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GENETIC VARIABILITY OF CARRION AND HOODED CROWS AND THEIR HYBRIDS ACCORDING TO RAPD-PCR DATA

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We conducted RAPD-PCR analysis of genetic diversity of hooded crow (*Corvus cornix*), carrion crow (*C. corone*), and their phenotypic hybrids from the Siberian zone where natural habitats of the birds overlap and hybridization of parental forms goes on. Genetic distances (G_N), polymorphism (P_{95}), average expected heterozygosity (H_e), gene fixation rate (F_{st}), and interpopulation differentiation (G_{st}) were estimated using variation of RAPD markers in both species and their hybrids. Our analysis demonstrated that genetic variation in hooded and carrion crows is lower than in their hybrids. We detected that hybrids are genetically closer among themselves ($D_N = 0.295$) than to each parental species ($D_N = 0.441$ and 0.397 with *C. corone* and *C. cornix* respectively). Individual birds of *C. cornix* studied by us had the minimum level of heterozygosity and polymorphism ($H_e = 0.12$ and $P_{95} = 27.8\%$) comparing to *C. corone* and crows from the hybrid zone ($H_e = 0.18$ and 0.20 ; $P_{95} = 42.6\%$ and 50.4% respectively). Wright's inbreeding ($F_{st} = 0.285$) and population differentiation ($G_{st} = 0.352$) indices indicate minor genetic differentiation among all birds analyzed in our study. Nevertheless, UPGMA and NJ dendrograms permit differentiating individual birds from habitats of carrion crow, hooded crow, and the hybrid zone and assigning them to different clusters. Our data agree with molecular-genetic studies conducted for *Corvidae* by other methods. Our data do not contradict to the concept considering carrion and hooded crows as semispecies within the superspecies.

INTRODUCTION

Elucidation of taxonomic status of hybridizing forms is an important problem in modern biology. This problem is tightly associated with the problem of species boundaries [1]. This problem remains unresolved for many biological forms forming stable hybrid zones. This is associated mostly with interpretation of species definition in different concepts. Causes and biological significance of elevated hybrid variation, the role of hybridization in evolution, estimation of taxonomic ranks of cross-forms represent special interest and need precise assessment through modern technologies.

A classic example of a messy taxonomic problem is interrelations between widely spread and well-known representatives of *Corvidae* family: hooded crow (*Corvus cornix* Linnaeus, 1758) and carrion crow (*Corvus corone* Linnaeus, 1758). Some ornithologists consider carrion and hooded crows as different biologic species [2], others hold the opinion that the species are conspecific [3–5] or classify them as superspecies together with three other species [6]. Elucidation of taxonomic status of these forms is complicated because these forms have European and Siberian hybrid zones. Madge [6] believe that European and East Asian subspecies of carrion crow are less close each to the other than to hooded crow whose habitat is located between habitats of these two subspecies. Hooded and carrion crows have similar body shapes, size, and localization. They easily substitute for each other geographically, and easily hybridize producing fertile progeny at sites of contact and in overlapping habitats [7, 8]. Phenotypes of hybrids are extremely variable in color. At least 11 hybrid versions are distinguished (Fig. 1) [7].

Combination of classic approaches and modern molecular-genetic techniques permits clarifying controversial issues and identify genetic discreteness of taxons in certain cases. Genetic analysis of isozymes

