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PHYLOGENETIC RELATIONSHIPS OF THREE SPECIES OF CROWS (CORVIDAE, AVES) BASED ON THE RESTRICTION SITE VARIATION OF NUCLEAR RIBOSOMAL RNA GENE

Southern blot analysis of nuclear ribosomal DNA (rDNA) was carried out to examine phylogenetic relationships between three species of crows: Corvus cornix, C. corone and C. macrorhynchos. In this purpose DNA samples of birds were digested by 12 restriction enzymes (EcoRI, HindIII, PstI, BamHI, DraI, PvuII, KpnI, XbaI, BglII, BclI, SacI and AatI) and hybridized with the clones of mouse rDNA probes (18S, 28S and INT). Based on the data obtained and Gallus gallus restriction map as a standard the restriction site maps of the main rDNA repeating unit types (repetypes) were constructed. The length of crow rDNA genes was estimated to be 22.5 kb for Jungle and 22.0 kb for Hooded and Carrion crows. C. corone and C. cornix shared a common repetype which differed, by presence of two restriction sites (XbaI and PvuII) in the spacer region, from that of C. macrorhynchos with the estimated sequence divergence of 0.26%. Restriction-size variation was revealed between individuals of C. corone and C. cornix, although the substantial meanings of this variation remain unclear yet. These data suggest that the crow species evolve with slower rate of molecular evolution, as generally observed in other avian species, compared with the higher extent in external morphology, ecological features and behavior.

Introduction. Corvidae belongs to one of the most advanced and numerous families among Aves [1]. However phylogenetic relations between its diverged taxa remain unclear [2]. Especially status of such common species as Carrion and Hooded crows is still under discussion. Some taxonomists treat them as the same species, *Corvus corone* L. [3], while others believe it consists of two species — *Corvus corone* with two subspecies: *C. c. corone* L. and *C. c. orientalis* Eversm., both black-colored, and gray-and-black colored *C. cornix* L. [4]. *Cornix* forms two stable hybrid zones with corone wherever their ranges contact, in Europe and Siberia [5–10], so they don't correspond to the biological species concept. In zoological literature, this example is considered as classical case of natural hybridization [11], and we studied just these two forms here. The third, distinct species Jungle crow C. macrorhynchos Wagler was used for comparison. It is semisympatric with carrion crow, also black-colored but clearly distinguished by larger and more strongly arched bill, and other morphological characters. Their behavioral properties such as calls, and some habitat preferences also differ [12].

Genetic studies of the crow's hybrid zone phenomenon have been started just recently. Results of investigation of allozyme variation [13, 14], the RFLP of repeated DNA [15] and hypervariable regions of genome [16] did not clear up the taxonomic rank of carrion and hooded crows. Some phylogenetic analysis was undertaken on Corvids using cyt b sequencing, but mainly on intergeneric level [17, 18]. It became obvious that it was necessary to find out the other molecular markers that able to provide estimation of the divergent level for the forms mentioned.

Such a marker would be nuclear rDNA. Similarly to the other animals, repeated sequence of the bird's rDNA consists of 28S, 18S and 5.8S genes, two internal transcribed spacer regions — ITS-1 and ITS-2, and two external — ETS and NTS, flanking, respectively, 18S and 28S coding regions [19]. Unlike conservative coding regions, the spacer regions (particularly nontranscribed) are known to evolve rapidly, therefore RFLP analysis of rDNA is applying widely for estimating of genetic relations and phylogenetic coher-

ence between the taxa of species and subspecies ranks [20–24]. It was argued that the pheno- and phylogenetic reconstructions based on the nuclear rDNA RFLPs do not need large sample sizes, and intragenomic heterogeneity of rDNA depends on the population number to which the sample belongs [22]. In this paper we undertook studying of genetic differentiation between hooded and carrion crows based on rDNA RFLPs data in purpose to clear up their phylogenetic relations. Also we tried to find some marker variants of digestions (restriction patterns) appropriate for analyzing of hybrid populations. Jungle crow was used for a comparison, as a sister group.

