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Inferring the phylogeny of disjunct populations of the azure-winged magpie *Cyanopica cyanus* from mitochondrial control region sequences†

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The azure-winged magpie (AWM), *Cyanopica cyanus*, is found in Asia and Iberia. This remarkable disjunct distribution has been variously explained by either the sixteenth-century introduction of birds into Iberia from the Far East, or by the loss of individuals from the central part of their range as a result of Pleistocene glaciations. We have used the mitochondrial control region to undertake a molecular phylogenetic analysis of the AWM, with sequences examined from individuals collected from across the current distribution range and incorporating representatives of all currently defined subspecies. The Western birds are genetically distinct from their Asian congeners and their divergence is basal in the phylogenetic tree. This indicates that the AWM is native to Iberia and not the result of a recent introduction from Asia. In Asia, two major mitochondrial DNA lineages were identified. These correspond to an Inland Asia group and a Pacific Seaboard group, and are separated topographically by the Da Hingan Ling mountains and the Yellow Sea. Molecular clock estimates suggest that these divergences are associated with Pleistocene glaciations. Furthermore, our data do not support the current classification of the AWM into 10 subspecies, as defined based on morphology and geographical distribution.

Keywords: azure-winged magpie; *Cyanopica cyanus*; mitochondrial control region; molecular phylogeny; phylogeography; taxonomy

1. INTRODUCTION

The azure-winged magpie (AWM), *Cyanopica cyanus*, has one of the most remarkable distributions among birds. It occurs widely in the eastern Palaearctic across much of China, Korea, Japan, northern Mongolia and southeastern Russia (figure 1). It is also found 9000 km away in a smaller geographical area in Spain and Portugal (Goodwin 1986). In the eastern Palaearctic, eight or perhaps nine subspecies have been recognized, based upon plumage variation and geographical distribution (Vaurie 1959). In Spain and Portugal (Iberia), two subspecies have been described, although their validity is questionable (Cramp & Perrins 1994). Differences are most marked between the Asiatic and Iberian forms. The former are slightly larger, with a longer tail, and have a paler blue plumage with a pallid tip to the central tail feathers (Goodwin 1986).

The extremely disjunct distribution of *C. cyanus* has attracted interest since the middle of the nineteenth century (Wallace 1881) and two alternative theories have been put forward to explain the origin of the Iberian birds. The first, the 'introduction theory', proposes that the Iberian population was established in the sixteenth century following the introduction of birds from the Far East by Portuguese sailors (Dos Santos 1968; Sacarrão 1974; Tyrberg 1998). The second, the 'refugium theory', proposes that the species had a continuous distribution across the Palaearctic prior to the Late Pleistocene, with the Asiatic and Iberian populations becoming isolated in refugia as the ice advanced (Sacarrão 1974; Harrison 1982; Goodwin 1986). There was no conclusive evidence in support of either theory until the recent discovery of AWM bones in cave deposits in Gibraltar that were 44 100 years old (Cooper & Voous 1999; Cooper 2000; though see comments by Voous, in Cooper & Voous (1999), for alternative explanations of the identity of the fossil bones). Assuming that these do not originate from an unknown species, they provide compelling evidence that *C. cyanus* is native to Western Europe and not a recent introduction.

Pleistocene climatic cycles have been invoked to explain disjunct populations of animals in habitat refugia and it is generally accepted that these climatic fluctuations have played an important role in intraspecific diversification and species range modification (Klicka & Zink 1997; Avise & Walker 1998). The disjunct distribution of *C. cyanus* is potentially one of the most extreme products of such climatic events. It is therefore pertinent that a genetic study of the AWM be undertaken in order to investigate the origin of the Iberian population and the extent (and possibly time) of divergence between the Asiatic and Iberian forms. It would be instructive to examine the genetic variation within the Asiatic and Iberian populations in parallel with this.

In this paper, we present, to our knowledge, the first molecular study of genetic variation and evolutionary relationships in the AWM. The control region (mtDNA CR; D-loop) has proved very informative in analysing genetic relationships among closely related species and for investigating intraspecific evolution (e.g. Avise 1994; Arctander *et al.* 1996; Bensch & Hasselquist 1999; Milot *et al.* 2000). Here, we use the mtDNA CR to investigate

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† We dedicate this paper to Professor Karel Voous (23 June 1920–31 January 2002), a giant of Palaearctic ornithology, who commented on this paper for us shortly before his death. He was admired and respected throughout the world for his leadership, scholarship and enthusiasm for the field that has become known as phylogeography.

