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Species delimitation in the Hwamei *Garrulax canorus*

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Due to the male's elaborate songs, the Hwamei *Garrulax canorus* is the most popular caged bird in the global Chinese community. Three allopatric Hwamei subspecies have been described: *G. c. canorus* in central and southern China and northern Indochina, *G. c. owstoni* from Hainan and *G. c. taewanus* from Taiwan. We sequenced the entire mitochondrial cytochrome *b* gene to reconstruct the molecular intraspecific phylogeny of the Hwamei. Molecular phylogenetic trees indicated that individuals of the three subspecies formed three monophyletic clades with high bootstrap support (> 95%). The basal clade was *G. c. taewanus*. According to a conventional molecular clock (2% divergence per million years), *G. c. taewanus* split from the other Hwamei taxa around 1.5 million years ago, and *G. c. owstoni* diverged from *G. c. canorus* around 0.6 million years ago. Considering the periodic connection between the Asian mainland and nearby continental islands during the glacial periods, habitat vicariance may have played a more important role than geographical vicariance in facilitating the differentiation of these taxa. Molecular diagnosability, population integrity, and concordance between the population ranges and the topology of the phylogenetic tree suggested that the Hwamei should be delimited into at least two full species: *G. canorus* and *G. taewanus*. Our work represents one of the first attempts to re-evaluate the intraspecific systematics for an eastern Asian bird species using molecular data.

The development of molecular techniques and phylogenetic theories has revolutionized our view of the process and mode of speciation (e.g. Avise 2000) and has led to the re-evaluation of avian systematics in recent decades. Species limits have been redefined and populations have been identified as full species by inspecting genealogy (e.g. Zink 1994, Baker *et al*. 1995, García-Moreno & Fjeldsa 1999). Unfortunately, the systematic status of bird species in eastern Asia, an area with high avian diversity, has hardly been examined using molecular approaches (e.g. Martens *et al*. 2004, Päckert *et al*. 2004, Olsson *et al*. 2005). Here, we re-evaluate the systematic status of an endemic Asian species, the Hwamei *Garrulax canorus*, using mitochondrial cytochrome *b* (CYTB) sequence data.

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As a common resident of the margins of lowland secondary evergreen woodlands, the Hwamei is widely distributed in central and southern China, northern Indochina, Hainan and Taiwan (MacKinnon & Phillipps 2000, Fig. 1a). Three allopatric subspecies are widely recognized: *G. c. canorus* in mainland Asia, *G. c. owstoni* in Hainan and *G. c. taewanus* in Taiwan (Berlioz 1930, Deignan 1964, Dickinson 2003). Morphologically, *G. c. taewanus* differs from other subspecies by lacking the distinct white eye-ring that continues behind the eye as a narrow streak. In addition, the upperparts of *G. c. taewanus* are more greyish than in the other subspecies, with more heavily streaked crown and nape. Compared with *G. c. canorus*, *G. c. owstoni* has paler underparts and more olive upperparts. Compared with the songs of *G. c. taewanus*, songs of the mainland subspecies *G. c. canorus* are more complex, containing significantly more syllable types, more variations in syllable changes, larger syntactic