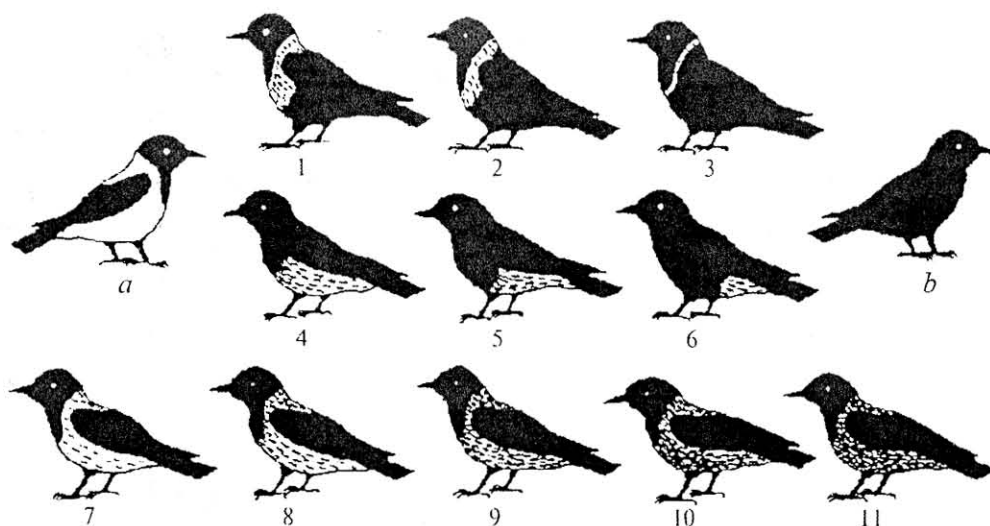


Fig. 1. Variability in hybrid phenotype: a) hooded crow; b) carrion crow; 1–3) "gray neck" hybrids; 4–6) "light belly" hybrids; 7–11) "diffuse" hybrids [7].

was conducted for cross-forms and their phenotypic hybrids from the European hybrid zone. Gene drive between populations and genetic polymorphism were assessed [4]. However, allozyme analysis [4, 9] and genome dactyloscopy [10], polymorphism analysis of restriction sites in highly repeated segments of genome DNA [11], sequencing of cytochrome *b* gene from mtDNA [12], and PDRF studies of ribosomal DNA (rDNA) of carrion and hooded crows [13] did not clarify the problem.

The aim of this study is to find molecular markers of carrion and hooded crows for their taxonomic diagnostics and analyze phylogenetic relations of the species. The following problems were the focus of our study: screening of primers and identification of taxon-specific molecular markers; comparison of genomes of carrion crows, hooded crows, and their hybrids from the hybrid zone using genetic variability (P_{95} , D_N) and polymorphism (H_S , H_T , n_a , n_e , H_e , F_{st} , G_{st}) indices; assessment of correlation between phenotypic characteristics in hybrid birds.

MATERIALS AND METHODS

Sixteen birds were used in this study: East Asian carrion crow *Corvus corone orientalis* from Krasnoyarsk, Primorie, Sakhalin and Kunashir islands ($n = 4$); European carrion crow *C. c. corone* from France ($n = 2$); hooded crow *C. cornix* from Moscow and Novosibirsk oblasts ($n = 5$); phenotypic hybrids from the hybrid zone of West Siberia ($n = 5$). Birds from the hybrid zone had the following phenotypes: two birds had pure black color, two birds were black with gray necks (according to classification given on the Fig. 1, type 2), and one bird was black-and-white.

DNA was isolated from fresh liver tissue according to the standard method [14] including consecutive treatments with: proteinase K, phenol, chloroform-isoamyl alcohol mix, and repelleting with isopropanol and ethanol. Samples of total cellular DNA were used as templates for RAPD-PCR. PCR was performed in 25 μ l reaction mix containing 60 ng total DNA, 1 \times buffer (67 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 0.01% Tween-20, 0.01 M 2-mercaptoethanol), 0.2 mM each dNTP, 0.5 μ l primer, 1 U *Taq*-polymerase. The following PCR parameters were applied: 2 min. denaturing at 94°C; 41 cycles included 1 min. denaturing at 94°C, 30 s annealing at 37°C or 15 s annealing at 45°C, and 2 min synthesis at 72°C. The final stage was 5 min. synthesis at 72°C. Twenty random decamer oligonucleotide primers with different sequences and 60–70% G+C content were used for RAPD analysis. Two specific primers were used to identify sex of birds [15] (Table 1). Sex identification was necessary, since it is impossible to identify sex of nestlings morphologically. RAPD-PCR products were resolved by electrophoresis on 2%-agarose gel and photographed in UV-light. *Pst*I-digested phage λ DNA was used as a mass ladder.

