic structure for the *C. pectoralis* group. The nuclear copies of *cytb* gene mtDNA discovered in *C. calliope* are similar to the mitochondrial copy of the *C. pectoralis* gene. How can one explain this homology of sequences from different genomes located in different taxa? Theoretically, there are two ways of forming divergent haplotypes of mtDNA. The first is a gradual accumulation of substitutions as result of a prolonged isolation of the population. The second is interspecific hybridization, as a result of which a new variant of the haplotype appears that is not typical of this taxon. However, the similarity of distribution of polymorphic sites between mtDNA and its nuclear copies cannot be explained by any of these pathways. The third path, which is considered here, is the common homologous recombination between the nuclear and mitochondrial genomes, which can lead to sudden change of the mt-haplotype to a new mtDNA paralog identical to the existing one in the nuclear genome.

Crossover between homologous sites of the nuclear and mitochondrial genomes and the transfer of a

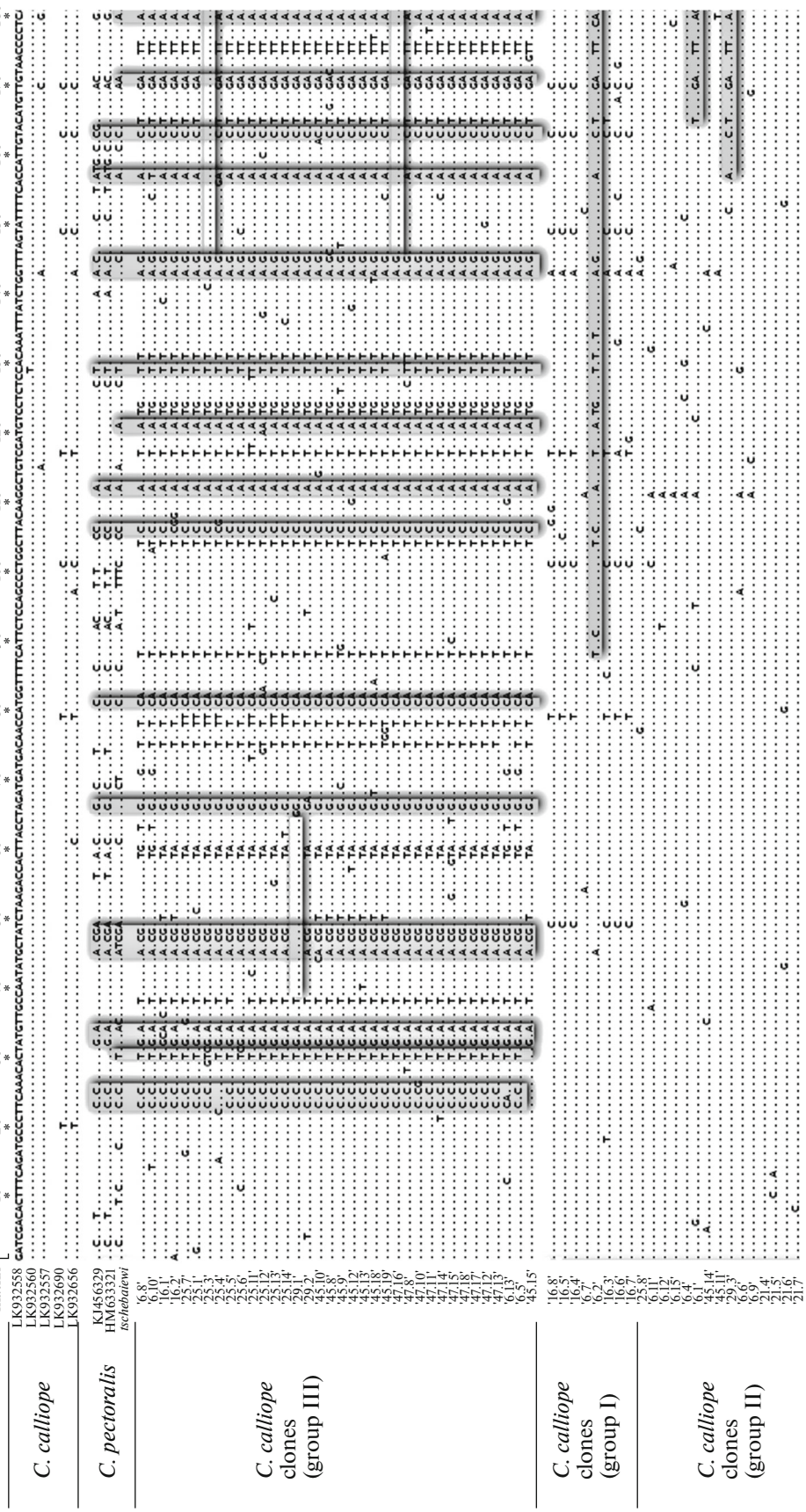

Fig. 3. Matrix of polymorphic sites of *cytb* gene mtDNA from *C. calliope* and *C. pectoralis* and cloned nuclear copies from *C. calliope*. In gray vertically are sites identical in *C. pectoralis* and nuclear copies of *C. calliope*; in gray horizontally are recombinant sites of nuclear pseudogene variants in *C. calliope*. Numbers indicate numbers of migrant birds of *C. calliope* and its clones.

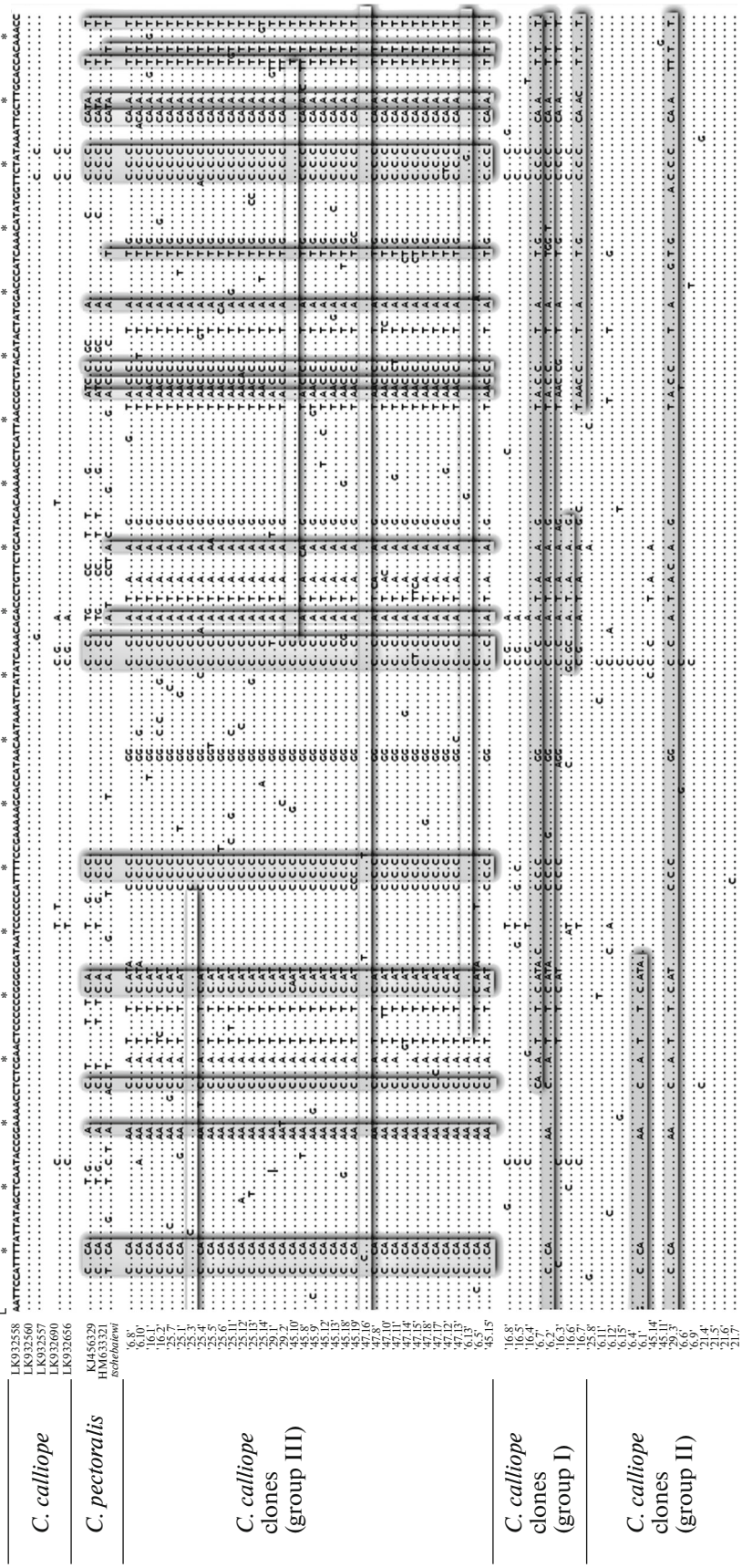
C. calliope
C. pectoralis
C. calliope clones (group III)
C. calliope clones (group I)
C. calliope clones (group II)
matrix
LK932558
LK932560
LK932557
LK932690
LK932656
K1456329
HM633323
tschebaiewi

