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ANIMAL GENETICS

Phylogenetic Relationships within the Corvine Assemblage (Aves, Corvidae) Based on Partial Sequencing of the Mitochondrial DNA Cytochrome b Gene

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Abstract—To establish phylogenetic relationships within the corvine birds at the interspecific and intergeneric levels, the sequence of the mitochondrial DNA cytochrome b gene was analyzed. The NJ, UPGMA, and MP trees showed similar clustering. Relationships between the jungle crow, on the one hand, and the rook and Australian raven, on the other hand, were closer than those between the jungle crow and the hooded and carrion crows. The mitochondrial genome of the Australian raven displayed the closest similarity to the ancestral genome of the genus *Corvus*. Populations inhabiting the eastern part of the carrion crow *C. corone orientalis* area were statistically significantly subdivided into two lineages. These data also confirmed the hypothesis on the location of the carrion crow's ancestral lineage in the southeastern part of the area. In general, the transition and transversion substitution levels, their relationships, and distribution over codon positions were similar to that already reported for birds. Synonymous transitions in the third codon position were the prevailing substitution type. Using standard calibration scales, the time of divergence between species and genera within the corvine family was estimated to be 3.1–4 and 3.8–8.8 My respectively. The divergence time between the examined corvine birds and birds of paradise was from 8 to 10 MYa.

INTRODUCTION

The family Corvidae belongs to the most prosperous group of birds. The members of this family vary in size, appearance, and coloration. They are distributed throughout the world and inhabit all landscapes. This family contains 113 species grouped in 25 genera [1, 2], including the young genus *Corvus*, which contains 38 species. DNA hybridization data showed that the family originated from Australia and colonized Eurasia 20–30 MY ago [3]. Evaluation of the cytochrome b gene sequence divergence confirmed that Corvidae and Paradisaeidae (birds of paradise) separated about 20 MYa[4]. To date, information on cytochrome b gene sequences for about 25 corvine species is available, permitting extended comparisons. Some authors use this marker, occasionally together with morphological characters [4, 6], to estimate intergeneric relationships [5]. However, phylogenetic relationships within the Corvidae family remain obscure and the status of some forms is still questionable [7].

In studies of molecular phylogeny and taxonomy, mitochondrial DNA markers are widely used. Their application has stimulated many evolutionary studies and provided solutions to problems unsolved by other methods [8]. Development of the polymerase chain reaction technique, along with the construction of universal primers, promoted sequencing of individual genes, including the mitochondrial gene encoding the cytochrome b protein. The high evolution rate of this gene makes it a promising marker for population studies; at the same time, its sequence is rather conservative [9]. Molecular phylogenetic studies of birds, characterized by low rates of molecular evolution, usually utilize the cytochrome b gene as the mtDNA marker. The sequence data obtained in different laboratories can be easily compared; gene banks contain over 3000 such sequences for vertebrates. It has been shown that mitochondrial haplotype-based trees are most probably congruent with the species trees. This is partly due to the small effective avian population size with respect to the mitochondrial genome [10]. Generally, analysis of cytochrome b gene haplotypes is considered to be a powerful tool for construction of avian phylogenies at taxonomic levels from subspecies to subfamilies [11].

In the present study, the divergence of crows and some other corvine birds at interspecific and intergeneric levels was examined. For this purpose, the sequence of the 1047-bp mtDNA cytochrome b gene fragment was determined. Based on it, we constructed matrices of genetic distances, phenograms, and phylogenetic trees and evaluated the divergence time for the taxa. The patterns of nucleotide substitutions at different levels of the taxonomic hierarchy were compared, making it possible to shed light on molecular bases of the divergence.

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MATERIALS AND METHODS

Samples. Experiments were carried out using liver samples from hooded, carrion, and jungle crows. For *C. corone*, four samples, each representing one of the lineages described in the preceding study [12], were examined. In addition to the samples prepared for this study, the data on the cytochrome b gene sequences for eight corvine species from the International Gene Bank were examined (cytochrome b sequences over 1000 bp in length are available only for these corvine species). The outgroup was represented by the corresponding Gene Bank sequences of two species of birds of paradise, which are considered a sister group of corvine birds [4]. The samples used are listed in Table 1.

