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Synchronic east–west divergence in azure-winged magpies (*Cyanopica cyanus*) and magpies (*Pica pica*)*

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Abstract

Morphometric and genetic analyses were performed to investigate the relationships between populations of the azure-winged magpie (*Cyanopica cyanus*). In the morphometric analysis 193 specimens were included representing seven of the nine currently accepted subspecies. Among eight characters analysed, four showed significant differences between samples from Spain and Asia. In contrast, the Asian populations/subspecies are not differentiated morphologically except *Cyanopica cyanus japonica*. The genetic analysis was based on two mitochondrial sequences (control region, *cytochrome b* gene). The results are in accordance with the morphometric analysis, showing a clear distinction between birds from the western and eastern distribution ranges. The differentiation of *C. c. japonica* is not found at the sequence level. Both genetic and morphological data support species status of *C. cyanus* and *Cyanopica cooki*. The magpie (*Pica pica*) was included in the phylogenetic study for comparing intraspecific variation. As in *C. cyanus*, two clearly separated groups are found, one of them containing the far-eastern populations (*Pica pica jankowskii* and *Pica pica sericea*) and the other the remaining subspecies studied. For both the azure-winged magpie and the magpie the sequence data imply an east–west differentiation, probably caused by long lasting isolation that may have even started in the Pliocene or repeated expansions/restrictions of distribution ranges during the Pleistocene.

Key words: Genetic analysis – morphometric analysis – *Cyanopica* – *Pica* – molecular phylogeny – phylogeography

Introduction

The azure-winged magpie *Cyanopica cyanus* Pallas, 1776 is the classical example of a species with disjunctive distribution range. Whereas the main part of the range stretches from Lake Baikal and central China to the Japanese islands, a much smaller region is inhabited by the species in Spain and Portugal (Fig. 1). Within these isolated ranges, several subspecies can be distinguished. The European populations of the western range comprise two subspecies: *Cyanopica cyanus cooki* and *Cyanopica cyanus gili*, although the latter is not generally accepted and usually lumped with *C. c. cooki*. Within the Asiatic (eastern) range, up to eight subspecies have been described: *Cyanopica cyanus cyanus*, *Cyanopica cyanus pallescens*, *Cyanopica cyanus stegmanni*, *Cyanopica cyanus koreensis*, *Cyanopica cyanus kansuensis*, *Cyanopica cyanus interposita*, *Cyanopica cyanus swinhoei*, and *Cyanopica cyanus japonica* (Vaurie 1959).

All Asian subspecies mentioned differ by minor details of plumage coloration, much more pronounced comparing birds from the western and eastern parts of the range. Asian azure-winged magpies have white tips on their central tail feathers. Furthermore, the mantle and rump in Asian birds are definitely greyish, while they are brownish in the European ones. However, no comparative analysis of morphological measurements of the various taxa has been carried out so far.

The existence of a wide geographic gap between the eastern and western range of the azure-winged magpie, spanning about 9000 km, attracted wide attention. Usually these isolates were treated as Tertiary relics, assuming subdivision of a former continuous range during the Pleistocene (Dementiev 1940; Goodwin 1986). This hypothesis was corroborated recently by the discovery of azure-winged magpie's bones in two caves near Gibraltar close to the southern tip of the Iberian Peninsula (Cooper 2000). Two left proximal humeri with well-preserved epiphyses were found, which are considered as one of the best diagnostic parts of the avian skeleton. Radiocarbon dating of one of these bones estimated its age at more than 44 000 years. In one cave, the fossils occurred together with tools of Neanderthal humans. However, another explanation for the disjunctive distribution range exists as well. It has been hypothesized that in the 16th century Portuguese or Spanish sailors might have brought these nice birds from Asia (China or less probably Japan): 'It may have been released voluntarily or escaped from cages and found local conditions to be suitable' (Dos Santos 1968, p. 24). According to this hypothesis, the descendants of these founder individuals spread successively over the Iberian Peninsula (Madge and Burn 1999).

In a first molecular analysis based on the mitochondrial control region (CR) of all currently defined subspecies Fok et al. (2002) found that the eastern forms are genetically distinct from the western populations. From the genetic differentiation they estimated a divergence time of 1.2 million years ago (mya). As a taxonomic consequence Fok et al. (2002) proposed to split *C. cyanus* into two species, namely *Cyanopica cyanus* Pallas, 1776 for the Asian forms and *C. cooki* Bonaparte, 1850 for the Iberian ones. Furthermore, the genetic data did not support the current classification into subspecies, neither within the western group (no genetic differentiation of the disputed subspecies *C. c. gili*) nor within the Asian group. For the latter they reported a genetic division into an 'Inland Asia' group and a 'Pacific seaboard' group.

In the present paper, we performed a combined morphological and genetic study to investigate the phylogeography of

*This paper is dedicated to Prof. Ernst Mayr on the occasion of his 100th birthday.

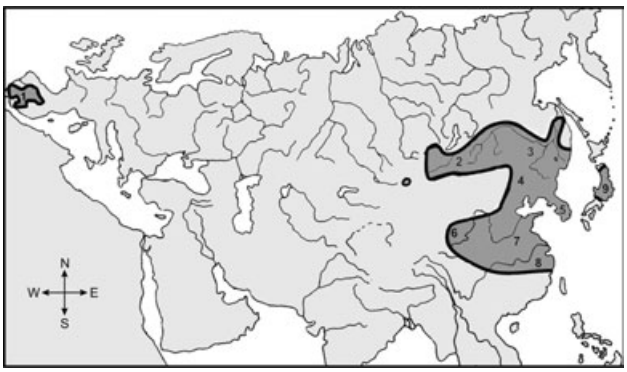

Fig. 1. Geographic distribution of *Cyanopica cyanus*. 1, *C. c. cooki*, 2, *C. c. cyanus*, 3, *C. c. pallescens*, 4, *C. c. stegmanni*, 5, *C. c. koreensis*, 6, *C. c. kansuensis*, 7, *C. c. interposita*, 8, *C. c. swinhoei*, 9, *C. c. japonica*. After: Cheng (1987); Fomin and Bold (1991); Cramp and Perrins (1994); Madge and Burn (1999); Babenko (2000)

the azure-winged magpie and to interpret the results with respect to taxonomy and divergence times. The morphological analysis of eight external metric characters of study skins representing seven subspecies of *C. cyanus* was intended to answer the following questions: (1) Is there, besides plumage coloration, any morphological differentiation corresponding to the genetic distinctness of the eastern and western populations? (2) To what extent are the Asian subspecies, for which no clear genetic differentiation was found so far, morphologically distinct? (3) Is the genetic difference between the two groups ('Inland Asia' and 'Pacific seaboard') proposed by Fok et al. (2002) paralleled by a morphological differentiation?