Fig. 3. (Contd.)

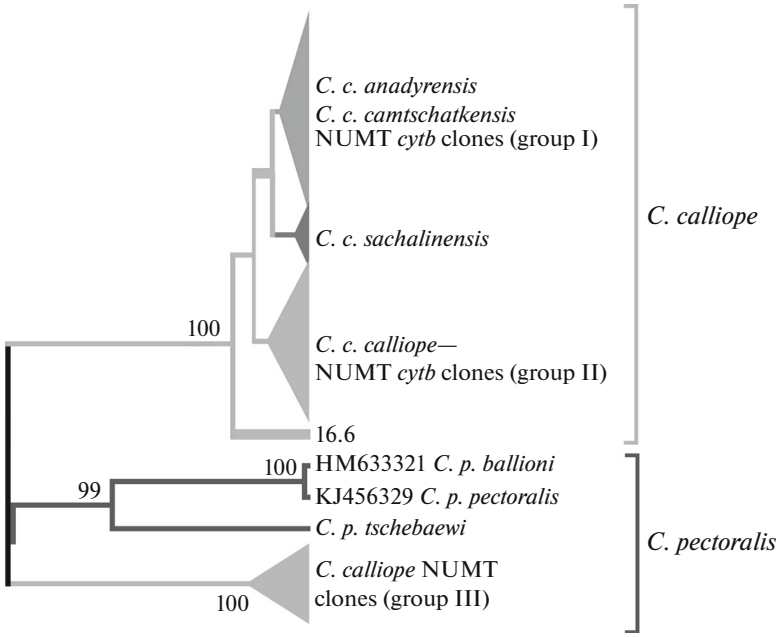

Fig. 4. Reconstruction of phylogenetic links of mitochondrial haplotypes of *C. pectoralis* and nuclear copies of mitochondrial genes in *C. calliope* built according to the maximum likelihood method. I, II, and III are clones variants.

nuclear copy of the *cytb* gene to mtDNA could have happened in the genome of a hybrid female that became the founder of the *C. pectoralis* species. Thus, *C. pectoralis* likely got its mt-haplotype from the nuclear pseudogenes of *C. calliope* (one of the parents). The transport of nucleic acids of various lengths into mitochondria and the mechanisms by which these processes occur are described in the literature [33, 34]. In the detected recombination event, we have not been able to determine the full length of the recombinant fragment. Its length could be very significant, as was established using the example of a domestic cat [35]. However, the presence of the *cytb* gene and control region (*CR*) in this recombination fragment was established experimentally.

