ANIMAL GENETICS

Phylogeography of Carrion, Hooded, and Jungle Crows (Aves, Corvidae) Inferred from Partial Sequencing of the Mitochondrial DNA Cytochrome b Gene

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Abstract—Distribution of mitochondrial DNA cytochrome b gene haplotypes in two crow species was examined by means of sequencing of the 336-bp gene fragment. The topology of the NJ and UPGMA trees showed that the carrion crow range was split into two parts due to the presence of significantly diverged ancestral lineages localized in the southeastern part of the range. The carrion crow populations, inhabiting a territory ranging from France to northern Sakhalin, along with interspersed hooded crow populations and hybrid Siberian populations, shared a common haplotype. The border between two carrion crow lineages is located in central Sakhalin. The subdivision of two weakly differentiated lineages within the jungle crow range, also observed within this territory, coincided with the subspecies division of this species. The estimated genetic distances indicate the isolation of the subgenus Coelocanus. These data also suggest the convergent similarity between the chough Pyrrhocorax pyrrhocorax and the Corvus genus, as well as the conspecificity of Corvus corone cornix and C. c. corone.

INTRODUCTION

The crow, one of most common birds in Russia, displays a quite uncommon distribution. The central part of Palearctic is occupied by hooded crow Corvus corone cornix populations that, in the east and west, are bordered by populations of carrion crow C. c. corone and C. c. orientalis (Fig. 1). These borders are represented by narrow and stable hybrid zones, which attract the special interest of evolutionists. The taxonomic interpretation of this situation depends on the evaluation of the hybridization range and the accepted species concepts. Some authors assign all crows to a single species [1], while others, in view of the conspecificity of western and eastern isolates of carrion crow, consider that the latter and the hooded crow belong to different species [2]. Genetic markers seem promising for clarification of taxonomic relationships and for determination of distribution limits of the lineages. However, reliable genetic markers for carrion and hooded crows have not yet been discovered either among the allozymes [3, 4] or among the RFLP [5] and minisatellite [6] markers. Another closely related species, jungle crow C. macrorhynchos, is widely distributed in Central and Eastern Asia. This species resembles the carrion crow in its coloration, but differs from it in other morphological characters, as well as in its behavior and ecology. The jungle crow is interesting not only in comparison with the carrion crow but also with respect to the analysis of geographic variability across its large discontinuous area, which includes island isolates.

In present-day molecular phylogeny and taxonomy studies, mitochondrial DNA markers are often used. Their advantages compared to the nuclear markers include conservative gene order and location of amino-acid substitutions, insertions, and deletions [7-9], along with high rates of synonymous substitutions [10-13]. The existence of silent substitutions increases the probability that the signs of synapomorphies, which shed light on recent common ancestors, are conserved in the mitochondrial DNA molecules. This fact is of particular importance for phylogenetic studies of birds, which are characterized by low rates of molecular evolution [14]. The maternal type of mtDNA inheritance permits determination of the gene flow direction upon hybridization. Phylogenetic studies of birds based on DNA sequencing usually utilize the cytochrome b gene as the mtDNA marker. This gene demonstrated high-resolution capacity on the species level, although sometimes it is used for interspecific comparisons [15-17].

The present study was focused on the search for new molecular markers of each geographical form and isolate of carrion, hooded, and jungle crows, and on determination of the limits of mitochondrial lineages distribution. Specific features of natural hybrid crow populations were examined. Patterns of nucleotide substitutions at the early stages of divergence were compared.

MATERIALS AND METHODS

Samples. Experiments were carried out using 43 liver samples from hooded (C. c. cornix), carrion

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(C. c. corone and C. c. orientalis), and jungle (C. macrorhynchos mandshuricus and C. m. japonensis) crows belonging to 20 populations inhabiting the territory extending from France to Sakhalin and the Japanese Islands (Fig. 1; table). In addition, the International Gene Bank database on corrin crow (C. c. corone) from France, raven (C. corax), daw (C. monedula), rook (C. frugilegus), and Australian raven (C. coronoides) were used. For better tree robustness, the outgroup was represented by three genera: magpie (Pica pica jankowskii from our collection and F. p. galliæ from the Gene Bank), plush built jay (Cyanocorax chrysops), and chough (Pyrrhocorax pyrrhocorax) from the Gene Bank. A total of 52 sequences were analyzed.

