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Comparative phylogeography of two widespread magpies: Importance of habitat preference and breeding behavior on genetic structure in China

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ABSTRACT

Historical geological events and climatic changes are believed to have played important roles in shaping the current distribution of species. However, sympatric species may have responded in different ways to such climatic fluctuations. Here we compared genetic structures of two corvid species, the Azure-winged Magpie *Cyanopica cyanus* and the Eurasian Magpie *Pica pica*, both widespread but with different habitat dependence and some aspects of breeding behavior. Three mitochondrial genes and two nuclear introns were used to examine their co-distributed populations in East China and the Iberian Peninsula. Both species showed deep divergences between these two regions that were dated to the late Pliocene/early Pleistocene. In the East Chinese clade of *C. cyanus*, populations were subdivided between Northeast China and Central China, probably since the early to mid-Pleistocene, and the Central subclade showed a significant pattern of isolation by distance. In contrast, no genetic structure was found in the East China populations of *P. pica*. We suggest that the different patterns in the two species are at least partly explained by ecological differences between them, especially in habitat preference and perhaps also breeding behavior. These dissimilarities in life history traits might have affected the dispersal and survival abilities of these two species differently during environmental fluctuations.

1. Introduction

During the last 3 million years, repeated glacial cycles have greatly affected the landform and fauna of the northern hemisphere, especially of the boreal and temperate biomes (Avise et al., 1998; Hewitt, 2000, 2004). Phylogeographical analyses are used to understand how these changes in landscape and climate have influenced species distributions and population demography (Avise, 2000). Major historical events are generally assumed to produce concordant genetic structure in species with similar distributions (e.g., Haring et al., 2007; Hewitt, 2000, 2004; Kirchman and Franklin, 2007). However, some studies have noticed that even sympatric species might respond differently to similar historical perturbations, resulting in species-specific distribution patterns and genetic structures (e.g., Polihronakis and Caterino, 2010; Taberlet et al., 1998; Zink et al., 2001). Therefore, by comparing phylogeographical structures of co-distributed species, one can obtain a better understanding of how underlying factors contributed to historical processes (Bermingham and Moritz, 1998).

Previous studies have found that recent population expansion generally results in shallow genetic divergence (Conroy and Cook, 2000; Hewitt, 2000, 2004; Polihronakis and Caterino, 2010; Zink, 1996). Others have shown that species with different social behaviors might respond differently to environmental changes, leading to distinct population structures (Chen et al., 2010; Hoelzel et al., 2010; Lange et al., 2010; McDonald et al., 1999; Whiteley et al., 2004). It is believed that complex breeding behaviors, such as

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cooperative breeding, could cause natal philopatry and limited dispersal, which might further contribute to genetic divergence (e.g., Blackmore et al., 2011; Fischer and Lindenmayer, 2007; Hatchwell, 2009). Furthermore, for species with similar dispersal ability, gene flow between populations is affected by habitat preferences. Habitat generalists with high ecological plasticity and wide distributions have better chances to survive and disperse when in adverse conditions than specialists with narrow niches and small ranges (Hamer and McDonnell, 2010; Lange et al., 2010; Michaux et al., 2005; Morris-Pocock et al., 2010).

The Eurasian Magpie *Pica pica* is distributed across large parts of Eurasia, including some areas north of the Arctic Circle and some desert areas in Central Asia (del Hoyo et al., 2009; Goodwin, 1976). Several subspecies have been described (Fig. 1). Studies of mitochondrial DNA have revealed a deep divergence between, on the one hand, subspecies from the Western to the Northeastern Palearctic (including the nominate subspecies) and, on the other hand, the subspecies from southeast Russia (*jankowskii*) and South Korea (*sericea*) (Haring et al., 2007; Kryukov et al., 2004; Lee et al., 2003; according to del Hoyo et al., 2009, *jankowskii* is a synonym of *anderssoni* and *sericea* should be spelled *serica*). Furthermore, Lee et al. (2003) showed that the West to Northeast Palearctic lineage is more closely related to the two North American congeners, Yellow-billed Magpie *P. nuttalli* and Black-billed Magpie *P. hudsonia*, than to the Southeast Palearctic lineage of Eurasian Magpie. Consequently, the mitochondrial data suggest that Eurasian Magpie is a non-monophyletic species, calling for a taxonomic revision. *P. hudsonia* was previously considered conspecific with the Eurasian Magpie (cf. American Ornithologists' Union, 2000). Lee et al. (2003) suggested that the ancestor of the North American magpies reached North America through the Bering land bridge during the Ice Ages .

The Azure-winged Magpie *Cyanopica cyanus* is also widespread throughout East Asia, but with a small isolated distribution in the South Iberian Peninsula, about 13,000 km away (del Hoyo et al., 2009; Goodwin, 1976). The latter is sometimes treated as a separate species, *C. cooki* (Iberian Magpie or Iberian Azure-winged Magpie), based on differences in mitochondrial DNA (mtDNA) and morphology (del Hoyo et al., 2009; Fok et al., 2002; Gill and Donsker, 2012; Haring et al., 2007; Kryukov et al., 2004). Fok et al. (2002) divided the Asian subspecies into two groups, inland Asia and Pacific, based on the mitochondrial control region. Kryukov et al. (2004) did not find any structure based on two mitochondrial loci, and they argued that there is gene flow among all Asian continental populations. Both studies used only a few samples from each subspecies, so there is still no clear understanding of the pop-