For the genetic analysis we employed two mitochondrial marker sequences: besides the CR used also by Fok et al. (2002) the *cytochrome b* (*cyt b*) gene was analysed. The CR is the most variable section of the mitochondrial genome, and therefore it should be suitable to detect variation among taxa for which the *cyt b* gene is still too conservative. On the other hand, the *cyt b* gene probably is the most commonly used marker gene and it is considered as the best choice for dating divergence times at the species/subspecies level. Our data set supplements the study of Fok et al. (2002) as it includes larger samples of the subspecies *cyanus* and *pallescens*. Furthermore, we included the magpie *Pica pica* into the genetic analysis for several reasons: (1) It should serve as outgroup to root the tree. (2) The intraspecific genetic variation found in *C. cyanus* should be compared with that of another polytypic corvid species. For this purpose, we selected *P. pica* as a species with a continuous distribution range throughout the Palearctic and the western part of the Nearctic. For *P. pica*, division into several species has been suggested recently on the basis of morphological and behavioural characters (Ebels 2003). Lee et al. (2003), analysing the rather conserved mitochondrial *16S* rRNA gene (16S) of three subspecies, detected a clear genetic differentiation between them. Using the more variable mitochondrial CR, the intraspecific variation and taxonomy of *P. pica* can be analysed in more detail. (3) In comparing the rates of three marker sequences (CR, *cyt b*, *16S*) we discuss the possibility to calibrate a molecular clock and to estimate divergence times.

Materials and methods

Morphological analysis

For the analysis of morphological variation we measured 193 skins of adult birds (94 males and 99 females) from the following avian collections: Zoological Museum of Moscow University, Russia (69 skins studied), Yamashina Institute for Ornithology, Chiba, Japan (75), Natural History Museum Paris (18), Natural History Museum Vienna (16), and Institute of Biology and Soil Science, Vladivostok, Russia (15). These 193 specimens represent the following subspecies: *C. c. cooki* from Spain, $n = 15$; *C. c. cyanus* from Transbaikalia and Mongolia, $n = 16$; *C. c. pallescens* from Ussuriland (Far East Russia), $n = 55$; *C. c. stegmanni* from North-East China (Mandzhuria), $n = 23$; *C. c. interposita* from East China, $n = 27$; *C. c. koreensis* from South Korea, $n = 17$; *C. c. japonica* from Japan, $n = 40$. The number of skins examined was actually much higher, but unsexed and young birds were not measured. Unfortunately, the various museums collections harbour only a limited selection of subspecies. Thus, in spite of the high number of specimens investigated, some of the subspecies are represented by only a few individuals and some could not be included at all.

The following measurements were taken from each skin: wing length (Wing-L), tail length (Tail-L), distance between the tips of longest primaries and secondaries (wing apex, Wing-A), distance between the tips of second and fifth primaries (Prim-Dist), distance between the tips of inner and outer tail feathers (Tail-Dist), bill length from the mouth angle (Bill-M) and from the frontal end of nostril (Bill-N), and bill height (Bill-H). Measurements were made with a digital calliper, by the first author only. For the multivariate canonical analysis and to test the significance of differences between means we used the program STATISTICA version 6.0 (StatSoft, Inc. 1995).

Phylogenetic analysis

For the genetic study tissue samples of 22 individuals of the azure-winged magpie *C. cyanus* (six subspecies) and 21 individuals of the magpie *P. pica* (six subspecies) were analysed (Table 1). Liver and muscle samples stored in ethanol as well as feathers were used for DNA extraction. DNA was extracted according to Haring et al. (1999). The following primers were used: CR sequences: CR-Cor+ ACCCTTCAAGTGCGTAGCAG, Phe-Cor− TTGACATCTT-CAGTGTCATGC; these primers amplify a partial sequence of the CR (positions 693−1308 of the reference sequence of *C. c. cyanus*, AJ458536) as well as 21 bp of the adjacent *tRNA-Phe* gene (length of PCR fragment ~680 bp). PCR was performed on a Master gradient thermocycler (Eppendorf) in 25 µl with 0.5 units Dynazyme DNA polymerase (Finnzyme OY), 1 µM of each primer and 0.2 mM of each dNTP (Boehringer Mannheim, Germany); annealing temperature: 58°C; 35 reaction cycles. Control reactions of both DNA extraction and PCR amplification were performed. PCR products were extracted from agarose gels using the Qiaquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands) and cloned (TOPO TA Cloning Kit; Invitrogen, Lofer, Austria). Sequencing of both strands was performed by MWG-Biotech (Ebersberg, Germany). Fragments of *cyt b* were amplified with the following primer set: L14827: 5′-CCACACT-CCACACAGGCCTAATTAA-3′, H16065: 5′-GGAGTCTTCAGTC-TCTGGTTTACAAGAC-3′ (Helm-Bychowski and Cracraft 1993). Conditions of the PCR reactions were described in Iwasa et al. (2002). Sequencing of *cyt b* fragments was performed with an automated DNA sequencer model 310 (Applied Biosystems) at Hokkaido University.

The two alignments had a length of 659 sites (CR) and 1136 sites (*cyt b*), respectively. For comparing our CR sequences with those of Fok et al. (2002) we produced an additional alignment of the overlapping sections comprising positions 693–1308 of the complete CR (reference sequence of *C. c. cyanus*, AJ458536). Editing and alignment of sequences was performed using the BioEdit software package version 5.0.9 (Hall 1999).

The software PHYLTEST (Kumar 1996) was used to test rate for constancy in both marker sequences using the relative rate test. For both *P. pica* and *C. cyanus* rate constancy between the eastern and western populations was not rejected at the 5% level. Maximum parsimony (MP), Maximum Likelihood (ML), and Neighbour-joining