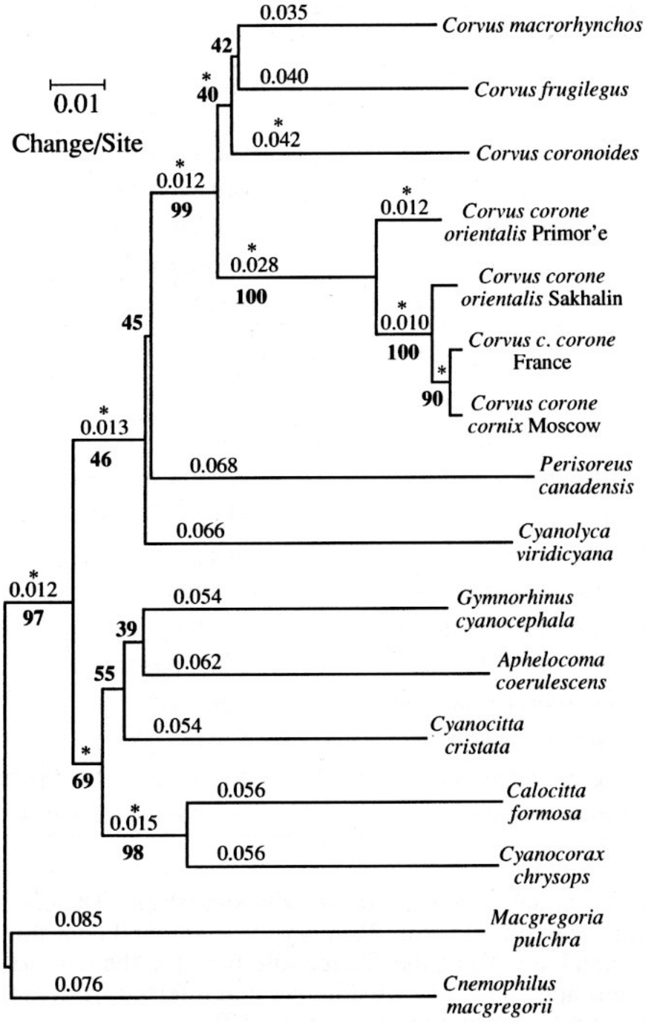
DNA extraction and amplification were carried out as described in [12].

Sequencing. The product of the second PCR reaction was purified by use of the QIA quick DNA purification kit (Qiagene). In some cases, the product of the first PCR reaction was used. Prior to sequencing, the concentration of the PCR product was determined. The Dye terminator cycle sequencing kit (Perkin-Elmer, United States) was used, and sequencing was performed with the use of an automated ABI PRSM DNA sequencer, model 377.

Phylogenetic analysis. To eliminate recognition errors, nucleotide sequences converted into letter files were first analyzed with the use of the DNASIS 2.0 computer program (Copyright Hitachi, 1996). For tree construction, the CLUSTAL W 1.6 (Macintosh) program [13] was utilized, and the trees were built by means of the neighbor-joining (NJ) method [14]. Branch significance was tested by means of bootstrap analysis [15]. The bootstrap index values were calculated at 1000 iterations. Transversions and transitions in each codon position were scored separately. For construction of trees using the maximum parsimony (MP) method and the unweighted pair-group method with arithmetic averages (UPGMA) [16], the MEGA 1.02 software package [17] was used.

RESULTS

The nucleotide sequence of the 1047-bp mtDNA cytochrome b gene fragment was determined. This sequence comprises the major part of avian cytochrome b gene, which is 1143 bp in size. The matrices of pairwise comparisons obtained represented the total amount of nucleotide substitutions, the ratios between the differing and common bases (P distances), 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. Some of these parameters are presented in Tables 2 and 3. No deletions or insertions were found. Synonymic transitions in the third codon position were the predominant substitution type.