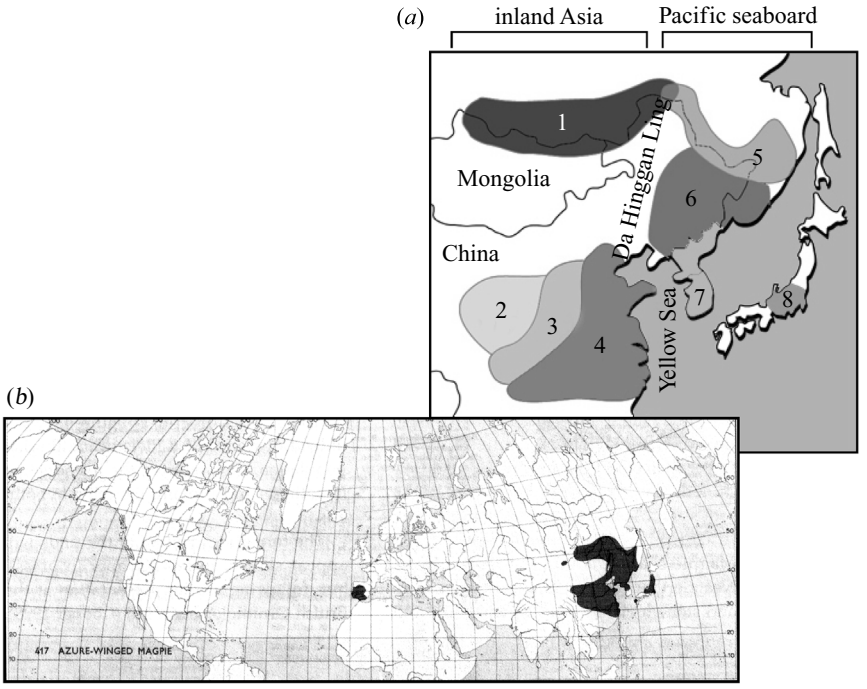

Figure 1. (*a*) An outline of the current distribution of *Cyanopica cyanus* according to Vaurie's (1959) descriptions. Asiatic subspecies: 1, *C. c. cyanus*; 2, *C. c. kansuensis*; 3, *C. c. interposita*; 4, *C. c. swinhoei*; 5, *C. c. pallescens*; 6, *C. c. stegmanni*; 7, *C. c. koreensis*; 8, *C. c. japonica*. (*b*) Current distribution of *C. cyanus* (from Voous 1960). The Iberian subspecies *C. c. cooki* and *C. c. gili* are distributed across the shaded area in Spain and Portugal (see shaded area, left).

genetic variation among all described subspecies of AWM in order to address the following questions.

(i) What is the extent of genetic divergence between the Asiatic and Iberian forms?
(ii) At what time did the Asiatic and Iberian forms diverge?
(iii) Do the Asiatic and Iberian populations merit designation as separate species?
(iv) What are the implications for the taxonomic status of currently recognized subspecies within the Asiatic and Iberian groups?

2. MATERIAL AND METHODS

(a) *Samples*

(i) *Fresh samples*

Blood samples were obtained from 24 adults and 93 offspring from a population in the south of Spain (labelled S1–S117 in electronic Appendix A, table 1, available on The Royal Society's Publications Web site). Six blood samples were collected from birds in an area near Beijing (C1–C6, electronic Appendix A, table 1). Six feather samples were collected from live birds trapped in various parts of central Japan (J1–J6, electronic Appendix A, table 1).

(ii) *Museum specimens*

Footpad samples were taken from study skins in museum collections to complete the sampling of all subspecies (samples 1–32, electronic Appendix A, table 1).

Blood samples were stored in Queen's lysis buffer (Seutin *et al.* 1991). Feathers were kept in re-sealable plastic bags with absorbent paper to limit moisture. The samples of foot tissue were stored dry.

(b) *DNA extraction*

Whole genomic DNA was isolated and purified from blood samples following standard proteinase-K/phenol extraction procedures (see Bruford *et al.* 1998). DNA was isolated from feathers by Chelex extraction of a single feather shaft. For the museum samples, DNA was extracted from a 3 mm piece of footpad using the QIAeasy Tissue Kit (Qiagen) following the manufacturer's instructions. For the museum samples, negative controls were performed at all stages in order to detect any contamination, and extractions and PCRs were undertaken in a different laboratory. All equipment and consumables were sterilized with ultraviolet (UV) light for at least 1 h before use.

(c) *PCR amplification and sequencing*

A *ca.* 1367 bp fragment of the mtDNA CR was amplified using PCR. Primers JCR01 and H417, R1SJ and CR2, and JCR13 and H1248 were used for the fresh blood and feather samples collected from Spain, Beijing and Japan (for primer details, see electronic Appendix A, figure 1, available on The Royal Society's Publications Web site). Since the DNA from the museum specimens was more degraded, the sequence data derived from the blood samples were used to design AWM-specific primers for the amplification of smaller fragments (see electronic Appendix A, figure 1). In order to confirm that no nuclear mitochondrial pseudogenes (Numts; see Bensasson *et al.* 2001) were co-amplified using these primers, we used a standard single-stranded conformation polymorphism analysis of the family groups collected in Spain to confirm the heritage of the amplified products (assuming maternal inheritance of the mitochondrial genes).