Binary matrixes were built according to RAPD patterns. Bands were designated as 1, gaps were designated as 0. All visually detectable bands were taken into consideration. The following indices were evaluated using POPGENE [16] and TFPGA [17] computer software: unbiased genetic distance D_N [18]; portion of polymorphous loci at 95% significance (P_{95}); the average number of alleles (n_a), and effective number of alleles (n_e) per locus; average expected heterozygosity (H_e) and genetic diversity h ; interpopulation

Table 1
Primers Used in the Study

Primer	Nucleotide sequence (5' → 3')
OPA-05	AGGGGTCTTG
OPA-13	CAGCACCCAC
OPC-02	GTGAGGCGTC
OPC-05	GATGACCGCC
OPC-09	CTCACCGTCC
OPC-10	TGTCTGGGTG
OPC-12	TGTCATCCCC
OPC-16	CACACTCCAG
OPE-20	AACGGTGACC
OPF-11	TTGGTACCCC
2717F	TAGAAGAAGATATTCTTGA
3088R	CTCAGCACCAAACCTCAAA

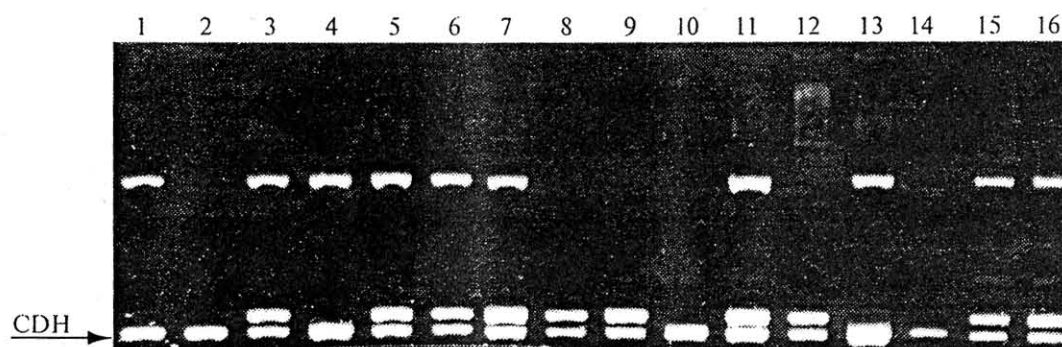


Fig. 2. Electrophoregram of CDH gene amplification. Sex identification for: 1–6) carrion crow; 7–11) hooded crow; 12–16) phenotypic hybrids (males, one band; females, two bands).

differentiation (G_{st}). The number of migrants per generation Nm was assessed for local populations using the last index. Population subdivision index F_{st} was evaluated according to Edwards [19]. Total genetic variability (H_T), average genetic diversity (H_S), and total genetic diversity between populations (D_{ST}) were evaluated as described [20]. The precise test for population differentiation including χ^2 , degrees of freedom (df), and probability (p) was conducted according to Raymond and Rousset [21]. Phylo- and phenograms were developed using TREECON ver. 1.3 [22] computer software.

RESULTS

The initial step of the study was to select primers suitable for differentiation between hooded and carrion crows [23]. For this purpose we tested 20 primers. Ten primers that reproduced most readable amplification patterns were selected and tested on parental forms and their phenotypic hybrids (Table 1). Four primers from that set revealed additional marker fragments only for hybrid birds. The other primers had uniform patterns with insignificant variation of fragments (band/gap or intensity of the band) in both hybrid and "pure" birds. The primers used in our study revealed from 6 to 12 amplicons per sample ranging from 0.2 to 2.14 kb. Each primer initiates synthesis of a specific pattern of DNA fragments that differ in molecular weight and band staining intensity. Finally we identified 115 traits. RAPD-PCR analysis did not reveal fixated differences or differences in frequencies between hooded and carrion crows. PCR analysis with sex-specific primers permitted us to identify six males and ten females among the birds used in our experiments (Fig. 2).