Molecular phylogeny of the Corvidae species as inferred from the sequence of the mtDNA cytochrome b gene 1047-bp fragment. The bootstrap levels in percent (1000 iterations) are shown below the branches of the neighbor-joining (NJ) tree. The branches confirmed by maximum parsimony analysis (MP) are designated by the arrows. The branch length is proportional to the number of substitutions per site.

Based on the primary data, three types of phylogenetic trees, NJ, UPGMA and MP, were constructed. Since all tree types displayed identical topology, only the NJ tree is demonstrated in the figure. The monophyletic character of the *Corvus* genus was highly statistically significant. (Unfortunately, long cytochrome b gene sequences, other than for the four examined representatives of this genus, have not yet been determined). One of the two clusters within this genus includes the hooded and carrion crows, while the other cluster is represented by the jungle crow, the Australian raven, and the rook. It is noteworthy that the closest relationships within the crow cluster were demonstrated between the hooded crow samples from Moscow and carrion crow samples from Paris. The difference between these and carrion crow samples from

Table 1. List of the samples studied

Species		Our samples	
		sample	collection site
Corvus c. corone	carrion crow	3346	Paris
C. c. orientalis	carrion crow	601	southern Primorye
C. c. orientalis	carrion crow	732	northern Sakhalin
C. c. cornix	hooded crow	715	Moscow
C. macrorhynchos mandshuricus	jungle crow	817	southern Primorye
Species		Samples from the Gene Bank	
		number	authors
Corvus frugilegus	rook	Y16885	Harlid A.
C. coronoides	Australian raven	AF197837	Cracraft J., Feinstein J.
Cyanocorax chrysops	plush built jay	U77334	Espinosa M.A., Cracraft J.
Calocitta formosa	magpie jay	U77336	the same
Cyanocyta cristata	blue jay	X74258	Cracraft J.
Aphelocoma coerulescens	scrub jay	U77335	Espinosa M.A., Cracraft J.
Cyanolyca viridicyana	white throated jay	U77333	the same
Gymnorhinus cyanocephala	Pinon jay	U77332	"
Perisoreus canadensis	Canada jay	U77331	"
Macgregoria pulchra	Macgregor bird of paradise	AF197861	Cracraft J., Feinstein J.
Cnemophilus macgregorii	scrub bird of paradise	AF197841	the same

northern Sakhalin was statistically significant. The carrion crow sample from Primorye was situated near the branch base. Thus, the clusterization within the *Corvus* genus appeared to be identical to that inferred from the data on the short gene sequences [12].

The members of other genera of the Corvidae family do not cluster in a similarly consistent fashion: only the *Cyanocorax* and *Calocitta* genera were grouped in one cluster. The Canada jay *Perisoreus canadensis* and the blue-green jay *Cyanolyca viridicyana* were positioned near the common root of jays and crows. Two species of the birds of paradise, serving as a sister group, were situated rather far from the corvine assemblage.

DISCUSSION

In our previous study, sequence analysis of the 336-bp gene fragment, which has proven to be sufficient for determination of haplotype distribution, was carried out [12]. On the basis of examination of longer sequences, we were able to conduct comparisons between the species and genera and to more accurately estimate the divergence times for certain lineages.

On the tree we constructed, the monophyletic character of the corvine assemblage in comparison with the sister group of the birds of paradise, and also that of the *Corvus* genus, were confirmed by the high bootstrap levels (over 97%, see figure). Intergeneric differentiation was expressed to a different extent. A group of jay genera endemic to the New World (*Cyanocorax*, *Calocitta*, *Cyanocyta*, *Aphelocoma*, *Cyanolyca*, and *Gymnorhinus*), known to have penetrated there from Asia through Beringia [6], is characterized by various coloration and sizes of its representatives. Nevertheless, these genera are considered to be close relatives and are usually placed on one branch [1]. Recent molecular phylogeny of these birds, partly confirmed by cranial analysis, has been described in detail [6]. The *Cyanolyca* genus from this group, along with the Canada jay *Perisoreus canadensis* belonging to another jay genera group, were shown to be closest to the common ancestor. The relationships between the *Calocitta* and *Cyanocorax* genera were the closest. The *Gymnorhinus* genus, considered earlier as a sister taxon for the jays of the New World, and for this reason placed on another branch of morphological tree [1], appeared to belong to the same jay group. Scarce molecular genetic data on the corvine genera hampers a comprehensive discussion of their phylogeny.