Reactions (25 µl) were carried out in 0.2 ml reaction tubes and overlaid with 20 µl of mineral oil. The reaction used 10–20 ng DNA (or 3–5 µl of the final eluate for the museum samples), 10 pM of each primer, 100 µM dNTPs, 2.5 mM $MgCl_2$, reaction buffer (0.75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$ and 0.01% [v/v] Tween20) and 0.5 units of Red Hot Taq DNA polymerase (Advanced Bioenzymes). Amplifications were run in a PTC 200 Thermal Cycler (MJ Research) with a typical profile of 30 cycles (45 for museum samples), each consisting of 60 s at 95 °C, 40 s at 57 °C and 60 s at 72 °C, with a final extension of 10 min at 72 °C.

Products were run on a 2% agarose gel to confirm amplification and lack of contamination. Fragments of amplified DNA were excised, and purified with the Qiagen Gel Extraction Kit as recommended by the manufacturer. Sequencing was carried out using 4 µl of purified PCR product, 2.5 µl of sterile distilled water, 0.5 µl of a single-sequencing primer (10 µM) and 3 µl of BigDye Sequencing reaction mix (ABI Dyeterminator chemistry). The sequencing cycles and purification of products were as described in the manufacturer's instructions and the products were run on an ABI PRISM-377 automated sequencer (Perkin Elmer).

(d) *Data analysis*

Sequences of the DNA fragments were assembled manually, and the complete CR sequence alignments were obtained using the CLUSTAL program in the BioEdit Sequence Alignment Editor (BIOEDIT v. 5.0.5; Hall 1999) with default parameters and subsequent manual adjustment. After the removal of ambiguous sequences near the priming sites at the 5′- and 3′-ends of the amplified fragment, 1314 sites were available for subsequent analyses.

AWMs, other jays and crows are part of the family Corvidae. Although *C. cyanus* has retained many similarities in structure with other magpies, such as the black-billed magpie *Pica pica*, they are generally thought to be more closely related to the jays because of their similarity in size and behaviour (Madge & Burn 1994). Consequently, the choice of a suitable outgroup for rooting the phylogeny is not obvious. We therefore sequenced the complete CR from a black-billed magpie collected as a road-kill near Nottingham, UK. We also obtained CR sequences of yellow-billed magpie *Pica nuttalli*, Siberian jay *Perisoreus infaustus* (an Old World jay), Steller's jay *Cyanocitta stelleri* (a New World jay) and rook *Corvus frugilegus* (an Old World corvid) from GenBank (reference numbers AF218936, AF131078, AF218922 and AF090341, respectively). The phylogenetic tree included all of these, and was rooted with the rook *C. frugilegus* (definitely the most distant). Phylogenetic trees were constructed using the neighbour-joining (NJ; Saitou & Nei 1987) and Fitch–Margoliash (FM; Fitch & Margoliash 1967) distance methods and the maximum-parsimony (MP) method within the PAUP* v. 4.0 package (Swofford 1998). In addition, maximum-likelihood (ML; Felsenstein 1981) trees were constructed using TREEPUZZLE v. 5.0 (Schmidt *et al.* 2002).

Evolutionary trees were constructed using four different methods because consistency of tree topology across methods provides insight into the robustness of phylogenetic hypotheses. Gaps and ambiguous alignment positions were removed prior to analysis resulting in datasets of 1174 bp with outgroup sequences and 1302 bp without outgroups. For the NJ and FM trees, distances were corrected for multiple hits using the general time-reversible model. The rate matrix, base frequencies, proportion of invariant sites (pinvar) and shape parameter (α) of the gamma distribution (based on 16 rate categories) were estimated using likelihood by iteration from an initial NJ tree. Tree searching for FM and MP methods was performed using a heuristic search procedure with tree-bisection–reconnection branch swapping. ML trees were constructed using quartet puzzling, with correction for multiple hits using the Tamura–Nei 1993 model (TN93; Tamura & Nei 1993) with a discrete gamma model of 16 categories. Parameters for among-site rate variation (α) and transition bias parameter (κ) were estimated from the data using ML in TREEPUZZLE. Bootstrap re-sampling was used to assign support to branches in the NJ and MP trees (1000 and 100 bootstrap replications, respectively). The internal node support values for the ML trees (which are analogous to bootstrap values) were based on 10 000 puzzling steps. Corrected nucleotide diversity (π) between all sequences was obtained using PAUP* v. 4.0 under the GTR model of evolution as above. Time of divergence was estimated using the equation $T = k/2r$, where k is the mean sequence divergence between lineages and r is the rate of nucleotide substitution (Li 1997).

(e) *Nucleotide sequence accession numbers*

Nucleotide sequences in this study have been given the GenBank accession numbers AJ458536 to AJ458573.

3. RESULTS

We have amplified part of the mitochondrial control region for over 149 specimens of the AWM. In general, the amplified region was 1367 bp in length, though in the Iberian birds we found that the 5′-end was *ca.* 10 bp shorter than in the Oriental taxa. Specimens were collected widely from across the geographical distribution of *C. cyanus* and included at least one representative of all currently recognized subspecies (electronic Appendix A, table 1). Museum specimens proved to be an excellent source of DNA in this study and we obtained complete CR sequences from 20 out of 32 samples (electronic Appendix A, table 1).