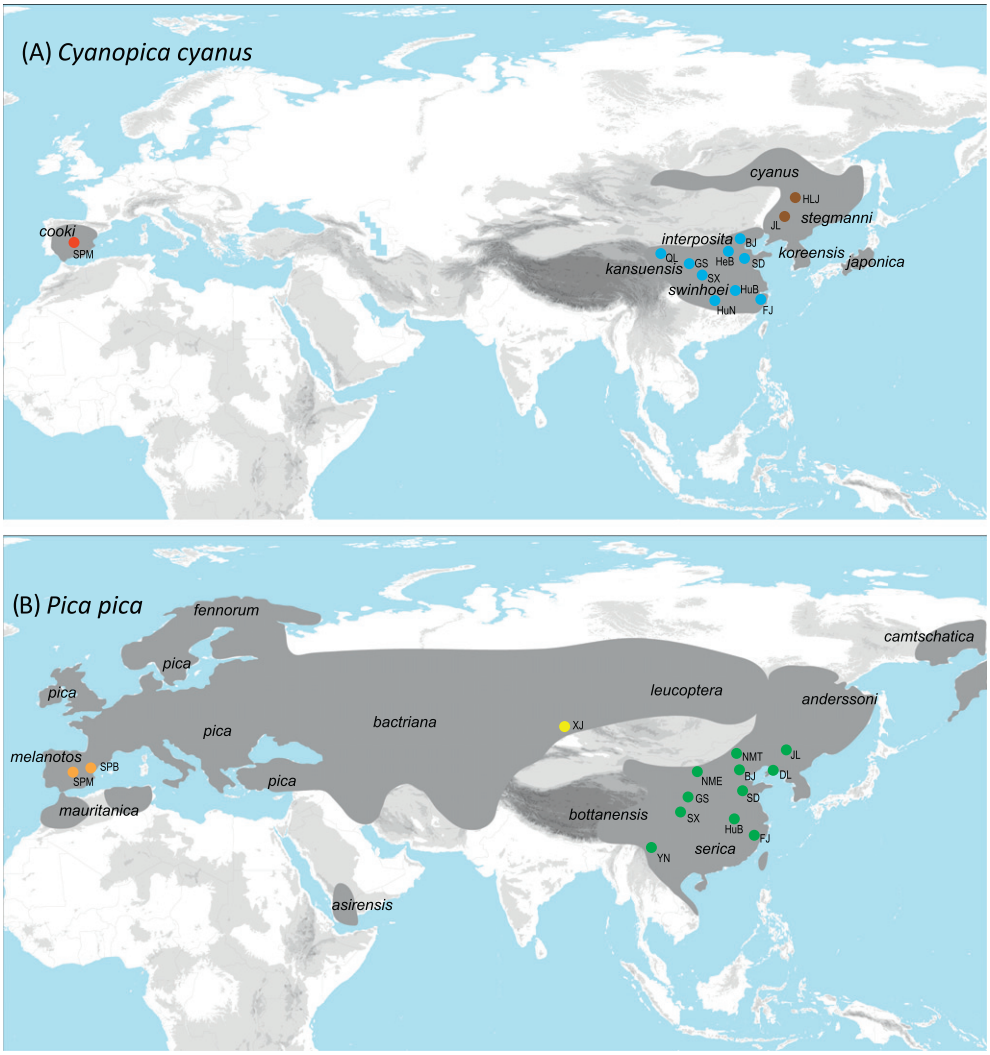

Fig. 1. Distributions of Azure-winged Magpie *Cyanopica cyanus* (A) and Eurasian Magpie *Pica pica* (B) according to del Hoyo et al. (2009). Sampling sites for the present study are marked by dots and coded names (Table S1). Colors represent clades defined in Figs. 2 and 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ulation structure and evolutionary background of *C. cyanus* in China.

As indicated above, previous studies based on mtDNA have found similar phylogenetic structure between "western" and "eastern" lineages in both *Pica* and *Cyanopica* (Fok et al., 2002; Haring et al., 2007; Kryukov et al., 2004; Lee et al., 2003). However, these studies included only one (*Pica*) or a few (*Cyanopica*) samples from China, where these two species have broadly overlapping distributions, so whether they have similar population structures and dynamics in East Asia is still unknown. Moreover, *P. pica* and *C. cyanus* differ in certain life history traits, which might have affected their population genetics according to previous studies on other species. *P. pica* has a wide environmental tolerance (Goodwin, 1976), it breeds mostly in rather open habitats and can adjust to harsh environments, but avoids closed forests and completely treeless areas (del Hoyo et al., 2009; Goodwin, 1976). It is usually found singly or in pairs, sometimes in flocks in the non-breeding season, and breeds solitarily (del Hoyo et al., 2009; Goodwin, 1976). In recent decades, it has been found to colonize urban habitats (del Hoyo et al., 2009). In contrast, *C. cyanus* is a highly gregarious and mostly arboreal breeding species with a complex cooperative breeding system (Canario et al., 2004; Valencia et al., 2003).

In this study, we used mitochondrial and nuclear markers to examine how past environmental changes and life history differences might have shaped population genetic structures in these species. First, we used samples from China and the Iberian Peninsula to test whether these two species have been affected similarly by geological events and environmental changes. Second, due to limited sample sizes from Iberia, we analyzed the genetic structure and demography of Chinese populations alone, to find out whether sympatric species have responded similarly to past environmental fluctuations. Finally, we considered both expansion histories, breeding behaviors and ecological traits, and tried to clarify how species' life history traits might have moulded the demographic responses to environmental change.

2. Materials and methods

2.1. Sampling and laboratory works

One hundred and twenty-eight Azure-winged Magpies and 179 Eurasian Magpies were collected and used for molecular analyses (Fig. 1, Table S1). Total genomic DNA was extracted from blood or muscle samples using the QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's protocol. Partial sequences of the mitochondrial cytochrome *b* (cyt*b*), NADH dehydrogenase subunit 2 (ND2) and control region (CR) genes were obtained through Polymerase Chain Reaction (PCR) amplification. We used primer pairs L14851 (5′-CCTACTTAGGATCATTCGCCCT-3′) and H16058 (5′-TTGGCTTACAAGACCAATGTT-3′) for cyt*b* (Groth, 1998). The alternative pair was L14731 (5′-AATTGCATCCCACTTAATCGA-3′) and H16067 (5′-CTTCAATCTTTGGTTTACAAGACC-3′) (Helm-Bychowski and Cracraft, 1993). The PCR program consisted of a pre-denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 46–48 °C for 45 s and 72 °C for 45 s, plus a final extension at 72 °C for 5 min. Primers for the control region (CR) were CR-Cor+ (5′-ACCCTTCAAGTGCGTAGCAG-3′) and Phe-Cor− (5′-TTGACATCTTCAGTGTCATGC-3′) (Kryukov et al., 2004), and for ND2 L5219 (5′-CCCATACCCCGGAAATGATG-3′) and H6313 (5′-CTCTTATTTAAGGCTTTGAAGGC-3′) (Sorenson et al., 1999). PCR conditions were similar to the above, but the annealing temperature was 56–58 °C for those two primers. For nuclear markers, β-fibrinogen intron 5 (Fib5) and transforming growth factor beta 2 intron 5 (TGFB2) were selected. Primer pairs Fib5 (5′-CGCCATACAGAGTATACTGTGACAT-3′) and Fib6 (5′-GCCATCCTGGCGATTCTGAA-3′) were used for Fib5 (Fuchs et al., 2004), and TGFB2.5F (5′-GAAGCGTGCTCTAGATGCTG-3′) and TGFB2.6R (5′-AGGCAGCAATTATCCTGCAC-3′) for TGFB2 (Kimball et al., 2009). For both of these, the PCR program was 3 min of pre-denaturation at 95 °C, then 35 cycles of 94 °C for 30 s, 55–56 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Sequencing was carried out using an ABI 377 automatic sequencer following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. Both strands were sequenced for all individuals using the same primers as for PCR. Sequences were aligned and checked in MEGA4 (Kumar et al., 2007). No stop codons or indels that could indicate the presence of nuclear pseudogenes (Sorenson and Quinn, 1998) were found in the mitochondrial protein-coding genes. All sequences have been submitted to GenBank (JQ393158–JQ394686). *Cyanocitta stelleri* (AY030113, AY030139, AF218924) and *Corvus frugilegus* (NC002069) were selected as outgroups following Fok et al. (2002).