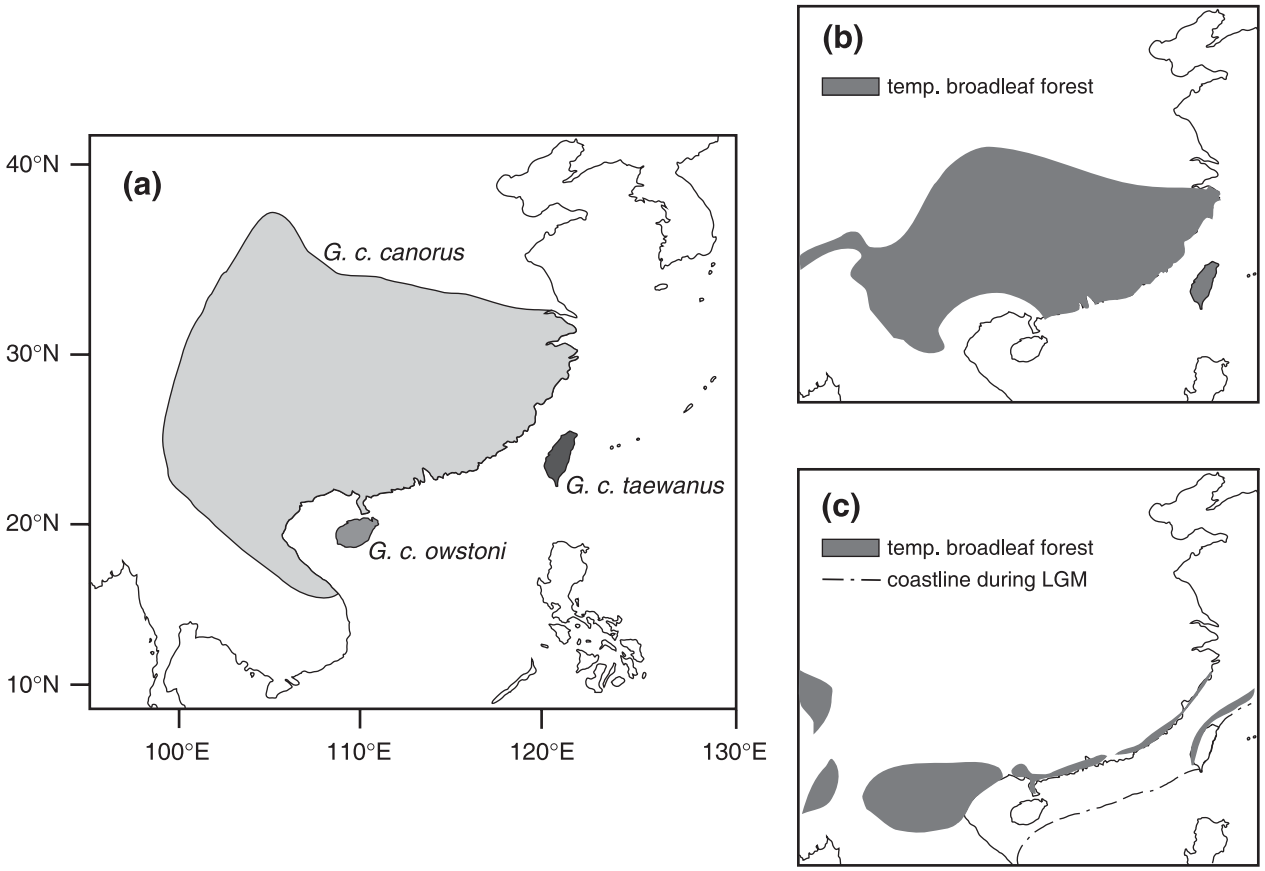

Figure 1. (a) Ranges of the three Hwamei subspecies. (b,c) Temperate broad-leaved forest in eastern Asia at present and during the last glacial maximum, respectively (modified from Harrison *et al.* 2001).

combinations and fewer repeated syllables per song (Tu & Severinghaus 2004).

The elaborate melodious song of male Hwamei has made it the most popular caged bird in China for many years. Large numbers of *G. c. canorus* have been collected to meet high market demand. Consequently, it was listed in Appendix III of CITES to regulate its international trade in 2000. In addition to over-collecting, potential hybridization (between subspecies) and introgression (between hybrids and their parental subspecies) may pose other threats to the survival of some Hwamei populations. Since the 1980s, when the nominate subspecies began to be imported into Taiwan in large quantities, potential hybrids between *G. c. taewanus* and *G. c. canorus* have frequently been observed in Taiwan (Tu & Severinghaus 2004). The viability of a population can be endangered by outbreeding depression, breakdown of locally adapted genomic configuration, and loss of genetic uniqueness through hybridization and introgression between evolutionarily distinct lineages (evolutionary significant units, ESUs; Ryder 1986, Moritz 1994). Consequently, *G. c. taewanus* has been protected by the Wildlife Conservation Law of Taiwan since 1989. As a result of the potentially severe genetic consequence of hybridization and introgression, identifying the ESUs (Barrowclough & Flesness 1996, Rhymer & Simberloff 1996) within the Hwamei and re-evaluating its intraspecific systematic status have become conservation priorities.