(a) *Mitochondrial inheritance*

The comprehensive blood samples obtained from individuals belonging to 19 broods from a population in the south of Spain (electronic Appendix A, table 1) afforded an opportunity to examine the inheritance of the mitochondrial control region in the azure-winged magpie. In the 19 broods examined, both parents were trapped from five broods, four had only the female parent trapped, four only the male, and the remaining six had neither parent trapped. Within each nest, all offspring possessed identical CR haplotypes. In all cases where the female parent was trapped (9 out of the 19 broods), the CR sequence of the offspring was identical to that of their mother. In all cases where the male parent was trapped (9 out of the 19 broods) the CR sequence of the offspring was different to that of the father. The data therefore support the maternal inheritance of the mitochondrial CR sequences in the AWM. Furthermore, there is no evidence that any CR sequences obtained in this study are paternal (or indeed nuclear) in origin.

(b) *Haplotypes*

After the removal of ambiguous sequences near the priming sites at both ends of the amplified fragment, 1314

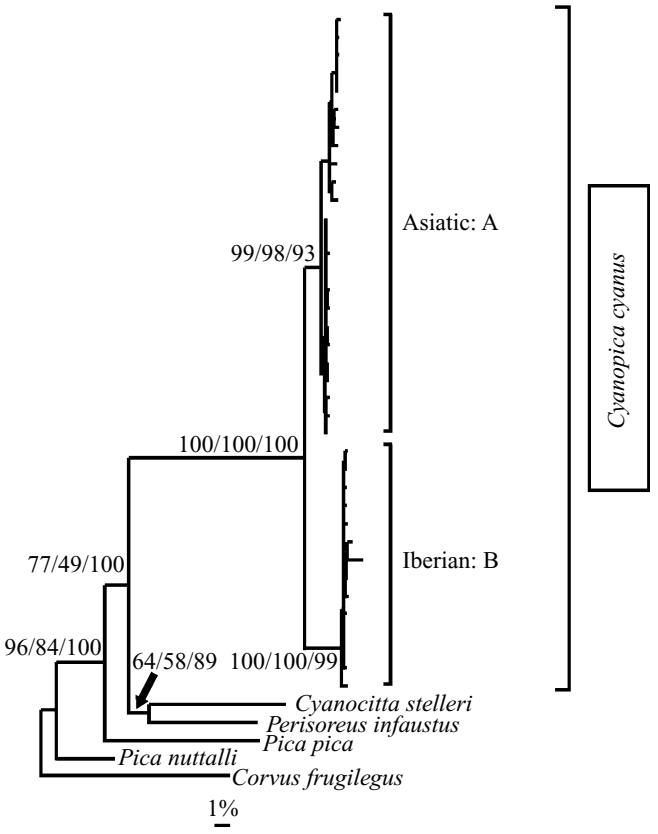

Figure 2. NJ phylogeny showing the relationship of *Cyanopica cyanus* to other Corvidae. The phylogeny is rooted on the rook *Corvus frugilegus*. Numbers on the branches represent the bootstrap support values using NJ/MP/ML methods of tree construction. The tree is based on 1174 nucleotide sites (proportion of invariant sites = 0.536; $\alpha = 2.994$). Trees constructed using transversions only are identical in the inferred relationships among the corvids.

sites remained available for use in subsequent analyses. We identified 37 different haplotypes among the *C. cyanus* specimens for which the complete 1314 bp CR fragment was obtained. Of the fresh samples, nine haplotypes were found among individuals from Spain, four haplotypes were found in the Japanese samples, and five haplotypes were found among the samples from Beijing. Nineteen further haplotypes were identified among the museum specimens.

(c) *Phylogenetic analysis*

An evolutionary tree showing the relationships of *Cyanopica* specimens to other corvids is shown in figure 2. The tree is rooted on the rook (*C. frugilegus*) and was based on 1174 nucleotide sites that were unambiguously aligned across all taxa. These included 373 polymorphic sites of which 227 were parsimony informative. All tree construction methods employed (NJ, FM, MP and ML) produced similar topologies. *Cyanopica* forms a clearly defined monophyletic group in the CR tree that is strongly supported in all analyses (100%; figure 2). Within the AWM clade, the *C. cyanus* haplotypes are principally divided into two groups, an Asiatic clade (A) and an Iberian clade (B). Both clades are strongly supported in all methods of tree construction, with bootstrap support values of ⩾93% and ⩾99%, respectively (figure 2). With all tree methods, *Cyanopica* is more closely related to the jays *Perisoreus* and *Cyanocitta* than to the magpie *Pica* (77% NJ, 49% MP and 100%; figure 2). However, caution must be exercised when interpreting this relationship as the control region sequences are highly divergent and there is some saturation of transitions. Nevertheless, the closer relationship of the AWM to the jays than to other magpies is also inferred in trees constructed using transversions only, where there is no evidence of saturation (data not shown).