2.2. Nucleotide polymorphism

The number of segregating (polymorphic) sites, haplotype diversity (Hd) and nucleotide (π) diversity for each population and all populations together were calculated with DnaSP 5.0 (Rozas and Librado, 2009) for both mtDNA (three loci combined) and the two nuclear markers. Fu's *Fs* and Tajima's *D* were used to test for neutrality, as implemented in DnaSP and Arlequin 3.5 (Excoffier et al., 2005). Additionally, the McDonald–Kreitman test (Mcdonald and Kreitman, 1991) for selective neutrality of the mitochondrial protein-coding fragments was done in DnaSP 5.0.

For the nuclear sequences, haplotype phasing to infer haplotypes from heterozygous sites was done using the Perl scripts CVhaplot version 2.0 (Huang and Zhang, 2010), which reformat the input files to several popular phasing software packages: PHASE (Stephens and Donnelly, 2003), HAPLOTYPER (Liu et al., 2002), HAPLOREC (Eronen et al., 2006), ARLEQUIN-EM (Excoffier et al., 2005), GCHAP (Thomas, 2003a,b), GERBIL (Kimmel and Shamir, 2005), and HAPINFERX (Clark, 1990). The integrated data were then obtained by comparing the consistency of results among these programs. Sequences with uncertain phasing results (probability threshold below 85%) were not used in further analysis.

2.3. Phylogenetic structure in mtDNA

The Maximum-likelihood (ML) algorithm implemented in the program PhyML version 3.0 (Guindon and Gascuel, 2003) and the Bayesian inference (BI) implemented in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) were used to reconstruct phylogenetic relationships among mtDNA haplotypes. MrModelTest2 (Nylander, 2004) was used to evaluate the models of molecular evolution based on the Akaike Information Criterion (AIC). Non-parametric bootstrapping (1000 replicates) performed on ML trees was further used to evaluate nodal support. The result was visualized by FigTree 1.3.1 (Rambaut, 2009).

For Bayesian analyses, two independent parallel runs of four incrementally heated Metropolis-coupled Monte Carlo Markov Chains (MCMCs) were run for 5 million or more generations, with tree sampled every 1000 generations, until the average standard deviation of split frequencies was below 0.01. The same evolution models as in the ML analyses were used, and the first 10% of generations was discarded as "burn-in" when summarizing the trees. The convergence of MCMC results was checked in Tracer 1.5 (Rambaut and Drummond, 2009).

A maximum parsimony based method, TCS version 1.21 (Crandall et al., 2000), was used to draw an unrooted network evaluating

haplotype relationships for mtDNA with 95% parsimoniously plausible branch connections.

Analysis of molecular variance (AMOVA) was performed in Arlequin 3.5 to test the geographic structure in the mtDNA dataset using pairwise differences (Φst). Two levels of AMOVA tests were done for both species, first with the full set of data with a subdivision between European and Asian clades; second with samples from East China only. For *C. cyanus*, the third test was done on the Central subclade (see Section 3).

2.4. Phylogenetic structure in nuclear introns

For the nuclear introns, we drew unrooted networks in TCS version 1.21 with 95% parsimoniously plausible branch connections for each nuclear marker separately. The loops were resolved following the rules suggested by Pfenninger and Posada (2002). Additionally, we used STRUCTURE version 2.3 (Pritchard et al., 2000) to detect population structure based on the information of the frequency of genotypes, and the distribution of rare genotypes of both markers. The admixture model with correlated allele frequencies was applied and sampling locality was set as a prior to magnify the signals as suggested by Hubisz et al. (2009). We also tuned the Lambda to 0.8 to fit the SNP-like data (Falush et al., 2003). The number of populations (K) was set from 1 to 10, and each K was run 10 times. The most likely K was identified according to ΔK (Goudet et al., 2005). CLUMPP (Jakobsson and Rosenberg, 2007) was used to merge results from replications of each K, and DISTRUCT (Rosenberg, 2004) was used for outputting bar plots for the combined results.

2.5. Divergence time estimate

A Bayesian framework based on the coalescent method was used to estimate divergence times for the cyt*b* dataset in BEAST 1.6.1 (Drummond and Rambaut, 2007). The site model selected for *C. cyanus* and *P. pica* was GTR + Γ following Weir and Schluter (2008). For each species, sequences of all individuals were used in the analysis excluding outgroups. The analysis was done under an expansion growth model and an uncorrelated lognormal relaxed clock (Drummond et al., 2006) with a fixed molecular clock rate of 2.1% per million years (MY) (Weir and Schluter, 2008). Default priors were used. Chains were run 100 million generations with sampling every 1000 generations. Tracer 1.5 was used to check the posterior distribution and the effective sample sizes (ESSs) of the MCMC output. We used TreeAnnotator 1.6.1 in the BEAST package (Drummond and Rambaut, 2010) to summarize trees with "Mean height", and discarded the first 25% trees as "burn-in", well after stationarity of chain likelihood values had been established. The tree and divergence times were displayed in FigTree 1.3.1.

2.6. Pattern of isolation by distance

The isolation by distance (IBD) pattern was tested by comparing the matrix correlation between the genetic pairwise Φst and the geographic distances, using a Mantel test in Arlequin 3.5. We tested the pattern with both mtDNA and two nuclear introns. For *P. pica*, the Mantel test was done on the East Chinese clade, whereas for *C. cyanus* only the samples from the Central subclade (see Section 3) was used.

2.7. Historical demography

An MCMC integration and coalescent-based method, the Bayesian skyline plot (BSP) (Drummond et al., 2005), was used in using BEAST 1.6.1 to reconstruct the effective population size fluctuations since the time to the most recent common ancestor (TMRCA). Only the cyt*b* dataset was analyzed. For each clade, we ran the chains for 100 million generations or more until the ESSs was more than 200, and the first 10% were discarded as "burn-in". The best-fit substitution model was set as estimated by MrModeltest2, and a relaxed uncorrelated lognormal molecular clock was applied with the mutation rate 2.1%/MY for both species (Weir and Schluter, 2008). Demographic history through time was reconstructed using Tracer 1.5.

3. Results

3.1. Genetic variation

Both mitochondrial and nuclear sequences were successfully obtained from 128 individuals of *C. cyanus*. We amplified 1122 bp of cyt*b*, 1014 bp of ND2 and 584–587 bp of CR, and 580 bp of Fib5 and 592 bp of TGFB2. The nucleotide diversities for the mtDNA dataset ranged from 0.00042 to 0.00158, and for nuclear markers from 0.00090 to 0.00405 for Fib5, and from 0.00149 to 0.00809 for TGFB2 (Table 1). The haplotype diversity ranged from 0.524 to 0.939 for mtDNA, and for Fib5 and TGFB2 from 0.242 to 0.870 and 0.358 to 0.813, respectively (Table 1).