Nomenclature. In the present study, we consider crows as a single species, Corvus corone sensu lato [1], and accept the following nomenclature: C. c. corinex for the hooded crow, and C. c. corone and C. c. orientalis for the western and eastern corrin crow isolates, respectively. For the jungle crow, its traditional name, C. macrorhynchos, was kept. Only western japonensis population groups [18] of the latter species were examined. For magpie, generally accepted nomenclature was used [2].

DNA extraction and amplification. DNA from either fresh liver samples or those fixed in the field (by freezing or ethanol) was isolated according to the standard methods including phenol–detergent deproteinization along with RNAse and pronase treatment [19]. The 336-bp fragment of the mtDNA cytochrome b gene was amplified by means of polymerase chain reaction (PCR). The reaction mixture contained 0.5 to 1% DNA; 10% 10× buffer; 2.5 mM MgCl₂; 0% dNTP mixture; 0.5% Taq polymerase; and 5% each of two primers in bidistilled water. For the first PCR round, the following primers were used [20]: L14827 (ND5) 5'-CCA-CACCTCCACACAGGCTAATTAA-3'; H16605 (tRNA-thr) 5'-GGAGTCTTTCAGTCTCTGTTTTACAAGAC-3'. In the second PCR round, the concentration of MgCl₂ was changed to 1.87%, and the L14827 and H15916 5'-ATGAAAGGATGTCTACTGTTG-3' [12] primers were used. Letters L and H designate light and heavy chains, and the figures represent the position of the primer's 3'-nucleotide in the complete sequence of chicken mtDNA [8]. The first two primers are specific to the genes adjacent to the both ends of the cytochrome b gene, the protein-encoding ND5 gene and the tRNA-thr gene [20]. In some cases, only one round of PCR was carried out using the SNL4, 5'-CATCTAC-CTACACATCGGCCGAGG-3' (constructed by us), and H15916 sequences as internal primers. The primer concentration was 1 pmol/μL. The reaction was run for 35 cycles of 96°C for 30 s; 50°C for 30 s; and 60°C for 30 s. The PCR products were analyzed in 1% agarose gels with subsequent ethidium bromide staining and visualization in UV light.
The list of samples studied

<table>
<thead>
<tr>
<th>Sample</th>
<th>Site number</th>
<th>Locality</th>
<th>Sample code</th>
<th>No. of individuals</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1 Paris</td>
<td>CC Paris</td>
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<tr>
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<td>HC Mosc</td>
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<tr>
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<td>HC Itat</td>
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<td>hybrids</td>
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<td>HBR Itat</td>
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<td>8 southern Primorye</td>
<td>JC Prim</td>
<td>3</td>
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</table>

Samples from the Gene Bank

<table>
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<th>Locality</th>
<th>Sample code</th>
<th>No. of individuals</th>
</tr>
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<tbody>
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<td>U86032</td>
<td>CC Fr GB</td>
</tr>
<tr>
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<td></td>
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<td>A. raven GB</td>
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<td>Magp. GB</td>
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<td>Jay GB</td>
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<td>chough</td>
<td></td>
<td>U86044</td>
<td>Ch. GB</td>
</tr>
</tbody>
</table>

Note: The point numbers correspond to the map in Fig. 1. The sample codes correspond to the trees in Figs. 1 and 2.

Sequencing. The product of the sond PCR was treated with Taq Dye Primer cycle sequence kit and subjected to amplification: 15 cycles at 96°C for 10 s; 55°C for 5 s; and 70°C for 60 s; followed by 15 cycles of 96°C for 10 s and 70°C for 60 s. The reaction products were precipitated with ethanol and, after thermal pretreatment, were sequenced using the automatic sequencer 373A, ABI. The DNA fragment was sequenced in both directions and, in the case of utilization of the SNL4 and H15916 primers, sequencing in one direction was sufficient. The lengths of the fragments sequenced constituted at least 336 bp.

