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## Article

### Complete taxon sampling of the avian genus *Pica* (magpies) reveals ancient relictual populations and synchronous Late-Pleistocene demographic expansion across the Northern Hemisphere

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Previous studies have suggested that bird populations in east Asia were less affected by Pleistocene climatic fluctuations than those in Europe and North America. However, this is mainly based on comparisons among species. It would be more relevant to analyse geographical populations of widespread species or species complexes. We analyzed two mitochondrial genes and two nuclear introns for all taxa of *Pica* to investigate 1) which Earth history factors have shaped the lineage divergence, and 2) whether different geographical populations were differently affected by the Pleistocene climatic changes. Our mitochondrial tree recovered three widespread lineages, 1) in east Asia, 2) across north Eurasia, and 3) in North America, respectively, with three isolated lineages in northwest Africa, Arabia and the Qinghai-Tibet Plateau, respectively. Divergences among lineages took place 1.4–3.1 million yr ago. The northwest African population was sister to the others, which formed two main clades. In one of these, Arabia was sister to Qinghai-Tibet, and these formed the sister clade to the east Asia clade. The other main clade comprised the North American and north Eurasian clades. There was no or very slight structure within these six geographical clades, including a lack of differentiation between the two North American species black-billed magpie *P. hudsonia* and yellow-billed magpie *P. nutalli*. Demographic expansion was recorded in the three most widespread lineages after 0.06 Ma. Asymmetric gene flow was recorded in the north Eurasian clade from southwestern Europe eastward, whereas the east Asian clade was rooted in south central China. Our results indicate that the fragmentation of the six clades of *Pica* was related to climatic cooling and aridification during periods of the Pliocene–Pleistocene. Populations on both sides of the Eurasian continent were similarly influenced by the Pleistocene climate changes and expanded concomitantly with the expansion of steppes. Based on results we also propose a revised taxonomy recognising seven species of *Pica*.

Keywords: lineage divergence, Palearctic, population expansion



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## Introduction

The Pleistocene glacial cycles have played a prominent role in shaping the population structure of species and in driving speciation (Klicka and Zink 1997, Lovette 2005). During the glaciations, large ice sheets covered northwestern Eurasia and northern North America, while east Asia was less extensively glaciated. During glacial maxima east Asia was largely covered by extensive shrub steppe vegetation with only local glaciers and some arboreal vegetation (Clark and Mix 2002, Dyke et al. 2002, Svendsen et al. 2004, Allen et al. 2010). The southeastern parts of Asia, especially the eastern part of China, were less affected (Williams et al. 1998). For example, during the Last Glacial Maximum (LGM), most parts of this area were warmer than Europe and North America at similar latitudes (Williams et al. 1998, Harrison et al. 2001). Comparative studies of phylogeography among these different areas would therefore be expected to discover regional differences due to varying glacial effects.

Recent studies on birds suggest that east Asian lineages generally have deeper population histories and more stable population sizes through the LGM than European populations (Song et al. 2009, Zhao et al. 2012, Li et al. 2016). Saitoh et al. (2010) pointed out that clades of northeastern Palearctic birds are generally older than those in the northwestern Palearctic and northern North America, probably as a result of longer survival in the eastern Palearctic due to less severe conditions in that region.

Although many phylogeographical studies have identified a relationship between glacial refuges and lineage diversification in widespread species across the Eurasian continent (Haring et al. 2007, Zink et al. 2008), few have focused on the regional variation of glacial effects on geographical populations. For two main reasons the east-west difference in glacial effects needs further study. First, previous studies are mainly based on comparisons among different species, while it would be more enlightening to compare regional differences in historical population dynamics within a species or a species complex with a shared ancestry. Second, species with different habitat requirements and life histories may have responded differently to climatic changes in space and time (Zink et al. 2008, Burbrink et al. 2016). The comparatively stable populations in east Asian lineages have mostly been found in forest-dwelling birds (Song et al. 2009, Liu et al. 2012, Zhao et al. 2012). In addition to the repeated retreat and regeneration of wooded habitats, range shifts of deserts and arid regions in central Eurasia have also been an important feature of the Pleistocene environment on the Eurasian continent (An et al. 2001, Guo et al. 2002, Ding and Chan 2005). We would therefore expect to find different patterns in population structure and historical dynamics in birds that favour open environments compared to forest birds.

The magpies (genus *Pica*) comprise three (Dickinson and Christidis 2014, Gill and Donsker 2017) to five species (del Hoyo et al. 2016), depending on taxonomy. The genus inhabits four biogeographical realms. It is widespread in the western Nearctic (black-billed magpie *P. hudsonia* and yellow-billed magpie *P. nutalli*), and in the Palearctic and Sino-Japanese

realms (various subspecies of Eurasian magpie *P. pica*), and has more marginal distributions in the Saharo-Arabian realm, with isolated populations in the Maghreb region of north Africa (*P. p. mauritanica*) and Saudi Arabia (*P. p. asirensis*) (Fig. 1). All species and subspecies are resident or short-distance dispersers. They inhabit various open and semi-open habitats, in some parts including urban areas, where they may be locally common in cities. They forage on the ground in mostly short-grass areas but depend on trees, tall bushes or man-made structures, such as pylons, for breeding. They are omnivorous and quite opportunistic foragers, although they are mainly insectivorous during summer, but rely mainly on scavenging at other parts of the year, and take advantage of human handouts and offal (Madge 2009).

Previous phylogenetic studies of *Pica* have achieved some knowledge about magpie systematics and taxonomy (Lee et al. 2003, Kryukov et al. 2004, 2017, Haring et al. 2007, Zhang et al. 2012). Lee et al. (2003) found that the North American *P. hudsonia* and *P. nutalli* were nested within *P. pica* based on mitochondrial DNA (mtDNA). The most recent study (Kryukov et al. 2017) found deep mtDNA splits into four major lineages: Europe-Siberia, southern east Asia, north Africa and North America. Although that is the most comprehensive study so far, it lacks samples from some key geographical regions, such as Iberia, Arabia and the Qinghai-Tibet Plateau and has only one sample each from China and North America (*P. hudsonia*; none of *P. nutalli*). A more comprehensive study of the magpies is therefore needed.

This paper conducts analyses at both inter- and intra-specific level within the genus *Pica* using two mitochondrial and two nuclear markers. With a more comprehensive sampling than any previous study, including all species and subspecies, we reconstruct the phylogeny, and study the genetic population structure and compare the historical demography among the major regional clades in order to test 1) what geological and geographical factors have shaped the lineage divergence of the magpies; and 2) whether east Asian populations were less affected by the glacial climate changes than its western counterpart. We also discuss taxonomic implications.