For *P. pica*, 179 sequences of mtDNA were obtained, while only 167 individuals of Fib5 and 159 of TGFB2 were available for further analysis after phasing. The sequence length of each mtDNA gene was similar as in *C. cyanus*, but for Fib5 and TGFB2 520 bp and 505–512 bp were obtained, respectively. The nucleotide diversities were 0.00133–0.00371 for mtDNA, from 0.00093 to 0.00380 for Fib5 and from 0.00289 to 0.00695 for TGFB2 (Table 1). The haplotype diversities ranged from 0.895 to 1.000 for mtDNA, and for Fib5 and TGFB2 from 0.339 to 0.803 and 0.699 to 0.948, respectively (Table 1).

In both species, the McDonald–Kreitman' tests detected no significant deviation from neutrality in the two mitochondrial protein-coding fragments ($P > 0.05$, Table 2). The results of the Fu's *Fs* test and Tajima's *D* test indicated an over-abundance of singleton mutations and rare alleles for most fragments (negative *D* or *Fs* values, Table 2).

3.2. Phylogenetic structure in mtDNA

Both ML and BI analyses produced similar tree topologies based on combined mitochondrial sequences (Fig. 2). *Cyanopica* samples were separated into two strongly supported major clades: an Iberian clade with Spanish population SPM, and an East Chinese clade with all Chinese populations. The latter was further divided into two subclades. Samples from HLJ and JL (Fig. 1) formed the Northeastern subclade, and samples from other localities constituted the Central subclade. No shared haplotypes were detected among any of these clades. All 56 haplotypes were used in TCS analysis and the result identified three independent networks, indicating long-term isolation (Fig. 2A). For *P. pica*, samples from East China were well separated from those from Iberia (SPM and SPB) and Xinjiang (XJ; Northwest China), both in the phylogenetic trees and TCS networks (Fig. 2B). In the East Chinese clade, no clear structure existed between geographic populations, while Iberian (including SPM and SPB) and XJ samples divided into two groups, with one sample from Madrid (SPM) clustering with the XJ samples (Fig. 2B).

For *C. cyanus*, AMOVA tests showed a significant genetic variance among groups (92.8%) when setting the number of populations to two, Iberian and East Chinese. The percentage of variance among groups was higher (96.49%) when further dividing the East Chinese clade into Northeastern and Central subclades. Within the East Chinese clade, 91.51% of the variance was among groups; when the Northeastern subclade was excluded, the value

Table 1
Nucleotide polymorphism in each population.

C. cyanus mtDNA	Sampling size	S	Nhap	π	Hd	*P. pica* mtDNA	Sampling size	S	Nhap	π	Hd
Whole set	128	213	56	0.01055	0.914	Whole set	179	292	125	0.02270	0.993
SPM	9	9	6	0.00090	0.889	SPM	15	36	10	0.00212	0.895
HLJ	8	9	5	0.00096	0.786	SPB	12	34	12	0.00364	1.000
JL	12	15	9	0.00173	0.939	XJ	16	23	10	0.00179	0.933
BJ	10	5	5	0.00047	0.756	JL	11	26	10	0.00292	0.982
HeB	8	6	5	0.00087	0.857	DL	12	24	10	0.00215	0.970
SD	13	12	7	0.00076	0.731	BJ	16	36	15	0.00303	0.992
SX	11	11	8	0.00098	0.927	SD	15	33	13	0.00267	0.981
GS	9	2	3	0.00039	0.722	NMT	10	23	8	0.00235	0.956
QL	13	6	6	0.00045	0.795	NME	10	30	8	0.00368	0.956
HuB	14	11	7	0.00080	0.802	GS	16	30	12	0.00297	0.950
HuN	14	7	7	0.00064	0.846	SX	7	12	5	0.00173	0.902
FJ	7	4	3	0.00042	0.524	HuB	16	25	11	0.00186	0.933
						FJ	11	25	13	0.00184	0.967
						YN	12	20	10	0.00168	0.955
C. cyanus Fib5	Sampling size	S	Nhap	π	Hd	*P. pica* Fib5	Sampling size	S	Nhap	π	Hd
Whole set	256	17	23	0.00423	0.820	Whole set	334	19	27	0.00362	0.718
SPM	18	1	2	0.00087	0.503	SPM	28	6	7	0.00384	0.775
HLJ	16	7	6	0.00391	0.817	SPB	18	5	6	0.00289	0.627
JL	24	9	11	0.00405	0.870	XJ	26	6	8	0.00297	0.803
BJ	20	6	7	0.00299	0.847	JL	20	3	4	0.00147	0.642
HeB	16	3	3	0.00102	0.242	DL	22	5	5	0.00133	0.528
SD	26	5	6	0.00271	0.677	BJ	26	5	6	0.00101	0.468
SX	22	5	6	0.00283	0.779	SD	28	4	5	0.00100	0.471
GS	18	5	4	0.00163	0.399	NMT	18	6	6	0.00204	0.686
QL	26	6	6	0.00318	0.511	NME	20	5	6	0.00129	0.574
HuB	28	6	6	0.00258	0.701	GS	32	4	5	0.00168	0.671
HuN	28	5	6	0.00169	0.693	SX	14	3	4	0.00148	0.648
FJ	14	5	5	0.00248	0.659	HuB	32	5	5	0.00093	0.339
						FJ	26	3	4	0.00122	0.554
						YN	24	4	5	0.00149	0.641
C. cyanus TGFB2	Sampling size	S	Nhap	π	Hd	*P. pica* TGFB2	Sampling size	S	Nhap	π	Hd
Whole set	256	16	20	0.00585	0.656	Whole set	318	18	41	0.00542	0.912
SPM	18	2	4	0.00125	0.634	SPM	30	7	8	0.00537	0.802
HLJ	16	10	4	0.00809	0.725	SPB	22	7	10	0.00612	0.874
JL	24	10	4	0.00778	0.714	XJ	22	5	7	0.00335	0.766
BJ	20	9	3	0.00288	0.358	JL	22	11	9	0.00418	0.870
HeB	16	11	5	0.00735	0.667	DL	18	11	12	0.00695	0.948
SD	26	10	5	0.00404	0.406	BJ	20	9	12	0.00474	0.947
SX	22	10	6	0.00411	0.476	SD	30	12	13	0.00477	0.892
GS	18	9	4	0.00238	0.595	NMT	18	9	8	0.00285	0.699
QL	26	9	4	0.00247	0.406	NME	20	10	11	0.00423	0.916
HuB	28	11	5	0.00659	0.519	GS	28	12	11	0.00450	0.857
HuN	28	10	4	0.00743	0.595	SX	12	8	7	0.00385	0.879
FJ	14	11	7	0.00802	0.813	HuB	28	5	7	0.00328	0.799
						FJ	28	11	13	0.00539	0.905
						YN	20	9	8	0.00385	0.826

S, number of segregating sites; Nhap, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity.

was 41.52%. For *P. pica*, when all populations were used in AMOVA, the largest variance was obtained from Eurasian and East Chinese grouping (93.00%). When only the East Chinese clade was included, 20.92% of the variance was explained by among-group variance and 75.82% by within-population variance (Table 3).