We examined the evolutionary identities of the three Hwamei subspecies by reconstructing their intraspecific molecular phylogeny with the full-length sequence of the mitochondrial CYTB gene. This is not only important to our understanding of the rate and mode of differentiation of these birds, but should also provide essential information to formulate conservation measures for this culturally important species. Taxonomic recommendations for the three Hwamei subspecies are made.

MATERIALS AND METHODS

Sample preparation

Liver, muscle or blood samples from *G. c. canorus* ($n = 30$), *G. c. taewanus* ($n = 13$) and *G. c. owstoni* ($n = 2$) were used to extract gross DNA. In addition, toe pad samples from three 30-year-old skin specimens of *G. c. owstoni* were used as sources of DNA. Samples of *G. c. canorus* were collected from the entire range of this continental taxon (four of each from Zhejiang, Anhwei, Shaanxi, Sichuan, Fujian, Guandong and Yunnan provinces of China and two from Hunan Province). One sample each of *G. chinensis*, *G. subunicolor*, *G. poecilorhynchus poecilorhynchus* and *G. p. berthemyi* was used as the composite outgroup to root the phylogenetic tree.

DNA was extracted from the tissue samples by traditional proteinase K digestion followed by LiCl extraction (modified from the procedure of Gemmell & Akiyama 1996). Samples were resuspended in ddH_2O and stored at –20 °C.

DNA amplification and sequencing

Two pairs of polymerase chain reaction (PCR) primers, L13653/H14296 and L14192/H14853 (Table 1), were used to amplify the entire CYTB gene in two overlapping fragments. For the toe pad samples of *G. c. owstoni*, ten additional primers (Table 1) were designed specifically to amplify and sequence CYTB gene fragments of length less than 400 bp. Less than 100 ng of genomic DNA was added to 12.5 μL of reaction mix containing 1× PCR buffer (Amersham Biological Sciences, Piscataway, NJ, USA), 80 μM of each of the four deoxynucleotide triphosphates, 0.5 μM of each PCR primer, 2 mM of $MgCl_2$ and 1 unit of *Taq* DNA polymerase (Amersham Biological Sciences). The PCR profile was 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The PCR reactions were carried out in iCyclers (Bio-Rad, Hercules, CA, USA). Both strands of the PCR products were sequenced. Sequencing was performed on a MegaBace 1000 automatic sequencer with DYEnamic ET dye Terminator kit (Amersham Biological Sciences). To ensure the accuracy of sequences, sequences from both strands were assembled into contigs and the chromatogram of each sequence was proofread by eye with the aid of the program Sequencher v.4.0 (GeneCodes, Ann Arbor, MI, USA).

Table 1. PCR primers used to amplify the mitochondrial CYTB gene in this study. Primers L13653, H14296, L14192 and H14853 were designed from the consensus sequence of the CYTB gene and its nearby regions of *Corvus frugilegus* (GenBank accession number: NC_002069, Harlid & Arnason 1999) and *Vidua chalybeata* (GenBank accession number: NC_000880, Mindell *et al.* 1999). The other ten primers were designed from sequences of the CYTB gene of *G. c. owstoni*.

Primer	Primer sequence
L13653	5′-TAGGATCTTTCGCCCTATC-3′
H14296	5′-TTGTTTGATCCTGTTTCGTG-3′
L14192	5′-CCTAGTAGAATGACTATGAGG-3′
H14853	5′-TTACAAGACCAATGTTTTTATA-3′
L13819	5′-GGTTCCCTACTAGGCATCTGTC-3′
L13821	5′-TACTAGCCCTCATAGCCA-3′
H13885	5′-CACATGTGAGCGACAGATTCGA-3
L14051	5′-AGACATGAAACGTCGGAGTCCTC-3′
H14090	5′-TTTGGCCTCAGGGTAGGACG-3′
L14273	5′-CCTAACGTCATCGCAGGACTC-3′
H14334	5′-GATTTTGTCGCAGTCTGA-3′
H14352	5′-TGGAGTAGTAGGGGTGGAATGG-3′
L14551	5′-GCCATCCTCCGATCTATCCC-3′
H14580	5′-AATAGGACTAGGACTGAGGCAGC-3′