Table 1. List of specimens analysed

Taxon	Sample code	Locality	Source	Tissue	Marker
Cyanopica cyanus cooki	Cccoo2	Spain, N. Burgillos, NE Sevilla	A. Gamauf	fe	CR
	Cccoo4	Spain, Badajoz	C. Cruz, J. Martinez	DNA	CR
	Cccoo5	Spain, Badajoz	C. Cruz, J. Martinez	DNA	*cyt b*
	Cccoo6	Spain, Badajoz	C. Cruz, J. Martinez	DNA	CR
	Cccoo10	Spain, Badajoz	C. Cruz, J. Martinez	DNA	CR
	Cccoo12	Spain, Badajoz	C. Cruz, J. Martinez	DNA	CR, *cyt b*
C. c. gili	Ccgil1	Portugal, Alvor, Algarve	B. Wylie, D. Radford	fe	CR
C. c. cyanus	Cccya23	Russia, Irkutsk	I. Fefelov	fe	CR
	Cccya24	Russia, Transbaikalia, Olowjannaja	S. Weigl, S. Wegleitner	mu	CR
	Cccya25	Russia, Transbaikalia, Olowjannaja	S. Weigl, S. Wegleitner	mu	CR
	Cccya26	Russia, Transbaikalia, Olowjannaja	S. Weigl, S. Wegleitner	mu	CR
	Cccya27	Russia, Transbaikalia, Olowjannaja	S. Weigl, S. Wegleitner	mu	CR
	Cccya28	Russia, Transbaikalia, Olowjannaja	S. Weigl, S. Wegleitner	mu	CR
C. c. pallescens	Ccpal1	Russia, South Ussuriland, Nadezhdinsk	A. Kryukov	fe	CR
	Ccpal2	Russia, West Ussuriland, Konstantinovka	A. Kryukov	li	CR, *cyt b*
	Ccpal3	Russia, West Ussuriland, Konstantinovka	A. Kryukov	li	*cyt b*
	Ccpal5	Russia, West Ussuriland, Konstantinovka	A. Kryukov	li	CR, *cyt b*
	Ccpal6	Russia, Lower Amur, Kutuzovka	A. Kryukov	li	CR, *cyt b*
	Ccpal7	Russia, Lower Amur, Kutuzovka	A. Kryukov	li	CR, *cyt b*
	Ccpal13	Russia, Ussuriland, Gaivoron	Ya. Red'kin	li	CR
	Ccpal21	Russia, Ussuriland, Arsenyev	Ya. Red'kin	li	CR
C. c. koreensis	Cckor1	South Korea, Chungnam prov., Sochongun	Hyongwook Park	li	CR
C. c. interposita	Ccint10	China, Beijing	A. Gamauf	fe	CR
C. c. japonica	Ccjap22	Japan, Kavaguchi, near Tokyo	T. Hiraoka	m	CR, *cyt b*
Pica pica pica	Pppic4	Russia, Smolenskaya reg.	Ya. Red'kin	li	CR, *cyt b*
	Pppic5	Russia, Smolenskaya reg.	Ya. Red'kin	mu	CR
	Pppic7	Turkey, Büyük Camlica	R. Kothbauer	fe	CR
	Pppic8	Turkey, Büyük Camlica	R. Kothbauer	fe	CR
Pica p. bactriana	Ppbac4	Russia, Kirov	Ya. Red'kin	li	CR
	Ppbac5	Russia, Ivanovo reg.	Ya. Red'kin	mu	CR
	Ppbac6	Russia, Kislovodsk	Ya. Red'kin	li	CR, *cyt b*
Pica p. hemileucoptera	Pphem1	Russia, Tuva republic, Muhur-Aksy	Ya. Red'kin	li	CR
	Pphem2	Russia, Tuva republic, Muhur-Aksy	Ya. Red'kin	li	CR
Pica p. leucoptera	Ppleu1	Russia, Transbaikalia, Ulan-Ude	S. Weigl, S. Wegleitner	mu	CR
	Ppleu2	Russia, Transbaikalia, Ulan-Ude	S. Weigl, S. Wegleitner	mu	CR
	Ppleu3	Russia, Transbaikalia, Schartal	S. Weigl, S. Wegleitner	mu	CR
	Ppleu4	Russia, Transbaikalia, Ulan-Ude	S. Weigl, S. Wegleitner	mu	CR
	Ppleu5	Russia, Transbaikalia, Ulan-Ude	S. Weigl, S. Wegleitner	mu	CR
Pica p. jankowskii	Ppjan1	Russia, Ussuriland, Nadezhdinsk	A. Kryukov	li	CR
	Ppjan2	Russia, Lower Amur, Solnechny	A. Kryukov	li	CR
	Ppjan3	Russia, Ussuriland, Gaivoron	Ya. Red'kin	li	CR
	Ppjan4	Russia, Ussuriland, Nadezhdinsk	A. Kryukov	li	CR, *cyt b*
	Ppjan5	Russia, Ussuriland, Nadezhdinsk	A. Kryukov	li	CR, *cyt b*
Pica p. sericea	Ppser1	South Korea, Chuncheon City	Jong Teak Kim	li	CR
	Ppser3	South Korea, Chuncheon City	Jong Teak Kim	li	CR

Sample codes: abbreviations are derived from genus (first letter), species (second letter), subspecies (fourth to fifth letter), and number of specimen.
CR, control region; *cyt b*, *cytochrome b*; Tissue: fe, feather; li, liver; mu, muscle.

(NJ; Saitou and Nei 1987), dendrograms were calculated with the software package PAUP (version 4.0b10; Swofford 2002). MP analyses were based on heuristic searches with the TBR (tree bisection reconnection) branch swapping algorithm with a random taxon addition sequence (1000 replicates) and delayed character transformation (DELTRAN). Gaps were treated as fifth character state for the CR data set. Bootstrap analyses were performed with 1000 replicates for MP (10 random addition replicates) and NJ trees. Parameters for the ML analysis were estimated by the hierarchical likelihood ratio test using MODELTEST version 3.06 (Posada and Crandall 1998). The optimal model for CR was the HKY + Γ model with empirical base frequencies ($A = 0.335$, $C = 0.231$, $G = 0.097$, $T = 0.337$), an estimated transition/transversion ratio of 3.32, and a gamma distribution shape parameter of 0.306. For *cyt b* the K81uf + Γ model was chosen with six substitution types, empirical base frequencies, and a γ-distribution shape parameter of 0.080. ML trees were calculated by TBR branch swapping using a NJ starting tree. Bootstrap support values for ML trees were calculated from 100 replicates with NNI branch swapping. For NJ trees HKY85 distances (Hasegawa et al. 1985) were used, since applying the substitution models selected by MODELTEST resulted in considerably lower bootstrap values. The sequences determined in the course of the present study are registered under the GenBank accession numbers AY701131–AY701185.

