



# DNA barcoding and phylogenetic relationships of genera *Picoides* and *Dendrocopos* (Aves: Picidae)

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**ABSTRACT.** *Picoides* and *Dendrocopos* are two closely related genera of woodpeckers (family Picidae), and members of these genera have long been the subjects of phylogenetic debate. Mitochondrial cytochrome c oxidase subunit I (*COI*) is a powerful marker for the identification and phylogenetic study of animal species. In the present study, we analyzed the *COI* barcodes of 21 species from the two genera, and 222 variable sites were identified. Kimura two-parameter distances were calculated between barcodes. The average interspecific genetic distance was more than 20 times higher than the average intraspecific genetic distance. The neighbor-joining method was used to construct a phylogenetic tree, and all of the species could be discriminated by their distinct clades. *Picoides arcticus* was the first to split from the lineage, and the other species were grouped into two divergent clades. The results of this study indicated that the *COI* genetic data did not support the monophyly of *Picoides* and *Dendrocopos*.

**Key words:** *Picoides*; *Dendrocopos*; Phylogenetic relationship; DNA barcoding

## INTRODUCTION

The avian genera *Picoides* and *Dendrocopos* are the largest of the nearly worldwide family of woodpeckers (Picidae) (Short, 1982). The interpretation of the evolutionary relationship between *Dendrocopos* and *Picoides* has frequently changed (Rutkowski et al., 2006). Picidae is currently a conglomerate of two earlier genera of “pied” woodpeckers, *Picoides* (the “three-toed” woodpeckers) and *Dendrocopos* (the “ladder-backed” woodpeckers) (Weibel and Moore, 2002a). Therefore, Peters (1948) concluded that the original members of *Picoides* included only two species, *P. tridactylus* and *P. arcticus*, which both have a reduced hallux instead of the distinct fourth zygodactyl toe seen in *Dendrocopos* species. Delacour (1951) united the *Dendrocopos* and *Picoides* into the single genus *Picoides* according to priority in the systematic nomenclature, which claimed that variance in toe length or number is not phylogenetically important among closely allied avian species. Both genera were then combined into the single genus *Picoides* (Short, 1982). However, del Hoyo et al. (2003) surmised that “spotted” woodpeckers (*Dendrocopos*) should be separated from *Picoides*. Rutkowski et al. (2006) used the control region to resolve the phylogeny of the woodpecker family, and demonstrated that the three-toed woodpecker (*Picoides*) was grouped within a single monophyletic clade that included members of *Dendrocopos*. Thus, the systematics of the two genera is in need of revision.

Estimating phylogenies from DNA sequence data has become the major methodology of molecular phylogenetics (Prychitko and Moore, 2003). Large-scale standardized sequencing of mitochondrial cytochrome c oxidase subunit I (*COI*) has made DNA barcoding an efficient tool for species identification in many animal groups (Hebert et al., 2003a,b). However, several studies have confirmed a clear gap (the so-called barcoding gap) between intra- and interspecific Kimura two-parameter (K2P) distance distributions (Bremner et al., 2013). Previous barcoding studies on birds mainly focused on the surveys of regional groups, such as the Korean (Park et al., 2011), North American (Kerr et al., 2007), Southeast Asian (Lohman et al., 2009), Neotropical (Kerr et al., 2009b), and Scandinavian groups (Johnsen et al., 2010). Moreover, *COI* barcoding was successfully used to reconstruct the phylogenies of several animal groups at the family level (Cai et al., 2010; Huang and Ke, 2014, 2015). However, few DNA barcoding studies have been conducted at the genus level (Bremner et al., 2013).

DNA barcoding studies on *Picoides* and *Dendrocopos* remain limited (Weibel and Moore, 2002a). In the present study, we examined a 652-bp *COI* fragment, and conducted phylogenetic analyses using sequences from *Picoides* and *Dendrocopos*. Our main aims were: 1) to test whether DNA barcodes allow the identification of *Picoides* and *Dendrocopos* species; and 2) to resolve the phylogeny of *Picoides* and *Dendrocopos*.