Materials and methods. Carrion crow Corvus corone and Jungle crow C. macrorhynchos were caught on the Kunashir Island, Kuril Islands, while Hooded crow C. cornix near Novosibirsk city. Both populations of Carrion and Hooded crows are distanced so far that they could be treated as genetically "pure". DNA was isolated from fresh or fixed in ethanol liver by a common [25] or specially developed method [26], respectively, with phenol-detergent deproteinization and treatment of DNA samples by pronase and RNAse. Southern blot-analysis was carried out as previously described [25]. The DNA was digested with 12 different restriction enzymes (EcoRI, HindIII, BglII, BclI, KpnI, AatI, SacI, XbaI, PvuII, BamHI, PstI, and DraI) and subjected to electrophoresis on 0,7 % agarose gels, at 2 v/cm for 13–15 hours in Tris-acetate buffer (40 mM Tris-acetate, 2mM EDTA, pH7.8). DNA fragments were transferred to nylon filters, baked at 80 °C for 2 hours. Labeling of probes was carried out using (γ^{32} P)-dCTP and random primers [21]. Specific radioactivity of probes was $4–8\times10^8$ cpm/μg. Prior to hybridization, the filters were incubated at 66 °C during 2–4 hours in 6xSSC buffer, which contained 0.01% (w/v) SDS and Denhardt's solution with denaturated salmon's sperm DNA (100 μg/ml). Hybridization was performed at the same temperature for 20 hours, and then filters were washed twice with 0.1 SSC for 30 min at room temperature. Autoradiography was conducted on Fuji-RX film for 12–24 hours at room temperature with an intensifying screen. After this procedure, filters were washed in distilled water at 95 °C for 5–10 min, dried and reused with other rDNA probes.

Genetic distances were calculated by correlation of common and taxon-specific rDNA fragments by Nei and Li [27]. Intragenomic heterogeneity was estimated using the equation: $h = 1-\Sigma X_i^2$, where X_i is frequency of an i-fragment [22].

Results. The restriction fragment length polymorphism (RFLPs) of ribosomal DNA (rDNA) was analyzed using blot-hybridization of digested by 12 restriction enzymes crow DNA samples with cloned 18S, 28S and INT rDNA probes of mouse (Fig. 1). No differences were revealed in the restriction enzyme patterns among the species, when DNA was digested by EcoRI and HindIII. Also DNA patterns of *C. cornix* and *C. corone* were similar, when KpnI, BglII, BclI, DraI, PstI and BamHI were used. Consequently, restriction enzymes pointed and regions of their location are the most conserved for rDNAs of the species studied. In fact, the conservative BclI site was found within the 5.8 rDNA gene in various avian and mammalian species investigated. Additionally, the same KpnI and DraI sites were also found in the internal spacer regions of their genomes [20, 21, 28, 29].

rDNA digested by both BclI and BglII was identified as a single band of 22.0 kb in Hooded and Carrion, but 22.5 kb in Jungle crow

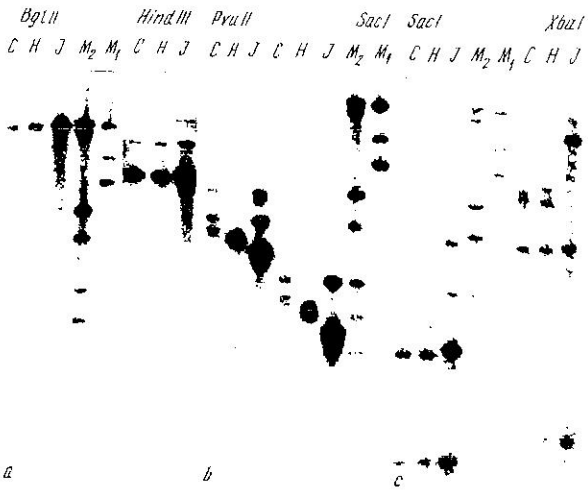

Fig. 1. Southern blot patterns of Carrion (C), Hooded (H) and Jungle (J) crows rDNA cleaved with restriction enzymes using the 1.9 kb 18S (a), 0.7 kb 28S (b) and 6.6 kb INT (c) rDNA probes. M — molecular weight markers