The phenotype of the Australian raven *Corvus coronoides* is considered as intermediate between the raven and crows. Among five representatives of the *Corvus* genus examined, this species appeared to lie closest to the genus node on the phylogenetic tree constructed in the present study, as well as on the tree based on shorter sequences [12]. These data are congruent with the hypothesis on the Australian origin of the corvine assemblage [3].

Table 2. Intergeneric, interspecific, and intraspecific pairwise genetic distances for some species of Corvidae and Paradisaeidae

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Cnemophilus macgregorii*	–	0.182	0.185	0.180	0.184	0.180	0.186	0.175	0.177	0.172	0.166	0.166	0.177	0.176	0.173	0.165
2. *Macgregoria pulchra*	0.101	–	0.200	0.197	0.201	0.196	0.183	0.201	0.197	0.173	0.162	0.180	0.182	0.181	0.181	0.179
3. *Cyanocorax chrysops*	0.098	0.124	–	0.143	0.149	0.163	0.123	0.134	0.176	0.157	0.152	0.148	0.159	0.161	0.156	0.155
4. *Cyanocitta cristata*	0.103	0.125	0.103	–	0.141	0.144	0.149	0.117	0.158	0.136	0.135	0.127	0.135	0.134	0.132	0.128
5. *Aphelocoma coerulescens*	0.101	0.123	0.107	0.112	–	0.151	0.163	0.129	0.158	0.158	0.156	0.150	0.158	0.157	0.156	0.145
6. *Cyanolyca viridicyana*	0.097	0.118	0.113	0.109	0.109	–	0.168	0.149	0.150	0.133	0.134	0.131	0.145	0.144	0.139	0.131
7. *Calocitta formosa*	0.094	0.100	0.091	0.103	0.115	0.110	–	0.156	0.175	0.148	0.142	0.145	0.158	0.154	0.154	0.148
8. *Gymnorhinus cyanocephala*	0.103	0.131	0.094	0.093	0.102	0.109	0.110	–	0.158	0.146	0.143	0.133	0.143	0.142	0.145	0.152
9. *Perisoreus canadensis*	0.098	0.109	0.104	0.108	0.105	0.100	0.101	0.112	–	0.143	0.141	0.128	0.129	0.128	0.131	0.130
10. *Corvus coronoides*	0.095	0.101	0.095	0.093	0.109	0.092	0.086	0.103	0.096	–	0.089	0.085	0.094	0.095	0.095	0.089
11. *C. frugilegus*	0.091	0.089	0.088	0.090	0.105	0.087	0.076	0.094	0.092	0.070	–	0.088	0.097	0.096	0.096	0.095
12. *C. macrorhynchos*	0.090	0.108	0.083	0.090	0.100	0.088	0.079	0.087	0.080	0.071	0.062	–	0.088	0.089	0.085	0.084
13. *C. corone cornix* (Moscow)	0.100	0.102	0.097	0.095	0.106	0.100	0.094	0.099	0.080	0.079	0.073	0.077	–	0.005	0.009	0.028
14. *C. corone corone* (France)	0.100	0.102	0.100	0.094	0.106	0.100	0.091	0.098	0.080	0.081	0.073	0.079	0.004	–	0.010	0.029
15. *C. c. orientalis* (Sakhalin)	0.096	0.101	0.094	0.091	0.105	0.094	0.090	0.100	0.082	0.080	0.072	0.075	0.009	0.009	–	0.025
16. *C. c. orientalis* (Primorye)	0.090	0.100	0.091	0.087	0.094	0.086	0.082	0.105	0.081	0.072	0.071	0.074	0.027	0.027	0.024	–

Note: Kimura's two-parameter distance matrices taking into account transitional substitutions in all codon positions (above the diagonal) and all substitutions (below the diagonal) are presented.