Sequence analysis

Four estimates of DNA polymorphism were calculated for each subspecies with the software DnaSP v.3.5.3 (Rozas & Rozas 1999): *K*, haplotype number; *S*, number of segregating sites; π, average pairwise nucleotide difference per site (nucleotide diversity; Li 1997, equation 9.1); and *h*, haplotype diversity (Nei 1987, equation 8.5).

Tajima's *D* statistics (Tajima 1989) was used to detect the deviation of CYTB sequences from evolutionary neutrality assuming the infinite allele model (software DnaSP v.3.5.3). The 95% confidence interval of *D* under neutrality was calculated by the grouped simulation algorithm implemented in DnaSP v.3.5.3. One thousand simulations based on the grouping process for a neutral infinite-sites model and assuming a large constant population size (Hudson 1990) were conducted.

Molecular phylogenetic reconstruction

Using only the distinct CYTB haplotypes, three different methods were used to reconstruct the intraspecific phylogeny of the Hwamei: neighbour-joining method (MEGA2, Kumar *et al.* 2001), maximum-parsimony and maximum-likelihood (PAUP* v.4.0b10, Swofford 2002). In reconstructing phylogeny using the neighbour-joining method, to avoid incorrect topology resulting from the larger variance of distances introduced by a complex DNA substitution model (Nei 1996), the Jukes–Cantor estimate of the number of nucleotide

substitutions per site (d) was used to evaluate the appropriate distance measure (Nei & Kumar 2000). Following their recommendations, if d is less than 0.5, the p-distance would be used. Otherwise, a more complicated distance model would be employed. A heuristic search was used for maximum-parsimony: using the stepwise-addition option, sequences were added randomly with ten replicates and the tree bisection–reconnection algorithm was used for branch swapping. In maximum-likelihood reconstruction, the best substitution model was chosen by the Akaike Information Criterion in Modeltest v.3.06 (Posada & Crandall 1998). Estimated parameters were used to construct the maximum-likelihood tree via the branch-swapping algorithm (tree bisection–reconnection) in PAUP*4.0b10. The bootstrap method (1000 replicates) was used to evaluate the nodal support of maximum-parsimony, neighbour-joining and maximum-likelihood trees.

Genetic distance among subspecies

To describe the genetic differentiation between the three Hwamei subspecies, a net genetic distance was calculated by subtracting the average of intrasubspecific distance within two subspecies from the uncorrected intersubspecific distance between two subspecies (Nei & Li 1979) to remove the effect of within-subspecies polymorphism. The standard error of net distances was calculated by the bootstrap method with 1000 replicates. Both the net genetic distance and its standard error were estimated using the program MEGA2.

RESULTS

Characteristics and genetic variation of the CYTB gene

The entire CYTB sequences for the Hwamei and outgroup species were deposited in GenBank (accession numbers AY333126–333155 for *G. c. canorus*, AY333156–333160 for *G. c. owstoni*, AY333161–AY333173 and AY333176 for *G. chinensis*, AY333175 for *G. subunicolor*, AY333174 for *G. poecilorhynchus poecilorhynchus* and AY333177 for *G. p. berthemyi*). Considering sequences of the Hwamei alone, there were, in total, 75 polymorphic sites and 36 haplotypes identified. Within each Hwamei subspecies, substantial variation in CYTB sequences was found (Table 2). A high number of haplotypes were found in each of the three subspecies (K = 4–24, Table 2). Levels of intrasubspecific divergence were similar (0.003, Table 2) among the three groups of *G. canorus*. Within the Hwamei, there were five, four and 20 diagnostic sites for *G. c. canorus*, *G. c. owstoni* and *G. c. taewanus*, respectively (Table 2).

