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First insights into a DNA sequence based phylogeny of the Eurasian Jay *Garrulus glandarius*

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The Eurasian jay *Garrulus glandarius* Linnaeus, 1758 is a widespread Palearctic species which includes 33-35 more or less differentiated subspecies. These subspecies, combined into eight groups (Stresemann 1940; Vaurie 1959) were later classified in five morphologically and geographically defined groups (Goodwin 1986). Besides *G. glandarius*, two monotypic species belong to the genus: Lidth's jay *Garrulus lidthi* Bonaparte, 1850 (restricted to some southern Japanese Islands) and Black-headed jay *Garrulus lanceolatus* Vigors, 1831 (Himalayas).

Up to now, molecular phylogenetic studies including jays were published mainly for inter-species and inter-genus comparisons. Cibois and Pasquet (1999) investigated the phylogenetic relationships of 11 genera of Corvidae using sequence of the mitochondrial (mt) *cytochrome b* gene *(cytb)* as a molecular marker. In that analysis a close relationship of *G. glandarius* and the Siberian jay *Perisoreus infaustus* was rejected, a result which was confirmed later by Ericson *et al.* (2005) in an analysis based on one *mt* and two nuclear genes. These authors revealed a closer relationship of jays with several genera of the Old World corvids than to the monophyletic group of New World jays.

Besides those studies, no phylogenetic analyses were performed concerning interspecific relationships within the genus *Garrulus* or the intraspecific variation within *G. glandarius*. Therefore, the aim of the study was to provide insights into the phylogeography of jays and to assess intraspecific genetic variation and phylogeographic patterns. We analyzed sections of two mt sequences with different substitution rates: the *cytb* gene and the control region (CR). The latter one is in general considered as the faster evolving sequence and therefore was supposed to be especially useful for intraspecific studies (e.g. Kryukov *et al.* 2004). On the territory of Russia and adjacent countries, five subspecies are recognized (Stepanyan 2003): *G. glandarius glandarius* (Linnaeus, 1758), *G. glandarius brandtii* Eversmann, 1842; *G. glandarius iphigenia* Sushkin et

Ptuschenko, 1914; *G. glandarius krynicki* Kaleniczenko, 1839; *G. glandarius hyrcanus* Blanford, 1873. Besides samples of these subspeices we included also *G. glandarius japonicus* Temminck et Schlegel, 1847 from Japan as well as *G. lidthi*. We wanted to find out whether the individuals cluster according to subspecies assignment (i.e., geographic origin) as well as the level of their genetic differentiation.

Material

The sample set included 26 specimens (Table 1) belonging to five subspecies covering a huge range from Western Europe to Japan: *G. g. glandarius* (distributed in Europe), *G. g. brandtii* (Asia), *G. g. krynicki* (Caucasus and Turkey), *G. g. iphigenia* (Crimea), *G. g. japonicus* (Japan), and one hybrid between *G. g. glandarius* and *G. g. krynicki* (taken from South Russia), and also *G. lidthi*. Samples of livers and muscles fixed in 96% ethanol and kept in -4°C were used. As an outgroup, we used the magpie *Pica pica* (sequence determined in a previous study: Kryukov *et al*. 2004) and the chough *Pyrrhocorax pyrrhocorax* (this study). Accession numbers of all sequences determined in this study as well as of published sequences are given in Table 1.

Methods

DNA-extraction was performed with the phenol-chloroform deproteinization method (Maniatis *et al*. 1982). The two marker sequences were analyzed in two different laboratories (IBSS, Vladivostok and NHMW, Vienna). A partial region of *cytb* (length of PCR product: 586 bp) was amplified at the IBSS employing several published primers used previously for corvid birds (L14827, H16065, Helm-Bychowski, Cracraft 1993; L14990, Kocher *et al*. 1989; H15916, Edwards *et al*. 1991; and SNL4 (L15196), Kryukov, Suzuki 2000). All these primers initiated amplification, but the most successful combination (yielding one clear fragment on the gel) proved to be L15916 (ATGAAGGGATGTTCTACTGGTTG) / H16065 (GGAGTCTTCAGTCTCTGGTTTACAAGAC). Polymerase chain reaction was carried out in a «Biometra» Thermocycler (USA) in 20 µl of reaction containing 2 µl of 10x buffer, 0.125 mM $MgCl_2$, 0.1 mM of each dNTP, 1 pmol of each primer, 60 ng of template DNA and 1 unit of Taq-DNA-polymerase. PCR was performed under the following conditions: 5 min of pre-denaturation at 94°C, 35 cycles of denaturation for 1 min at 95°C; primer annealing for 2 min at 55°C; elongation for 2 min at 72°C, and finally an elongation step for 7 min at 72°C before cooling to 4°C. The amplification products were analyzed by electrophoresis in 1.5% agarose gels before sequencing.