Table 3. Intergeneric, interspecific, and intraspecific divergence levels of the 1047-bp cytochrome b gene fragment the corvine assemblage (Corvidae)

		All substitution types	Ts	Tv	Ts/Tv	K-2p	K-2p(Tv)	K-2p(Tv3)	K-2p(Ts3)
Intergeneric	range	121–163	70–103	24–72	1.1–3.6	0.12–0.18	0.02–0.07	0.02–0.06	0.05–0.09
($n = 8$)	mean	136.9	87.9	49.0	1.9	0.15	0.049	0.041	0.073
Interspecific	range	82–94	60–76	10–24	2.8–7.6	0.08–0.1	0.01–0.02	0.01–0.02	0.05–0.07
Corvus ($n = 4$)	mean	87.9	70.8	17.1	4.6	0.09	0.017	0.014	0.063
Intraspecific	range	5–30	4–27	0–3	9–13.5	0.01–0.03	0–0.003	0–0.002	0.003–0.026
C. corone s. l. ($n = 4$)	mean	18.2	16.7	1.8	9.5	0.02	0.0	0.002	0.016

Note: The data presented include the total number of substitutions; the number of transitions (Ts); the number of transversions (Tv); the ratio between them (Ts/Tv); sequence divergence, calculated with the use of Kimura's two-parameter distance matrices (Kimura, 1980) taking into account all substitutions (K–2p), of transversions (K–2p(Tv)), transversions in the third codon positions (K–2p(Tv3)), and transitions in the third codon positions (K–2p(Ts3)).

Our data were compared with the short gene sequence for the American crow *C. brachyrhynchos*, which recently appeared in the Gene Bank. The latter species is considered to be a close relative of the carrion crow. Furthermore, many ornithologists describe both species as conspecific [1]. According to our data, the American crow was closer to the raven than to the carrion crow. These results, however, should be considered as preliminary, because homologous sequences only 99 bp in size were compared.

All of the trees constructed showed the subdivision within the hooded and carrion crow cluster. One part of the cluster includes the carrion and hooded crows inhabiting the territory from France to Moscow; the second cluster part is represented by the carrion crow lineage from northern Sakhalin, while the third part is comprised by the carrion crow lineage from the southeastern edge of the continental range. On the NJ trees constructed on the basis of the short sequences, the latter population group lay close to the branch base [12]. For this reason, it was suggested to be the initial group for the entire cluster. Phylogenetic analysis carried out using the maximum parsimony method confirmed this assumption (see figure).

Substitutions at the third codon position are known to be the most informative for phylogenetic analysis [9, 18–21]. Thus, 78% of informative sites in corvine birds are at this position [5]. The data in Table 3 showed that maximum information for the genetic distances was obtained from the third codon positions.

The prevalence of the transitions among the substitution types confirmed the trend revealed in the analysis of the early stages of animal molecular phylogeny [22]. In consecutive pairwise comparisons from species to orders, the number of transitions first increases more rapidly, then this increase slows down and the transition number reaches a plateau, while the number of transversions continues to grow [11, 23]. Because of this, the number of transversions is informative for the evaluation of the genetic distances between remote taxa, whereas transitions are more suitable for analyzing young branches [4, 24]. According to our data, the ratio between transitions and transversions increases from 1.1–3.6 for intergeneric comparisons to 9–13.5 for intraspecific comparisons (Table 3). In the corvine birds studied, the proportion of transitions varied from 0.3–2.6% for intraspecific comparisons, through 5.7–7.2% for interspecific comparisons, to 6.7–9.8% for intergeneric comparisons (Table 3). These values are in agreement with the scarce and rather tentative evidence on birds available so far [11]. Most of the recorded transitions were synonymic, and, similarly to other birds and mammals, these mutations were represented by the C to T substitutions [25–29].