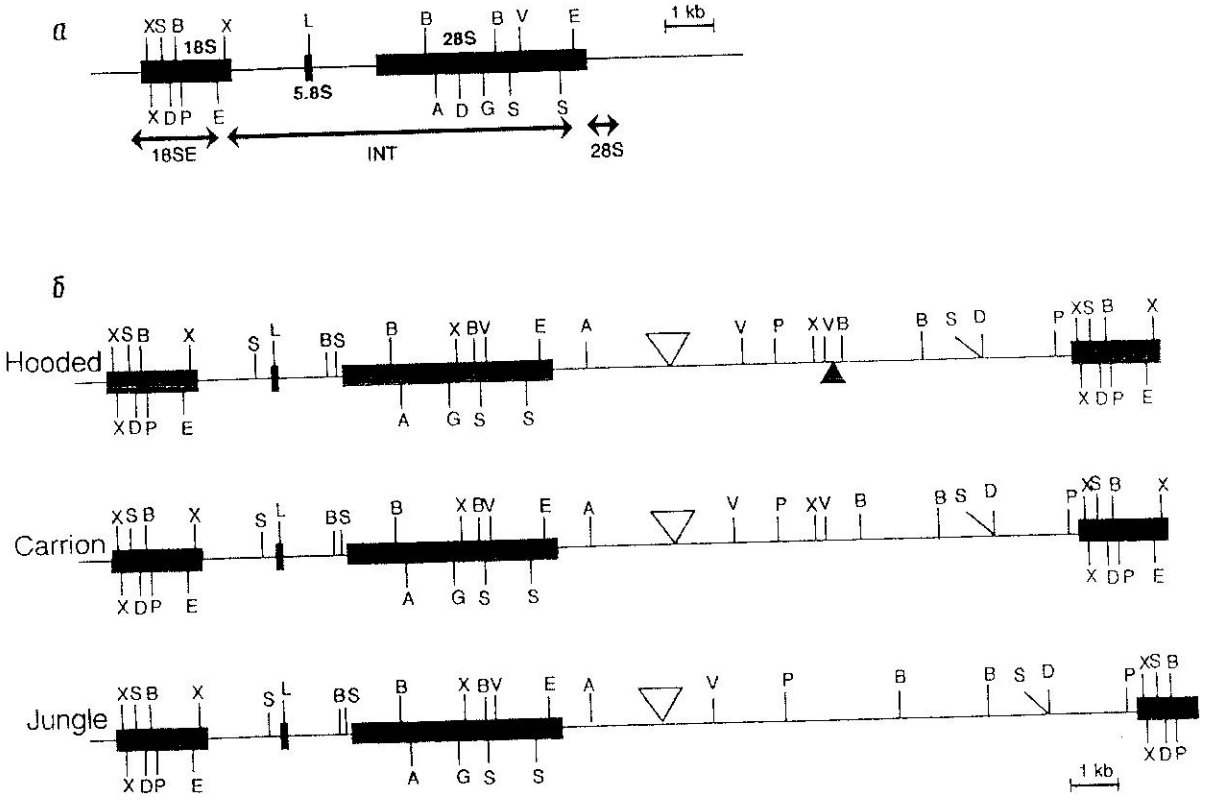

Fig. 2. Restriction maps of the major rDNA repetypes of crows (b) and Gallus gallus as a "standard" (a). The short and long black boxes indicate the genes encode 18 and 28 rDNA, respectively. Position of probes are shown with arrows. Both open and black triangles indicate size variation (see text). A = AatI, B = BamHI, D = DraI, E = EcoRI, G = BglII, H = HindIII, K = KpnI, L = BclI, S = SacI, P = PstI, V = PvuII, X = XbaI

genomes. This result means that for both restriction enzymes there is a single site along the whole rDNA repeated unit in crow genomes, and the length of this unit should be not less than 22.0 kb.

Patterns of BamHI fragments with the 18S and INT probes pointed out that there would be no size variation in the rDNA coding regions in crows. It is known for 18S and 28S rDNA genes that each of them maintains the conservative BamHI site [30, 31]. Sites of the remaining restriction enzymes, XbaI, PvuII, SacI and AatI, revealed a high level of size variation in crow rDNAs, particularly with 28S probe, i.e. they can locate in the most variable region. Nevertheless, only 2 of 36 DNA patterns can be used for population analysis of the crow hybrid zone: PvuII and SacI digests hybridized with 28S-rDNA probe, because the highest differences between Hooded and Carrion crows were revealed under these conditions (see Fig. 1).

Based on the data obtained and restriction map of *Gallus gallus* as a "standard" [32], restriction maps of crow's rRNA genes were constructed (Fig. 2). The reconstruction was performed with minimum changes of standard map. Since rDNA continues to evolve concertedly within genomes and within populations [33, 34], we assumed that the each map of rDNA, displayed in Fig. 2, represents the major type of repeating unit for rDNA (repetype) in each species. rDNA of crows has the prominent restriction size variation (Fig. 2, open triangles). No differences in arrangement of restriction sites for the main rDNA repetype were revealed between the two individuals of *cornix* and *corone*, but substantial size variation found between them (Fig. 2, black triangle). Their rDNA units were estimated to be of an equal length, about 22.0 kb. Jungle crow differs by an absence of two

restriction sites, XbaI and PvuII, and longer size of rDNA repeat, that is approximately 22.5 kb.

The level of genome divergence for the pairs *C. cornix/C. corone, C. cornix/C. macrorhynchos and C. corone/C. macrorhynchos*, according to rDNA RFLPs and calculated by number of common and different restriction fragments, was estimated to be 1.52, 7.95 and 7.26%, respectively. However, when we took into account only site variations, no genetic differences were revealed between C. cornix and C. corone, and their divergence with C. macrorhynchos reached 0.26 %, only (Table). In contrast, the level of intragenomic heterogeneity (h) proved to be high and almost identical among the all genomes studied - about 0.7.