The identified recombination event may have occurred during or shortly after interspecific hybridization on the periphery of the breeding range of *C. calliope beicki* and the breeding range of *C. obscura*. Low numbers of individuals, most likely in the area where their modern zone of contact with the *C. p. tschebaiewi* subspecies is located, apparently contributed to the subsequent spread of the recombinant mt-haplotype by the mechanism of the founder effect (Fig. 1). This made it possible for the new haplotype to gain a foothold and become taxon-specific as new territories were resettled. The inverse assumption about the introduction of the mitochondrial haplotype of *C. p. tschebaiewi* into the nuclear gene of *C. calliope* seems unlikely for several reasons. The first reason is because of the considerable geographic remoteness of the sites where the nuclear pseudogene was discovered (Russian Far East), and the second reason is due to the high breeding philopatry of these rubythroats species.

Figure 5 shows the relationship of *Calliope* species according to two nuclear genes taken from the paper of Liu et al. [21]. It is important to note the separation of the *C. pectoralis*-complex into two phyletic lines. One of these lines is closely related to *C. calliope*, and the second slightly diverged, but both are in the clade of the latter species. This result is fully consistent with our data and confirms the hypothesis of the hybrid origin of the *C. pectoralis* species from *C. calliope*.

Interspecific hybridization in birds occurs quite often and has been reported for approximately 850 species [36]. However, isolation of interspecific hybrids into a separate species and the occurrence of precopulation isolating mechanisms are rare. Not long ago, a new type of Darwin-finch was formed as the result of natural hybridization over the course of several generations of Espanola Cactus-finch *Geospiza conirostris* with Medium Ground-finch *G. fortis* [37]. Individuals of the new species of birds differ from the parental species in the shape and size of the beak, have different vocalizations, and mate only with representatives of their species. It is likely, that the *C. pectoralis* complex developed in the same way.

According to the mitochondrial markers data, the divergence time in the "molecular clock" between the *C. calliope* and *C. pectoralis* haplotypes is estimated at 4 million years [21]. The results of our study cast doubt on the suitability of the "molecular clock" estimate for this event not only because of the abrupt haplotype change but also due to the fact that accumulation of