Automated sequencing was perfomed with an ABI Prism 310 (Applied Biosystems). Cycle sequencing of purified PCR products was performed with the BigDye Terminator kit (Applied Biosystems) and the primers SNL4 and H15916 at a final concentration of 1 pmol/µl. Conditions of cycle sequencing: 25 cycles of denaturation for 30 sec at 96°C, annealing for 10 sec at 55°C, and elongation for 4 min at 60°C, and finally cooling to 4°C. A partial section of the CR was amplified at the NHMW with the primers CR-Cor+ (ACCCTTCAAGTGCGTAGCAG) and Phe-Cor- (TTGACATCTTCAGTGTCATGC) as described previously (Kryukov

et al. 2004). PCR products (length: ~ 680 bp) were extracted from 1% Agarose gels using the Quiaquick Gel Extraction Kit (Qiagen) and cloned using the TOPO TA cloning Kit (Invitrogen). Sequening of both strands was performed by MWG Biotech (Germany) using M13 universal primers.

Table 1. Specimens and sequences from GenBank used in the study.

Labcode	Geographic origin	Source / collection number	Marker sequences	Accession numbers
		Garrulus glandarius glandarius		
Gglagla1	Russia, Kirov Region	V.Sotnikov / 174	cytb	EF602118
Gglagla2	Russia, Kirov Region	V.Sotnikov / 227	cytb	EF602119
Gglagla3	Russia, Kirov Region	V.Sotnikov / 228	cytb	EF602120
Gglagla4	Russia, Kirov Region	V.Sotnikov / 233	cytb	EF602121
Gglagla5	Russia, Kirov Region	V.Sotnikov / 235	cytb	EF602122
Gglagla6	Russia, Kirov Region	V.Sotnikov / 239	cytb, CR	EF602123, EF602136
Gglagla7	Russia, Kirov Region	V.Sotnikov / 243	cytb	EF602124
Gglagla8	Russia, Kirov Region	V.Sotnikov / 226	CR	EF602137
Gglagla9	Russia, Smolensk Region	Ya.Red'kin / 126	cytb	EF602125
Gglagla10	Russia, Tula Region	Ya.Red'kin / 128	CR	EF602138
Gglagla11	Russia, Moscow Region	V.Korbut / 175	cytb, CR	EF602126, EF602139
Gglagla12	Russia, Moscow Region	M.Konovalova / 518	cytb	EF602127
Gglagla13	France	E.Pasquet / 381	cytb, CR	EF602128, EF602140
Gglagla14	Austria, Upper Austria	A.Gamauf	CR	EF602141
Gglagla15	Austria, Upper Austria	A.Gamauf	CR	EF602142
Gglagla16	GenBank	Cibois, Pasquet 1999	cytb	U86034
		Garrulus glandarius brandtii		
Gglabra1	Russia, Primorsky Region	A.Kryukov / 354	CR	EF602146
Gglabra2	Russia, Primorsky Region	A.Tsvetkov / 347	cytb	EF602131
Gglabra3	Russia, Primorsky Region	Ya.Red'kin / 346	cytb, CR	EF602132, EF602147
Gglabra4	Russia, Amurskaya Region	N.Kolobaev / 239	cytb, CR	EF602133, EF602148
Gglabra5	Russia, Primorsky Region	V.Sotnikov / 351	cytb	EF602134
		Garrulus glandarius iphigenia		
Gglaiph1	Russia, Crimea pen.	Ya.Red'kin / 747	CR	EF602144
Gglaiph2	Russia, Crimea pen.	V.Arhipov / 748	CR	EF602145
		Garrulus glandarius krynicki		
Gglakry2	Russia, Kislovodsk	Ya.Red'kin / 125	cytb, CR	EF602130, EF602149
		Garrulus glandarius krynicki × *G. glandarius glandarius*		
Gggxk1	Russia, Rostov Region	G.Bahtadse / 375	cytb, CR	EF602129, EF602143
		Garrulus glandarius japonicus		
Gglajap1	Japan, Honshu	W.Neuner	cytb, CR	EF602135, EF602150
		Garrulus lidthi		
Glid1	Japan, Riukiu Isl.	E.Pasquet / 383	CR	EF602151
Glid2	GenBank	Cibois, Pasquet 1999	cytb	U86035
		Pica pica jankowskii		
Ppicjan5	Russia, Primorsky Region	A.Kryukov / 714	cytb, CR	AY701183, AY701171
		Pyrrhocorax pyrrhocorax brachypus		
Gpyrbra1	Russia, Tuva Rep.	Ya.Red'kin / 104	CR	EF602152
Gpyrbra2	Russia, Tuva Rep.	A.Tsvetkov / 133	CR	EF602153