A potential problem with phylogenetic reconstruction based on mitochondrial DNA data is PCR amplification of mitochondrial DNA homologues from the nuclear genome (numts) (e.g. Sorenson & Fleischer 1996, Kidd & Friesen 1998). In our dataset, the substantial intraspecific CYTB sequence variation is evidence that no numts had been amplified and sequenced. In addition, all the CYTB sequences we obtained could be functionally translated without any premature termination. Furthermore, amplification of nuclear sequences from skin samples is highly unlikely, given the relative copy numbers of mitochondrial to nuclear DNA, and the severe degradation experienced by nuclear DNA during this type of preservation. Therefore, there was no evidence suggesting that subsequent phylogenetic analysis could have been plagued by the presence of numts.

Tajima's D statistics were used to detect whether the variation of CYTB sequences of Hwamei significantly deviated from neutrality. Including the outgroup, Tajima's D was −1.431. The grouped estimate of the 95% confidence interval of Tajima's D was −1.796 to 1.801. Therefore, we were unable to reject ($P > 0.05$) evolutionary neutrality of the CYTB gene in these taxa using our dataset.

Table 2. Nucleotide variation and diagnostic sites of three *G. canorus* subspecies.

Taxon	n	K	H (± se)	S	π (± se)	Diagnostic sites
G. c. canorus	30	24	0.972 ± 0.021	33	0.003 ± 0.000	$T_{31,\ 726,\ 786}$; A_{705}; G_{1035}
G. c. owstoni	5	4	0.900 ± 0.161	6	0.003 ± 0.001	$T_{648,\ 691}$; $A_{525,\ 729}$
G. c. taewanus	13	8	0.910 ± 0.056	11	0.003 ± 0.000	$T_{165,\ 189,\ 235,\ 285,\ 432,\ 570,\ 606,\ 985}$; $A_{90,\ 565,\ 702}$; $G_{504,1137}$; $C_{33,\ 264,\ 381,\ 501,\ 583,\ 1020,\ 1077}$

n, sample size; K, number of haplotypes; H, haplotype diversity; S, number of segregating sites; π, nucleotide diversity. For diagnostic sites, positions of sites were subscripted to the type of nucleotides.