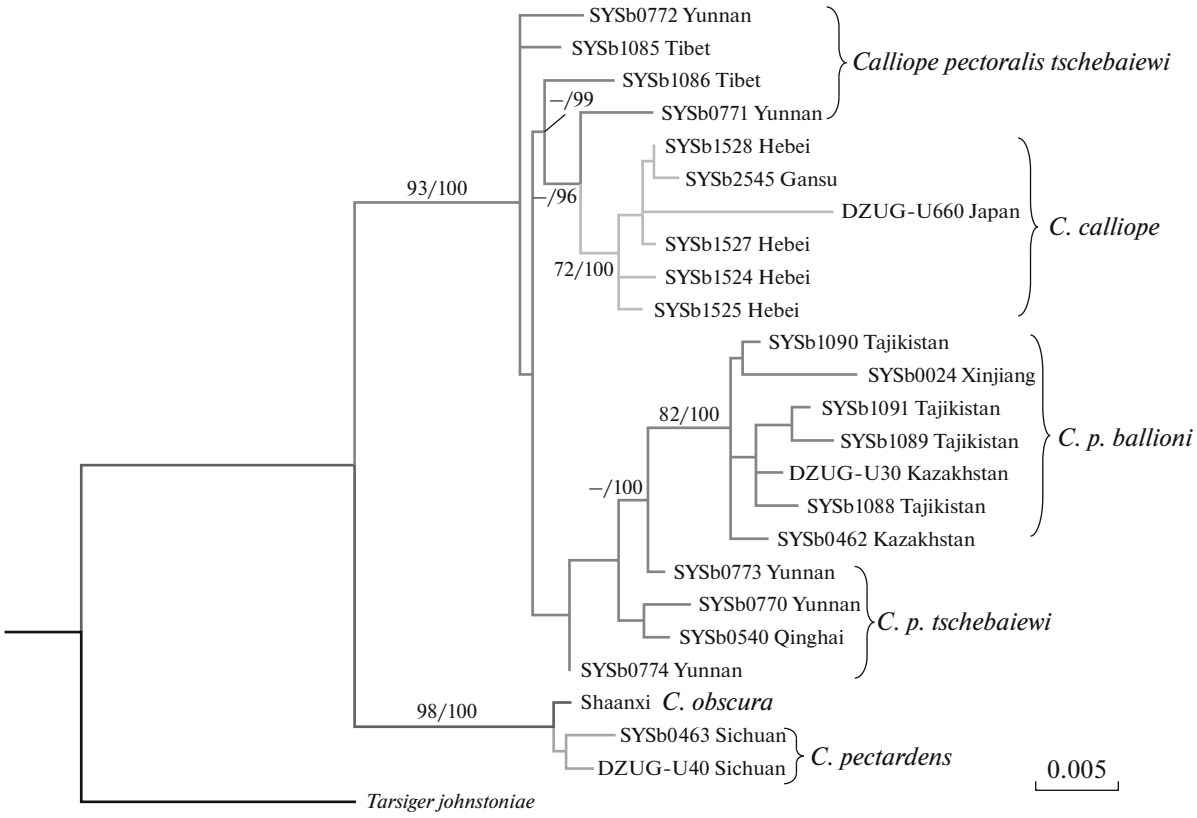

Fig. 5. Phylogenetic relationships between four *Calliope* species constructed on the basis of combined sequences of nuclear myoglobin and ODC gene (1364 bp) [21].

mutations occurred first in the nuclear genome. Comparing the genetic similarity (96%) of nuclear copies of the mtDNA in *C. calliope* and mitochondrial *cytb* haplotype in *C. pectoralis* with the case described in our previous work [11], we can state that formation of the latter species as a result of hybridization occurred earlier than the emergence of *C. calliope* subspecies. This is supported by the fact that similarity between the *cytb* gene and its nuclear copies in *C. calliope* is higher (up to 100%) compared with those of *C. pectoralis*.

In addition to genetic data pointing to the hybrid origin of *C. pectoralis*, we conducted a detailed phenotypic comparison of possible participants in hybridization resulting in the appearance of this species. Representatives of the genus *Calliope* can be distinguished quite clearly on the basis of the external morphological features primarily owing to the characteristics of the color of their plumage (Fig. 6). The characteristic traits of the Blackthroat *C. obscura* include blackish-blue above in adult males, absence of depigmented parts on the sides of the crown ("white supercilium") and the sides of the throat light submoustachial stripe ("whiskers"), presence of broad white part at the base of the tail-feathers (except for the central pair), and a relatively short tail. In addition, in adults, pigmented parts of the tail feathers are completely black. For males of the Blackthroat, a completely black color is characteristic on the sides of the head, throat, and breast, forming a wide black plastron, which contrasts sharply with the predominantly white color of the abdomen.

Siberian rubythroat *C. calliope* differs in the presence in males of a sharply expressed colored plastron (formed by feathers with bright red tops and white bases), covering the throat, upper part of the breast, sharply contrasted by the black feathers of lateral throat-stripe on the sides of the throat and turning into a gray tint on the sides of the goiter and breast. For both sexes, there is a peculiar topography of the head color due to the presence of clearly defined light supercilium (above the eye and ear-coverts), as well as light sub-moustachial stripe. Both males and females are characterized by olive brown coloring of the upper side of the head and body, monochromatic (without white parts) coloring of the rectrices feather of the same tint, and a relatively long tail length.