Results

Morphometric analysis

We analysed samples from seven populations corresponding to seven subspecies from both distribution ranges of the azure-winged magpie *C. cyanus*. For all measurements, values of males were larger than those of females, so we did not combine data for both sexes, but the female and male samples were

Table 2. External morphometric measurements of azure-winged magpies from different populations (in mm)

		Wing-L		Wing-A		Prim-Dist		Tail-L		Tail-Dist		Bill-M		Bill-N		Bill-H	
Population	Sample size	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Males	94																
C. c. cooki	7	138.9	6.5	20.23	3.62	25.91	2.64	189.8	5.71	102.68	3.25	29.04	0.88	16.71	0.59	8.00	0.47
C. c. cyanus	12	143.9	5.6	21.39	2.80	24.85	3.31	222.9	13.82	125.50	12.54	31.13	1.19	18.37	0.81	8.07	0.59
C. c. pallescens	25	142.9	3.5	21.17	2.52	24.99	3.25	222.0	14.68	125.09	11.89	30.48	1.57	18.52	0.97	8.13	0.51
C. c. stegmanni	9	143.7	5.2	22.08	4.22	24.33	3.48	225.2	14.50	127.48	13.91	30.09	1.28	18.41	1.08	7.92	0.79
C. c. interposita	10	141.9	5.0	25.74	3.10	23.9	3.06	220.4	11.15	124.52	10.02	30.71	2.06	18.26	1.36	8.02	0.37
C. c. koreensis	8	138.4	1.9	19.83	2.23	24.94	3.67	214.6	6.50	116.96	10.77	31.21	2.16	17.84	1.69	8.04	0.28
C. c. japonica	23	135.9	3.5	22.86	3.86	22.84	2.10	214.1	12.04	130.97	9.06	31.00	2.39	18.95	1.10	8.66	0.62
Females	99																
C. c. cooki	8	130.8	4.3	17.75	2.66	23.36	3.76	179.0	9.32	95.13	7.78	27.39	1.93	15.56	0.77	8.21	0.59
C. c. cyanus	4	139.0	5.8	20.23	2.11	25.13	3.47	208.0	13.88	109.93	13.35	31.30	2.47	19.10	2.03	8.03	0.85
C. c. pallescens	30	139.2	5.2	20.89	4.22	23.82	3.48	211.8	14.50	113.88	13.91	29.95	1.28	17.94	1.08	7.70	0.79
C. c. stegmanni	14	137.6	4.3	18.68	1.51	24.86	2.68	214.3	13.35	117.83	9.54	28.91	1.80	17.92	1.24	7.54	0.47
C. c. interposita	17	137.5	4.1	22.57	2.93	22.96	3.55	211.6	8.34	116.52	7.58	30.35	1.61	17.82	1.11	7.80	0.65
C. c. koreensis	9	134.6	4.9	21.40	4.16	24.67	3.86	208.7	10.70	114.14	8.36	29.69	1.34	17.41	0.94	7.82	0.69
C. c. japonica	17	131.3	2.8	20.72	2.01	22.93	2.64	199.7	9.71	116.02	10.54	29.37	1.45	17.81	1.64	8.21	0.45

M, mean; SD, standard deviation.

treated independently (Table 2). For example, in the male sample from Ussuriland (subspecies *pallescens*) – the sample with the highest number of skins studied – average Wing-L was 3.7 mm longer and average Tail-L was 10.2 mm longer than in females. Furthermore, males have a more graduated tail and a longer bill.

Both sample sets (males and females) demonstrate a clear differentiation between the western population from Spain (*C. c. cooki*) and the Asian populations. Among eight characters analysed, at least four showed significant differences between samples from Spain and Asia. Significance was tested statistically by Student's *t*-test, with 5% probability level. According to these results *C. c. cooki* differed in both sexes from almost all Asian populations with respect to Tail-L, Tail-Dist, Bill-M, and Bill-N. In addition, for females differences in Wing-L were observed between *C. c. cooki* and each of the four Asian subspecies, and for males between *cooki* and *pallescens* only. The birds from Japan and Korea have comparatively short wings, similar to those of the Spanish specimens, in both sexes.

In contrast to the differentiation between Spanish and Asian populations, which is found in most of the external characters, differences between the Asian samples are not significant in most cases. Only *C. c. japonica* differs significantly from most others in Wing-L and Prim-Dist in males, while in females it differs in Wing-L from all Asian subspecies ($p < 0.05$). It should be mentioned that we found four unusual skins in the Natural History Museum Paris: Birds collected in Japan, Honshu Island (from the collection of N. Kuroda, one male and three females) lacked the white tail spots, whereas in all other aspects those skins were characteristic for the subspecies *C. c. japonica*.

For a more comprehensive presentation of our data, we conducted a multivariate canonical analysis. Among seven canonical roots, the two statistically most significant roots were calculated. The corresponding scatter diagrams for males and females are shown in the Figs 2 and 3, respectively. In both diagrams it can be seen that the population from Spain differs clearly from the others, while all Asian populations share almost the same cluster. The only exception is the Japanese subspecies, which occupies a distinct space, not

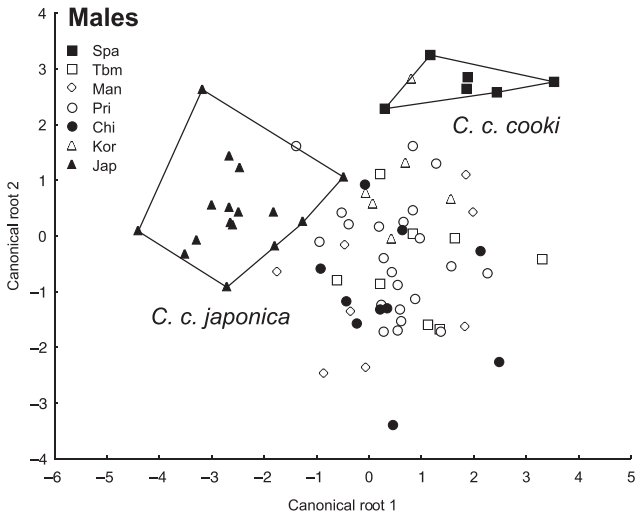

Fig. 2. Scatter-diagram of canonical variates analysis of eight measurements for males of *Cyanopica cyanus*. *Abbreviations*: Spa, Spain; Tbm, Transbaikalia and Mongolia; Man, Mandzhuria; Pri, Primorye; Chi, China; Kor, Korea; Jap, Japan

overlapping with the others in the case of males and partly overlapping in females. The proposed subdivision into two Asian subgroups ('Inland Asia' and 'Pacific seaboard') according to Fok et al. (2002) is not corroborated by morphological differentiation. In total, the morphological analysis revealed a very clear distinctness between the Iberian and Asian populations but failed to distinguish among the Asian subspecies with the exception of *C. c. japonica*.