## Material and methods

### Sampling and laboratory work

The taxon sampling has been selected to cover the major distribution range of magpies and includes all three species of *Pica* as well as all recognized subspecies following the taxonomy of Gill and Donsker (2017). We included sequences of seventy-eight individuals from Zhang et al. (2012), in addition to 60 fresh tissue and blood samples, 5 feather samples and 40 toepad samples sequenced for this study (Fig. 1, Supplementary material Appendix 1 Table A1). The samples were obtained from the following institutions: American Museum of Natural History; Ewha Woman's Univ., Museo Nacional

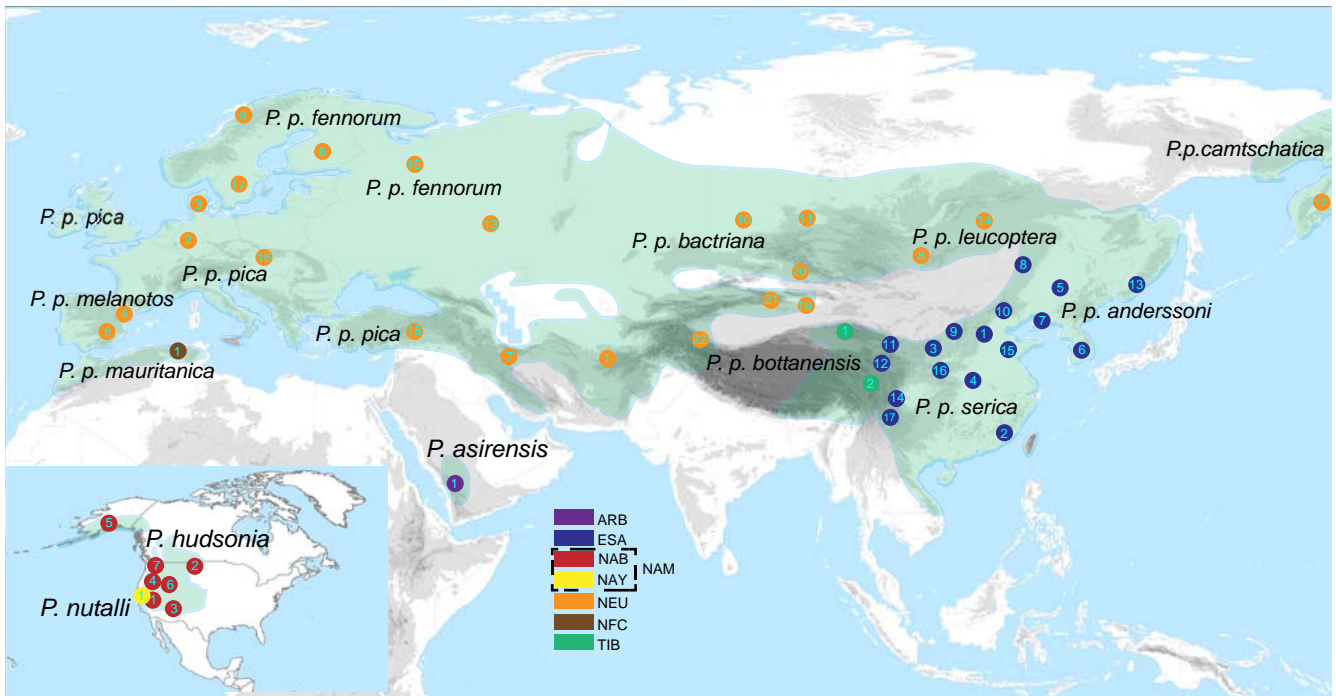


Figure 1. Distributions and sampling sites. Sampling sites are marked by coloured dots with numbers referring to locality names (explained in Supplementary material Appendix 1 Table A1). Colours represent clades defined in Fig. 2.

de Ciencias Naturales; Museu de Zoologia de Barcelona; Novosibirsk State Univ.; The Natural History Museum, UK; Swedish Museum of Natural History; Inst. of Forest Ecology of Slovak Academy of Sciences; Inst. of Zoology, Chinese Academy of Sciences; Univ. of Washington Burke Museum; and the Natural History Museum of Denmark, Univ. of Copenhagen. We selected *Cyanocitta stelleri*, *Cyanopica cyanus*, and *Corvus frugilegus* as outgroups based on the Corvidae phylogeny by Ericson et al. (2005).

Total genomic DNA was extracted from blood and muscle samples using the QIAamp DNA Mini Kit (QIAGEN). For feather and toepad samples, we first bathed sample in ddH<sub>2</sub>O for an hour, then the chopped samples were digested in 180 µl ATL buffer with 20 µl proteinase K and 20 µl of 1% DTT in 56°C overnight. The same DNA extraction kit was used as for the tissue and blood samples. The mitochondrial cytochrome *b* (Cytb) and NADH dehydrogenase subunit 2 (ND2) were obtained by PCR amplification. We also amplified the two nuclear markers, β-fibrinogen intron 5 (FIB5) and transforming growth factor beta 2 intron 5 (TGFB2). For toepad and feather samples, primers were designed for short fragments (180–200 bp). All primers and their annealing temperature are shown in Supplementary material Appendix 1 Table A2.

The PCR products were purified using the QIAquick™ PCR Purification Kit (QIAGEN). Sequencing was carried out using an ABI 377 automatic sequencer. Both strands were sequenced for all individuals with the same PCR primers. Sequences were aligned and checked in MEGA 5 (Tamura et al. 2011). All sequences are accessible in GenBank (MG640603–MG641033).

## Nucleotide polymorphism

The number of segregating sites (*S*), haplotypes (*H*), haplotype diversity (*H<sub>d</sub>*), and nucleotide diversity (*P<sub>i</sub>*) were calculated by DnaSP 5.0 (Librado and Rozas 2009) based on mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) datasets, respectively. We used Fu's *F<sub>s</sub>* and Tajima's *D* parameters calculated in DnaSP 5.0 to test evolutionary neutrality. The McDonald and Kreitman's test (McDonald and Kreitman 1991) in DnaSP 5.0 was used to detect selective neutrality of the mtDNA protein-coding fragments. For two nuclear introns, heterozygous sequences were phased in DnaSP 5.0. Sequences with uncertain phasing results (probability < 80%) were removed from further analyses.