3.3. Phylogenetic structure in nuclear DNA

In *C. cyanus*, none of the nuclear introns showed any shared haplotypes between the Iberian clade and the East Chinese clade, but the network was not as well structured as for mtDNA. In the East Chinese clade, samples from Northeast China and Central China shared haplotypes (Fig. 3A). In *P. pica*, only one haplotype of Fib5 was shared between the Eurasian and East Chinese clades, indicating long term separation. In contrast, TGFB2 showed considerable haplotype sharing between clades (Fig. 3B).

In both species, the results of STRUCTURE (Fig. 4) detected similar patterns as for mtDNA. Although the best-fit *K* for *C. cyanus* was three, and for *P. pica* was two, we compared the results from $K = 2$ to 10. For *C. cyanus*, when setting $K = 2$, the Iberian population (SPM) clustered with the Northeast China samples (HLJ and JL), and the Central samples formed the other cluster. At $K = 3$, SPM was isolated from the other populations, and the second cluster contained the Northeastern subclade and some samples from the Central subclade. At *K* higher than three, the Iberian clade remained as a single cluster and the Northeastern subclade split out, while the central populations fell into the rested clusters with no clear relation to geographic locations (Fig. 4A). For *P. pica*, the bar plots showed a clear two-clade structure when *K* was set to 2, and XJ separated from the Iberian clade at $K = 3$. At $K > 3$, the two Eurasian clusters remained, while the East Chinese clade showed no significant structure (Fig. 4B).

3.4. Divergence time estimate

The divergence time between the Iberian and East Chinese clade of *C. cyanus* was estimated to be 2.9 MY ago (MYA) (95% HPD: 0.7–4.6 MYA), and the divergence between the Northeastern and

Table 2
Nucleotide polymorphism and results of neutrality tests at each gene and mitochondrial gene combined.

C. cyanus	cytb	ND2	CR	mtDNA combined	Fib5	TGFB2
Length(bp)	1122	1014	587	2723	580	592
S	103	69	41	213	17	16
Nhap	34	26	12	56	23	20
Hd	0.872	0.685	0.431	0.914	0.82	0.656
π	0.01368	0.00852	0.00806	0.01055	0.00423	0.00585
Fu's *Fs*	0.417	−0.504	2.528	−2.192	−7.248*	−2.002
Fu and Li's *D*	0.75186	−0.14688	0.17305	0.37039	1.04872	1.04872
Tajima's *D*	−0.71492	−1.07577	−1.12921	−0.94473	−0.29887	0.6266
MK test	1.751	0.794				
P. pica						
Length(bp)	1122	1014	584	2720	520	512
S	130	96	66	292	19	18
Nhap	82	56	39	125	27	41
π	0.967	0.875	0.860	0.993	0.718	0.912
Hd	0.02143	0.02017	0.02952	0.02270	0.00362	0.00542
Fu's *Fs*	−16.033*	−2.80	1.235	−23.926*	−14.421*	−26.454*
Fu and Li's *D*	−1.43512	−1.12925	−0.40295	−1.27007	−0.47358	1.18586
Tajima's *D*	0.05955	0.67055	1.34825	0.57195	−1.1017	−0.34114
MK test	0.876	1.595				

S, number of segregating sites; Nhap, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; Fu's *FS*, statistics of Fu's *FS* test (*$P < 0.01$); Fu and Li's *D*, statistics of Fu and Li's *D*-test (*$P < 0.05$); Tajima's *D*, statistics of Tajima's *D*-test (*$P < 0.05$);MK test, statistics of McDonald and Kreitman's test (*$P < 0.05$).

Central subclade 0.9 MYA (95% HPD: 0.4–1.4 MYA). For *P. pica*, the Eurasian clade and the East Chinese clade was estimated to have separated at 2.3 MYA (95% HPD: 0.8–4.2 MYA).

3.5. Pattern of isolation by distance

The results of the Mantel test showed that the Central subclade of *C. cyanus* was in accordance with an IBD pattern in both mtDNA ($r = 0.48$, $P = 0.02$) and TGFB2 ($r = 0.47$, $P = 0.01$), while Fib5 did not show this pattern ($r = -0.20$, $P = 0.88$). In *P. pica*, pairwise genetic distances were not significantly correlated with geographic distances in mtDNA ($r = 0.05$, $P = 0.28$) or nuclear markers ($r = -0.19$, $P = 0.79$ for Fib5; $r = -0.03$, $P = 0.55$ for TGFB2).

3.6. Historical demography

Past population dynamics were shown by the Bayesian skyline plot (BSP) (Fig. 5). The East Chinese clade of *P. pica* had a considerable expansion at about 0.10 MYA, with TMRCA of 0.25 MYA (95% HPD: 0.11–0.41 MYA). For *C. cyanus*, the Northeastern subclade has undergone slow population growth since 0.05 MYA, while the Central subclade expanded at 0.04 MYA. The TMRCA for these subclades was 0.09 MYA (95% HPD: 0.02–0.18 MYA) and 0.08 MYA (95% HPD: 0.03–0.15 MYA), respectively.

4. Discussion

Based on our results, *C. cyanus* and *P. pica* showed concordant genetic patterns between Iberian and East Chinese populations in both mtDNA and nuclear datasets, confirming previous studies using mtDNA only (Fok et al., 2002; Haring et al., 2007; Kryukov et al., 2004; Lee et al., 2003). For both species, the estimated divergence time between the two major clades was dated to the late Pliocene/early Pleistocene. This date is earlier than the time estimated in *C. cyanus* by Fok et al. (2002) using the control region and a molecular clock rate of 5%/MY. While Kryukov et al. (2004) argued that it is not proper to calculate divergence time with CR, and gave a time range from 2.04 to more than 3 MY estimated by several different molecular clock assumptions, this timescale was further supported by our results.

The climatic changes and the development of ice sheets in the Pleitocene are believed to have played an important role in intra-specific diversifications (e.g., Hewitt, 2000, 2004; Weir and Schluter, 2004). Marked divergences between western and eastern populations have been reported from other birds with wide distributions across the Eurasian continent (e.g., Haring et al., 2007; Olsson et al., 2010; Pavlova et al., 2003; Zink et al., 2006, 2008, 2009). Haring et al. (2007) suggested, based on analyses of eight widespread corvid species or species pairs, that the similar magnitude of divergence between western and eastern clades in different species implied common mechanisms and similar divergence times. The congruent genetic patterns in the two magpie species imply that they have been similarly affected by the past environmental changes.