Fig. 3 represents RAPD-patterns of *C. corone*, *C. cornix*, and their hybrids. Parental forms have less variable patterns than hybrids. Patterns of parental forms are more similar each to the other than to patterns of hybrids. Specific markers were identified for birds from the hybrid zone: OPC-05₄₄₈ and OPC-02_{260, 577}.

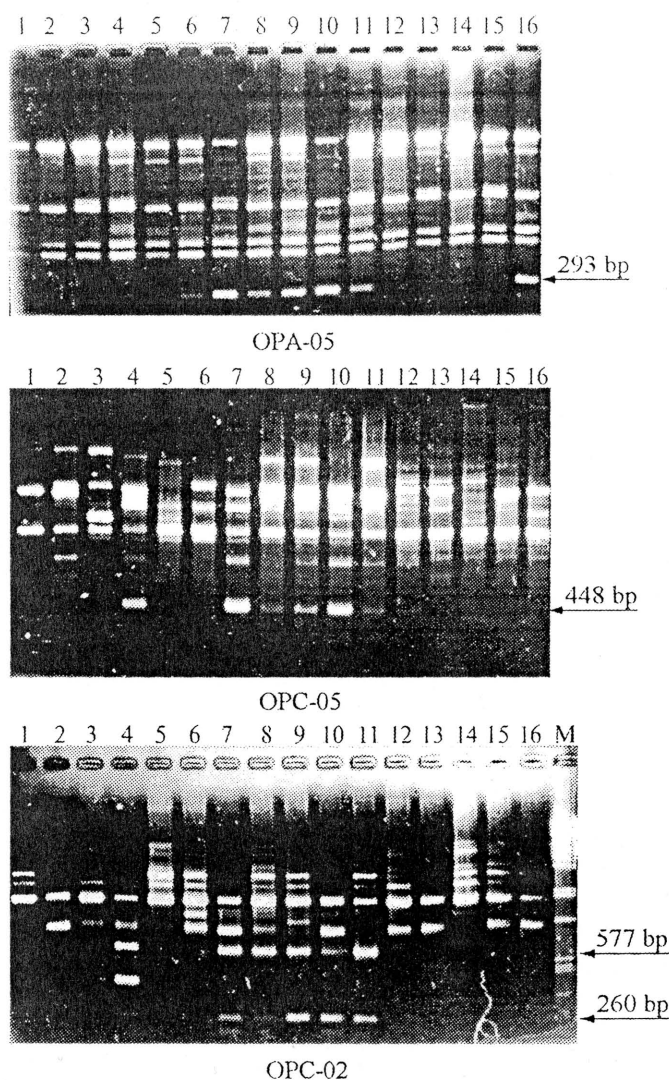


Fig. 3. RAPD-PCR DNA patterns of: 1–6) carrion crow; 12–16) hooded crow; 7–11) their phenotypic hybrids. M, phage λ DNA digested with *Pst*I.

Molecular fragments OPC-02_{805, 3621} were detected in all hybrid birds and several carrion crows, i.e., this trait was characterized by partial polymorphism. The fragment OPA-05₂₉₃ was well pronounced in patterns of hybrid birds and distinguishes them from parental forms. The parental forms either did not have that fragment or it was less expressed.

