biology letters

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Biol. Lett. 2006 **2**, 466-469
doi: 10.1098/rsbl.2006.0490

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doi:10.1098/rsbl.2006.0490
Published online 16 May 2006

Mid-Pleistocene divergence of Cuban and North American ivory-billed woodpeckers

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We used ancient DNA analysis of seven museum specimens of the endangered North American ivory-billed woodpecker (*Campephilus principalis*) and three specimens of the species from Cuba to document their degree of differentiation and their relationships to other *Campephilus* woodpeckers. Analysis of these mtDNA sequences reveals that the Cuban and North American ivory bills, along with the imperial woodpecker (*Campephilus imperialis*) of Mexico, are a monophyletic group and are roughly equidistant genetically, suggesting each lineage may be a separate species. Application of both internal and external rate calibrations indicates that the three lineages split more than one million years ago, in the Mid-Pleistocene. We thus can exclude the hypothesis that Native Americans introduced North American ivory-billed woodpeckers to Cuba. Our sequences of all three woodpeckers also provide an important DNA barcoding resource for identification of non-invasive samples or remains of these critically endangered and charismatic woodpeckers.

Keywords: *Campephilus principalis*; ivory-billed woodpecker; Pleistocene divergence; phylogenetics; DNA barcoding

1. INTRODUCTION

The ivory-billed woodpecker (*Campephilus principalis*) is a spectacular bird that was thought to be extinct until publication of recent reports (Fitzpatrick *et al.* 2005). Ivory-billed woodpeckers occurred in two disjunct regions, with *C. p. principalis* found in primary hardwood forest throughout much of the southeastern United States and *C. p. bairdii* on the island of Cuba in mature lowland hardwood and hill pine forests. Populations in Cuba seriously declined over the past century and may now be extinct (Stattersfield & Capper 2000; Jackson 2002). The related imperial woodpecker (*Campephilus imperialis*) also underwent a major population decline in the highlands of Mexico and may also be extinct (Stattersfield & Capper 2000).

Cuban and mainland ivory-billed woodpeckers differ only slightly in plumage and size (Jackson 2002), and, although originally described as separate species by Cassin (1863), have been considered conspecific since at least 1976 (AOU 1976). In fact, Jackson (2002) suggested that ivory-billed woodpeckers in Cuba are a recent human-assisted introduction, citing evidence of extensive trade in birds between Native Americans in Cuba and Florida, a high value placed on ivory-billed woodpecker artefacts by Native Americans, and a presumed low propensity for dispersal by a forest-dwelling woodpecker over open water.

Here, we compare mitochondrial DNA sequences from museum specimens to estimate the level of genetic divergence between the two forms, and between each and the related imperial woodpecker (*C. imperialis*) and other members of their genus. We evaluate their taxonomic status and test whether the Cuban ivory-billed woodpecker is a recent introduction. Our sequences also provide an important DNA barcoding resource for the ivory-billed woodpecker and relatives. We find that the Cuban ivory-billed woodpecker is mitochondrially distinct from the North American form, a result that has implications for the conservation of the species in both regions.

2. MATERIAL AND METHODS

(a) *Samples*

We used scalpels to pare small samples of skin from the toe pads of museum specimens collected between 1861 and 1923 of seven North American and three Cuban ivory-billed woodpeckers, and three imperial woodpeckers. These museum specimens of *C. p. principalis*, *C. p. bairdii* and *C. imperialis* are listed in the electronic supplementary material table 1, along with accession number, locale and date of collection, sex, amount of sequence obtained and GenBank (NCBI) accession number. We also obtained frozen tissues of seven South and Central American species of *Campephilus*, along with two species each of *Dryocopus* and *Picumnus* woodpeckers as outgroups. Details concerning tissue specimens of *Campephilus* and other outgroups analysed at Wayne State University are shown in the electronic supplementary material table 2.