Phylogenetic analysis

Experimental sequences and those obtained from the GenBank were aligned with the software SeaView (Galtier *et al.* 1996). Phylogenetic trees (NJ, MP and ML) were calculated with PAUP, version 4.0b10 (Swofford 2002). NJ trees were calculated with p-distances. MP trees were calculated by a heuristic search with a random taxon addition sequence (1000 replicates) and the TBR (tree bisection reconnection) branch swapping algorithm and delayed character transformation (DELTRAN). Gaps were treated as missing character. ML trees were calculated by a heuristic search with a NJ starting tree and TBR branch swapping using the GTR+Γ model. For all tree calculating algorithms (NJ, MP and ML) a bootstrap analysis was performed with 1000 (NJ, MP) or 100 (ML) repeats, respectively. Pairwise P-distances and Kimura 2-parameter distances were calculated with the program DNASA (Kryukov *et al.*, in press). Average distances were calculated by hand from the tables obtained from DNASA.

Results and Discussion

The partial *cytb* sequence was isolated from 18 individuals comprising the four subspecies: *G. g. glandarius*, *G. g. brandtii*, *G. g. krynicki*, and *G. g. japonicus*. The partial sequence of the CR was determined from 16 *Garrulus* individuals. Besides the four subspecies mentioned above, the CR data set includes also two samples of *G. g. iphigenia*. Moreover, two *Garrulus* sequences from GenBank were included: Gglagla16 and Glid2 *(cytb)*. *Pica pica* and *Pyrrhocorax pyrrhocorax* were used as outgroup: Ppicjan5 (*ctyb*, CR; sequences from Kryukov *et al.* 2004) and Ppyrbra1, 2 (CR; this study). Lengths of the alignments were 586 bp for *cytb* (about half of the total gene, 1143 bp) and 677 bp for the CR.

Within the *cytb* sequences no insertions or deletions were found. Nucleotide frequencies for the *cyt b* gene were: A = 0.2942, T = 0.2272, G = 0.1120, C = 0.3666. The G-criterion was calculated as 1.0922 which means equal nucleotide dispersion within the section studied. As expected, synonymous transitions at the third codon positions were found as the most common substitution type, which is in agreement with published data for many animals. Among birds, up to 78% of informative substitutions are located in the third codon position (Helm-Bychowski, Cracraft 1993).

The neighbor-joining tree based on the *cytb* sequences is shown in Fig. 1. The MP and ML trees calculated from this data set have in general the same topology with the four subspecies of *G. glandarius* separated in four clusters (bootstrap values of all analyses are shown in Fig. 1 as NJ/MP/ML). Among them, the most basal split separates *G. g. japonicus* from the mainland taxa. *G. g. brandtii* splits off from the next node. Finally, a clade comprising the 12 individuals of *G. g. glandarius* is the sister group of the *G. g. krynicki* clade which includes also a hybrid between *G. g. glandarius* and *G. g. krynicki*. The hybrid origin of this phenotypi-

cally intermediate bird was approved by Ya.Red'kin (pers. comm.). Within subspecies, haplotype variation is rather low which is especially interesting for the nominate race the samples of which originated from quite distant European regions, i.e. Kirov (East European Russia), Austria and France.