In order to investigate the relationships within the AWM clade in greater detail, sequences of other corvids were excluded from the analysis. Their removal permits the inclusion of a further 128 unambiguously aligned sites in analyses of the AWM alone. This increases the number of sites used in the phylogenetic analyses to 1302 (including 103 polymorphic sites of which 72 are parsimony informative). The AWM tree (figure 3) is rooted on the Iberian clade (shown to fall outside of the Asiatic clade in figure 2). Based upon this alignment, the Iberian birds (B) are separated from the Asiatic birds (A) by a mean genetic distance of 6.06% (s.d. = 0.32), with intraclade distances of 0.99% (s.d. = 0.61) and 0.35% (s.d. = 0.18) for the Asiatic and Iberian clades, respectively (table 1). In the Asiatic clade, the CR haplotypes are further separated into two distinct groups, A1 and A2. Both of these are strongly supported with all methods of tree construction, with bootstrap support values of ⩾91% and ⩾87%, respectively (figure 3). The genetic distance within the Asiatic clades A1 and A2 are 0.64% (s.d. = 0.27) and 0.18% (s.d. = 0.09), respectively, and they are separated by a mean genetic distance of 1.47% (s.d. = 0.13; table 1). Finally, within clade A1, the Japanese haplotypes form a distinct monophyletic group supported in ⩾97% of bootstrap support values. The mean genetic distance among the Japanese haplotypes is 0.16% (s.d. = 0.06) and they are separated from their mainland clade A1 congeners by a distance of 0.78% (s.d. = 0.12; table 1).

In addition to these analyses, we examined the phylogenetic placement of the further five haplotypes for which only a partial fragment was available (PCR fragment B and/or H; electronic Appendix A, table 1). The placement of these sequences into clades A1, A2 and B were consistent with other members of the species from the same localities (data not shown).

4. DISCUSSION

(a) *Nuclear pseudogenes*

Nuclear copies of mitochondrial genes have been documented in a wide variety of organisms (for a review, see Bensasson *et al.* (2001)) and there is widespread concern over their effects on studies of molecular systematics and population biology (Sorenson & Quinn 1998). We are confident that all of the CR sequences obtained in this study are mitochondrial in origin. We detected no evidence of multiple copies of the CR in any of our sequences suggesting that our primers had not amplified a mixture of mitochondrial and nuclear copies. Furthermore, our studies of Spanish broods demonstrated that the CR was inherited maternally with all nest-mates having the same maternal CR haplotype.