(b) *Laboratory analysis*

All pre-PCR ancient DNA labwork was completed in isolated ancient DNA laboratories at the Smithsonian, University of Florida, California Academy of Sciences and Harvard Museum of Comparative Zoology, following stringent protocols to avoid and detect potential contamination. DNA was isolated from museum specimen toe pads using phenol–chloroform extraction and centrifugal dialysis following the methods outlined in Fleischer *et al.* (2000). Short fragments of specific regions of mitochondrial DNA (*COI*, cytochrome *b*, *ND2*, ATP6/8, 12S rDNA) were amplified from the museum specimen extracts using PCR and specific primers. Amplification of these small fragments used primers listed in the electronic supplementary material table 3, developed in the case of *COI* from existing sequence for related species (DeFilippis & Moore 2000). Up to 1730 nucleotide sites were obtained, and at least 1079 bp were sequenced for at least two individuals of each taxon; smaller amounts of sequence were obtained for additional individuals. Nearly half of the sequences were at least partially and independently replicated by more than one laboratory (electronic supplementary material table 1). Analysis of

The electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2006.0490 or via http://www.journals.royalsoc.ac.uk.

Received 4 March 2006
Accepted 18 April 2006

DNA from outgroups was conducted at Wayne State University, and limited to *COI*, cytochrome *b* and 12S rDNA sequences, with somewhat different primers and methods. Detailed methods and primers for amplification and sequencing of these outgroup taxa are available for cytochrome *b* in Moore & DeFilippis (1997), for *COI* in DeFilippis & Moore (2000) and for 12S in Webb & Moore (2005).

(c) *Phylogenetic analyses*

Phylogenies were reconstructed using three approaches: maximum likelihood (ML), maximum parsimony (MP) and Bayesian. We conducted an unweighted analysis with a heuristic search and 10 random addition repetitions under the MP criterion in PAUP* (Swofford 2002), and a subsequent bootstrap analysis with 1000 replications. The Akaike information criterion (AIC) in MODELTEST (Posada & Crandall 1998) was used to select the most appropriate model of sequence evolution for subsequent ML and Bayesian approaches. The AIC selected the GTR+I+G model, with a gamma shape parameter $\alpha=5.55$ and the proportion of invariable sites $I=0.657$. The ML analysis involved a heuristic search, and a bootstrap of 100 heuristic repetitions to indicate nodal support in PAUP*. Bayesian analyses were run in MRBAYES (Huelsenbeck & Ronquist 2001). We did a chain of 1 000 000 generations MCMC with the parameters as selected by MODELTEST (above). We excluded the first 500 000 generations as burn-in, and computed a consensus from 50 000 trees in the remainder. *Campephilus* taxa were rooted to *Picumnus* and *Dryocopus* outgroups.

(d) *Dating of nodes*

A likelihood-ratio test comparing a molecular clock-constrained and an unconstrained tree in PAUP* (Swofford 2002) indicated no significant departure from a molecular clock for this dataset ($\chi^2_{17}=25.23$, $p>0.05$). Dating of key nodes was estimated with MEGA using an ultrametric tree (clock-like) approach (Kumar *et al.* 2004), and with R8S (Sanderson 2003) using variable clock approaches (although the lack of significant among-lineage rate variation suggests the latter corrections are not necessary). For both of these methods, we used two calibrations. First, we used the rate of 2.0±0.5% sequence divergence per million years (Myr) calibrated for cytochrome *b* in woodpeckers from Moore *et al.* (1999), based on a fossil *Colaptes* woodpecker. This rate is similar to those calibrated for many other avian taxa (e.g. Fleischer *et al.* 1998; Paxinos *et al.* 2002). Second, because of the basal position of *Campephilus haematogaster* in our phylogeny, we make an assumption that the ancestor of *Campephilus* clades A and B (see figure 1) occurred in South America, and was able to colonize North America only after the formation of the Isthmus of Panama about 3.1 Myr ago (Coates & Obando 1996). Once this colonization occurred, the two lineages could diverge, and the range of *Campephilus guatemalensis* would represent a secondary range expansion from South America into Central America.

3. RESULTS AND DISCUSSION

Phylogenetic analyses of these mtDNA sequences show that the Cuban and mainland North American ivory-billed woodpeckers are clearly distinct lineages. The analysis is ambiguous with regard to their status as sister taxa (figure 1), statistically a polytomy containing both ivory-billed woodpecker taxa and the imperial woodpecker. Our MP search resulted in three trees, the consensus of which matched in topology the ML and Bayesian trees, as did the consensus tree resulting from a 1000-repetition bootstrap with an MP search. The MP and ML trees have high bootstrap support values and the Bayesian tree has high posterior probabilities for many nodes, including for monophyly of the northern *Campephilus* taxa (*C. imperialis*, *C. p. principalis* and *C. p. bairdii*; clade A, figure 1). Clade A is sister to a clade containing all other *Campephilus* woodpeckers (clade B), except for *C. haematogaster*.