Unlike *C. cyanus*, which has a highly disjunct distribution, *P. pica* has a continuous distribution from Northwest Africa and West Europe to East Asia, with two congeners in North America (del Hoyo et al., 2009; Goodwin, 1976). Although the geographic distance between the XJ and Iberian populations is more than 13,000 km, the genetic distance (mtDNA combined) between them (0.8%) is much smaller than between XJ and GS (5.6%), which are separated by only about 2000 km. This is in agreement with previous studies (Haring et al., 2007; Kryukov et al., 2004; Lee et al., 2003), which showed a lack of differentiation between samples from Europe to northeast Russia, whereas southeast Russian and Korean samples were much more divergent. This pattern, with shallow genetic divergence over a large area in the northern Eurasian continent and deep divergence between northern and southern regions in Eastern Asia, has been reported in several other species (e.g., Haring et al., 2007; Päckert et al., 2007, 2010; Saitoh et al., 2010). Geographical barriers are believed to have led to vicariant divergence of populations in the past between, on one hand, much of the northern Palearctic and, on the other hand, Russian Far East and Japan (Lee et al., 2003; Saitoh et al., 2010).

Within the East Chinese clade, for *C. cyanus*, both phylogenetic and AMOVA analyses indicated a divergence in mtDNA between populations in Northeast China and Central China. This agrees with an earlier study based on the control region, which showed that the Central China subclade of the current study is part of the "Inland Asia" population from south Russia and north Mongolia to west of the Da Hinggan Ling mountain range, and that the Northeast China subclade of the present paper is part of the "Pacific seaboard" population from Japan, South Korea and southeast Russia (Fok et al., 2002). Our STRUCTURE analysis of the nuclear markers

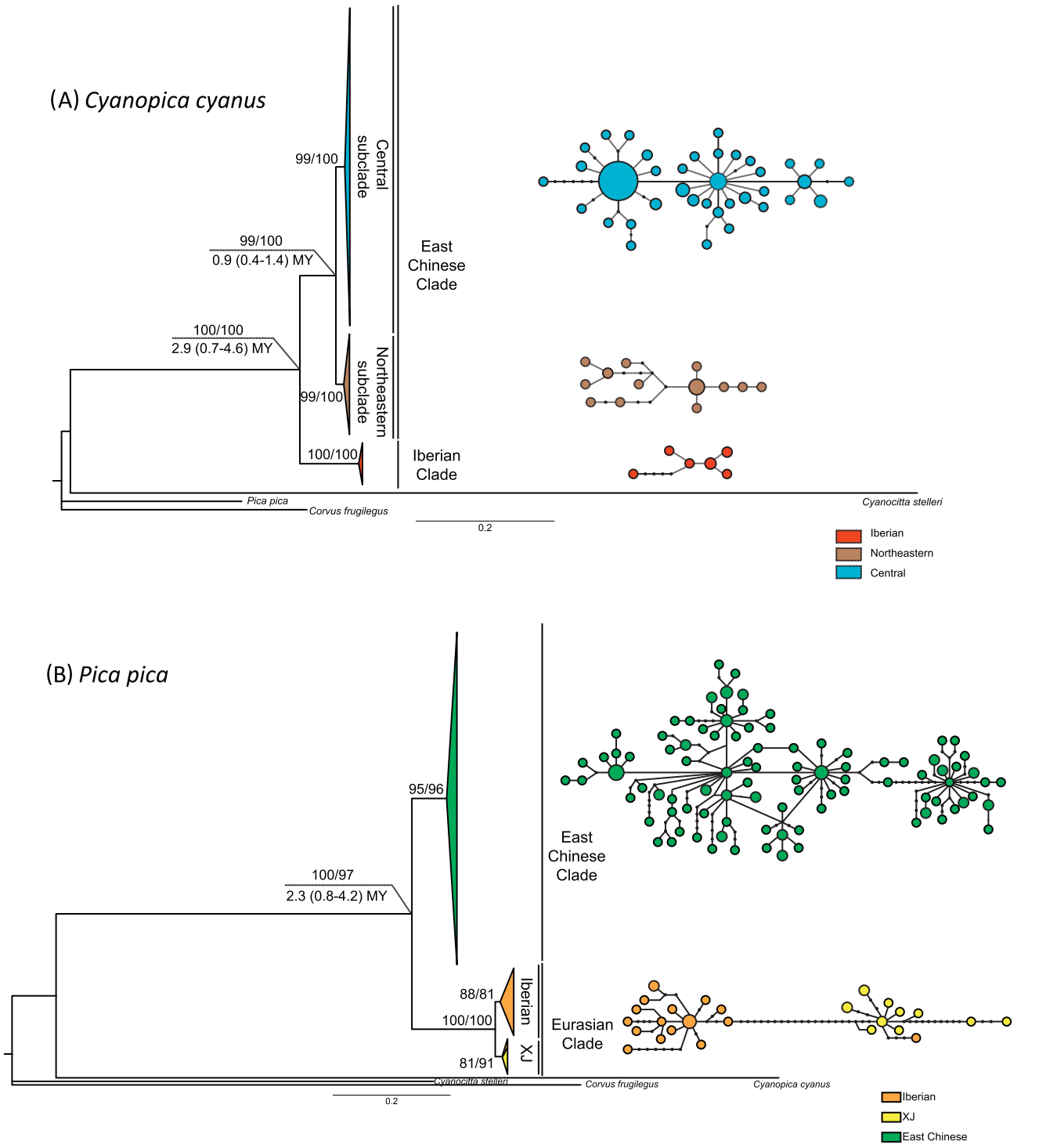

Fig. 2. Maximum likelihood tree and nested clade networks based on mtDNA. Values above branches indicate ML bootstrap support and Bayesian poster probability, while the values below the branches are the divergence time estimated by BEAST. Black dots refer to missing steps intermediate between observed haplotypes.

Table 3
AMOVA for *C. cyanus* and *P. pica* with mtDNA. I, whole set; II, within East Chinese clade; III, within Central clade (for *C. cyanus*).