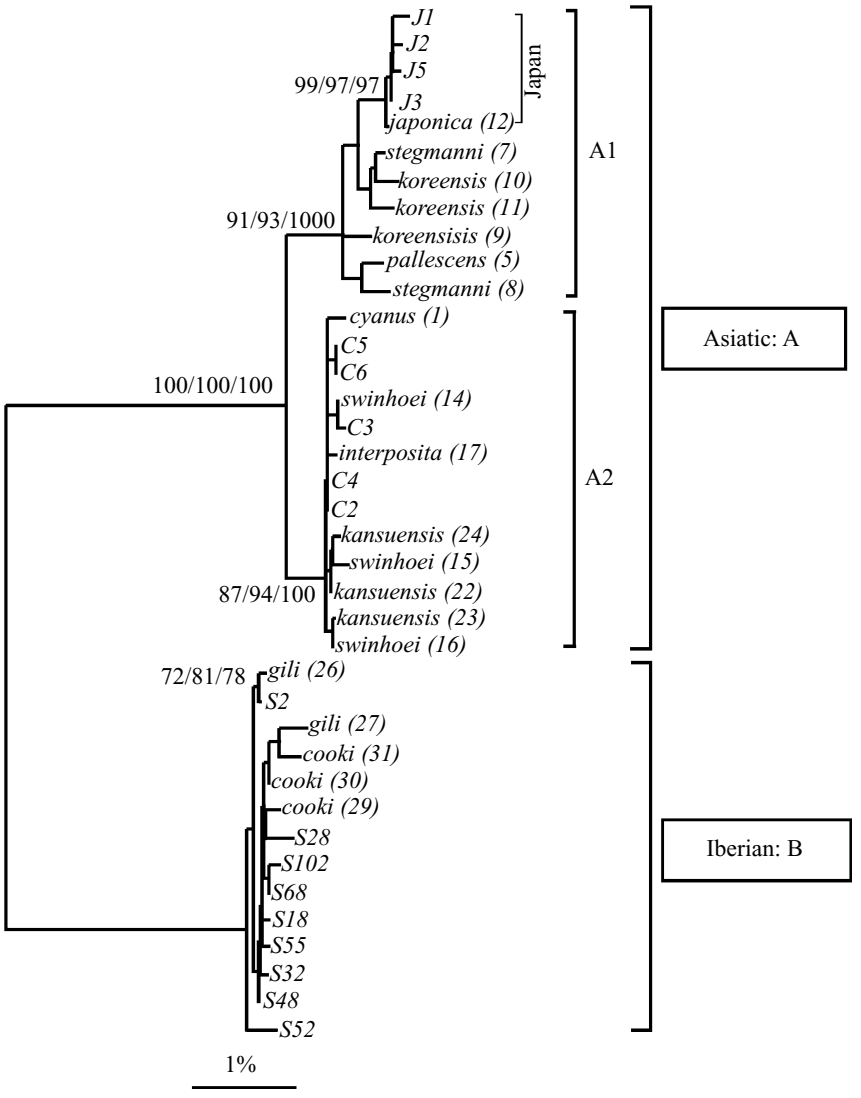

Figure 3. NJ tree showing the relationships among *Cyanopica cyanus* haplotypes. The phylogeny is rooted on the Iberian clade (shown to fall outside the Asiatic clade in figure 2). Numbers on the branches represent the bootstrap support values using NJ/MP/ML methods of tree construction and are shown for only those branches that have at least 70% support with all three methods. *C. cyanus* haplotypes are detailed in table 1 in electronic Appendix A. The tree is based on 1302 nucleotide sites (proportion of invariant sites = 0.344; $\alpha = 0.335$).

(b) *Evolutionary origins and phylogeography of* Cyanopica cyanus

The relationship of the AWM to the jays and magpies has long been a subject of debate (Madge & Burn 1994). Our analyses suggest that the AWM is more closely related to the Old and New World jays, such as *Perisoreus* and *Cyanocitta*, than to magpies of the genus *Pica* (figure 2), though this finding must be interpreted with caution (see § 3). This apparent relationship is consistent with some morphological and behavioural studies (see Goodwin 1986; Madge & Burn 1994).

The AWM CR haplotypes fall into two significant phylogenetic units corresponding to the Asiatic birds (clade A) and Iberian birds (clade B; figure 2). This forms the basal division within the *C. cyanus* clade and provides the first molecular evidence that the western birds are genetically distinct from their Asian congeners. If the Iberian birds were the result of a sixteenth-century introduction from the Far East, we would expect little or no genetic differentiation between eastern and western forms. The molecular data therefore conclusively demonstrate that *C. cyanus* is native to Iberia and not the result of a relatively recent introduction. This supports the 'refugium theory' (Sacarrão 1974; Harrison 1982; Goodwin 1986), which invokes Late Pleistocene glaciation events as an explanation for the current distribution. The role of Pleistocene glaciations in the differentiation of species and populations is still debated (Klicka & Zink 1997; Avise & Walker 1998). However, it is generally accepted that these events play an important role in intraspecific diversification and species range modification (see Hewitt 1996, 1999) and they have been proposed as an explanation for the disjunct distributions observed in many taxa (see Selander 1971; Gill 1995). Nevertheless, it is probable that the Asiatic and Iberian birds were already undergoing genetic differentiation as a result of isolation by distance (Wright 1943) and possibly topographical barriers to dispersal even before the populations of AWM became extinct across the central part of its range. Whatever the cause, there is no doubt that the Iberian populations long

Table 1. Genetic distances (%) between and within clades of *Cyanopica cyanus*.
(Distances were calculated based on 1302 nucleotide sites and corrected for multiple hits using the GTR model incorporating a proportion of invariant sites [pinvar = 0.344] and gamma distribution [$\alpha = 0.335$]. Approximate divergence times were estimated using an evolutionary rate of 5% per million years. This corresponds to a nucleotide substitution rate of 0.025 per site per million years.)

region	mean	s.d.	range	divergence (years)	range (years)
within clades					
Iberian B	0.35	0.18	0.08–0.89	70 000	16 000–178 000
Asiatic	0.99	0.61	0.08–1.76	198 000	16 000–352 000
A1	0.64	0.27	0.08–0.91	128 000	16 000–182 000
A1 less Japan	0.68	0.2	0.32–0.91	136 000	64 000–182 000
within Japan	0.16	0.06	0.08–0.24	32 000	16 000–48 000
A2	0.18	0.09	0.00–0.39	36 000	0–78 000
between clades					
Asiatic A–*Iberian* B	6.06	0.32	5.49–6.83	1 212 000	1 098 000–1 366 000
A1–B	6.33	0.2	5.77–6.83	1 266 000	1 154 000–1 366 000
A2–B	5.82	0.19	5.49–6.38	1 164 000	1 098 000–1 276 000
A1–A2	1.47	0.13	1.15–1.76	294 000	230 000–352 000
Japan–A1 less Japan	0.78	0.12	0.56–0.98	156 000	112 000–196 000

predate any possible introduction by sixteenth-century sailors.