## MATERIAL AND METHODS

Seventy-five *COI* sequences were obtained from GenBank, and 14 *Picoides* and 7 *Dendrocopos* species were analyzed (Table S1). *COI* sequences (652 bp) were aligned using Clustal X (Thompson et al., 1997). DnaSP v5.0 (Librado and Rozas, 2009) was used to define the variable sites, and sequence divergence among species and genera was calculated using the K2P (Kimura, 1980) distance model in MEGA 6.0 (Tamura et al., 2013). The neighbor-joining method (NJ) (Saitou and Nei, 1987) was used to reconstruct the phylogenetic tree (based the K2P model) using MEGA6.0. Statistical support for the internodes in phylogenetic tree was tested by bootstrap percentages (BP) with 1000 replicates (Felsenstein 1985).

## RESULTS

### Barcoding analysis

The 652-bp *COI* sequences were aligned, and the gene fragment corresponded to the *Dryocopus pileatus* mitochondrial gene that started at position 5456 and stopped at position 6107 (Gibb et al., 2007). Analyses were performed on 1 to 5 specimens per species (3.5 on average), and 222 variable sites were identified. Of these variable sites, 196 were parsimoniously informative (30.06% of the entire sequence). All of the analyzed species had distinct *COI* sequences, and the average nucleotide composition was 24.04% T, 34.90% C, 24.74% A, and 16.32% G.

### Genetic distance

#### *Picooides*

K2P within-species genetic distances had a small range (0 to 2.25%), with more than 90.09% of the observations below a genetic distance of 1.00%. Pairwise among-species comparisons were distributed from 2.02% (between *P. nuttallii* and *P. scalaris*) to 16.99% (between *P. mixtus* and *P. arcticus*). Most of the observed comparisons were between 7 to 16% K2P genetic distance, and the K2P genetic distance peaked at 90.11% (N = 91). The average interspecific genetic distance of the *COI* sequences (10.82%) was 26 times higher than the average intraspecific genetic distance (0.41%).

#### *Dendrocopos*

K2P within-species genetic distances ranged from 0.09% (*D. major*) to 1.13% (*D. medius*). Pairwise comparisons among-species varied from 4.06% (between *D. leucotos* and *D. major*) to 14.98% (between *D. canicapillus* and *D. major*). The average interspecific genetic distance of the *COI* sequences (11.53%) was 22 times higher than the average intraspecific genetic distance (0.51%).

### Phylogenetic relationships

NJ was used to reconstruct the phylogenetic tree based on the K2P model, and *Jynx torquilla* was used as the outgroup. All of the species could be discriminated by their distinct clades (Figure S1). The black-backed woodpecker (*P. arcticus*) was the first to split from the lineage (BP = 99%, Figure S1), and the other species were grouped into two divergent clades (A and B, Figure S1). *COI* analysis strongly supported the placement of *P. tridactylus* with *P. dorsalis* in subclade A<sub>1</sub>. The results of the NJ analysis suggested the inclusion of *D. kizuki* + *D. maculatus* + *D. canicapillus* in subclade A<sub>2</sub>. Clade B contained 15 species, including two subclades (B<sub>1</sub> and B<sub>2</sub>). Furthermore, *D. medius*, *D. mahrattensis*, *D. leucotos*, and *D. major* formed subclade B<sub>1</sub>. Clade B<sub>2</sub> contained the other *Picooides* species (*P. villosus* + *P. arizonae* + *P. albolavatus* + *P. fumigatus* + *P. borealis*, *P. mixtus* + *P. lignarius* + *P. nuttallii* + *P. scalaris* + *P. minor* + *P. pubescens*) (Figure S1).

## DISCUSSION

The interspecific genetic distance of *Picooides* (2.02 to 16.99%; average 10.82%) and *Dendrocopos* (4.06 to 14.98%; average 11.53%) corresponded to *COI* genetic distances greater

than 2.00%, which is indicative of valid species (Hebert et al., 2003b). Several studies support the conclusion that distance-based DNA barcoding can provide sufficient information to identify and delineate a large majority of bird species (Kerr et al., 2007; Lohman et al., 2009; Kerr et al., 2009b; Johnsen et al., 2010; Park et al., 2011; Breman et al., 2013). Hebert et al. (2003a) proposed a “10X rule” to define species boundaries, which is defined as a sequence threshold that is 10 times the mean intraspecific variation of the group under study. However, the rate of *COI* gene evolution is subject to variation in different clades of birds (Pereira and Baker, 2006). Therefore, Huang and Ke (2015) postulated that it might be inappropriate to suggest a universal distance criterion for different species, and the *Picooides* and *Dendrocopos* results supported this opinion.