Genetic divergence between the North American and Cuban ivory-billed woodpeckers was calculated in MEGA (Kumar *et al.* 2004), and averaged 0.021±0.007 substitutions per site. This value is similar to that between each ivory-billed woodpecker taxon and the imperial woodpecker (0.017±0.008 and 0.017±0.004, respectively). Applying the woodpecker calibrated rate of 2.0% sequence divergence per Myr for cytochrome *b* sequence (Moore *et al.* 1999) to the cytochrome *b* distance between the Cuban and North American ivory-billed woodpeckers results in an estimated split date of 1.0 Myr. Notably, the sequence divergence rate for cytochrome *b* calculated from our dataset using the date of the separation of the Isthmus of Panama was 1.9% per Myr.

Assuming clades A and B began to diverge 3.1 Myr ago, our estimates either with or without a molecular clock resulted in rates for our entire mtDNA dataset of between 0.011 and 0.014 substitutions per site per Myr. The predicted dates of divergence by penalized likelihood (PL, TN model) in R8S (Sanderson 2003) were 1.15 Myr ago for the split between *C. p. principalis* and (*C. p. bairdii*, *C. imperialis*), and 0.91 Myr ago for the split between *C. p. bairdii* and *C. imperialis*; by non-parametric rate smoothing (Powell model, Sanderson 2003): 1.57 and 1.27 Myr; by Langley–Fitch (Sanderson 2003): 1.05 and 0.83 Myr; and by MEGA (Kumar *et al.* 2004): 1.37 and 1.14 Myr. Thus, applying these different methods to the divergence of the three North American *Campephilus* lineages indicates that their split occurred sometime between about 0.8 and 1.6 Myr ago, although coalescent effects could push the species split closer to the present by as much as 200 000 years (Moore 1995; Edwards & Beerli 2000).

Our data suggest that, like *C. imperialis*, both *C. p. principalis* and *C. p. bairdii* should also be considered separate species. This contention is supported by the effectively unresolved trichotomy among the three northern taxa, and the degree of genetic divergence among them. Such a classification would appear to be supported under either a traditional biological species concept (i.e. allopatric, divergent lineages that appear reproductively isolated) or a phylogenetic species concept (i.e. allopatric, diagnosable lineages).

Our dating analyses reveal that the Cuban and North American ivory-billed woodpeckers and the imperial woodpecker diverged sometime in the Mid-Pleistocene. This period corresponds to the Mid-Pleistocene revolution (when global temperature oscillations changed from a 41 ky period to a 100 ky period), and was a period of global cooling and lowered sea-levels (Maasch 1988; Raymo *et al.* 1997). Reported sea-level differences of more than 30 m (Wright & Flower 2002) at this time would have increased the size of the Yucatan Peninsula and reduced the *ca* 176 km current distance between the Yucatan Peninsula and Cuba, and thus may have favoured colonization of Cuba by a woodpecker presumably averse to flying over water. Our data clearly do not support the hypothesis that Native Americans recently introduced the ivory-billed woodpecker from North America into Cuba (Jackson 2002).

Our discovery of three genetically divergent lineages of northern *Campephilus* that each may be a different species increases the already urgent need for