## Phylogenetic reconstruction and population structure analyses

We used MrModelTest2 (Nylander 2004) to select the best-fit models of molecular evolution based on the Akaike information criterion. The best model was HKY+I+G for Cytb, GTR+I+G for ND2 and FIB, and SYM+I for TGFB2. We first applied the maximum-likelihood (ML) algorithm implemented in PhyML 3.0 (Guindon and Gascuel 2003) to reconstruct the phylogenetic relationship of the concatenated mtDNA sequences, and used non-parametric ML bootstrapping (1000 replicates) to evaluate nodal support among branches. Then we applied the Bayesian inference (BI) in BEAST 1.8 (Drummond et al. 2012) to the mtDNA dataset, with the exponential growth coalescent tree



prior for the phylogenetic reconstruction. We ran the Markov Chain Monte Carlo (MCMC) with 100 million generations with the relaxed log-normal clock model. Convergence to the posterior distributions of the parameter estimates was evaluated by monitoring the effective sample size ( $ESS > 200$ ) and trace plots in TRACER 1.6 (Rambaut et al. 2014).

We also tried a coalescent-based species tree method using \*BEAST in BEAST 2 (Bouckaert et al. 2014), with three partitions: mtDNA and the two nuclear introns, respectively. We unlinked substitution models across the three loci (concatenated *Cytb* and ND2, FIB5, and TGFB2) and set the substitution parameters for each locus according to the MrModeltest results. We implemented a Piecewise linear and constant root model as the species tree prior and used default molecular clock settings (strict clock model, the rate of the first locus = 1.0 and the rates for other loci were set to be estimated) in major lineage identification. MCMC chains ran for 500 million generations, sampling every 5000 generations. The convergence of the MCMC chains was examined in TRACER 1.6 (Rambaut et al. 2014), and the first 10% of the samples were discarded as 'burn-in', well after stationarity had been reached.

A parsimony based method in TCS ver. 1.21 (Clement et al. 2000) was used to draw unrooted networks evaluating haplotype relationships for nuclear introns with 95% parsimoniously plausible branch connections.

The corrected average pairwise distance between populations was computed in MEGA 5 based on mtDNA, then a UPGMA tree was constructed to visualize the distance matrix. The geographical structure was also investigated by analysis of molecular variance (AMOVA) in Arlequin 3.11 (Excoffier et al. 2005) based on mitochondrial sequences. We tested different grouping arrangements referring to species and subspecies delimitation, major geographical features and phylogenetic results. The statistical significance of variance components in the AMOVA was tested with 10 000 permutations.

### Dating of lineage divergence and historical demography

Divergence times were estimated in BEAST 1.8. The nucleotide diversity of the two mitochondrial fragments were found to be rather similar (*Cytb*: 0.029; ND2: 0.030; combined: 0.030) so we applied the substitution rate (0.0105 substitutions/site/million years) of the standard molecular clock (Weir and Schluter 2008) to the combined mtDNA sequences. The analyses were run with a relaxed log-normal molecular clock model and a coalescent exponential growth tree prior. MCMC chains were run for 100 million generations, sampling every 1000 generations. We used TRACER 1.6 to check the posterior distribution and effective sample sizes (ESSs) of the MCMC output. We used TREEANNO-TATOR 1.8 in the BEAST package to summarize trees with 'mean height', and discarded the first 10% of the trees as 'burn-in'. The tree and divergence times were displayed in FIGTREE 1.4.

We used extended Bayesian skyline plots (EBSPs; Heled and Drummond 2008) to reconstruct the evolution of ancestral population sizes for major regional clades. EBSP is based on gene genealogies to estimate population sizes at different points in time. For each clade, we built intraspecific tree with extended Bayesian skyline plot tree prior based on both mtDNA and nuclear intron sequences. We unlinked substitutions models, clock models, and trees among genes. A strict molecular clock was enforced for all genes and the rates were estimated relative to the combined mtDNA (0.0105). The MCMC chain was run for 50 million steps or more until the ESS was larger than 200, sampling every 1000 steps. The first 10% samples were discarded as burn-in. The changes of population size were visualized with ggplots2 (Ginestet 2011) in R.

Gene flow among populations and parameters measuring effective population size were estimated in Migrate 3.2.19 (Beerli 2009). Initial values were generated from a  $F_{ST}$  calculation, and the uniform priors and slice sampling were set for both  $M$  (0–1 000 000, mean = 500 000) and  $\Theta$  (0.0–10.0, mean = 5.00) with the full migration model. We ran four chains with static heating scheme (1.0, 1.5, 3.0, 10 000). The long chain was run 50 000 steps with the sampling increment 100, and the first 5000 trees were discarded as the 'burn-in'. We ran the program with the same parameter setting three times with different starting seeds to ensure the consistency of the results.

### Data accessibility

Sample information and GenBank accession numbers for all sequenced individuals are given in Supplementary material Appendix 1 Table A1.

## Results

### Nucleotide polymorphism and evolutionary properties of mitochondrial and nuclear sequences

We amplified 905 bp of *Cytb* and 712 bp of ND2 from 183 individuals, 320 bp of FIB5 from 170 individuals and 324 bp of TGFB2 from 153 individuals. The  $Hd$  was higher for *Cytb* (0.95) than for ND2 (0.90), while the  $Pi$  was more or less similar (0.0292 and 0.0307, respectively). The combined mtDNA sequences generated 93 haplotypes with the  $Hd$  and  $Pi$  0.98 and 0.030, respectively. The two nuclear introns showed lower levels of genetic diversity than mtDNA, and FIB5 had a higher nucleotide diversity ( $Pi = 0.00773$ ) than TGFB2, whereas the latter had the higher haplotype diversity ( $Hd = 0.898$ ). Tajima's  $D$  showed positive values in mtDNA but not in nuDNA. Fu's  $F_s$  were significantly negative in all four genes. The McDonald-Kreitman's tests detected no significant deviation from neutrality in the two mitochondrial protein-coding fragments (Table 1).

Table 1. Nucleotide polymorphism and results of neutrality tests for mitochondrial and nuclear genes.

	Cytb	ND2	Combined mtDNA	FIB5	TGFB2
Length (bp)	905	737	1617	320	324
N	183	183	183	170	153
S	125	114	239	21	25
Nhap	64	52	93	32	40
Pi	0.029	0.030	0.030	0.00773	0.00749
Hd	0.946	0.901	0.979	0.795	0.898
Fu's Fs	-4.808*	-1.969*	-11.296*	-16.812*	-30.489*
Tajima's D	0.411	0.0201	0.318	-0.870	-1.302
MK test	1.242	1.004	/	/	/

N, sample size; S, number of segregating sites; Nhap, number of haplotypes; Hd, haplotype diversity;  $\pi$ , 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 (\* $p < 0.05$ ). /, the values were not computed for the combined mitochondrial sequences and the two nuclear introns.

