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DNA barcoding and evolutionary relationships of the Phasianidae family in China

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ABSTRACT. A DNA barcode is a short sequence of standardized genomic region that is specific to a species. According to studies of bird species, the 694-bp sequence of the mitochondrial gene encoding cytochrome c oxidase 1 (COI) is extremely useful for species identification and phylogeny. In the present study, we analyzed the COI barcodes of 31 species from 18 genera belonging to the Phasianidae family in China. Kimura two-parameter (K2P) distances were calculated between barcodes. We found that the average genetic distance between congeneric species was 24 times higher compared to the average genetic distance within species. Each bird species had a barcode that was distinct to all other bird species. The neighbor-joining method was used to construct a phylogenetic tree, which grouped all of the genera into 2 divergent clades. In conclusion, DNA barcoding is an effective molecular tool for Phasianidae species identification and phylogenetic inference.

Key words: DNA barcoding; Cytochrome c oxidase I; Phasianidae; Phylogeny

INTRODUCTION

DNA barcoding using mitochondrial cytochrome c oxidase subunit I (COI) is regarded as a standard method for species identification (Hebert et al., 2003; Arif et al., 2011). Large-scale standardized sequencing of COI has made DNA barcoding an efficient species identification tool for many animal groups (Hebert et al., 2003). Previous barcoding studies of birds have mainly focused on the survey of regional groups, in areas such as Korea (Yoo et al., 2006; Park et al., 2011), North America (Kerr et al., 2007), Southeast Asia (Lohman et al., 2009), the Neotropics (Vilaca et al., 2006; Kerr et al., 2009), and Scandinavia (Johnsen et al., 2010). However, DNA barcoding studies on specific taxa, such as Galliformes birds, remain limited.

Mitochondrial DNA (mtDNA) has been widely employed in phylogenetic studies of animals, because it evolves much more rapidly compared to nuclear DNA, resulting in the accumulation of differences among closely related species (Brown et al., 1979; Moore, 1995). Previous studies have successfully used the COI gene to discriminate bird species (Kerr et al., 2009; Johnsen et al., 2010; Ong et al., 2011; Arif et al., 2011; Breman et al., 2013). Recent reports have also extended the application of COI gene analysis into phylogenetic research (Arif et al., 2011; Breman et al., 2013).

The avian family Phasianidae is one of the most important groups of birds for both human society and research purposes. China boasts 56 species of Phasianidae belonging to 21 genera, with many of the species being distributed in the southwestern mountains and southeastern Himalayas of China (Lei and Lu, 2006). There have been numerous attempts to reconstruct the phylogenetic relationships of the Phasianidae (Kimball et al., 1999, 2001; Armstrong et al., 2001; Dimcheff et al., 2002; Bush and Strobeck, 2003; Dyke et al., 2003; Pereira and Baker, 2006; Kaiser et al., 2007; Kriegs et al., 2007; Kimball and Braun, 2008; Huang et al., 2009; Liu et al., 2012). However, the taxonomic status of some species and genera within the Phasianidae family remain controversial. Different markers are required to resolve the taxonomic status of these species. However, research remains limited on the DNA barcoding of Phasianidae (Cai et al., 2010).

In the present paper, we examined the 694-bp of the COI gene of Phasianidae birds in China. A 694-bp region of the COI gene is now, by convention, used as the standard genetic marker to assist with identifying animal species (Breman et al., 2013). This study aimed to 1) test whether DNA barcodes allow the identification of Phasianidae species, 2) resolve the molecular phylogenetic relationships of Phasianidae, and to compare the results with other genetic markers.

MATERIAL AND METHODS

Taxon sampling

Sixty-eight COI sequences were obtained from the GenBank. A total of 31 species from 18 genera belonging to the Phasianidae family were analyzed (Table 1).

Sequence analysis

Sequences were aligned by the CLUSTAL X procedure (Thompson et al., 1997). A total of 694 bp of the mtDNA COI genes were analyzed, which corresponded to the *Coturnix chinensis* mitochondrial genome gene start point at position 6601 and stop point at position 7294 (Nishibori et al., 2002). DnaSP v5.0 (Librado and Rozas, 2009) was used to define the variable sites. Nucleotide composition was calculated using MEGA5.0 (Tamura et al., 2011).

Table 1. Species examined in the present study and the data sources of sequences.