Discussion. The total length of rDNA increased during the process of vertebrate evolution from 10–24 kb in amphibians to 33–44 in mammals, mainly due to complicated and variable region of rDNA, NTS [19, 30, 31]. It is indicated that species of birds represented by Gallus (28 kb) [30, 32] and Corvus (22.0–22.5 kb; this study) shows intermediate size of the length of the repeating unit. Among the three species of Corvus there may be no considerable variation and there may be no reason for phylogenetic consideration.

We revealed that there was only one site of restriction variation between the jungle and hooded-carrion crows along the rDNA spacer (see Fig. 2). However, hooded and carrion crows shared the common rDNA repetype and we can not obtain any useful information to discriminate them each other with such variation. On the other hand, each individuals of our samples showed somewhat high extent of intragenomic restriction size variation of the rDNA external spacer (Fig. 1) as was observed in those of other vertebrate taxa [e.g., 19, 21, 30, 31, 33]. The patterns observed in the crow samples seemed like species-specific but we need some extensive populational survey to illustrate the significance of the size variation as a diagnostic marker for each population and/or species in crows.

Concerning intragenomic rDNA heterogeneity, its high level was reported for mammals of the genus Felis: from h = 0.41 for domestic cat to 0.63 for the Leopard cat. However no variations were found within Iriomote cat, probably because of small population number of the species inhabiting restricted island area [22]. Authors concluded that magnitude of the rDNA variation within a population or an individual is thought to be depend on population size [22]. Thus, the high level of intragenomic heterogeneity for three crow species (about 0.7) would correspond to their large areas and comparable high population numbers for these species.

Divergence (%) of crow rDNA, based on both site (above diagonal) and size (below diagonal) variations, calculated by number of common and different sites of restriction fragments

Species	*C.cornix*	*C.corone*	*C.macrorhynchos*
C.cornix	—	0	0.26
C.corone	1.52	—	0.26
C.macrorhynchos	7.95	7.26	—

The interspecies distances for the crows (0–0.26 %) inferred from site variation data are so low in comparison with those of mammalian species examined. For example, genetic distances for 3 species of Mustela varied between 0.65 and 1.59 [20], Apodemus mice — 1.5–8.5 [21], 4 species of Felis — 1.2-2.5 % [22]. In the same time, intraspecies genetic distances within Mus were considerably smaller: 0.44–0.62 % [23]. Unfortunately, no analogous data are available in a literature on bird's nuclear rDNA. However it was reported less molecular divergence of avian taxa studies on average than in other vertebrates of the same rank [35, 36]. In other words, this observation may be due to, at least partly, slow rate of molecular evolution in Aves which was reported many times, as for nuclear as mitochondrial genes, whereas their external morphology, behavior and ecological adaptations evolved quite fast.

Thus, we obtained the new data on restriction polymorphism of avian rDNA. Our results support phylogenetic distinctness of jungle crow, evident even on external properties, ecology and behavior. The data also show that taxonomic relationships of hooded and carrion crows remains to be unclear, and only a few variants of digested rDNA could be used for population analysis of their hybrid zone.

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РЕЗЮМЕ. При помощи метода ПДРФ генов ядерной рРНК проведен анализ филогенетических связей врановых птиц: серой *Corvus cornix*, черной *C. corone* и большеклювой *C. macrorhynchos* ворон. Сконструированы рестрикционные карты, определен размер основного повтора рДНК (22 тпн для серой и черной, 22,5 тпн — для большеклювой ворон). На фоне выраженного ПДРФ обнаружено высокое сходство распределения рестрикционных сайтов в генах рРНК ворон: серая и черная вороны не отличаются, их дивергенция с большеклювой вороной составляет лишь 0,26 %. Предполагается, что такой результат может быть обусловлен низкими скоростями молекулярной эволюции у врановых.

РЕЗЮМЕ. За допомогою методу ПДРФ генів ядерної рРНК виконано аналіз філогенетичних зв'язків ворон: сірої *Corvus cornix*, чорної *C. corone* і великодзьобої *C. macrorhynchos* ворон. Сконструйовано рестрикційні карти, визначено розмір основного повтору рДНК (22 тпн для сірої і чорної, 22,5 тпн — для великодзьобої ворон). На фоні вираженого ПДРФ виявлено високу подібність розподілу рестрикційних сайтів в генах рРНК ворон: сіра і чорна ворони не відрізняються, їх дивергенція з великодзьобою вороною становить лише 0,26 %. Припускається, що такий результат може бути зумовлений низькими швидкостями молекулярної еволюції у ворон.

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