### Phylogenetics and population structure of the *Pica* species complex

The tree based on concatenated mtDNA sequences revealed six well supported primary clades in both the ML (Supplementary material Appendix 3) and BI analyses (Fig. 2). All individuals from Europe through western and central Asia, northwest China, Siberia and Mongolia to Transbaikal and an isolated population in Kamchatka grouped together as the north Eurasia clade (NEU). All individuals from southern eastern Asia, including China (except the Qinghai-Tibet Plateau), South Korea, and Primorsky Krai in the Russian Far East formed another major clade referred to as the east Asia clade (ESA). A deeply divergent Qinghai-Tibet clade (TIB) was observed for the populations from northeast Qinghai (TIB1) and southeast Sichuan (TIB2), spanning the eastern rim of the Qinghai-Tibet Plateau. *Pica nutalli* and *P. hudsonia* together formed a deeply divergent North American clade (NAM), within which *P. nutalli* formed a clade (NAY), whereas *P. hudsonia* (NAB) was paraphyletic with respect to *P. nutalli*, although this was not strongly supported. The divergences within the North American clade were shallower than in the north Eurasian and east Asian clades. Three individuals from Algeria (*P. p. mauritanica*) and two from Saudi Arabia (*P. p. asirensis*) formed two deeply diverged clades (northwest African, NFC, and Arabian, ARB, respectively). The relationships among the primary clades were not well resolved in the ML tree analysis, but the BEAST reconstruction obtained high support values for most internal nodes. According to the BEAST tree, the northwest African clade was sister to the others; the Arabian and Tibetan clades were sisters; and the North American and north Eurasian clades were sisters. Only the node suggesting a sister relationship between the east Asia and Arabia/Qinghai-Tibet clade was poorly supported and effectively unresolved. Although some tip nodes of the phylogenetic tree obtained high posterior probabilities, there was no notable geographical structure within the regional clades except for the shallow clusters of individuals from Iberia (NEU4 and NEU5) and Kamchatka (NEU12),

respectively (Fig. 2). For populations from north Africa and Saudi Arabia, sample sizes were too small to investigate geographical variation.

\*BEAST did not reach parameter convergence, not even after 500 million generations neither for the three partitions (mtDNA, FIB5, and TGFB2), nor for the two partitions (FIB5 and TGFB2). In the FIB5 network (Fig. 3), the majority of individuals from the east Asian clade shared a single haplotype (2), whereas two haplotypes (4, 6) were dominant in numbers in north Eurasian individuals. The individuals from the North American and Qinghai-Tibetan clades were close to those from north Eurasia, and samples from the east Asia clade were close to those from Arabia and northwest Africa as well as some north Eurasian haplotypes. The TGFB network had no apparent geographical structure, as individuals from different geographical regions were mixed and shared dominant haplotypes (Fig. 3).

The AMOVA showed the largest variance when populations were arranged into eight groups (ARB, EAS, NAM, NAY, NEU, NFC, TIB1 and TIB2) according to the phylogenetic results (Table 2). The UPGMA tree based on corrected average pairwise distances showed that the regional clades grouped similarly to the BI tree. The two Iberian populations (NEU4 and NEU5) formed a sub-clade within the north Eurasia clade, and the population from Kamchatka (NEU12) was also more differentiated than the rest of the populations. Within the east Asia clade, a population from northeast Qinghai-Tibet Plateau (ESA12) was more differentiated than other geographical populations (Supplementary material Appendix 2).

### Divergence time, historical population expansion and gene flow

The time to the most recent common ancestor (TMRCA) for all *Pica* populations was dated to 3.13 [95% highest posterior distribution (HPD) 2.20–4.29] million yr ago (Ma). The TMRCA for the east Asian clade was estimated at 0.37 (95%HPD 0.22–0.62) Ma, and for the north Eurasian clade

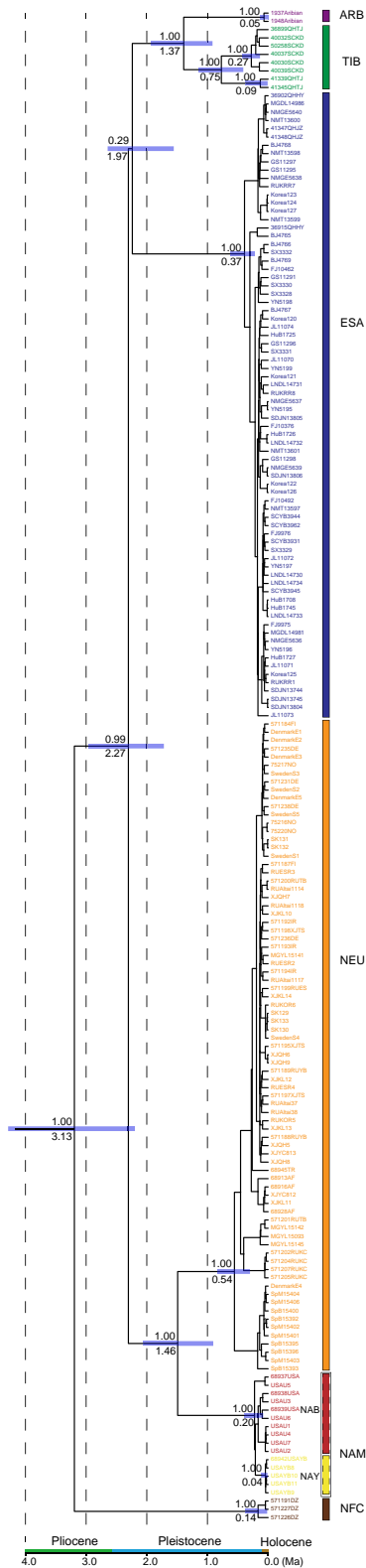


Figure 2. Bayesian tree based on mtDNA. Values above branches at the nodes indicate Bayesian posterior probabilities (PP), and values below indicate the median value of the branch heights. Blue bars at nodes represent 95% highest posterior distribution (HPD). Nodes without values or 95% HPD bars are poorly supported (PP < 0.90). The geological history classification refers to Lewis and Maslin (2015).

0.54 (95%HPD 0.30–0.84) Ma. The TMRCA for the Arabian, North American, Qinghai-Tibetan and northwest African clades were 0.05 (95%HPD 0.01–0.17), 0.20 (95%HPD 0.08–0.38), 0.75 (95%HPD 0.41–1.15) and 0.14 (95%HPD 0.02–0.38) Ma, respectively. The northwest African clade separated from the others at the earliest split, then the next split into two major clade groups took place around 2.27 (95%HPD: 1.72–2.96) Ma. The Arabian and Qinghai-Tibet clades were separated from the east Asian clade soon after the eastern/western divergence, at 1.97 (95%HPD: 1.56–2.65) Ma, although the low nodal support renders this divergence time uncertain. The Arabian and Qinghai-Tibetan clades were separated at 1.37 (95%HPD: 0.92–1.94) Ma. The divergence of the North American clade from the north Eurasian one was estimated around 1.46 (95%HPD: 0.90–2.06) Ma. The TMRCA of the *P. nutalli* clade (ANY), which was embedded within the *P. hudsonia* clade, was 0.04 (95%HPD: 0.01–0.1) Ma (Fig. 2).