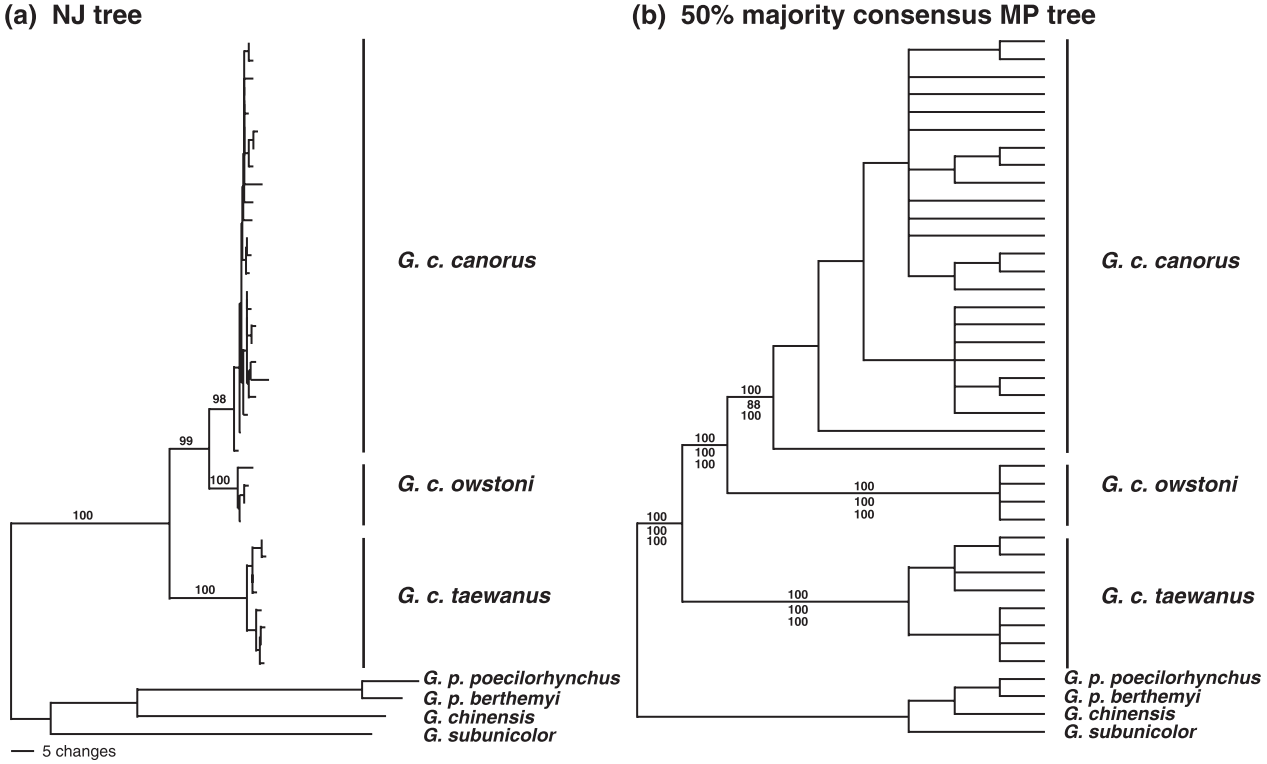

Figure 2. Molecular intraspecific phylogeny of the Hwamei. (a) The neighbour-joining tree with bootstrap support values for the major clades. (b) The 50% majority consensus tree of 2808 equally parsimonious trees; the three numbers above and below each node of the major clades are percentage consensus, and bootstrap value for the maximum-parsimony and maximum-likelihood tree.

Molecular phylogeny

Because *d*, the mean Jukes–Cantor distance, was 0.034 ± 0.003 se (< 0.05) for our sequence dataset, the *p*-distance was used to reconstruct the neighbour-joining tree (Nei & Kumar 2000). The topology of the neighbour-joining tree indicated that the three Hwamei subspecies formed three monophyletic clades with *G. c. taewanus* as sister to the other two (Fig. 2a). There was a high bootstrap support (> 98%) for each clade.

In the parsimony analysis, we found 2808 equally parsimonious trees (tree length = 367; consistency index = 0.837; retention index = 0.904) in a single tree-island. The majority consensus tree of these equally parsimonious trees (Fig. 2b) showed that, as in the neighbour-joining tree, samples from the three Hwamei subspecies formed three monophyletic clades, respectively, with high bootstrap support (> 95% for all three clades); *G. c. canorus* and *G. c. owstoni* also appeared to be sister taxa and *G. c. taewanus* was sister to them (Fig. 2b).

The TVM + Γ model (two transversion rates + one substitution rate + unequal base frequencies + Gamma distribution) was found to be the best model for the maximum-likelihood reconstruction. The maximum-likelihood estimates of the base composition were A = 28.2%, C = 35.5%, G = 12.4% and T = 23.9%. The estimated symmetrical substitution rate among these nucleotides was 3.3281 for A/C, 14.9767 for A/G and T/C, 1.2011 for A/T, 0.6662 for C/G and 1.0000 for G/T. The shape parameter (α) of the gamma distribution was 0.23. With the above parameters, we obtained a maximum-likelihood tree ($-\ln L = 3445.897$) that was identical to one of the most parsimonious trees for which the three subspecies formed three monophyletic clades and the clade of *G. c. taewanus* was located basally. Three major nodes were supported by high bootstrap values (100%; Fig. 2b).

Genetic distances between subspecies

The net genetic distance between subspecies was lowest between *G. c. canorus* and *G. c. owstoni* (0.012 ± 0.003

sd). Between *G. c. canorus* and *G. c. taewanus*, the net genetic distance was 0.029 (± 0.005 se), and the net genetic distance between *G. c. owstoni* and *G. c. taewanus* was 0.030 (± 0.005 se).