Mitochondrial DNA has been frequently used in woodpecker studies in order to resolve the phylogeny of the family, particularly the evolutionary relationships between Old and New World species (Weibel and Moore, 2002a; Winkler et al., 2005). The results of this study clearly showed the discriminative power of *COI* barcodes for the identification of *Picooides* and *Dendrocopos* species. Each woodpecker had distinct *COI* sequences, and the analysis separated the different species into distinct branches. Furthermore, none of the species shared sequences or exhibited overlapping clades with another species. The taxonomic position of the black-backed woodpecker was controversial (Winkler et al., 2005). In most cases, the black-backed woodpecker was considered to be the sister species of the Eurasian three-toed woodpecker (*P. tridactylus*) (Weibel and Moore, 2002a). The surprising inference that the woodpecker was a basal lineage in the phylogeny was supported (Figure S1). Using *cyt b*, Winkler et al. (2005) also found that this species split off from the basal node. The evolutionary relationships deduced from our sequence data indicated that the black-backed woodpecker was likely a basal ancestor of *Picooides* and *Dendrocopos*. However, additional taxon sampling and different markers are needed to resolve the taxonomic status. The general topology of the phylogenetic tree was strongly supported; however, the *COI* analysis indicated that the two genera might be polyphyletic.

## ***Picooides***

*Picooides* is the most diverse genus within Picidae, and member species are found on most major land masses (Weibel and Moore, 2002a). *Picooides* has long been treated as a single group by most taxonomists. However, Weibel and Moore (2002a) concluded that *Picooides* was para phyletic with two related genera, *Veniliornis* and *Dendrocopos*. Our results also supported the proposal that *Picooides* was not monophyletic. The *COI* phylogeny showed that *Picooides* species formed three distinct groups: *P. arcticus*, *P. tridactylus* + *P. dorsalis*, and the New World species. Several studies proposed that member species of New World *Picooides* were not monophyletic, because all analyzed Eurasian species were interspersed among the New World species (Short, 1971; Weibel and Moore, 2002a). The New World species formed three distinct subgroups: the “large” North American group (*P. villosus*, *P. arizonae*, *P. albolarvatus*, *P. fumigatus*, and *P. borealis*), the South American group (*P. lignarius* and *P. mixtus*), and the “small” North American group (*P. nuttallii*, *P. scalaris*, and *P. pubescens*). It is important to note that the “small” North American group also included *P. minor* (a Eurasian species), and this result was consistent with the results of Weibel and Moore (2002a). The lesser spotted woodpecker (*P. minor*) was often placed in *Dendrocopos* (MacKinnon et al., 2000), and the smoky-brown woodpecker (*P. fumigatus*) was merged into *Veniliornis*. Although we did not include *Veniliornis* sequences, the results of this study supported the placement of the lesser spotted woodpecker and the smoky-brown woodpecker in *Picooides*. Moreover, Weibel and Moore (2002a,b) also placed the lesser spotted woodpecker within the *Picooides*.

## ***Dendrocopos***

The systematic classification of *Dendrocopos* species was controversial. *Dendrocopos* was sometimes merged into *Picoides*, but this was neither generally accepted nor well supported. Our results indicated that monophyly of *Dendrocopos* was not supported. The NJ tree grouped members of *Dendrocopos* into two subclades, A<sub>2</sub> and B<sub>1</sub> ([Figure S1](#)). An interesting aspect of the phylogeny was the rather unexpected close relationship between the white-backed woodpecker (*D. leucotos*) and the great spotted woodpecker (*D. major*) (also see Weibel and Moore, 2002a; Winkler et al., 2005). The genetic distance between the two species was only 4.06%. These two woodpeckers both occupy vast ranges, and they are sympatric, with the exception of some East Asian islands (Winkler et al., 2005). *D. leucotos* + *D. major* were sister taxa to *D. medius* + *D. mahrattensis* ([Figure S1](#)), which indicated that the white-backed woodpecker and the great spotted woodpecker should not be placed into the *Picoides* (however, see Weibel and Moore, 2002a,b). Weibel and Moore (2002b) found that *D. leucotos* + *D. major* grouped with *D. fuscescens* + *D. griseocephalus*. Moreover, some researchers also placed the white-backed woodpecker and the great spotted woodpecker within *Dendrocopos* (MacKinnon et al., 2000; Winkler et al., 2005).

## **Conflicts of interest**

The authors declare no conflict of interest.

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## **[Supplementary material](#)**

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