To search genetic markers in crow genome, another version of RAPD-PCR analysis with two random primers (under standard conditions these primers do not permit discriminating between hooded and carrion crows) was also used. This approach was previously used for other objects that are morphologically different but indistinguishable by RAPD-analysis with one primer [24]. Four of 11 randomly selected paired variants permitted discriminating between hooded and carrion crows. In two cases (OPA-05 + OPB-20 and OPE-20 + + OPF-11), qualitative differences between hooded and carrion crows were found: *C. corone* had additional 514 bp marker fragments (Fig. 4). In other two variants (OPF-05 + OPC-02 and OPF-05 + OPC-12), only well pronounced quantitative differences were observed: major fragments of 805 and 1093 bp. These fragments were characteristic of *C. corone*, whereas they were less pronounced in *C. cornix*. In general, RAPD-PCR patterns of amplicons of hooded and carrion crows were very similar.

Preliminary assessments of genetic diversity criteria were different for *C. corone*, *C. cornix*, and birds from the hybrid zone. The level of genetic polymorphism for hybrid birds and *C. corone* was considerably higher ($P_{95} = 50.4$ and 42.6% respectively) than for *C. cornix* ($P_{95} = 27.8\%$) (Table 2). This indicates insignificant variability in *C. cornix*. We detected that 68% loci were polymorphous for all birds analyzed in our study.

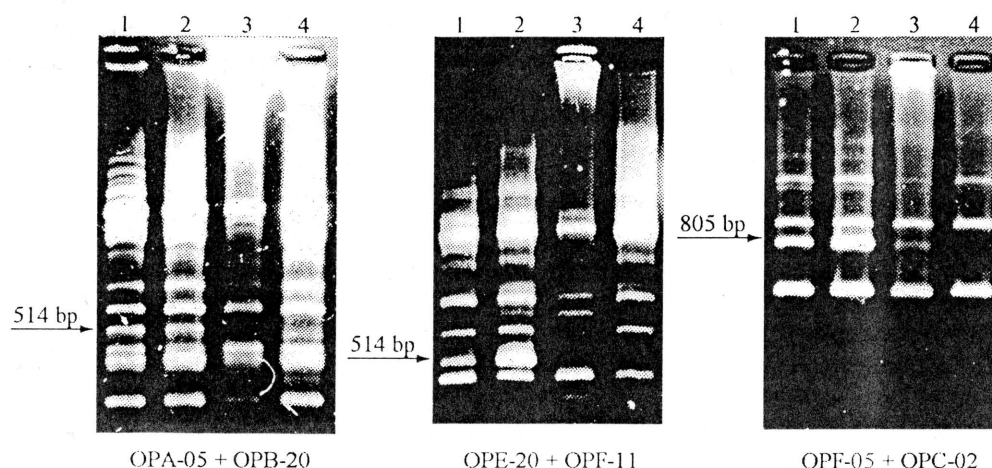


Fig. 4. Electrophoregram (2% agarose gel) of *Corvidae* DNA amplification products. PCR was done using two primers. 1, 2) *C. corone*; 3, 4) *C. cornix*.

Table 2

Genetic Diversity of Carrion Crow, Hooded Crow, and Their Phenotypic Hybrids

Species	P ₉₅ , %	H _d	n _a	n _e	h	D _N
<i>C. corone</i> (n = 6)	42.6	0.18	1.51 ± 0.50	1.29 ± 0.36	0.18 ± 0.19	0.157
<i>C. cornix</i> (n = 5)	27.8	0.12	1.28 ± 0.45	1.19 ± 0.35	0.11 ± 0.18	0.143
<i>corone</i> × <i>cornix</i> (n = 5)	50.4	0.20	1.49 ± 0.50	1.36 ± 0.41	0.20 ± 0.21	0.295
Total (n = 16)	67.8	0.27	1.71 ± 0.45	1.42 ± 0.36	0.25 ± 0.19	0.325

Table 3

Values of Genetic Differentiation of Carrion Crow, Hooded Crow, and Their Hybrids