The Himalayan rubythroat subspecies show external features that can be considered transitional, in varying degrees, between Blackthroat and Siberian rubythroat. Subspecies *C. p. pectoralis*, *C. p. ballioni*, and *C. p. confusa* have an olive-bluish-gray coloration of the upper side of the body; males, on the chin and throat, show a relatively small red spot, surrounded on all sides by a completely black plastron (feathers of the

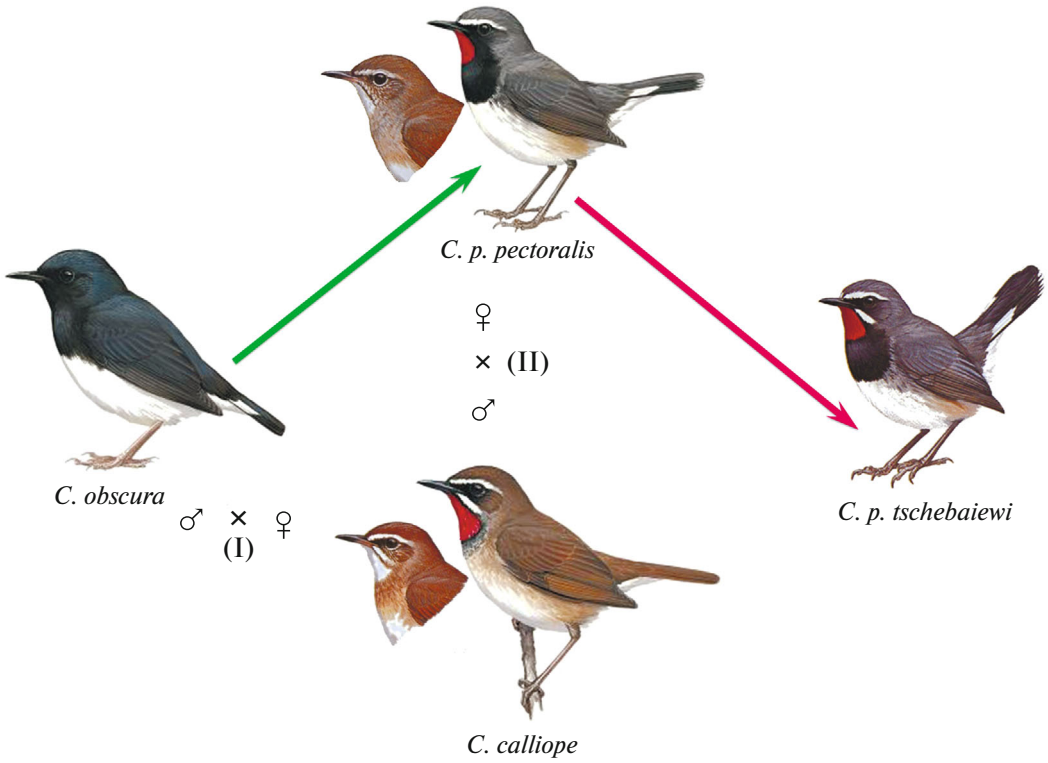

Fig. 6. Scheme of the two proposed events of interspecific hybridization (I and II), with indication of the sex of birds of each parental species, that resulted in the emergence of *C. pectoralis*. Green arrow indicates the form resulting from hybridization I; red arrow indicates the form resulting from hybridization II. For color photos, please see the article in electronic form.

moustachial stripe, front and lower parts of the ear-covers, sides of the throat, and most of the breast); both sexes have a white supercilium, which is noticeably shorter than that of *C. calliope* and ends immediately behind the eye. The tail is longer than in *C. obscura*, but on average shorter than in Siberian rubythroat. In males, the central rectrices are entirely brownish gray. Pigmented parts of other tail feathers are black. In turn, the bases of most tail feathers are white, with a width of depigmented areas on average less than that in Blackthroat. A unique feature of coloration of all Himalayan Rubythroat is the presence of white spots at the tips of most tail feathers (with the exception of the central pair), less pronounced or absent in females and young birds [16].

The second parental species of *C. pectoralis*, judging by the morphological features, could have been *C. obscura*. Among all of the species of *Caliope* genus, only this species has a black throat and chest. *C. pectoralis* retained phenotypic traits of both prospective parents. Males have red throats and white eyebrows (also, white sub-moustachial stripe in *C. p. tschebaiewi*) from *C. calliope* and black chest and black, with white bases, rectrices from *C. obscura*. Females look like females of *C. calliope*, but are darker and gray. Young birds look like those of *C. calliope*. Notably, *C. pectoralis* birds differ from both *C. calliope* and *C. obscura* in the white tips of the rectrices, characteristic only of representatives of the *C. pectoralis*-complex.