Phylogenetic analysis

The results of the phylogenetic analyses reveal a clear pattern concerning the grouping of haplotypes derived from the eastern and western isolates of the azure-winged magpie. European populations are represented by Spanish and Portuguese samples, while the Asian ones originate from Lake Baikal, the South of Far East Russia, East China, South

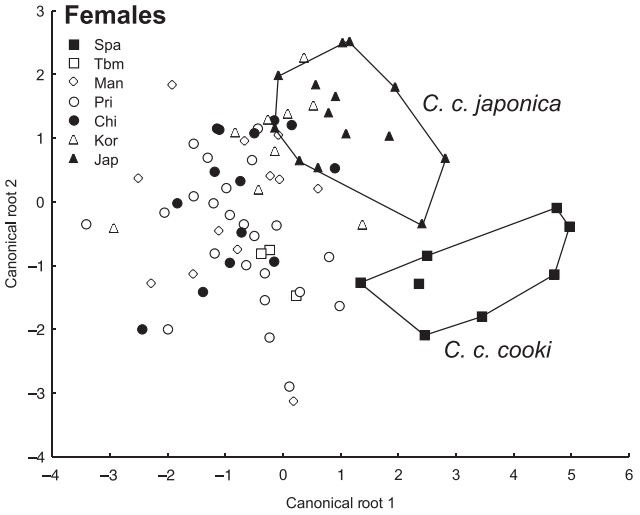

Fig. 3. Scatter-diagram of canonical variates analysis of eight measurements for females of *Cyanopica cyanus*. Abbreviations: see Fig. 2

Korea, and Japan (Table 1). The present analysis includes seven of the nine currently recognized subspecies. The NJ tree based on CR sequences (Fig. 4) reveals two major clades, one representing *C. cyanus*, and the other one *P. pica*. Each of the two clades is further subdivided into two subclades, which are supported by high bootstrap values. The ML and MP trees (not shown) have the same topology as the NJ tree with respect to the four major clades. Only minor rearrangements were found within subclades which are not relevant for the interpretation of the results. The MP analysis yielded four equally parsimonious trees (184 variable sites, 169 parsimony informative; tree length 219, CI = 0.936, CI excluding uninformative sites = 0.931, RI = 0.995, RC = 0.931, bootstrap values are included in Fig. 4).

The two subclades of the *Cyanopica* clade correspond to European and Asian subspecies, respectively. Differences between the two subclades are between 32 and 40 substitutions (average p-distance 5.3%, range 5.0–6.3%), while within subclades the respective values were 0–9 substitutions (0.5%, range 0–1.3%) for the eastern subclade and 0–4 substitutions (0.3%, range 0–0.6%) for the western subclade. The differences between representatives of the subspecies *C. c. cyanus*, *C. c. pallescens*, *C. c. stegmanni*, *C. c. interposita*, *C. c. koreensis*, and *C. c. japonica* are in the same range as those within *C. c. cyanus* (Fig. 4). A similar situation is found within the western subclade where distances between presumed subspecies (i.e. one sample of *C. c. gili* versus others belonging to *C. c. cooki*) are in the same range as all other within-subclade distances (Fig 4).

In the trees of Fok et al. (2002), based on the complete CR (1136 bp), the eastern group of *Cyanopica* is further subdivided into an 'Inland Asia' group with the subspecies *cyanus*, *kansuensis*, *interposita*, and *swinhoei*, and a 'Pacific seaboard' group with *pallescens*, *stegmanni*, *koreensis*, and *japonica*. In our tree, based on the 659 bp 3′-section, this subdivision is not visible. However, combining the published CR sequences with our data set (overlapping region of 638 bp), the two clusters can be distinguished but are separated by only a single

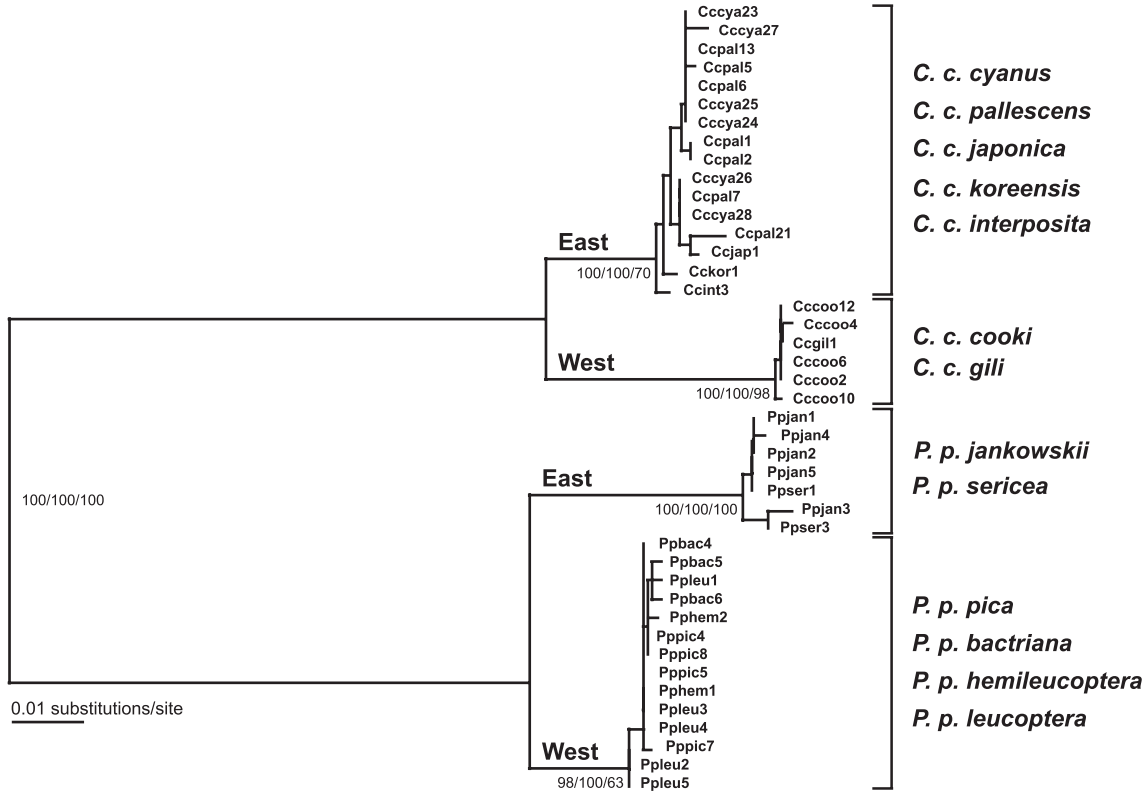

Fig. 4. NJ tree (calculated from HKY85 distances) for several subspecies of *Cyanopica cyanus* and *Pica pica* based on sequences of the mitochondrial control region (659 sites). Bootstrap values (1000 replicates) are given at the four major nodes within main clades (from left to right: NJ, MP, ML)