EBSPs were only done for the ESA, NEU and NAM clades due to sample size limitations for the other clades. The results showed a significant population growth in all three clades (Fig. 4). The population expansion times of the ESA and NEU clades were begun around 0.04–0.06 Ma, and there was no obvious difference in population expansion time between the east Asian and north Eurasian clades. The population size of the North America was small than the other clades, and the population expansion time was estimated even later (around 0.02 Ma). The north Eurasian clade had an earlier TMRCA than the two others, consistent with the BI tree.

We also computed the genetic diversity parameters ( $H_d$  and  $P_i$ ) and indicators of neutral evolution (Fu's  $F_s$  and Tajima's  $D$ ). Among regional clades the Qinghai-Tibet clade had the highest genetic diversity ( $H_d=1$ ,  $P_i=0.00601$ ,  $n=8$ ) in mitochondrial genes. The north Eurasian clade had the highest haplotype diversity in both FIB5 and TGFB ( $H_d=0.722$ ,  $P_i=0.00552$ ,  $n=146$ ;  $H_d=0.887$ ,  $P_i=0.00823$ ,  $n=78$ ), and also had a higher nucleotide diversity than the East Asian and North American clades. Fu's  $F_s$  and Tajima's  $D$  values were mostly negative in major regional clades and were significant for east Asian (both indices) and north Eurasia (Fu's  $F_s$ ) for mitochondrial genes (Table 3).

Limited by small sample sizes for some of the populations, we conducted gene flow analyses only among populations from the north Eurasia and east Asia clades, respectively. Substantial migration rates among geographic populations were noted (Supplementary material Appendix 1 Table A3). To detect asymmetric gene flow and its directions, we compared the immigration and emigration rates for each population. Immigrant/emigrant ratios > 10 and their directions were illustrated (Fig. 5). The migration matrixes suggested different patterns of gene flow within these two clades. In the north Eurasian clade, gene flow was mainly derived from southwest and northwest toward the east. In the east Asian clade, the pattern was predominantly from the southwest and central parts towards the peripheral areas.

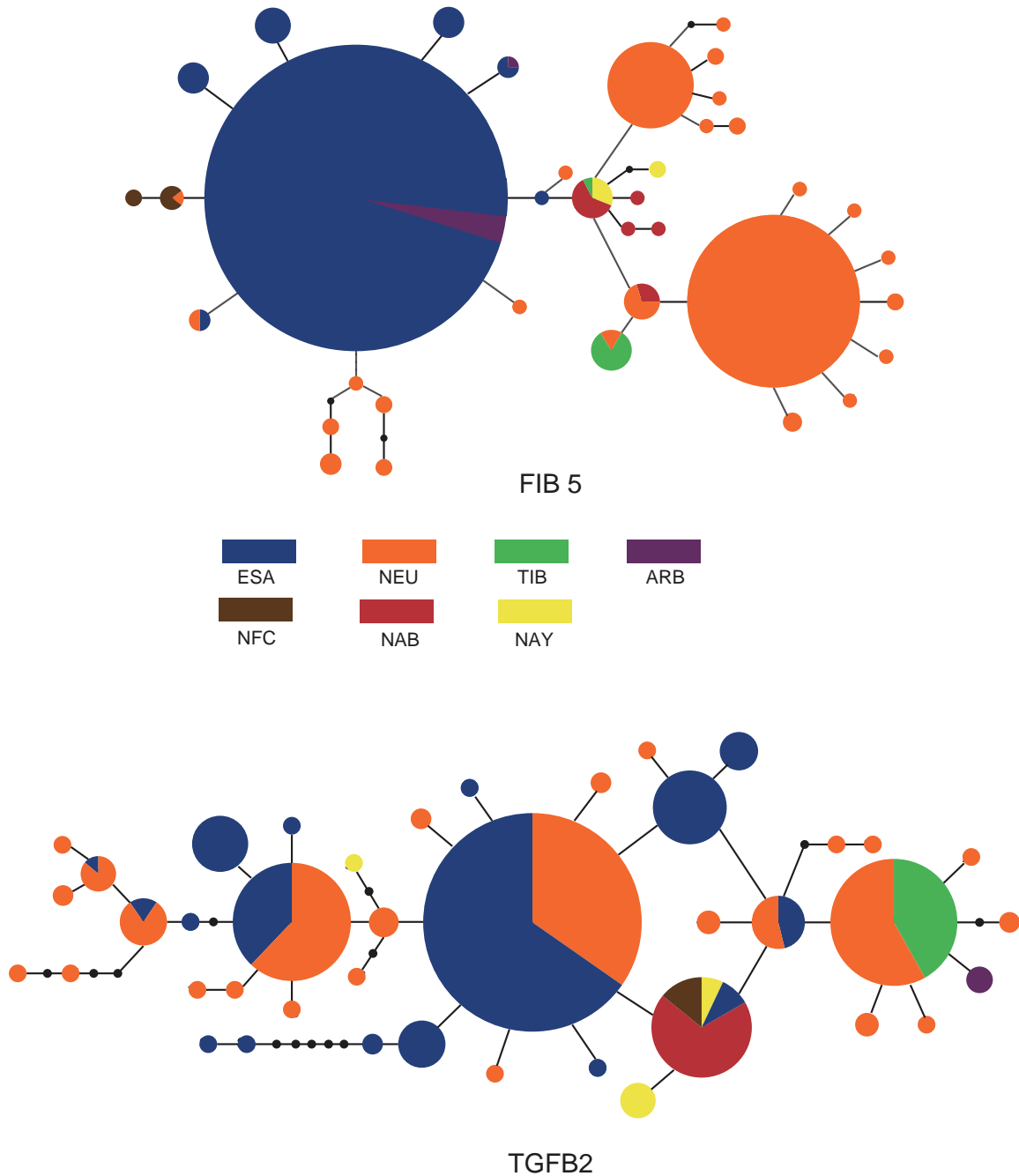


Figure 3. Nuclear networks based on haplotypes of the FIB5 and TGFB2. The colours correlate to the primary clades in the mitochondrial tree (Fig. 2).

Table 2. AMOVA result with mtDNA.