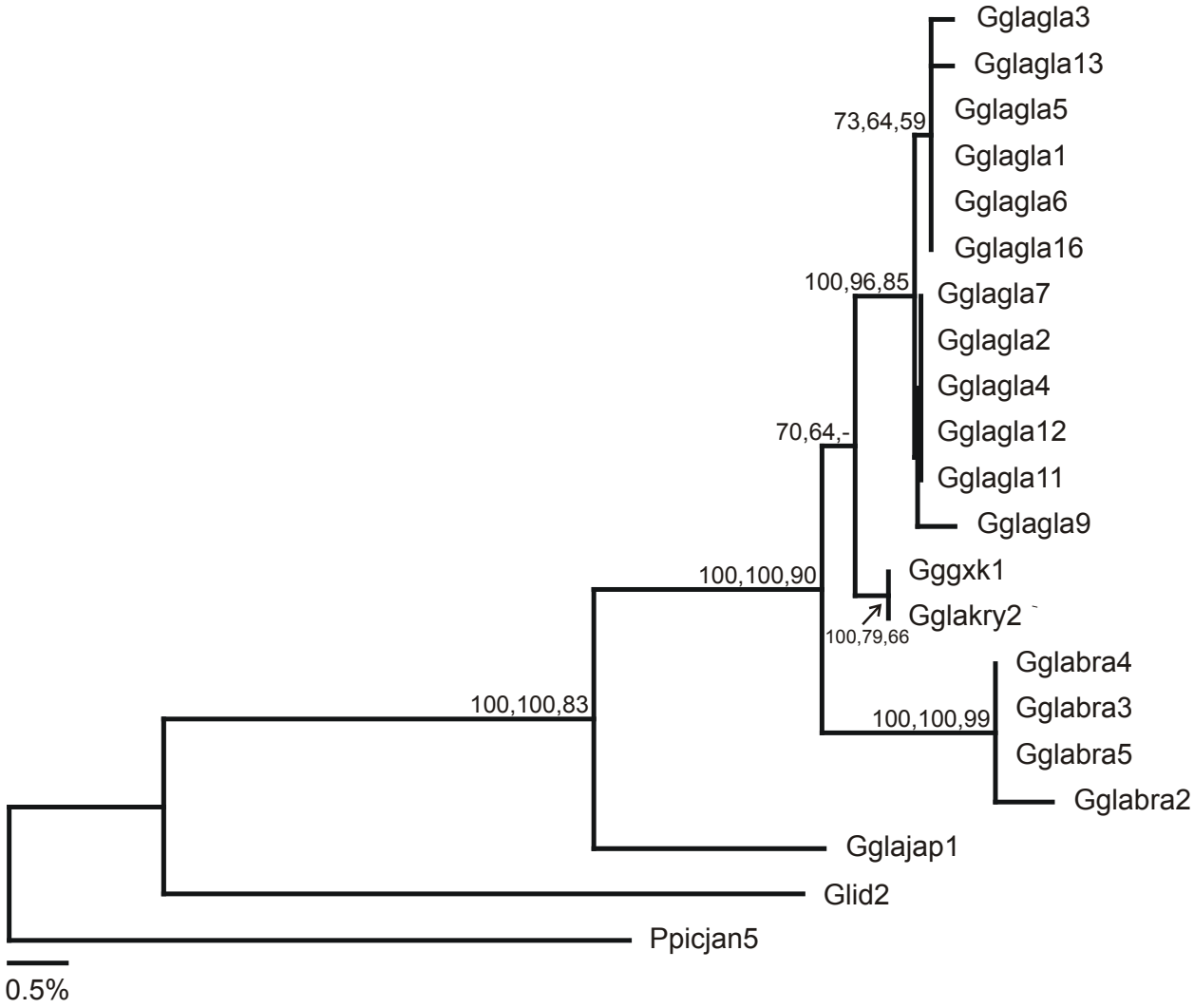

Fig. 1. NJ tree based on *cyt b* sequences of *Garrulus glandarius*, *G. lidthi* and *P. pica* (outgroup). Bootstrap values >50% are depicted at the nodes (NJ, MP, ML). Labcodes of specimens correspond to those in Table 1.

The NJ tree calculated on the basis of the CR data set is very similar to the *cytb* tree (Fig. 2). It includes also *G. g. iphigenia* which is part of the *G. g. glandarius* clade. The order of splits is the same as in the *cytb* tree.

The fact that the Japanese subspecies splits from the basal node in both trees is in accordance with the hypothesis about the first appearance of corvids in South East Asia from Australia (Sibley, Ahlquist 1985). In this context the origin of the genus *Garrulus* itself may have been located in South East Asia.