Phylogenetic analysis. For removal of recognition errors, nucleotide sequences converted into letter files were first analyzed by use of the DNAsis 2.0 program (Copyright Hitachi, 1996). For construction of the trees by means of neighbor–joining (NJ) method [21], the CLUSTAL W 1.6 (Macintosh) program was utilized. Branch significance was tested by means of bootstrap analysis with 1000 iterations. For construction of the phenograms according to the unweighted pair group method with arithmetic averages (UPGMA) [22] from Kimura's two-parameter distance matrix [23], as well as some other distance matrices, the original technique was applied. Transversions and transitions in each codon position were scored separately.

RESULTS

Nucleotide sequences of the 336-bp mtDNA cytochrome b gene fragments were determined. The matri-
ces of pairwise comparisons represented the total amount of nucleotide substitutions, the ratios between the differing and common bases, as well as some other genetic distances along with the numbers of transitions and transversions in each of three codon positions and the ratios between them. No deletions or insertions were found. Synonymic transitions in the third codon position were the prevailing substitution type. The lowest number of transitions was observed in the corone-coronix cluster. Transversions between the jungle crow populations were not detected, and only a few of these were observed in the corone-coronix population cluster. The ratio between the transitions and transversions in the pairwise species comparisons varied from 9 to 13.5.

Based on primary data, we constructed two types of phylogenetic trees: the NJ (Fig. 2) and the UPGMA (Fig. 3) trees. With the exception of some details, both tree types displayed similar topology. Each branch represented either a species or a geographically significant association of the populations. The significance of branching on the NJ trees was different: the bootstrap estimates ranged from 39 to 100%. The Australian raven C. corone s.l., jungle crow, and rook almost simultaneously radiate from the main stem. They are followed by daw and the species of the outgroup. The distinction between the raven and crows and that between the jungle crow and rook were highly statistically significant. The daw was statistically significantly distinct from other representatives of the genus.

The cluster corresponding to the carrion and hooded crows, i.e., C. corone s.l., was the largest in respect to the number of samples. Intergroup differences within this cluster constituted from 0 to 8 substitutions per 336 nucleotides, and the P distances reached the value of 0.02. Surprisingly, the subdivision within this cluster was inconsistent with the division of the species into carrion and hooded crows. Instead, we obtained a common subcluster that comprised populations of carrion crow from France in the West, populations of hooded crow from Moscow to Novosibirsk, as well as from the Siberian hybrid zone, and populations of carrion crow inhabiting the territories from Krasnoyarsk to northern Sakhalin in the East. Another group united included samples of carrion crow ranging from southern Sakhalin and Kunashir to Hokkaido, Honshu, and Primorye. Thus, the border between the two found haplotypes runs through the central part of Sakhalin and Eastern Siberia, where its position has not yet been determined due to a lack of material. The level of differences within each subcluster was not high and constituted only from 1 to 4 substitutions. The division of the C. corone s.l. cluster into two parts was statistically significant, since the bootstrap estimates for the NJ tree reached 95%, and the distance of 0.01 corresponded to 4–8 substitutions. All four UPGMA phenograms based on four different distance matrices displayed similar subdivision into two clusters (Fig. 3).

Within the jungle crow cluster, a slight subdivision into two groups at the level of four substitutions (transitions) was observed. Most of the tree topologies (the UPGMA trees with the Kimura distances, shown in Fig. 3, and the trees with the Jukes-Cantor and P distance matrices, data not shown) displayed subdivision into two groups, which corresponded to the populations of Primorye and northern Sakhalin, on the one hand, and to the populations of southern Sakhalin, Kunashir, Hokkaido, and Honshu, on the other hand. However, this differentiation was not reflected in the topology of the NJ tree and the UPGMA tree with the Tajima-Nei distance matrix. Probably, within the examined part of the range, this species is more homogenous than the carrion crow.