DISCUSSION

Rate and mode of differentiation for the Hwamei complex

The Pleistocene epoch (last 1.8 million years) was characterized by periodic glacial cycles. Glaciations have occurred every 100 000 years for the last 1 million years and every 41 000 years for the period 1–1.8 million years before the present (reviewed in Williams *et al.* 1998). Such climate oscillations have long been considered as important factors facilitating avian speciation in North America (Rand 1948). Although the role of very recent glaciations (especially the last two glacial cycles) in avian speciation is critically challenged by Klicka and Zink (1997), biological diversification during the Pleistocene is widely supported by genetic data from various vertebrates including birds (reviewed by Avise & Walker 1998, Avise *et al.* 1998). Following a conventional molecular clock for the avian mitochondrial CYTB gene (2% per million years; reviewed in Klicka & Zink 1997), *G. c. taewanus* probably diverged from *G. c. owstoni* and *G. c. canorus* about 1.5 (± 0.25 se) million years ago. *Garrulax c. owstoni* and *G. c. canorus* probably separated from each other about 0.6 (± 0.15 se) million years ago. Therefore, molecular dating suggests that the Hwamei complex diversified within the Pleistocene. Nevertheless, the differentiation of the Hwamei population cannot be attributed to the most recent climate oscillations of the Pleistocene epoch.

Although not explicitly emphasized, geographical isolation has been considered to be the key factor for allopatric speciation on continental islands (e.g. Grant & Grant 1998). However, continental islands were frequently connected to the continent when the continental shelf was exposed during periods of glaciation. During the last glacial maximum (LGM), sea-level was about 150 m lower than present (Rohling *et al.* 1998, Lambeck & Chappell 2001), and the continental shelf, including islands on it, became part of the continent. Such processes would have been at work periodically during the entire Pleistocene. For the gene pools on the continent and its peripheral islands, isolation by a geographical barrier (i.e. ocean) should be restricted largely to interglacial periods. Therefore, we suggest that, in addition to geographical isolation, habitat vicariance (the discontinuity of suitable habitat) during glacial periods would have been another important factor shaping genetic distinctness in the three groups of Hwameis. Two lines of evidence support this argument: molecular dating for the diversification of the Hwamei and the reconstructed palaeovegetation of East Asia during the LGM.

Based on the frequency of Pleistocene glaciations, the molecular dating in this study implies that the isolation of *G. c. canorus* and *G. c. owstoni* vs. *G. c. taewanus* and *G. c. canorus* vs. *G. c. owstoni* occurred over multiple glacial cycles (more than 20 cycles for the former pair and more than six cycles for the latter; Williams *et al.* 1998). Therefore, oceanic barriers between the continental and island Hwamei populations must have been absent several times since their divergence.

For organisms of lower altitude and warmer climate, the dry and cool climate during glacial periods repeatedly reduced and fragmented their ranges, pushing them towards the equator (reviewed by Davis & Shaw 2001); this helped to structure their gene pool and facilitated local differentiation (reviewed in Hewitt 2000). A reconstructed palaeovegetation map of East Asia during the LGM (Harrison *et al.* 2001) suggests that this might be the case for the Hwamei distributed at low elevation. On this map, the temperate evergreen broad-leaved forest in the Hwamei's current range (Fig. 1b) was largely replaced by the temperate deciduous broad-leaved forest and steppe during the LGM, with species of the former habitat retreating to northern Indo-China and south and southwestern China (Fig. 1c). The temperate deciduous broad-leaved forest and steppe could have easily become ecological barriers between fragmented temperate evergreen broad-leaved forest biota. Unsuitable habitat for Hwamei should have been enough to keep the ancestral Hwamei populations isolated, and such a scenario of habitat vicariance probably occurred to different extents during most of the Quaternary ice ages.

Potential hybridization and introgression

Phylogenetic analysis indicated that the three allopatric Hwamei subspecies could be regarded as three independent ESUs. Hybridization and genetic introgression between ESUs have posed a serious threat to the persistence of populations or species integrity (reviewed by Rhymer & Simberloff 1996). In birds, for example,

the range expansion and introduction of the Common Mallard *Anas platyrhynchos* has created opportunities for this holarctic species to hybridize with several locally distributed duck species, driving some of these (e.g. the Grey Duck *A. superciliosa superciliosa* of New Zealand, Rhymer *et al.* 1994) into endangered status.