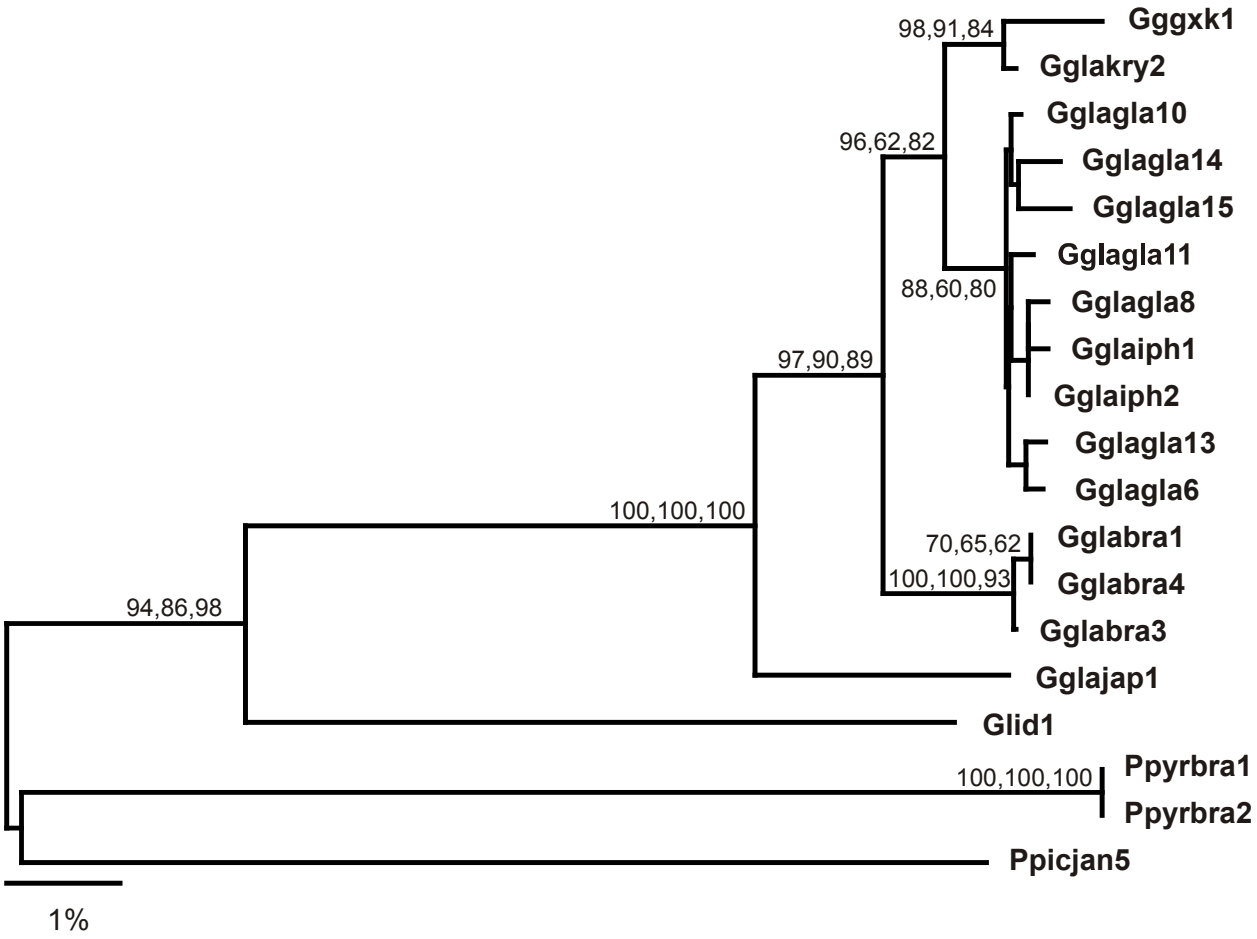

Fig. 2. NJ tree based on CR sequences of *Garrulus glandarius*, *G. lidthi* and the outgroup species *Pica pica* and *Pyrrhocorax pyrrhocorax*. Bootstrap values >50% are depicted at the nodes (NJ, MP, ML). Labcodes of specimens correspond to those in Table 1.

For both data sets we calculated average K2P distances between the four clades: *G. g. glandarius* including *G. g. iphigenia* (its distance to *G. g. glandarius* of 0.3% in the CR is neglectable), *G. g. krynicki*, *G. g. brandtii*, and *G. g. japonicus* (Tables 2 and 3). For comparison the p-distances are also shown. The differentiation of *G. g. japonicus* and *G. g. brandtii* is quite pronounced in both data sets. Average K2P distances between *G. g. japonicus* and the mainland subspecies range from 5.3-6.1 *(cytb)* and 4.4-4.9 (CR), for *G. g. brandtii* the respective values are 2-2.7 *(cytb)* and 2.6-2.8 (CR). This differentiation corresponds well with the distinct geographic distribution of these two subspecies in the Russian Far East and Japan respectively. In contrast, the differentiation between *G. g. krynicki* and *G. g. glandarius* is lower (1% in *cytb* and 2% in CR).