Source of variation	df	Variance components	Percentage of variation
Among groups	7	34.83046 Va	92.24
Among populations within groups	37	1.13848 Vb	3.01
Within populations	138	1.79197 Vc	4.75

## Discussion

### Phylogeny and historical biogeography

Based on a more completed taxon sampling and a coalescent model, we obtained the most comprehensive and best resolved phylogenetic hypothesis for magpies so far, admitting that the tree is only based on mtDNA. Unfortunately, the nuclear loci did not provide much support, as they



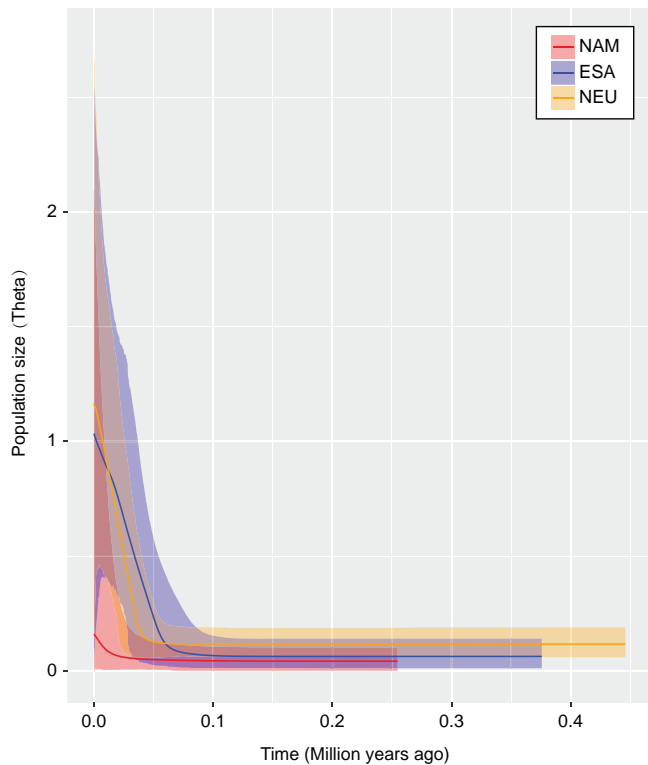


Figure 4. Extended Bayesian skyline plot for historical demographic trends in three regional clades: blue, east Asia; orange, north Eurasia; and red, North America. The x-axis is the timescale in units of million years ago, and the y-axis is the estimated theta value of the population size. Estimated median are joined by a solid line, while the shades delineate the 95% posterior density limits.

contained too little phylogenetic information. The identification in the mtDNA tree of six primary regional clades is consistent with the results of Kryukov et al. (2017) which, however, did not include samples from the Arabian and Qinghai-Tibetan clades. In agreement with Kryukov et al. (2017) we found the clade from northwestern Africa to be the most divergent single-taxon lineage, and the North American clade to be sister to the north Eurasian one. However, our mtDNA tree also strongly supported that the Arabian and Qinghai-Tibetan populations form sister clades, and that they are possibly most closely related to the east Asian clade. These results support that in addition to the widely distributed clades in northern Eurasia, east Asia and North America, there are some ancient isolated populations, such as *P. p. mauritanica* in the Maghreb region of northwest Africa, *P. p. asirensis* in Saudi Arabia, and *P. p. bottanensis* on the Qinghai-Tibet Plateau. These relictual populations occur in the same biogeographical regions as some relatives of magpies, such as ground jays *Podoces* spp. in arid parts of central Asia, and piapiac *Ptilostomus afer* and Stresemann's bushcrow *Zavattariornis stresemanni* of dry woodland savannas in or just south of the deserts of northern Africa (Ericson et al. 2005). The separation of the northwest African population from the others around 3.1 Ma may therefore be related to the climatic and

environmental change since the end of the Miocene when the Saharo-Arabian region was covered by grassland savanna, but with periods of desertification of varying intensity up through the Pliocene and Pleistocene (Bobe et al. 2002, DeMenocal 2004).

Our dating analyses suggest that the diversification of the primary clades took place during the late Pliocene to the middle Pleistocene. The second deepest split, separating the Arabia, Qinghai-Tibet, and east Asia clade from the north Eurasia and North America clade, was estimated to the early Pleistocene. This divergence was probably caused by environmental changes due to the glacial developments at the beginning of the Pleistocene. At that time, the central part of the Eurasian continent experienced cooling and drying climates (An et al. 2001, Yang et al. 2006, Wang et al. 2016).

The sister relationship between the North American and north Eurasian clades is consistent with previous studies of mtDNA (Lee et al. 2003, Kryukov et al. 2017). The divergence of North American populations from north Eurasian ones was dated to the middle Pleistocene. This indicates that an earlier connection and disconnection, likely via the Bering Land Bridge, shaped this pattern (Brigham-Grette 2001). This result is consistent with paleontological records of the oldest fossils of magpies from North America, from Colorado, which are ca 1 Ma (Emslie 2004).

The differentiation between the North American *P. nutalli* and *P. hudsonia* is poor according to both our mtDNA and nuclear intron data, and although the individuals of the former form a clade in the mtDNA tree, this is suggested to be embedded within the latter, although the support for this is poor. Moreover, the divergence between these two species in the mtDNA tree is very shallow, and more on par with individual variation within the other primary clades. This pattern indicates that the unique phenotypic characters of *P. nutalli* (notably the yellow bill and facial skin) have evolved within a short time span, alternatively that the lack of divergence is the result of past introgression.

### Geographical barriers promoted lineage divergence

In addition to climatic and environmental changes, geographical barriers in different parts of the Northern Hemisphere might also have had great influence on gene flow. Deep splits between the geographically close regions of Iberia and north Africa have been recorded in birds (Pons et al. 2011, Stervander et al. 2015, Li et al. 2016) and other organisms (Veith et al. 2004, Salicini et al. 2013). The last breakout of the Gibraltar Strait was at the start of the Pliocene, about 5 Ma (Krijgsman 2002), and we propose that the separation of the northwest African clade was also related to this event. Being a sedentary species, which only exceptionally flies across water bodies, it is not surprising that the Mediterranean Sea has effectively blocked dispersal between Iberia and Maghreb, and the dramatic climatic perturbations in the Pleistocene around the Mediterranean (Hooghiemstra et al. 1992, Correia et al. 2015) may have magnified the geographical barrier effect between Iberia and north Africa.



Table 3. Nucleotide polymorphism and results of neutrality tests for mitochondrial and nuclear genes.