In Asia, the eastern group is further divided into two monophyletic groups (clades A1 and A2; figure 3). Clade A1 includes birds from the Pacific seaboard (North East China, Korea and Japan) and comprises subspecies *pallescens*, *stegmanni*, *koreensis* and *japonica*. Clade A2 includes birds from inland Asia (North Central China–Mongolia) comprising subspecies *cyanus*, *kansuensis*, *interposita* and *swinhoei*. Although the sample sizes are small, there is no evidence of overlap between the Pacific seaboard and inland Asia haplotypes. Phylogeographically, the separation of the two major eastern clades is concordant with the topography of the region. They occur on opposite sides of the Da Hinggan Ling (Greater Khingan) mountains and the Yellow Sea (see figure 1). This indicates that the topography continues to form a serious barrier to east–west gene flow in *C. cyanus*, leading to the populations on either side evolving in comparative isolation. This process may have been exacerbated by range modification associated with Late Pleistocene glaciations (see § 4c), and it is possible that similar distribution patterns will be observed in other taxa. Within clade A1, the Japanese birds form a distinct group, presumably having evolved in isolation since their arrival in the islands.

(c) *Genetic variation and divergence times*

In order to place approximate estimates of time on divergences in the AWM phylogeny we have used a molecular clock approach. Likelihood ratio tests show that evolution in the AWM clade is approximately clock-like, with no significant difference in likelihood scores between trees constructed with or without the assumption of a clock (log-likelihood clock: −2608.696 26, log-likelihood non-clock: −2589.851 65, χ^2 ($-2\log\Lambda$) = 37.69, d.f. = 36, $p > 0.1$; see Huelsenbeck & Rannala 1997). We do not have appropriate data to permit the independent calibration of a molecular clock for the *C. cyanus* control region. However, it is possible to adopt molecular clock calibrations obtained from closely related taxa. The rate of divergence of mtDNA is generally accepted to be about 2% per million years (Brown 1984; Shields & Wilson 1987; Klicka & Zink 1997) and this rate has been applied to the control region in several studies of avian genera (e.g. gnatcatchers *Polioptila*, Zink & Blackwell 1998; guillemots *Cepphus*, Kidd & Friesen 1998; and towhees *Pipilo*, Zink *et al.* 1998). However, in many organisms, the CR has been shown to evolve more rapidly than the surrounding genes. Among avian taxa, Marshall & Baker (1997) demonstrated that the mitochondrial CR has a slightly higher evolutionary rate than the adjacent genes in the finches *Fringilla* and *Carduelis*. More recently, the rate of divergence of the CR has been estimated to be 6% per million years in Old World leaf warblers *Phylloscopus* (Irwin *et al.* 2001) based on a partial fragment of the CR, and 5% per million years in Darwin's finches *Geospizinae* (Freeland & Boag 1999) based on the complete CR. In this study, we have adopted a divergence rate of 5% per million years (following Freeland & Boag 1999) to estimate *approximate* divergence times in our studies of complete CR sequences of the AWM.

Using this value, the Asiatic and Iberian birds appear to have been isolated for as long as 1.2 million years (table 1). In Asia, the Pacific seaboard clade (A1) and the inland Asia clade (A2) are estimated to have diverged 294 000 years ago and within clade A1, the Japanese taxa are estimated to have diverged from their mainland sisters 156 000 years ago.

These divergence times are only estimates but, even allowing for their imprecision, we can be fairly certain that these divergences are during the Mid- to Late Pleistocene. The impact of the cold Pleistocene climatic cycles on the fauna and flora of the Palaearctic was substantial, and many species became extinct or lost from large parts of their former ranges, promoting substantial microgenetic diversification in many species (Hewitt 1996; Klicka & Zink 1997; Avise & Walker 1998). We hypothesize that the azure-winged magpie persisted in three refugia (Iberia,

Central China and East China/Korea/Japan) during one or more recent glacial advances, with these individuals becoming the founders of modern-day populations.

The extent of nucleotide diversity within clades (0.08–0.91; table 1) is broadly comparable with that found in greenfinches *Carduelis chloris* across Europe (0.39%; Merila *et al.* 1997) and rock ptarmigan *Lagopus mutus* in the Bering region (0.36%; Holder *et al.* 2000), but rather less than the 1.1% reported for ravens *Corvus corax* (Omland *et al.* 2000). Among the inland China subspecies (clade A2), the haplotype divergence is 0.18%, corresponding to a divergence time of about 36 000 years. A broadly similar value of 32 000 years was obtained for divergence among Japanese birds. Conversely, the coalescence times for the other two clades were slightly older. Within Spain and Portugal, the corrected average nucleotide divergence between haplotypes was 0.35% corresponding to 70 000 years of evolution. A rather higher within-clade average was observed among the Pacific Seaboard subspecies (clade A1; 0.64%), suggesting that the coalescence time could be 128 000 years. However, all of these estimates of within-clade coalescent times are consistent with the last Ice Age.