Table 3. Average distances (p = p-distance, d = HKY85 distance), ranges, and dates of divergence (mya) estimated from d-values

	CR/5.0				*cyt b*/2.0				*16S*/1.6	
Gene/rate (% per my)	p^1	Range	d^2	Ma	p	Range	d	Ma	p	Ma
Cyanopica E/W	5.3	5.0–6.3	5.2	1.04	6.3	6.1–6.4	6.7	3.35	–	–
Cyanopica E	0.5	0–1.3	0.5	0.10	0.2	0–0.5	0.2	0.10	–	–
Cyanopica W	0.3	0–0.6	0.1	0.02	0.0	–	–	–	–	–
P. pica E/W	6.6	6.2–7.7	4.7	0.94	5.1	4.7–5.4	5.4	2.70	3.3	2.06
P. pica E	0.6	0–1.4	0.4	0.08	0.3	–	0.3	0.15	1.0	0.63
P. pica W	0.3	0–0.6	0.2	0.04	0.6	–	0.6	0.30	0.4	0.25
Cyanopica/*P. pica*	20.3	19.3–22.5	–	–	12.3	11.6–13.1	–	–	13.8	–

The *16S* data for *P. pica* are taken from Lee et al. (2003).
Substitution rates according to Freeland and Boag (1999) and Fok et al. (2002) for CR, Klicka and Zink (1997) for *cyt b*, and Fleischer et al. (1998) and Lee et al. (2003) for *16S*. The p-distances between *Cyanopica* and *Pica* are given for comparison, but dating of divergence is not reasonable in this case.
[1]*p*-Distances for CR (gaps treated as fifth state).
[2]HKY85 distances for CR (substitutions only).
E, eastern subgroup; W, western subgroup.

substitution (combined tree, not shown). Further analysis of the data of Fok et al. (2002) reveals that the split 'Inland Asia' versus 'Pacific seaboard' is supported by 12 sites in the 5′-section, which has not been used in our study. The combined tree reveals another major discrepancy between our results and those of Fok et al. (2002) with respect to the assessment of the subspecies *C. c. cyanus*. The single representative analysed by Fok et al. (2002) (cyanus 1; Mongolia) belongs to the Inland Asia group, whereas the six individuals from the present study (Cccya23–28; Transbaikalia) are placed in the 'Pacific seaboard' group.

The degree of differentiation between the two isolates of the azure-winged magpie can be compared with that between subspecies of the magpie *P. pica* represented by the second main clade, which is also divided into two groups. One of them contains the far-eastern populations (*Pica pica jankowskii* and *Pica pica sericea*), whereas the other contains populations from eastern Europe and western Russia (*Pica pica pica*, *Pica pica bactriana*, *Pica pica hemileucoptera*) and from one central Siberian population (*Pica pica leucoptera*). With 40–50 substitutions the distance between the two groups is similar to that found for the two *C. cyanus* groups (average p-distance 6.6%, range 6.2–7.7%). Average distances within each of the two groups are very low (Table 3). Thus, within each group, distances between subspecies investigated are in the same range as those between representatives of the same subspecies (Fig. 4).

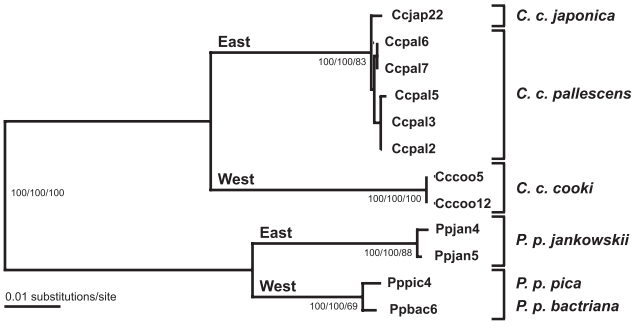

Fig. 5. NJ tree (calculated from HKY85 distances) for several subspecies of *Cyanopica cyanus* and *Pica pica* based on sequences of the mitochondrial *cytochrome b* gene (1137 sites). Bootstrap values (1000 replicates) are given at the four major nodes within main clades (from left to right: NJ, MP, ML)

The trees calculated from the *cyt b* sequences are congruent to the CR trees, yet the sample size is much smaller and each species is represented by three subspecies only. The topology of the NJ tree (Fig. 5) is identical to that of the ML tree and one of the two shortest MP trees (197 variable sites, 192 parsimony informative; TL = 232, CI = 0.922, CI excluding uninformative sites = 0.921, RI = 0.963, RC = 0.888). The other MP tree differs only with respect to the branching pattern within the eastern *Cyanopica* clade. As in the trees based on the CR, there is a clear distinction between the western and eastern groups. Within each group less genetic variation is found compared with the CR sequences (Table 3).

Discussion

Divergence pattern and taxonomy

In previous studies, the phenotypic differentiation between European and Asian azure-winged magpies was based on colour and feather size variation. An extensive morphometric analysis of azure-winged magpies had not been carried out so far. We found only one set of measurements published for a sample of Portuguese birds (Dos Santos 1968), where data for 22 specimens (14 males, eight females) were reported. Average wing length of males/females was 140.8 mm/129.8 mm, and average tail length was 192.3 mm and 183.6 mm, respectively. In some other publications, also Wing-L values were reported for two subspecies only (Rustamov 1954) or Wing-L and Tail-L for *C. c. pallescens* only (Nechaev 1974). All these values were similar to our data.

When comparing DNA sequence analyses and morphological analyses, one should be aware that morphological characters are changed under the influence of natural selection while DNA sequence data are more likely to be primarily evolving in the space of neutral drift. Selection may severely constrain morphological variation and cause parallelism. Nevertheless, our multivariate morphometric analysis shows a clear differentiation of Iberian azure-winged magpies in comparison with the Asian ones, which proved to be different in several measurements and in the space of two main canonical roots. The morphological data are in accordance with the genetic results (Fok et al. 2002; present study), where sequence divergence between the western and eastern isolates was found to be much higher than that among the Asian

populations. Both data sets clearly reject the hypothesis of a recent introduction of azure-winged magpies into Europe (Dos Santos 1968). The taxonomic status of such isolates is usually controversial as reproductive isolation, which according to the Biological Species Concept (Mayr 1963) defines species status, cannot directly be proved. The genetic analyses are exclusively based on mitochondrial sequences. Since the morphological characters mainly represent the nuclear genome, this analysis can be regarded as an important complement to the genetic investigations which corroborates the suggested subdivision into the two species *Cyanopica cyanus* Pallas, 1776 and *Cyanopica cooki* Bonaparte, 1850 (Fok et al. 2002).