The birds belonging to one population often shared common nucleotide sequences. For instance, in the carrion crow populations from France, Primorye, and southern Sakhalin, two shared haplotypes were revealed. The same situation was observed in the hooded crows from the Moscow population and in the jungle crows from Honshu and northern Sakhalin. The examined set of eleven crows from the Siberian hybrid zone, including phenotypically gray, black, and birds of intermediate color, was found to be homogenous with only one substitution. However, cases of intrapopulation haplotype polymorphism were also observed. Two samples of jungle crow from Kunashir and two of these from Primorye differed by one transition, whereas two jungle crow samples from northern Sakhalin differed by two transitions. The differences within the samples of carrion crow from Primorye, southern Sakhalin, France, and Krasnoyarsk, and the hooded crow from Novosibirsk constituted 1 to 2 substitutions. On the other hand, some cases of the sequence coincidence among the birds from different populations were described (Fig. 2). For more concise data presentation, consensus sequences (majority) (one for each of the clusters, distinguished on the NJ tree) were obtained. Based on these data, the matrices and the corresponding tree repeating the topology of the more detailed NJ tree were obtained (data not shown).

DISCUSSION

Sequence analysis of the 336-bp gene fragment was sufficient for estimation of certain haplotype distribution. An analysis of longer sequences allowed us more detailed interspecific and intergeneric comparisons and estimation of the divergence time, which will be the subject of the forthcoming paper (currently in preparation).

All the trees constructed displayed subdivisions within the cluster of hooded and carrion crows. One subcluster included the carrion crow populations inhabiting the territory extending from France to northern Sakhalin and the hooded crow populations, while the other subcluster included the carrion crow populations from the southeastern margin of the range. The latter
Fig. 2. Molecular phylogeny of the crows and the species of the outgroup as inferred from the sequence of the mtDNA cytochrome b gene 336-bp fragment. The bootstrap levels in percents over 1000 trials are shown below the branches of the neighbor-joining tree. The branch lengths are proportional to the number of nucleotide substitutions per site. The sample codes correspond to the table.

group of populations is located close to the node of the NJ and UPGMA trees. Thus, it can be considered as the initial group of the whole cluster.

The studies of molecular genetic variability among the true hybrid corone × cornix populations from Siberia are of particular interest. The majority of the animal hybrid zones studied so far were characterized by increased variability and by the appearance of new alleles manifested as new electromorphic variants, called hybrizymes [24, 25]. Hybrid crow populations are distinguished by unique phenotypic variability [26]. They also contain some new alleles [4]. However, within the fragment of the mitochondrial genome studied, no differences between the hybrids and the parental forms were observed, and no increase in variability was detected. Therefore, the marker used appeared to be unpromising in respect to the hybrid zone analysis.

The interspecific relationships within the corvine assemblage will be described in a separate publication. However, some aspects of the issue are worth men-
ing in the present paper. The trees constructed were partly congruent with the views on the phylogeny of the *Corvus* genera, which is based on morphological and ecological characters [27]. The chough *Pyrrhocorax pyrrhocorax* is similar to the true crows with respect to some morphological traits [28]. However, the behavioral and ecological differences of this species from those of the *Corvus* genus [29] are reflected in the data on cytochrome b gene sequencing ([30] and our data). These findings suggest the convergent similarity between these genera. The position of daw *Corvus monedula* on the molecular tree was quite distant from the other corvine birds (Fig. 2): its divergence inferred from cyt b data constituted 10 to 11%, which is significantly higher than the level of interspecific differences. These data are in agreement with the ecological features distinguishing this bird from the *Corvus* species, e.g., nesting in hollows, smaller size, and a weak beak. The daw display some other morphological differences, the most important of which is the structure of its digestive system [31]. In total, this evidence suggests a separate subgenus *Coleos*, to which the daw was ascribed [32], or even a separate genus of this name [33].