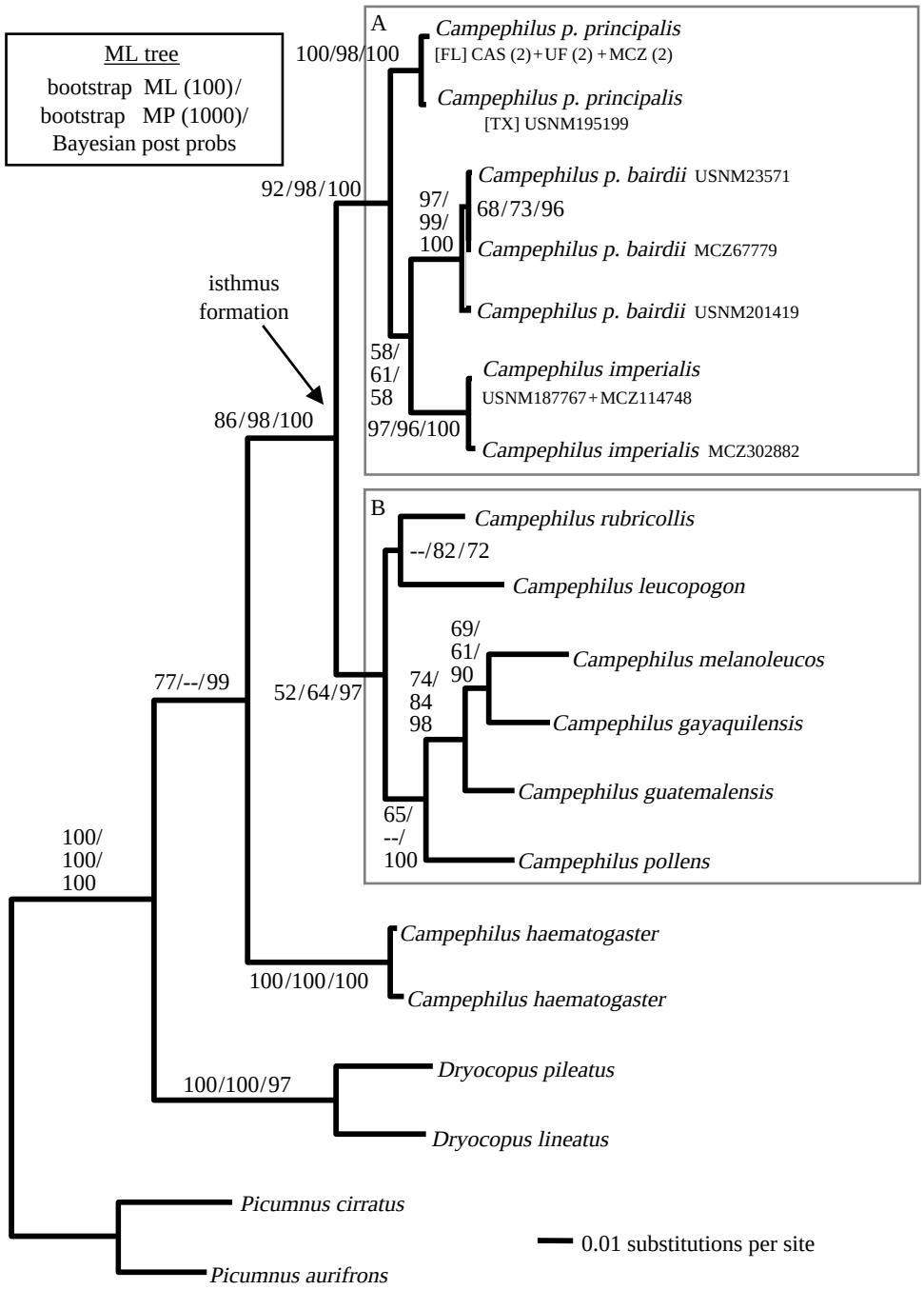

Figure 1. Phylogram of *Campephilus* species and outgroups produced by heuristic search with maximum-likelihood (ML) criterion. Topologies recovered from maximum-parsimony (MP) and Bayesian approaches were identical other than minor rearrangements of internal nodes in the three MP trees recovered. Numbers at nodes are ML (100-repetition) and MP (1000-repetition) bootstrap percentages and Bayesian posterior probabilities. Tree was rooted with *Dryocopus* and *Picumnus* outgroups. Note that the boxed clade A contains northern *Campephilus* taxa (i.e. ivory-billed and imperial woodpeckers), while the boxed clade B contains southern *Campephilus* taxa (with putative secondary northern expansion by *C. guatamalensis*). The basal position of *C. haematogaster* suggests a South American origin of *Campephilus* and colonization of North America after closure of the Isthmus of Panama. See text for details of phylogenetic and dating analyses.

rediscovery and conservation of this critical branch of the woodpecker tree. Our results will also provide an important DNA barcoding resource that could facilitate the discovery of living ivory-billed and imperial woodpeckers by identification of shed materials, such as feathers and faeces sampled from the wild.

We thank David Steadman and Gary Graves for permission to sample specimens under their care, and Rebecca Kimball, Kristy L. Deiner, Jeremiah Trimble, Carl E. McIntosh, Jennifer Reed and Dawn Reding for facilitating or assisting in laboratory work. We appreciate insights from Storrs Olson, Jim Hanken, Jerome Jackson and Per Ericson. The Smithsonian Institution and NSF DEB#0452732 provided funding.

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