Species	H _T	H _S	D _{ST}	F _{ST}	G _{ST}	Nm	D _N	Ext χ ² / df / p
<i>C. corone</i> / <i>C. cornix</i>	0.180 ± 0.04	0.122 ± 0.03	0.06	0.328	0.318	1.079	0.304 ± 0.038	117.02 / 230 / 1.000
<i>C. corone</i> / hybrids	0.235 ± 0.04	0.161 ± 0.02	0.07	0.283	0.314	1.091	0.441 ± 0.077	222.91 / 230 / 0.619
<i>C. cornix</i> / hybrids	0.243 ± 0.04	0.154 ± 0.02	0.20	0.452	0.345	0.872	0.397 ± 0.062	251.85 / 230 / 0.154
Total	0.251 ± 0.04	0.162 ± 0.02	0.09	0.285	0.352	0.911	0.325 ± 0.080	427.23 / 230 / 0.000

All birds were characterized by a quite high level of heterozygosity. Values of average expected heterozygosity was $H_e = 0.27$ for all crows. Thus, approximately 27% genes in the sample population were polymorphous. It should be noted that the lowest level of heterozygosity was characteristic of hooded crows ($H_e = 0.12$) (Table 2). Unfortunately, this heterozygosity cannot be detected through analysis of RAPD-patterns, because dominant homozygotes could not be distinguished from heterozygotes due to dominant characteristic of RAPD-loci. Average (n_a) and effective (n_e) numbers of alleles per locus had similar values. Nevertheless, they were higher in birds from the hybrid zone and carrion crow as opposite to hooded crow (Table 2). Gene diversity of carrion crow and hybrid birds was $h = 0.18$ and 0.20 respectively; this is two times higher than for hooded crow ($h = 0.11$) (Table 2).

Proceeding from 115 RAPD indices the genetic distance between hooded and carrion crows ($D_N = 0.304$) twice exceeded intraspecies values ($D_N = 0.143$ and 0.157 for *C. cornix* and *C. corone* respectively). It is interesting that values of genetic distances between phenotypic hybrids ($D_N = 0.295$) are similar to those between parental species (Tables 2 and 3).