	N	Hd	Pi	Fu's Fs	Tajima's D
<b>Combined mtDNA</b>					
ARB	2	1.000	0.00124	NA	NA
ESA	76	0.971	0.00364	-2.223**	-2.223**
NAB	10	0.956	0.00164	-1.274	-1.116
NAY	5	0.400	0.00025	-0.771	-0.816
NEU	79	0.914	0.00410	-2.589*	-2.589
NFC	3	1.000	0.00206	NA	NA
TIB	8	1.000	0.00601	-1.420	-1.259
<b>FIB5</b>					
ARB	4	0.500	0.00156	-0.478	-0.612
ESA	150	0.383	0.00132	-1.13011	-1.466
NAB	14	0.659	0.00264	-0.0788	-0.315
NAY	6	0.533	0.00333	1.2748	1.032
NEU	146	0.722	0.00552	-1.681	-1.138
NFC	6	0.533	0.00167	1.029	0.851
TIB	14	0.473	0.00501	1.250	0.907
<b>TGFB2</b>					
ARB	4	0.000	0.000	NA	NA
ESA	138	0.798	0.00517	-0.716	-0.446
NAB	20	0.000	0.000	NA	NA
NAY	10	0.511	0.00295	-1.245	-1.294
NEU	78	0.887	0.00823	0.324	0.262
NFC	2	0.000	0.000	NA	NA
TIB	16	0.000	0.000	NA	NA

N, sample size; S, number of segregating sites; Nhap, number of haplotypes; Hd, haplotype diversity; Pi, 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). NA, four and more sequences or haplotypes are needed to compute Tajima's and Fu's tests. The three-letter codes represent the regional clades: Arabia (ARB), east Asia (ESA), North America black-billed magpie (NAB), North America yellow-billed magpie (NAY), north Africa (NFC), north Eurasia (NEU), and Qinghai-Tibet (TIB).

Besides water bodies, other geographical barriers, such as deserts and montane forests, may have contributed to the lineage diversification of magpies. The clear split between the north Eurasian and east Asian clades has been recorded in previous studies on *Pica* (Lee et al. 2003, Haring et al. 2007, Zhang et al. 2012) as well as in other birds such as the *Phylloscopus borealis*, *Motacilla alba* and *Aegithalos caudatus* complexes (Saitoh et al. 2010, Song et al. 2015, Li et al. 2016), indicating a general geographical or climatic barrier blocking population dispersal and gene flow between the northern boreal zone and more mesic regions in eastern China and neighbouring areas (Song et al. 2016). The inferred dating suggests that this divergence began around 2.3 Ma in the magpies, concordant with the environmental cooling and drying at the early stage of the Pleistocene. The arid landscape in central Asia, such as the Gobi, Taklamakan and Gurbantunggut Deserts, were dramatically expanded due to climatic cooling during this period (Yang et al. 2006, Holmes 2007, Cai et al. 2012, Wang et al. 2016).

The suggestion in the mtDNA tree that the populations from the Qinghai-Tibet Plateau are more closely related to the Arabian populations than to the geographically adjacent east Asian clade is surprising. However, it seems plausible that magpies were widespread from western to central Asia during cooler periods of the Pliocene, when open grassland were extensive, and that they later became isolated in small areas

in Saudi Arabia and on the Qinghai-Tibet Plateau due to the development of deserts, high altitude meadows and montane forests during the early Pleistocene (Cosson et al. 2005). We also assume that the dense forests along the eastern rim of the Qinghai-Tibet Plateau may have acted as an ecological barrier between the more open habitats to the west and the lowlands to the east. Based on the timing of lineage diversification discussed above, we conclude that besides large water bodies, both extremely arid areas and dense montane forests may have constrained dispersal among magpie populations.

### Late Pleistocene population expansion in the three regional clades

The divergence time between the North American and northern Eurasian clades is dated to the early Pleistocene (ca 1.5 Ma), and the oldest fossils from North America are ca 1 Ma (Emslie 2004). According to fossil data, magpies were more widespread in North America in ancient times, and were widely distributed in the southeastern part of the continent as late as the latest glaciation, at ca 12 000 yr ago (Emslie 1998). These molecular and fossil data indicate that the North American magpies might have diverged from their Eurasian relatives earlier than the disruption of the Bering Land Bridge during the late Pleistocene. However, the EBSPs analysis



Figure 5. Map of the asymmetric gene flow. The gene flow among geographical populations within north Eurasia (orange circles) and east Asia (ESA; blue circles) were estimated independently. The population codes are consistent with those in Fig. 1 and Supplementary material Appendix 1 Table A1 except that some populations from ESA were combined (numbers with \*) due to close geographical distance. Arrows indicate directions of the asymmetric gene flow in cases where the immigrant/emigrant ratio is  $> 10$ . The topographical background map was produced by the Ovitalmap (Beijing Ovital software) based on google satellite.

suggested a considerably younger most recent common ancestor for the North American populations compared to that estimated by BEAST. These results imply that the glacial development during the late Pleistocene has probably influenced the demographic dynamics of the North American populations, but did not drive the lineage isolation from their Eurasian relatives.

The TMRCA of the north Eurasian clade according to the EBSP is longer than for the east Asian and North American clades, and it experienced a long period of population stability before the late Pleistocene expansion. This result is different from previous studies of other passerines, according to which east Asian lineages usually have older population histories than those from the western part of the Eurasian continent (Song et al. 2015, Li et al. 2016). We suggest that during the late Pleistocene there were more open steppe and scrub habitats and a more homogenous landscape across northern Eurasia than further south in eastern Asia. The cooler and drier climate during the late Pleistocene (Allen et al. 2010) was probably not too harsh for magpies to survive in most of northern Eurasia. East Asia features more topographical complexity, with more mesic climate and forests that were maintained through the glacial cycles (Harrison et al. 2001). This shortage of favourable habitats may have constrained the magpie populations in east

Asia into more localized areas, resulting in a shorter population history than in north Eurasia.

It could be expected that populations with large distributions would have experienced variable climatic effects in different types of habitats. Magpies thus provide an ideal study case of how climatic changes have affected lineages inhabiting different habitats through time. Previous studies have suggested that east Asian birds generally experienced Pleistocene population growth earlier than European and North American birds, and that their population dynamics were less affected by the glaciations in the late Pleistocene (Song et al. 2009, Lei et al. 2015). By contrast, our study records demographic expansion in all the three regional clades (east Asia, North America, and north Eurasia) within the time scale 0.02–0.06 Ma. The consistent expansion pattern indicates that magpies across the Eurasian continent and North America experienced a population growth at the same cooling transition time between the last interglacial and the last glacial maximum.