Considering their phylogenetic affinity, it is reasonable to suspect that the reproductive isolation between *G. c. canorus* and *G. c. owstoni* is also incomplete. In Taiwan, single nucleotide polymorphism data have further suggested that the frequency of hybridization and backcross between two Hwameis is at an alarming level (S.-H.L. unpubl. data) as a consequence of unregulated bird trades and uninformed release practices. Without the aid of molecular markers, Hwameis could be sexed only by their vocalization, often causing female Hwamei to be imported into Taiwan by mistake; less desirable female Hwameis are then released by owners or traders to reduce costs. In addition, for religious reasons large quantities of caged birds are released regularly in Taiwan as tokens of piety. Efforts clearly have to be taken to minimize the release or escape of the Hwamei and to regulate the import of *G. c. canorus* into Taiwan and Hainan.

In order to make conservation informed and effective, another important task is to evaluate the current status of hybridization and introgression between the Hwamei groups. The diagnosable sites within the maternally inherited mitochondrial CYTB gene revealed in this study can provide a set of genetic markers for detecting hybridization and introgression between Hwamei subspecies. Nuclear markers, such as microsatellite DNA (Huang *et al.* 2004) and single nucleotide polymorphism (S.-H.L. unpubl. data), will be needed to detect hybridization and genetic introgression of Hwameis from both maternal and paternal lineages.

Taxonomic recommendations

Diagnosability and integrity of the genotype and phenotype are considered to be two criteria to assign species status in birds (Helbig *et al.* 2002). Following these criteria, the systematic status of the three Hwamei subspecies should be revised.

Diagnosability of phenotypic and genetic characters suggests long-term interruption of gene flow between populations. Although the definition of species may vary when adopting different species concepts (e.g. the biological species concept, Mayr 1942, 1963; the evolutionary species concept, Wiley 1978; the phylogenetic species concept, Cracraft 1983), diagnosability is the essential element to all species concepts proposed at the operational level. The nominate *G. c. canorus* of mainland Asia was scientifically described in 1758 by Linnaeus. Just over 100 years later, the Taiwanese Hwamei was named as a distinct species, *G. taewanus*, by Swinhoe (1859). Despite its distinctive plumage (e.g. lack of the eye stripe), the Taiwanese Hwamei has been treated subsequently as a subspecies of *G. canorus* (e.g. Berlioz 1930, Deignan 1964). In contrast to the Taiwanese Hwamei, the Hainan Hwamei was named as a subspecies of *G. canorus* when it was described in 1903 (*Trochalopteron canorus owstoni*, Rothschild). Here, we have clearly demonstrated that the three Hwamei subspecies could be identified by diagnosable DNA characters (Table 2). The diagnosability of these birds indicates the lack of recent genetic exchange among these taxa. However, the diagnosability of *G. c. owstoni* may be an artefact of our small sample size ($n = 5$).

The distance between Hainan Island and the mainland is about 20 km. This is equivalent to the distance between mainland and island populations of the Grey-crowned Babbler *Pomatostomus temporalis* in Australia. Significant levels of gene flow have been found for this sedentary babbler (Edwards 1993). By contrast, the genetic divergence between the *G. c. owstoni* haplotypes and those from the mainland indicate that long-distance dispersal might be restricted for the Hwamei. Significant genetic distance between three allopatric populations suggests that the integrity of the three Hwamei subspecies has been maintained historically regardless of frequent land connections and should persist in the future unless human activity intervenes.

In view of the phylogenetic species concept, it is clear that at least two full species currently exist under the same species name, *G. canorus*. To represent the diversity of these birds more appropriately, we recommend that the systematic status of *G. canorus* be revised as follows: *G. canorus* reserved for the mainland Hwamei; and *G. taewanus* (Swinhoe 1859) for the Taiwanese Hwamei. Given the small sample size of Hainan Hwamei in this study, the systematic status of *G. c. owstoni* should remain unchanged until more genetic or behavioural data become available.

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