C. p. tschebaiewi more differs in the morphologic characteristics from other *C. pectoralis* races, and it is similar to *C. calliope*. The red spot in males of this form is noticeably larger than that of all other subspecies of *C. pectoralis* (in addition to the chin and throat, it covers the upper part of the breast) and is similar in size to that of *C. calliope*. The black plastron on the breast is significantly smaller than in other *C. pectoralis* subspecies. The light supercilium above the eye is on average longer than that of the *C. pectoralis* subspecies. The white sub-moustachial stripe is well expressed on the sides of the throat, quite similar to *C. calliope*. The coloration of the upper side of the body is grayish-olive in males and olive-brown in females, most closely approaching the coloring of the upper side of the body of Siberian Rubythroat. White spots at the tips of the rectrices are smaller than those of other subspecies of *C. pectoralis* and are often absent in females and young birds [16]. The tail is longer than that of other races of Himalayan rubythroat. Overall, in its proportions, *C. p. tschebaiewi* is very like to the Chinese subspecies *C. calliope beicki*. Thus, the form *tschebaiewi* objectively has a greater morphological and phenotypic similarity to *C. calliope* than other subspecies of *C. pectoralis*, which indirectly indicates its hybrid origin.

In our opinion, the separation of *pectoralis* in two phylogenetic lines can be explained by two facts of hybridization, the scheme of which is shown in Fig. 6. At the first event, female *C. calliope* carrying a recombinant mtDNA haplotype hybridized with a male *C. obscura* (Fig. 6). The result of this incident was emergence of the form that became ancestral to *pectoralis*, *ballioni*, and *confusa* subspecies, which have a wider black plastron on the breast, similar to *C. obscura* and apparently inherited from it, as well as a red spot on the throat and white supercilium acquired from *C. calliope*. At the second event, participants in hybridization were a male *C. calliope* and a female *C. pectoralis*. The offspring of this pair already carried a double dose of the nuclear genome of *C. calliope*, which is reflected on a tree constructed from nuclear genes (Fig. 5) [21]. The result of this incident was the emergence of the form *tschebaiewi*, in which the morphological characteristics of *C. calliope* were manifested significantly more strongly than in other forms of *C. pectoralis*.

Thus, we discovered new facts that point to a homologous recombination between the portions of mitochondrial and nuclear genomes in the Siberian Rubythroat, which resulted in the emergence of new mitochondrial haplotypes in the hybrid species of *C. pectoralis* on the basis of the uncovered nuclear pseudogene sequences. According to the molecular data and morphological characters, *C. pectoralis* is an interspecific hybrid of *C. calliope* and *C. obscura*, and its mitochondrial haplotype has a nuclear origin. Thus, we established a novel case of intergenomic recombination in the *Calliope* genus. Detected variants of nuclear copies of the *C. calliope cytb* gene (Fig. 3) are a potential source of the new mtDNA haplotypes and open a grand hidden stock of molecular variability in mtDNA, which can be implemented through intergenomic recombination.

Our data show the potential importance of using NUMT to correctly analyze phylogenetic reconstructions. Future studies of nuclear paralogs will promote a rethinking of their role in the formation of genetic diversity in nature and the assessment of the rate of microevolutionary processes using mitochondrial markers. A new fact of the transfer of nuclear copies of mtDNA into the mitochondrial genome through symmetric recombination confirms the interaction of the nuclear and mitochondrial genomes as a single conjugate genetic system of the cell.

The novel molecular and morphological data presented in this report strongly supports the hybrid origin of *C. pectoralis* based on the mechanism of symmetric recombination of DNA fragments between the nucleus and mitochondria and the role of NUMT—nuclear copies of mtDNA—as a potential source of novel taxon-specific mitochondrial haplotypes.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. 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 the care and use of animals were followed.

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