The average K2P distance between *G. lidthi* and *G. glandarius* is 12.3-14.9 in *cytb*, which is in the same range as the divergence of the outgroup genus *Pica* (14-16.6%). This underlines (1) that the mt lineages of the two species have split long ago and (2) that the *cytb* gene clearly has reached saturation for this level of divergence. This obviously is not the case in the CR data set as becomes apparent when comparing the dis-

tances between *G. glandarius*, *G. lidthi*, and the outgroup taxa (Table 3). For example, the distances between *Pica pica* vs. ingroup are twice as high as those found for *G. glandarius* vs. *G. lidthi*.

Table 2. Average genetic distances between the four clades of *Garrulus glandarius*, *G. lidthi* and the outgroup *Pica pica*, calculated from cytochrome b sequences. Kimura 2-parameter distances are above diagonal, p-distances below diagonal. All codon positions considered

	Gglagla	*Gglakry*	*Gglabra*	*Gglajap*	*Glid*	*Ppic*
Gglagla		1.0	2.72	5.34	14.24	15.28
Gglakry	0.99		2.04	5.01	14.32	15.54
Gglabra	2.66	2.0		6.08	14.90	16.58
Gglajap	5.09	4.78	5.75		12.34	14.88
Glid	12.57	12.63	13.11	11.09		13.99
Ppic	13.62	13.82	14.64	13.31	12.63	

Table 3. Average genetic distances between the four clades of *Garrulus glandarius*, *G. lidthi* and the outgroup *Pica pica* and *Pyrrhocorax pyrrhocorax*, calculated from Control Region sequences. Kimura 2-parameter distances are above diagonal, p-distances below diagonal

	Gglagla	*Gglakry*	*Gglabra*	*Gglajap*	*Glid*	*Ppyrbra*	*Ppic*
Gglagla		2.03	2.79	4.69	18.53	30.48	35.97
Gglakry	1.99		2.62	4.86	18.06	30.69	36.37
Gglabra	2.72	2.56		4.36	18.18	29.26	36.40
Gglajap	4.51	4.66	4.19		18.65	28.67	35.77
Glid	16.34	15.97	16.07	16.42		30.22	33.64
Ppyrbra	25.0	25.14	24.21	23.82	24.85		31.31
Ppic	28.53	28.77	28.8	28.4	27.07	25.59	

Which general conclusions can we draw from our data? There is a deep interspecific divergence of the two Palearctic jays, *G. glandarius* and *G. lidthi*, which is in the range of differentiation among many bird genera (Moore, DeFilippis 1997). Within *G. glandarius* there is a subspecific differentiation at the genetic (mt) level with the exception of *G. g. iphigenia*. The differentiation of *G. g. japonicus* and *G. g. brandtii* could be easily explained with a longer lasting (or repeated) isolation of these subspecies during the Pleistocene. Nevertheless, the differentiation is not so pronounced as found for the west-east divergence in other corvid taxa (e.g., *P. pica*, *Corvus corone*, *C. frugilegus* – Haring *et al.* submitted). Concerning the western subspecies one could interpret the trees at first sight in the following way: *G. g. krynicki* and *G. g. glandarius* are differentiated and the hybrid bird (Gggxk) has originated from a cross between a female *G. g. krynicki* and a male *G. g. glandarius*. Nevertheless, the sample size is too low to draw such conclusions, especially concerning the differentiation between the *G. g. glandarius* and *G. g. krynicki* clades, which appear very closely related. It also has to be taken into consideration that some mem-

bers of both clades come from geographic localities that are rather close compared to the rest of the European samples: Crimea (Gglaiph1, 2) and Rostov Region (Gggxk) in South Eastern Europe and Kislovodsk (Gglakry) in the North Caucasus region, respectively. Without analyzing much bigger samples of the subspecies one cannot rule out the possibility that the differentiation of *G. g. glandarius* and *G. g. krynicki* on the basis of mt sequences is just a sampling artifact.

The present data can be regarded as a first survey of the genetic diversity within *G. glandarius*. Our future aim is to analyze a broader sample covering the whole Palearctic which probably will be possible only with the inclusion of museum specimens into the study. This will enable us to elucidate the phylogeographic history of this species in more detail and to compare it to genetic patterns found in other widespread corvid birds.

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