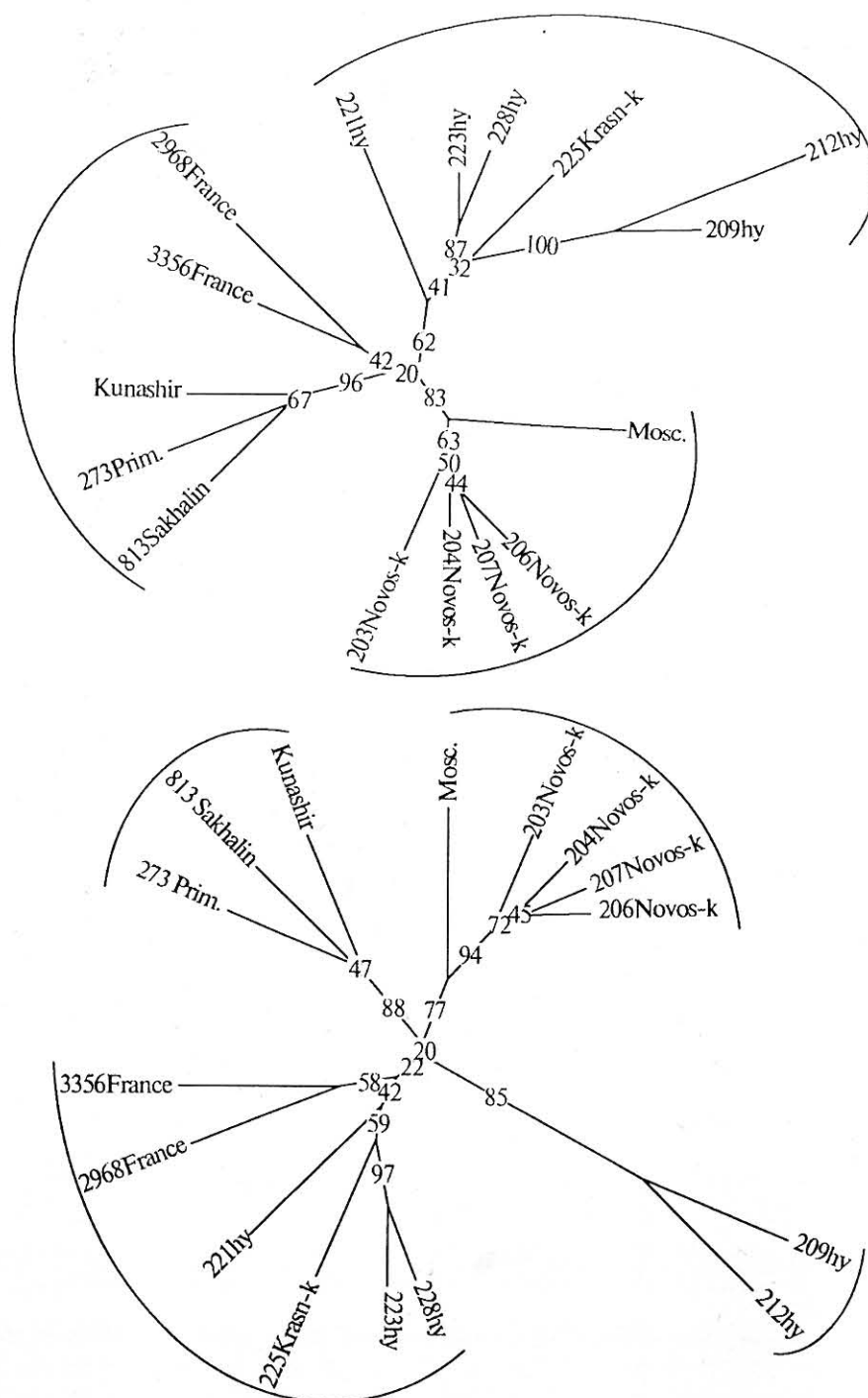


Fig. 5. *NJ* (upper) and *UPGMA* (lower) dendrograms of carrion crow (No. 273 Prim., 225 Krasn-k, 813 Sakhalin, Kunashir, 3356 France, 2968 France), hooded crow (No. (203, 204, 206, 207) – Novosibirsk, Moscow), and their phenotypic hybrids (No. 209hy, 212hy, 221hy, 223hy, 228hy). Dendrograms were built using TREECON computer software.

Total gene diversity H_T comprised 0.18 for carrion and hooded crows, whereas the population average gene diversity was $H_S = 0.12$ (Table 3). Total gene diversity between hooded crow and hybrid birds was the highest one ($D_{ST} = 0.2$).

The level of genetic subdivision of *C. cornix* and *C. corone* was assessed through gene fixation rate (F_{st}) and interpopulation diversity (G_{st}) (Table 3). The greatest values of F_{st} (0.452) at correspondingly minimum level of gene drive Nm (0.872) were detected between hooded crows and hybrids. The Nm values

between carrion crow *C. c. orientalis* and hybrids were slightly greater (1.091). This possibly indirectly reflects greater introgression of its traits into the hybrid zone.

The precise test for differentiation between *C. cornix*, *C. corone*, and their phenotypic hybrids indicated their genetic discreteness ($\chi^2 = 427.33$, $df = 230$, $p = 0.000$). However, we failed to detect considerable distinctions between hooded and carrion crows ($\chi^2 = 117.02$, $df = 230$, $p = 1.000$). At the same time, differences between hooded crow and hybrid birds are significant ($\chi^2 = 251.85$, $df = 230$, $p = 0.154$).