In contrast to the clear morphological distinctness of the eastern and western populations, almost no differentiation between the Asian populations investigated was found. Again the morphometric data are in accordance with the genetic results, i.e. considerable genetic homogeneity among the continental subspecies. Only the Japanese subspecies is somewhat distinct and its acceptance as distinct subspecies seems justified. Its divergence may be because of genetic drift as a consequence of geographic isolation on the Japanese islands. With the exception of *C. c. japonica*, a correspondence between clustering and subspecific division was not observed in the molecular trees. Yet, it has to be taken into account that not all subspecies have been included in our study. Thus, the morphological analysis is in complete accordance with the sequence data (this study and Fok et al. 2002) and suggests that gene flow occurs between all continental Asian populations, which to some extent may be favoured by migration. The supposed subdivision of the Asian lineage into two genetically distinct groups, Pacific seaboard versus Inland Asia, is not supported by our morphological data and thus a subspecific division into these two groups as suggested by Fok et al. (2002) appears not justified. Furthermore, the assignment of the subspecies *cyanus* to one of these groups remains ambiguous. Its position within the Inland Asia group as determined by Fok et al. (2002) is not in accordance with our data, where it clusters with *pallescens*, *stegmanni*, *koreensis*, and *japonica*. Geographically this makes more sense, as the range of *cyanus* is adjacent to that of *pallescens* but separated by a large distribution gap extending from Central Mongolia over Northern China to the Yellow Sea.

The inclusion of *P. pica* into the study was intended to provide reference values for intraspecific variability and sequence divergence. Despite the completely different distribution patterns of the azure-winged magpie (*C. cyanus*/*C. cooki*: disjunct) and the magpie (*P. pica*: continuous) the patterns of genetic differentiation are strikingly similar. In both cases, we found two highly diverged geographic groups (east versus west). Within groups genetic variation is low and there is no genetic differentiation of subspecies. In their *16S* based gene tree Lee et al. (2003) found a close relationship of *Pica pica camtschatica* from Kamchatka and *P. p. pica* from Europe, which appeared as sister group of the two North American species *Pica (p.) hudsonia* and *P. nuttalli*, whereas the individuals of *P. p. sericea* (Korea) were more distantly related. However, their study did not include other subspecies located between *P. p. camtschatica* and *P. p. pica*. According to our data, the widely distributed 'western haplogroup' comprises not only *P. p. pica* but also the subspecies *P. p. bactriana* (western Russia), *P. p. hemileucoptera* (Republic of Tuva, GUS), and *P. p. leucoptera* (Transbaikalia), whereas *P. p. jankowskii* (Primorskyi and Khabarovskyi Krai, Far East Russia) and *P. p. sericea* (Korea) belong to the 'eastern haplogroup'. This means that the borderline between these two mtDNA lineages should be located somewhere between *P. p. leucoptera*, and both *P. p. sericea* and *P. p. jankowskii*, probably the Amur River basin, a region assumed as an important biogeographic border.

Which taxonomic consequences concerning *P. pica* should be drawn from the genetic results? The differentiation between the two haplogroups in *P. pica* is similar to that found in *C. cyanus*/*C. cooki*. Nevertheless, a certain level of sequence divergence (even if it is surprisingly high) does not justify *per se* the division of *P. pica* into two species. Furthermore, no detailed morphometric analyses have been carried out yet and no data exist about gene flow between taxa. Even Lee et al. (2003) refrained from splitting *P. pica* although it appeared paraphyletic in their tree with respect to *P. hudsonia* and *P. nuttalli*. They rather suggested as a more conservative solution to include the two North American species into *P. pica*. To address this question and to determine the geographic borderline between the two phylogenetic lineages more precisely, and to assess the occurrence of gene flow between the two groups, further comprehensive studies (genetic and morphological) should be carried out.

History of the distribution ranges

The azure-winged magpie represents a rare but not unique example of discontinuous distribution. Similar cases have been reported for several pairs of bird taxa: e.g. *Ciconia ciconia*/*C. boyciana*, *Parus palustris*/*P. p. brevirostris*, *Prunella modularis*/*P. rubida*, and others (Madge and Burn 1999). About 20 such pairs with disrupted ranges were listed by A. Nazarenko (pers. comm.). Only a few phylogeographic studies were conducted on Eurasian birds (Kryukov and Suzuki 2000; Zink et al. 2002). It both cases, in crows (*Corvus corone*) and in woodpeckers (*Dendrocopos major*), a clear genetic differentiation was found between western and south-eastern population groups. The most plausible interpretation for all these cases would be a wide distribution before the Pleistocene followed by geographic isolation because of climatic changes during the ice ages. However, divergence by distance throughout a continuous distribution may also have taken place. Some observations on the current distribution of magpies may shed light on this matter.

In the Baikal region, a rather recent expansion of the range of the azure-winged magpie towards the west was observed. Until the middle of the last century it was distributed eastward from the south end of Lake Baikal (Rustamov 1954), but over the last 30 years it has spread to the west of the lake. It nests within Irkutsk city, Belaya river, Kimil'tei (Irkutsk region), and out of the nesting period it has been observed up to the western borders of the Irkutsk region and even Kuznetsk Alatau (Durnev et al. 1996; I. Fefelov, pers. comm.). In northwest Mongolia, Uliasutay, there is a small isolate about 400 km west from the main part of the range (Bannikov and Scalon 1948). This may originate from a recent colonization rather than being a relic population. In Japan, the size of the population in Honshu is increasing, whereas in Kyushu the population has become extinct after 1950 (Madge and Burn 1999). All these observations suggest a dynamic character of the range. In this view, it is still unclear why the European population remained restricted to a rather narrow area, while

the population of the Asian refuge expanded its range considerably. On the Iberian Peninsula, this species shows a fragmented distribution in southern Spain and Portugal. Although it is mainly insectivorous, it also eats fruits and thus was considered as a pest for agriculture and its nests were destroyed by farmers (Dos Santos 1968). This may probably explain why it did not extend its range towards the east.

At first sight it could be assumed that similar phylogeographic processes involving two isolated glacial refuges (in the east and the west, respectively) shaped the genetic structure of both *C. cyanus*/*C. cooki* and *P. pica*. The latter might just have expanded rather fast after the end of the Pleistocene leading to the present day continuous distribution. This fast expansion is implied by the genetic homogeneity found in such a huge area. Nevertheless, when we consider the data of Lee et al. (2003), the scenario appears more complicated. It could be hypothesized that the process of shrinking and expanding may have occurred repeatedly and the North American offshoots *Pica hudsonia* and *Pica nuttalli* may have derived from one of these former expansion phases. In this context, it would be important to analyse samples from the northernmost regions of North America in order to investigate, if the current Eurasian haplotype group has again reached North America (either postglacially or in the course of an earlier expansion).