Following the retreat of the glacial ice at the end of the Pleistocene, many Northern Hemisphere species have been shown to have expanded northwards from their refugia back into their former ranges (e.g. Hewitt 1996, 1999). In Iberia, *C. cyanus* does not seem to be expanding to re-establish its former range across the Palaearctic, but is instead apparently restricted to parts of Spain and Portugal. Similar patterns of postglacial dispersal have been observed in other species in the Iberian region (e.g. Bilton *et al.* 1998; Hewitt 1999) which indicates the possible existence of physical barriers to expansion. By contrast, in Asia, the current ranges of subspecies *pallescens* (A1) and *cyanus* (A2) partially overlap in an area of introgression at the northern edge of the Da Hinggan Ling mountains (Vaurie 1959; figure 1). This region (west of Dzhalinda) is probably due to secondary contact consequent upon the northward spread of two refugia populations following the last Ice Age (20 000 years before present; Adams 1997). Subsequently, the northern '*cyanus*' population seems to have become isolated from the southern populations and this could be a result of the eastward expansion of the north central China desert during the past 4000 years (Adams & Faure 1997). Our data are somewhat limited from north of Da Hinggan Ling, and it would be very interesting to examine the genetic structure and history of these populations in greater detail.

(d) *Taxonomic implications*

This study has important implications for the taxonomy of the AWM. Our findings do not support the current classification of *C. cyanus* into ten subspecies, defined on the basis of morphological characters and geographical distribution patterns (see Vaurie 1959). Sequences of the Asiatic subspecies *stegmanni*, *koreensis*, *swinhoei* and *kansuensis* are polyphyletic in the evolutionary tree (figure 3) and the molecular data therefore do not support their taxonomic distinction. Currently, only *japonica* is supported as a valid taxon in the Asiatic molecular tree, although we are unable to comment on the taxonomic validity of *pallescens*, *cyanus* and *interposita* since we only have sequences from a single individual for these subspecies. These findings are similar to other molecular studies of bird taxa, which have demonstrated that morphological and behavioural traits, such as plumage coloration, size and acoustic characters, do not always correlate with the evolutionary history of a group (e.g. song sparrow *Melospiza melodia*, Zink & Dittmann 1993; bluethroat *Luscinia svecica*, Questiau *et al.* 1998; and common crossbill *Loxia curvirostra*, Questiau *et al.* 1999).

In this study, we have demonstrated that *C. cyanus* is principally divided into two distinct groups, an Asiatic clade (A) and an Iberian clade (B). Their divergence forms the basal split in the phylogeny and is dated at 1.2 million years from our molecular clock estimates. The relatively deep genetic divergence between the European and Asian lineages allows us to conclude that Iberian *C. cyanus* are indeed a native population and the extent of their genetic divergence suggests that speciation has already taken place since their isolation. This view is supported by morphological and behavioural data (Madge & Burn 1994; Cramp & Perrins 1994). Most obviously, Iberian birds have a slightly darker blue plumage than those from Asia, and lack the pallid tips to the central tail feathers (Goodwin 1986). The two clades are separated by a molecular genetic distance of 6.06%, which is greater than comparable interspecific distances in other avian genera (e.g. gnatcatchers *Polioptila*, Zink & Blackwell 1998; tragopans *Tragopan*, Randi *et al.* 2000). On the strength of these morphological and molecular differences, and in agreement with present-day usage of narrowing the geographical species concept, we recommend that the European populations of *Cyanopica* are treated as a separate species to those from the Orient. The name *cyanus* was originally adopted by Pallas in 1776 to a type specimen from Dauria (Transbaikalia), and *cooki* by Bonaparte in 1850 to a specimen from Madrid, Spain (Vaurie 1959). We recommend, therefore, that the eastern birds continue to be named *Cyanopica cyana* (Pallas) and that Iberian birds be recognized as a separate species, *Cyanopica cooki* (Bonaparte), perhaps with the English-language name of Iberian magpie. The Iberian subspecies (*C. c. gili* and *C. c. cooki*) are at best only marginally distinguishable (Cramp & Perrins 1994; J. H. Cooper, personal communication) and since there is complete intermingling of the mitochondrial sequences within the molecular phylogeny, we can see no reason for their continued acceptance.

Among the Asiatic birds, we have found evidence for two distinct lineages: a Pacific seaboard lineage (A1) and an inland Asia lineage (A2). In general, our data do not support current subspecific definitions among the eastern birds and only the Japanese subspecies *japonica* (which falls within clade A1) appears to be sufficiently isolated to be regarded as a distinct subspecies. We are presently investigating the morphological differentiation of the currently recognized subspecies. However, from the results reported here, it might seem appropriate that the mainland Asiatic birds are reduced to two subspecies corresponding to the east–west division of the A1 and A2 lineages across the Da Hinggan Ling mountains and Yellow Sea. However, there is a need for a more comprehensive studies of genetic variation among the eastern birds

before a final taxonomy can be established for the Asiatic group.

In conclusion, this study has confirmed that European populations of the azure-winged magpie *Cyanopica cyanus* are distinct from Asiatic populations. European birds are native to Iberia and are not the result of recent introduction from Asia. The Asiatic birds are divided into two major mtDNA lineages that are likely to be the result of isolation and divergence during the Late Pleistocene climatic changes.

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