The intraspecific transversion level within the *Corvus* genus did not exceed 0.2%. The interspecific transversion level varied from 0.9 to 2.3%, while, in intergeneric comparisons, it reached a value of 6.9% (Table 3). These results agreed with the data on birds published elsewhere. For instance, the proportion of intrageneric transversions among the *Grus* cranes varied from 0.3 to 1% [11]. In the *Brachyramphus* murrelets, the transversion level varied from 0.4 to 1.1% [30]. In two lyrebird species (*Menura*), the maximum number of transversions, constituting 4.7% at the total substitution level of 12.8%, was observed [31].

The information on mitochondrial genome differences is traditionally used for estimation of the divergence time in different taxa. It should be noted, however, that these estimates are valid only within a single phylogenetic lineage and only when certain conditions are fulfilled. The most important of the latter is the constant rate of mutation accumulation. Transversions are considered to be more suitable for estimation of the divergence time, since a linear dependence between the transversion number and the divergence time of the two taxa has been postulated [19, 22]. This correlation in mammals was confirmed by fossil dating [19]. Similarities in the transversion and transition patterns revealed between mammals and Corvinae birds [4] permit transversion-based estimation of the divergence time in these birds. However, upon the slight divergence levels in the young branches, either the total number of substitutions or the number of transitions is to be scored.

For the avian cytochrome b gene, the following calibrations taking the fossil dating into account were suggested: an average divergence of 2% over 1 MY on scoring of the total substitution number for geese [32] and of 0.7 to 1.7% for cranes characterized by a longer life span and longer maturation [20]. In our work we used the standard calibration accepted for birds of 2% of among-lineage differences over 1 MY [33]. Transitions in all codon positions calculated with the use of the two-parameter Kimura method were analyzed (Table 2).

Under this assumption, the time of divergence of the jungle crow from the other species of the genus was 3.1 to 4.0 MYago. The value of this parameter for the rook was 3.1 to 3.6 MY ago, while for the Australian raven it was 3.5 to 4.0 MY ago. These data are congruent with the average species divergence time of 3.9 MY ago, inferred from the *cyt b* gene data in 88 avian genera [34]. If this is so, then the divergence between the carrion and hooded crows, as well as that between two carrion crow haplotypes, dates back to about 0.2 to 1.3 MY. These findings agree with the notion on a considerable distance between the jungle crow and rook on the one hand and the carrion and hooded crows on the other hand, based on morphological, behavioral, and ecological characters. However, the distance between the hooded crow and the carrion crow populations neighboring it in the east and west corresponds to a divergence time of only 0.2–0.5 MY. The difference levels observed point to the possible conspecificity of the hooded and carrion crows, including both parts of the discontinuous range of the latter species. Surprisingly, our data showed that the carrion crow lineage from the southern part of the Far East continental region diverged from the others even earlier, about 1.2 to 1.3 MY ago. The presumptive pathway of the development of the crow's areas was discussed in [12]. The dating obtained suggested that the separation of carrion crow lineages occurred in the early Pleistocene. These data agree with modern ideas on the early divergence of many avian groups [35]. The divergence time of the other Corvidae genera examined dates back to 3.8–5.7 MY, i.e., to the Pliocene. Since these evaluations were based on scoring of transitions only, both for intra- and interspecific lineages, underestimation of the divergence time at the intergeneric level, caused by the saturation effect, can occur [11, 23]. The intergeneric divergence time for the corvine birds estimated by scoring of all substitutions, including transversions, will be equal to 5.8 to 8.8 MY. The divergence between the corvine assemblage and the birds of paradise dates back to 8–10 MY. Construction of a more detailed phylogenetic scheme of the family requires further investigations utilizing the data on the cytochrome b gene and other molecular markers.

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