NJ-tree and *UPGMA* dendrogram were built for the total matrix (for 10 primers). These dendrograms have grouped the birds in different ways (Fig. 5). *NJ*-dendrogram distinguishes three bird branches for birds from habitats of carrion and hooded crows, and the hybrid zone respectively, with medium bootstrap support for hybrids (62%) and hooded crows (83%), and low bootstrap for carrion crows (20%). In the last case, only clustering of Eastern Asian subspecies could be considered as significant one (96%). The *UPGMA* dendrogram of genetic similarity revealed four branches. Three of them have significantly high bootstrap values: hooded crows (77%), Eastern Asian carrion crows (88%), and black-and-white hybrids (85%). The fourth branch had extremely low bootstrap (22%); Western carrion crow was separated from phenotypic carrion hybrids with medium significance. It is interesting that carrion crows from Krasnoyarsk (primarily considered as an initial parental variant) was in the same cluster with phenotypic carrion hybrids in both *UPGMA* and *NJ* trees. It should be noted that correlation between hybrid phenotypes and their distribution on both dendrograms was observed: hybrid crows that have black-and-white phenotype (No.212hy and 209hy) and high bootstrap values (85–100%) form individual subcluster.

DISCUSSION

RAPD-PCR analysis permits evaluating DNA polymorphism through a great number of various genome patterns and is widely used for assessment of species variability [25–27].

Our analysis demonstrated that genomes of phenotypic crow hybrids differ from parental species by additional DNA fragments and hence the increased level of intragenome heterogeneity. Similarly, unique alleles were detected in gopher hybrids *Spermophilus major* and *S. erythrogenys* from the zone of their overlapping habitats [28], hybrids between European and Asian butterflies [29], crossbred sheep [24], and interbreed hound hybrids [30]. Additional fragments detected in amplification patterns were not observed in genomes of parental species. This can indicate reorganization of genome (intragenome recombination between different alleles of parental forms) at various types of crosses and development of point mutations at primer annealing sites [30, 31].

Phenotypically pure crows from populations divided by hybrid zone, i.e., carrion crows from Krasnoyarsk and hooded crows from Novosibirsk, possibly could be classified as genotypic hybrids according to analysis of DNA amplification products. These birds contain fragments typical for phenotypic hybrids and untypical for birds from populations that are distant from the hybrid zone (Fig. 2). Thus, genetic boundaries of the zone of crow hybridization are wider than phenotypic ones as it has been described for hybridization of other species [32].

Distinctions between parental species and their phenotypic hybrids express themselves not only as bands/gaps, but also as intensities of amplicons under similar PCR conditions. This indicates that a particular sequence of a parental species or hybrid could be found more or less frequently in genome. Therefore, its amplification concentration could be higher or lower. Moreover, development of altered sequences in primer annealing sites is possible. This can induce variation in efficacy of product amplification.

Relatively small size of experimental population do not permit us making reliable assessment of genetic diversity of carrion and hooded crows. However, our preliminary analysis indicates that carrion crows and hybrid birds are characterized by an increased level of genetic diversity comparing to *C. cornix*.

Values of gene fixation rate ($F_{st} = 0.328$) and interpopulation diversity ($G_{st} = 0.318$) actually coincide for carrion and hooded crows and their values are low. Interpopulation differentiation comprises about 32% total genetic variability of investigated RAPD loci, while its specific portion comprises 68%. G_{st} and F_{st} indices indicate negligible interpopulation differentiation between carrion and hooded crows.

Our studies permit us to conclude that it is more reasonable to follow the opinion of Madge [6] and consider carrion and hooded crows as “a complex in the course of divergent evolution.” On one hand, evolution of hooded and carrion crows as species within a biologic concept is not over. Boundaries of hybrid zones as well as low values of genetic distances between hybridizing taxons support this viewpoint. However, distances themselves could not serve as a speciation criterion. There are examples of pronounced differences between species associated with substitution of only one gene and several modifiers [33]. On the other hand, considerable morphologic and ethologic differentiation permits zoologist to consider carrion and hooded crows at least as semispecies within the concept of superspecies. Genetic data indeed demonstrate that RAPD-

PCR patterns do not reflect satisfactorily genome differences of phenotypic parents. Development of unique amplicons in hybrid birds indicates that they developed through integration of two different genotypes.

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