It is well known that using transitions versus transversions is a more useful method for evaluating genetic distances between young branches [20, 35]. This was confirmed by the tree constructions. The topologies of the UPGMA trees constructed based on the P. Jukes–Cantor, and Kimura distance matrices and transitions and transversions were generally identical. The use of transitions alone resulted in the construction of simplified, but principally indistinguishable, trees. The transition-based trees, however, do not distinguish lineages within the jungle crow species. This finding confirms the greater value of transitions for analyzing low divergence. At the same time, the existence of transversion substitutions between carrion crow lineages of the Far East indicates ancient radiation.

The revealed subdivision of the *Corvus corone orientalis* range into two parts motivates examination of the history of this species. Based on the available data, it is possible to suggest that the modern crow phenotype originates from Southeast Asia. After separation of the northern part of the east Asian range, presumably in early Pleistocene, this lineage had spread to the west to Western Europe. The range is assumed to have shifted and to have undergone subsequent fragmentation caused by the climatic changes that took place in the Pleistocene several hundred thousand years ago. The development of the contemporary phenotype of the hooded crow with the preservation of the initial mitochondrial haplotype has occurred in one of these refugiums. The younger evolutionary age of the hooded crow is confirmed by biogeographical evidence [27]. Development of the modern-day forest-steppe landscape was accompanied by an extension of the area of each of three isolates to the north with the formation of secondary zones of overlapping and hybridization between the carrion and hooded crows [17]. Europe and Siberia. By analogy, the contact between the two carrion crow lineages in eastern Siberia and Sakhalin is also likely to be secondary.

The discrepancies between morphological and molecular genetic intraspecific differentiation have been repeatedly described. For birds, the following examples can be cited. In the dusky seaside sparrow *Ammodramus maritimus*, geographical subdivision into two mtDNA clusters differing by 1% was found [35]. Analysis of mtDNA restriction polymorphism in two Australian bird species *Zosterops lutea* and *Z. lateralis* showed that two closely located populations belonging to different species had a similar haplotype, and, conversely, that the haplotype differences within each species reached about 2.3% [36]. It is suggested that interspecies haplotype similarity resulted from hybridization between these species. In the morphologically homogeneous population of European blue tits, two mtDNA lineages differing by 1.2%, were described. This fact was explained either by presump-
tive hybridization between the Parus caeruleus and P. cyanus or by post-glacial fusion of two P. caeruleus isolates [37].

The subdivision of the jungle crow mtDNA haplotypes described in the present study is in good agreement with the fragmentation into two subspecies: the continental form, C. m. mandshuricus, also inhabiting northern Sakhalin, and the island form, C. m. japonensis, which inhabits southern Sakhalin and Japanese Islands. These forms differ in some morphological traits. For example, beak height varies from 24 to 27 mm in the first form, and from 28 to 32 mm in the second form [38]. The variation of this parameter in the birds analyzed for mtDNA sequences in the present study lies within the same limits and constituted 26.4 and 27.8 mm, respectively. Interestingly, the ecological conditions of the two forms are different. In the north of the island, they inhabit the seashores and the marshes; while in the south, they live near settlements (V.A. Nечаев, personal communication). The dissociation of the two forms during overwintering and migrations was observed. The mandshuricus birds migrate from Sakhalin to the mainland, and the japonensis crows move to islands situated farther south [39]. These data present indirect evidence supporting different histories of the colonization of Sakhalin by these forms. The existence of two foci, and correspondingly, two pathways of the C. macrorhynchos range formation can be suggested. One of these runs from the Ussuri-Korean subcenter in the south [40], through the Korean Peninsula and Japan Islands to southern Sakhalin. Another pathway begins in the north and passes through the mainland to northern Sakhalin. Our data, however, still cannot explain the relationships between these two forms in central Sakhalin, where either overlapping or limited hybridization, i.e., secondary contact [39], exists. It is noteworthy that, due to the lack of distinct morphological differences between the two forms, it is difficult to isolate and analyze this zone. The subdivision of the mtDNA lineages revealed for carrion and hooded crow species in the central Sakhalin confirms the location of the zoological-geographical border, which was earlier described for insects [41] and some other birds [39], in this particular territory. The zones of secondary contacts of recent migrants to islands of the Far East and to the eastern extremity of Asia require further investigation.

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REFERENCES