Within the north Eurasian and east Asian clades, the gene flow is notable among geographical populations, as expected for birds of opportunistic habits inhabiting climatically unstable regions at high latitudes. According to the matrixes of immigrant/emigrant ratios, there was an eastward population

expansion within the north Eurasian clade originating from southwestern Europe (Iberia). This direction of population expansion coincided with the expansion of sparsely wooded habitats across the continent from west to east, and the vegetation change promoted the spread of cold-tolerant herbivores such as woolly mammoth *Mammuthus primigenius* and giant deer *Megaloceros giganteus*, as well as *Homo sapiens* (Finlayson and Carrion 2007). As scavengers, magpies could follow carnivore predators and humans to feed on carcasses of large mammals during harsh times. With regard to the east Asia clade, asymmetric gene flow from the southwestern and central parts of the range towards the periphery implies expansion of non-forest environment in central and eastern China due to the cooler and drier climate during the cooling transition time (Ni et al. 2010).

### Taxonomic implications

Our results do not support the classification of *Pica* as three (*P. pica*, *P. hudsonia* and *P. nutalli*; Dickinson and Christidis 2014, Gill and Donsker 2017), four (also *P. asirensis*; Madge 2009), or five (also *P. mauritanica*; del Hoyo et al. 2016) species, as all of these treatments are inconsistent with the mtDNA tree. The six primary clades in our mtDNA tree have been separated for considerable time (1.4–3.1 Ma). In contrast, the TMRCA of the North American clade (0.2 Ma), which includes *P. hudsonia* and *P. nutalli*, is actually younger than the divergences within three of the other primary clades.

The northwest African endemic *P. p. mauritanica* has recently been given species status because of its bright blue facial skin in combination with slight plumage and more pronounced structural differences (del Hoyo et al. 2016). It might also have distinctive vocalizations (reviewed by Ebels 2003, Kryukov et al. 2017), although this needs further study. In our mtDNA tree, it is sister to the other taxa, and hence most divergent. The recognition of the geographically isolated Saudi Arabian endemic *P. p. asirensis* as a distinct species was recently proposed based on its geographical isolation and differences in structure and plumage and probably also vocalizations from other *P. pica* taxa (Madge 2009, del Hoyo et al. 2016). Others have noted its vocalizations to be highly distinctive (reviewed by Ebels 2003). Its distinctness is supported by our mtDNA data.

*Pica nutalli* has long been considered a distinct species because of its morphological distinctness (mainly yellow bill and facial skin), whereas *P. hudsonia* was only rather recently split from *P. pica* based on morphological, behavioural and mitochondrial restriction fragment profiles (Banks et al. 2000). According to our mtDNA data, *P. hudsonia* and *P. nutalli* are not clearly separable, although the mtDNA indicates some differentiation between them. More research is needed to evaluate whether the poor genetic differentiation is due to recent divergence or introgression. As they have non-overlapping ranges, and no hybrid zone between them is known (Marzluff and de Juana 2017, Marzluff and Sharpe 2017), recent introgression is unlikely to explain the pattern. It seems likely that the striking bare part differences between

*P. nutalli* and the others could have evolved rapidly. The bare facial skin might be considered a pedomorphic feature, as it is often shown (though not brightly coloured) by juveniles of several subspecies of *P. pica* (Madge 2009, unpubl.).

The east Asian clade, comprising *P. p. serica* and *P. p. anderssoni*, is highly distinct from the other taxa in the mtDNA tree, in agreement with previous studies (Lee et al. 2003, Haring et al. 2007, Zhang et al. 2012, Kryukov et al. 2017; though Haring et al. 2007 and Kryukov et al. 2017 used the name *jankowskii* for the population we referred to as *anderssoni*). We found no evidence of genetic differentiation between *P. p. serica* and *P. p. anderssoni*, which differ from each other in average size and colour hues (Madge 2009), while these two have been found to differ vocally from the others (Kryukov et al. 2017) as well as in plumage and structure (Madge 2009).

Also the Qinghai-Tibet clade, which comprises only *P. p. bottanensis*, represents a rather anciently diverged lineage. Both the mtDNA and nuclear introns data support that it is highly distinct from the geographically adjacent east Asia clade (*P. p. serica*), from which it also differs morphologically (Madge 2009) and vocally (Kryukov et al. 2017). Moreover, these two clades are mainly or entirely ecologically separated, one inhabiting the high Qinghai-Tibetan plateau and the other the lowlands of east China and neighbouring areas. Based on examination of museum specimens, Kryukov et al. (2017) suggested that *P. p. bottanensis* and *P. p. serica* do not interbreed, although it is unknown whether their ranges are in contact.

The six subspecies in the north Eurasian clade are not significantly differentiated according to our data, except for marginal divergence of the geographically and morphologically distinct *P. p. melanotos* and *P. p. camtschatica* (cf. Madge 2009, del Hoyo et al. 2016). This is partly in agreement with Kryukov et al. (2017).

All of the *Pica* taxa have allo-/parapatric distributions (Madge 2009, del Hoyo et al. 2016). Kryukov et al. (2017) reported a geographical gap between *jankowskii* (i.e. *anderssoni* in our study) and *leucoptera*, which is shrinking due to range expansion. Sporadic recent contact between these taxa shows indications of partial reproductive isolation.

Based on the available data, any taxonomic treatment of the *Pica* complex is subjective and open to discussion. As suggested by Lee et al. (2003) and Madge (2009), a conservative classification could treat all taxa as a single species, *P. pica*. However, recognition of *P. asirensis*, *P. mauritanica*, *P. hudsonia* and *P. nutalli* as separate species from *P. pica* sensu stricto, as proposed by del Hoyo et al. (2016), would require further splitting of *P. pica*. Kryukov et al. (2017) suggested that the four deeply diverged lineages found in their study (corresponding to our north Eurasian, east Asian, *P. p. mauritanica* and *P. p. hudsonia*; *P. p. nutalli* not analyzed by Kryukov et al.) could plausibly be treated as four species, further supported by differences in vocalizations. We agree, provided also the Qinghai-Tibetan clade is treated as a distinct species, which is also supported by morphology and vocalizations. With respect to *P. nutalli*, we provisionally support species rank,



although it could equally well be treated as a subspecies of *P. hudsonia*.

In summary, we advocate the following revised taxonomy: *Pica pica* (Linnaeus, 1758) sensu stricto (comprising the six subspecies in the north Eurasian clade); *P. mauritanica* Malherbe, 1845 (monotypic); *P. asirensis* Bates, 1936; *P. bottanensis* Delessert, 1840 (monotypic); *P. serica* Gould, 1845 (with subspecies *P. s. serica* and *P. s. anderssoni* Lönnberg, 1923); *P. hudsonia* (Sabine, 1823); and *P. nutalli* (Audubon, 1837).

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Supplementary material (Appendix JAV-01612 at <[www.avianbiology.org/appendix/jav-01612](http://www.avianbiology.org/appendix/jav-01612)>). Appendix 1–6.