	Source of variation	I (%)	II (%)	II (%)
C. cyanus	Among groups	96.49	91.51	41.52
	Among populations within groups	0.55	1.35	3.3
	Within populations	2.96	7.14	55.18
P. pica	Among groups	93.00	20.92	
	Among populations within groups	2.34	3.26	
	Within populations	4.67	75.82	

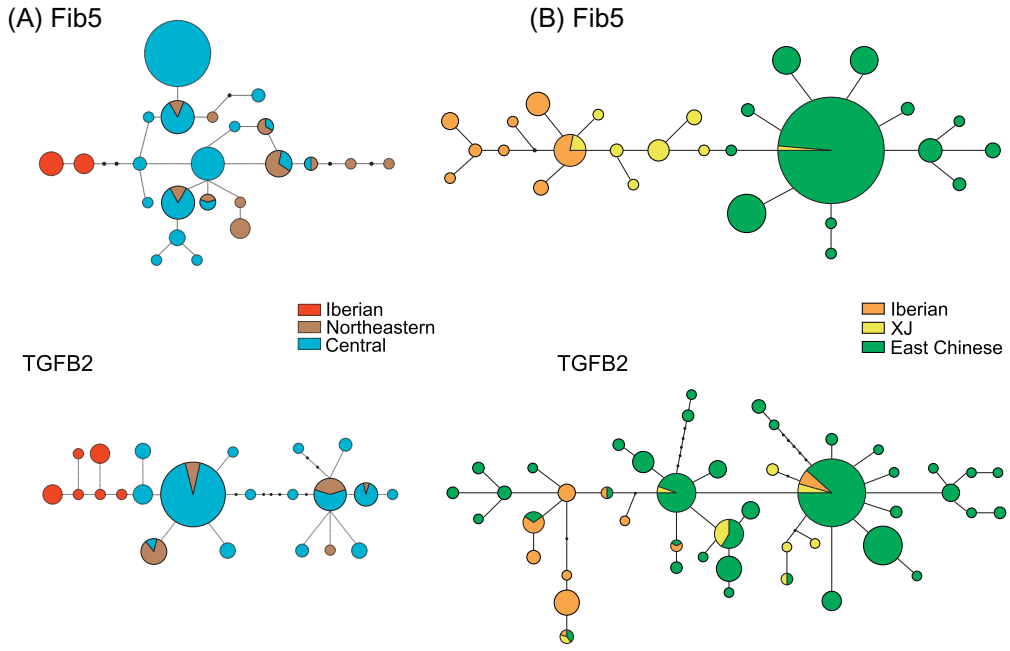

Fig. 3. Networks based on nuclear markers for *Cyanopica cyanus* (A) and *Pica pica* (B). Black dots refer to missing steps intermediate between observed haplotypes.

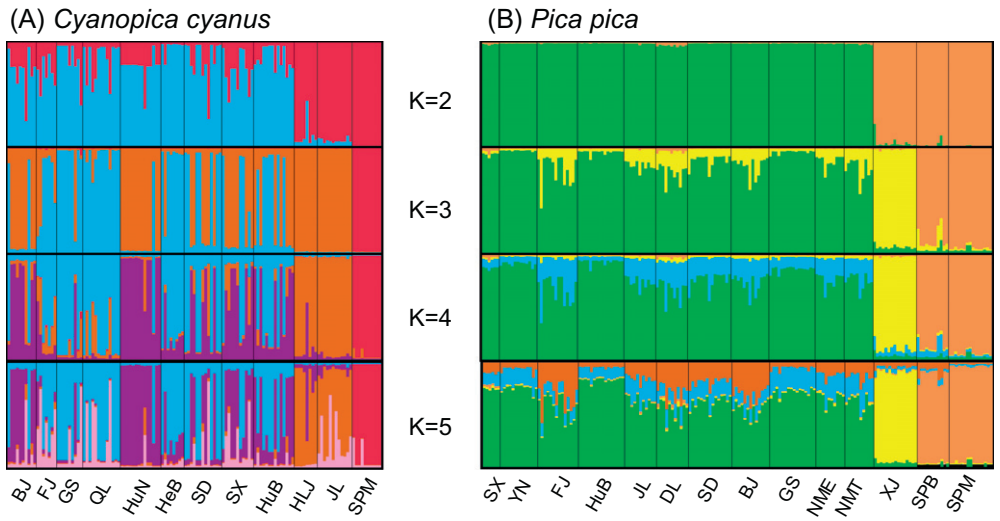

Fig. 4. Bar plot derived from Bayesian-based cluster analysis in STRUCTURE. Different colors represent preset clusters (*K*). Each individual is represented by a column with estimated proportions to each given cluster along the *Y*-axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

also supported this split. The divergence time was estimated to early mid-Pleistocene. During that period, with colder and drier climate, the forest in north China was replaced by steppe from the north and the desert expanded from the west (An et al., 2001; Holmes, 2007; Yang et al., 2006). The environmental transformation might have forced *C. cyanus* to retreat into several refugia with suitable habitats through the cold period. In Northeast China, Changbai Mountain was considered as a cryptic refugium for many species because the less disturbed southern slope could provide suitable habitats (Bai et al., 2010; Qiu et al., 2011). Milder environments of the mountainous areas in South China might have protected southern populations during cold periods (Lopez-Pujol et al., 2011). The pattern of two refugia, the Changbai Mountain and South China, has also been noted in some reptiles, frogs and mammals (Ding et al., 2011; Sakka et al., 2010; Zhang et al., 2008). After post-glacial expansion, Fok et al. (2002) suggested that the Da Hinggan Ling Mountains and the Yellow Sea acted as a geographical barrier to gene flow between these two separated populations, finally leading to their genetic divergence. During the breeding season, *C. cyanus* in Northeast China is reported to favor coniferous forest at an elevation of 1100–1800 m, while in lower latitude of East China it prefers broad leaved forest at lower elevation (Chen and Luo, 1998). This adaptation to local habitats might act as an ecological constraint to gene flows between these two populations.

Although covering a similar geographical range as *C. cyanus*, the East Chinese clade of *P. pica* did not show the same pattern of divergence. Shared haplotypes and unresolved trees indicate that all populations come from the same gene pool. This is in agreement with the fact that most populations in northeastern China and