Divergence times

Despite the fact that the molecular clock hypothesis has been a matter of discussion for many years (Lovette 2004), calculating divergence times of closely related taxa, for which similar substitution rates can be assumed, is widely applied. In many cases, where the fossil record is absent, a molecular clock is the only possible approach to explain genetic results in a temporal context.

When applying a rate of 5% per million years (myr) for CR sequences according to Freeland and Boag (1999), Fok et al. (2002) calculated a divergence time of 1.2 mya for the separation of Asian and Iberian populations representing *C. cyanus* and *C. cooki* (based on the corrected distance value of 6.06%). The divergence times based on this substitution rate and estimated from our data set (HKY85 distances) are compiled in Table 3. The estimated date for the *Cyanopica* east–west divergence (1.04 mya) is similar to the estimate of Fok et al. (2002), which is based on the complete CR. Nevertheless, in general the CR might be a bad choice for calculating divergence for various reasons. (1) In many species different sections of the CR differ considerably in substitution rates, e.g. the 3′ section is known to be the most variable part. Thus, divergence times computed from different sections of the CR may vary considerably. In the case of *Cyanopica* the difference (average p-distances) between *C. cyanus* and *C. cooki* is 4.9% in the 3′-section of the CR (present study) and 4.3% in the 5′-section calculated from data of Fok et al. (2002). (2) Despite the high variability of the CR, distances may be comparatively low when positions with gaps are excluded from the calculations. This is because variation is often found within length-variable regions. (3) Distances among CR sequences highly depend on the alignment, which may be ambiguous in length-variable sections (although this is certainly not a problem in our case, since sequence divergences are comparatively low). (4) Evolutionary rates of the CR may vary in different lineages. Taking these facts together, any estimation of rate seems highly questionable for CR sequences. Thus, the *cyt b* gene may appear as the better choice for calculating divergence times. At least it is often argued that the results are better comparable with other groups of organisms since most of the above mentioned problems do not arise. Applying the widely used value of 2% per myr (review: Klicka and Zink 1997) on our *cyt b* data set results in considerably higher divergence times, e.g. >3 mya between the Asian *C. cyanus* and the European *C. cooki* (Table 3). This would support recent considerations about more ancient speciation events in many birds assumed to have occurred in the Pliocene or in the earlier Quaternary, while geographic barriers posed by glaciations might only have completed the speciation processes and finally shaped the present phylogeographic patterns (Klicka and Zink 1997; Avise and Walker 1998). According to this hypothesis the phenotypic and genotypic differentiation of the azure-winged magpie has started already in the Pliocene, whereas repeated glacial expansions/restrictions of distribution ranges during the Pleistocene might have completed the process and enforced the differences. Nevertheless, we cannot decide which of these highly different estimates is more plausible. We rather want to exemplify that application of substitution rates is problematic, even if they are widely used and generally accepted. Similar contradictory conclusions can be drawn for *P. pica*. Lee et al. (2003) found a 3.3% p-distance in the 16S gene between the two mitochondrial lineages, and assumed a substitution rate of 1.6% per myr [adopted from Fleischer et al. (1998) who used Kimura two parameter distances]. According to this rate the divergence can be dated at 2.06 mya. This value is 24% less than the estimate for the two clades of *P. pica* on the basis of *cyt b* assuming a rate of 2% per myr (2.70 mya).

The obvious contradiction between divergence times derived from the two genes shows how inaccurate such estimations are. The assumed evolutionary rates of the two marker genes *cyt b* and CR are more than twice as high for the CR (5% versus 2% per my), but the results of the present study indicate that at least for the species analysed the rates of the two genes are in the same range. This disagreement is also found (although not so pronounced) when comparing the *16S* and CR data. Table 3 illustrates how arbitrary these datings appear. Although these comparisons show clearly that the problem comes from the lack of accurate calibration, there seems to be no solution for this problem since in the majority of investigations no fossils are available to calibrate the molecular clock, and if they were available, additional problems would arise from assigning such fossils to specific lineages. Unfortunately, for lack of such calibration points, the widely used practice is to apply substitution rates taken from the literature, and in the long-term these estimated rates are perpetuated.

Nevertheless, the pattern of genetic differentiation can help to sketch a hypothetical scenario about the phylogeographic history of the two species. For both the azure-winged magpie and the magpie the sequence data imply a synchronic east–west differentiation, probably caused by long lasting isolation that may have even had started in the Pliocene or repeated expansions/restrictions of distribution ranges during the Pleistocene.

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Zusammenfassung

Synchrone Ost-West-Divergenz in der Blauelster (Cyanopica cyanus) und der Elster (Pica pica)

Mittels morphometrischer und phylogenetischer Analysen wurden die Verwandtschafts verhältnisse zwischen Populationen der Blauelster (*Cyanopica cyanus*) untersucht. Die morphometrische Analyse umfasste 193 Individuen, welche sieben der neun gegenwärtig anerkannten Unterarten repräsentieren. Unter den acht untersuchten Merkmalen zeigten vier signifikante Unterschiede zwischen den Populationsstichproben aus Spanien und Asien. Im Gegensatz dazu waren die asiatischen Populationen/Unterarten bis auf *C. c. japonica* morphologisch nicht differenziert. Die genetische Analyse basierte auf zwei mitochondrialen Sequenzen (Kontrollregion, *Cytochrom b* Gen). Die Ergebnisse stimmen mit der morphometrischen Analyse überein und zeigen einen klaren Unterschied zwischen Vögeln des westlichen und des östlichen Verbreitungsgebietes. Die Differenzierung von *C. c. japonica* ist auf Sequenzebene nicht erkennbar. Sowohl die genetischen als auch die morphologischen Daten unterstützen den Artstatus für *Cyanopica cyanus* und *C. cooki*. Die Elster (*Pica pica*) wurde in die phylogenetische Untersuchung mit einbezogen, um das Ausmaß der innerartlichen Variation zu vergleichen. Wie in *C. cyanus* gab es auch hier zwei klar getrennte Gruppen, von denen eine die fernöstlichen Populationen umfasst (*P. p. jankowskii* and *P. p. sericea*), die andere die übrigen Unterarten. Sowohl für die Blauelster als auch für die Elster ergeben die Daten eine Ost-West-Differenzierung, die wahrscheinlich durch eine lang dauernde Isolation verursacht wurde, die möglicherweise im Pliozän begann, oder durch wiederholte Expansionen/Einengungen des Verbeitungsgebietes im Pleistozän.

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