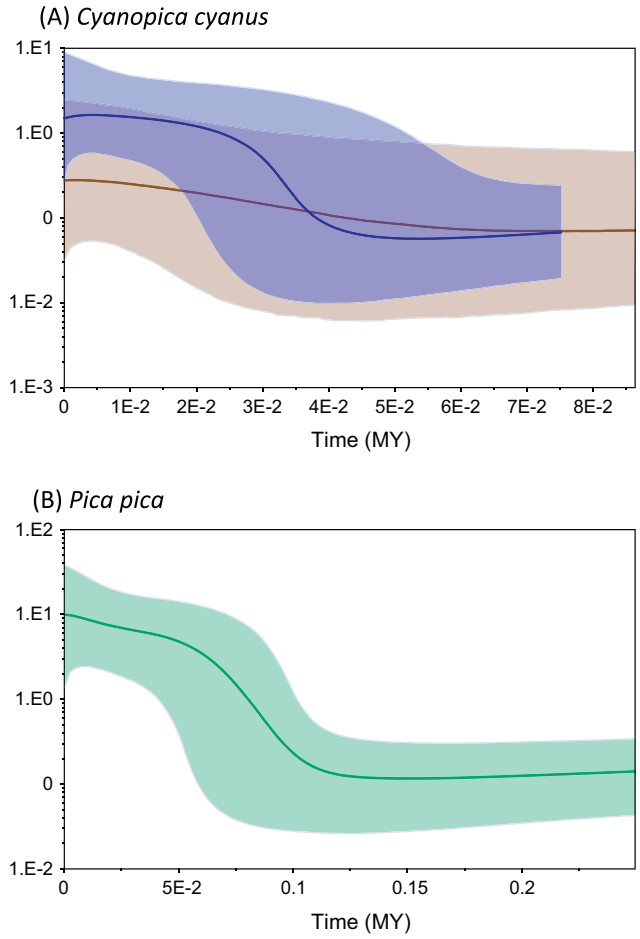

Fig. 5. Historical demographic trends of the East Chinese clade in each species represented by Bayesian skyline plot (BSP) for mitochondrial DNA. The *X* axis is the time scale before present in units of million years ago, and the *Y* axis is the estimated effective population size in units of $N_e\tau$, i.e. the product of effective population size and generation length in years (log transformed). Estimates of means are joined by a solid line while the shaded range delineates the 95% HPD limits. (A) *C. cyanus*, blue represents the Central clade and brown is the Northeastern clade; (B) *P. pica*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

eastern China are defined as the subspecies *serica* based on morphological characters (Chen and Luo, 1998; del Hoyo et al., 2009; Goodwin, 1976). These results suggest that *P. pica* possibly has expanded from one refugium and re-colonized East China after the glacial period.

Beside the major divergence, the pattern of genetic variance within clade/subclade is different between the two species. In the Central subclade of *C. cyanus*, nearly half of the genetic variance could be explained by differences among populations and among groups (44.8%, AMOVA; Table 3), and isolation by distance was detected in both mtDNA and nuclear introns. These results imply limited gene flow between these geographical populations. In contrast, in the East China clade of *P. pica*, both the results of STRUCTURE and Mantel test indicate that the population is genetically homogeneous and the main variance is among individuals.

Recent population expansion is generally used to explain poor population genetic structure (e.g., Conroy and Cook, 2000; Hewitt, 2000, 2004; Polihronakis and Caterino, 2010; Zink, 1996). According to the BSP results, both the Northeast and Central subclades of *C. cyanus* underwent recent expansion around the late Pleistocene. The East Chinese clade of *P. pica* experienced a considerable population growth in the same time range, but even earlier than *C. cyanus*. Since *P. pica* has had longer expansion time through the same geographical landscape in East China, the absence of genetic structure in *P. pica* is probably not due to recent expansion, but more likely due to more abundant gene flow.

Dispersal ability is considered to be one of the main factors affecting gene flow between populations (e.g., Bailey et al., 2007; Chen et al., 2010). Both *C. cyanus* and *P. pica* are mostly resident without long distance flight capability (Eden, 1987; Goodwin, 1976; Lee et al., 2011), but the availability of dispersal routes may not be the same. *C. cyanus* prefers woodland and forest edge, and avoids dry areas and dense forests (del Hoyo et al., 2009; Goodwin, 1976). Unlike some colony-nesting species which nest at high densities, *C. cyanus* usually requires more than 15 m between nests (del Hoyo et al., 2009; Goodwin, 1976). Although it occurs in city parks, *C. cyanus* avoids urban sprawl in Spain (Palomino et al., 2011), and to our knowledge, it does not nests on man-made constructions in East Asia. As *C. cyanus* requires forest habitat and substantial breeding territories, it is unlikely to survive in areas with scant forest cover. Moreover, the complex cooperative breeding behavior of *C. cyanus* (Canario et al., 2004; Valencia et al., 2003) and the territorial behavior among family flocks (del Hoyo et al., 2009; Hosono, 1989) might also result in low dispersal levels (Griesser et al., 2008; Hamilton, 1964a,b). Accordingly, open, treeless areas might act as an ecological barrier to gene flow, and accelerate divergence between different refugia, further leading to lower genetic diversity and isolation by distance within the Central subclade.

P. pica is more of a habitat generalist than *C. cyanus*, with less specific requirements on resources and with more plasticity in nest-site selection (del Hoyo et al., 2009). As examples of its adaptability, it can adjust its diet and can nest on human constructions, such as buildings or electricity pylons (Edwards et al., 1994; Kenney and Knight, 1992; Lu et al., 2008; Wang et al., 2008). Being an urban colonizer, the population size of *P. pica* has more than tripled after the 1980s in European cities (del Hoyo et al., 2009). Successfully adapting to urbanized environments, *P. pica* may benefit from abundant food sources and decreased predation risks, and even from long distance dispersal along electricity lines. These facts make *P. pica* more tolerant than *C. cyanus* to treeless environments and disturbances in their natural habitats. Moreover *P. pica* nests solitarily, and it does not hold stable family flocks (del Hoyo et al., 2009; Goodwin, 1976). All these characteristics might contribute to more abundant gene flow and more homogenization of genetic structure in *P. pica* than in *C. cyanus*.

Species with narrow niches are known to be sensitive to habitat disturbance (Driscoll and Weir, 2005; Kotiaho et al., 2005). The dependence on forest habitats, and perhaps also the complex breeding behavior of *C. cyanus* may restrict its movements in regions without continuous woodland habitats, and have a strong impact on its genetic structure. Both *C. cyanus* and *P. pica* are likely to have been continuously distributed across Eurasia sometimes in the past (Haring et al., 2007). During the glacial cycles, suitable forests retreated southward into separated refugia. *C. cyanus* seems to have been severely affected by the shrinkage of forest areas, leading to widespread local extinction between Iberia and East Asia, and resulting in the present disjunct distributions. In contrast, *P. pica* might have been less impacted due to its better adaptability, and could more easily re-colonize the Eurasian continent from two or more refugia after the retreat of the ice.

5. Conclusions

By comparing genetic structures estimated from mtDNA and nuclear introns, we found that the two sympatric species *C. cyanus* and *P. pica* have faced similar historical events that have formed concordant major lineage divergences between Iberian and East

Chinese clades, estimated to the late Pliocene/early Pleistocene. However, the two species showed different genetic patterns within their respective East Chinese clade. We suggest that differences in habitat preference and, to some extent, breeding behavior might have contributed to the different genetic patterns. Higher dependence on forest habitats, and possible also tight kin-groups, might have limited gene flow between areas in *C. cyanus* during periods of disturbances of its natural habitats. In contrast, with more plastic ecological requirements, better adaptability and less complex breeding behavior, *P. pica* has had better chances to expand its distribution, resulting in less geographically structured genetic patterns. These results indicate that species with different life histories may respond differently when facing environmental fluctuations during ice ages as well as present-day forest fragmentation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.07.011.

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