Genus	Species	Code	Sample size	Accession No.	Source
Tetraophasis	*Tetraophasis obscurus*	Tob	1	NC018034	Cai et al., 2010
	Tetraophasis szechenyii	Tsz	3	GQ922645	Cai et al., 2010
				GQ922646	Cai et al., 2010
				GQ922647	Cai et al., 2010
Tetraogallus	*Tetraogallus altaicus*	Tal	1	GQ482760	Kerr et al., 2009
Alectoris	*Alectoris chukar*	Ach	3	FJ752426	Shen et al., 2010
				JF498827	Kerr and Dove, unpublished data
				JF498828	Kerr and Dove, unpublished data
Francolinus	*Francolinus pintadeanus*	Fpi	2	NC011817	Shen et al., 2009
				EU165707	Shen et al., 2009
Perdix	*Perdix dauurica*	Pda	3	FJ752431	Shen et al., 2010
				GQ482330	Kerr et al., 2010
				GQ482331	Kerr et al., 2010
	Perdix perdix	Ppe	3	GU571528	Johnsen et al., unpublished data
				GU571529	Johnsen et al., unpublished data
				DQ433893	Kerr et al., 2007
Coturnix	*Coturnix chinensis*	Cch	1	AB073301	Nishibori et al., 2002
	Coturnix japonica	Cja	3	GQ481651	Kerr et al., 2009
				GQ481653	Kerr et al., 2009
				AP003195	Nishibori et al., 2002
	Coturnix coturnix	Cco	2	GQ481648	Kerr et al., 2009
				GQ481649	Kerr et al., 2009
Arborophila	*Arborophila rufogularis*	Aru	2	GQ922643	Cai et al., 2010
				GQ922644	Cai et al., 2010
	Arborophila gingica	Agi	1	FJ752425	Shen et al., 2010
Bambusicola	*Bambusicola thoracica*	Bth	2	EU165706	Shen et al., 2009
				NC011816	Shen et al., 2009
	Bambusicola fytchii	Bfy	1	FJ752423	Shen et al., 2010
Ithaginis	*Ithaginis cruentus*	Icr	1	GQ922649	Cai et al., 2010
Tragopan	*Tragopan caboti*	Tca	2	NC013619	Kan et al., 2010
				GU187969	Kan et al., 2010
	Tragopan temminckii	Tte	3	FJ752427	Shen et al., 2010
				GQ922634	Cai et al., 2010
				GQ922635	Cai et al., 2010
Pucrasia	*Pucrasia macrolopha*	Pma	2	FJ752429	Shen et al., 2010
				GQ922648	Cai et al., 2010
Lophophorus	*Lophophorus lhuysii*	Llh	2	NC013979	Ma et al., 2010
				GQ871234	Ma et al., 2010
Gallus	*Gallus gallus*	Gga	2	AY235570	Froman and Kirby, 2005
				GQ922621	Cai et al., 2010
Lophura	*Lophura nycthemera*	Lny	3	EU417810	Shen et al., 2009
				NC012895	Shen et al., 2009
				GQ922620	Cai et al., 2010
	Lophura ignita	Lig	2	NC010781	Kato et al., unpublished data
				AB164627	Kato et al., unpublished data
Crossoptilon	*Crossoptilon auritum*	Cau	3	GQ922639	Cai et al., 2010
				GQ922640	Cai et al., 2010
				NC015897	Li and Kan, unpublished data
	Crossoptilon crossoptilon	Ccr	3	GQ922613	Cai et al., 2010
				GQ922614	Cai et al., 2010
				NC016679	Zhao and Zou, unpublished data
Syrmaticus	*Syrmaticus reevesii*	Sre	2	AB164623	Kato et al., unpublished data
				NC010770	Kato et al., unpublished data
	Syrmaticus humiae	Shu	2	NC010774	Kato et al., unpublished data
				AB164625	Kato et al., unpublished data
	Syrmaticus soemmerringi	Sso	2	AB164622	Kato et al., unpublished data
				NC010767	Kato et al., unpublished data
Phasianus	*Phasianus colchicus*	Pco	3	GQ482363	Kerr et al., 2009
				NC015526	Kan and Li, unpublished data
				JF739859	Kan and Li, unpublished data
Chrysolophus	*Chrysolophus pictus*	Cpi	3	NC014576	Qin and Shi, unpublished data
				FJ752433	Shen et al., 2010
				HQ221859	Qin and Shi, unpublished data
	Chrysolophus amherstiae	Cam	3	FJ752434	Shen et al., 2010
				GQ922606	Cai et al., 2010
				GQ922608	Cai et al., 2010

Sequence divergence among species and genera was calculated using the Kimura two-parameter (K2P, Kimura, 1980) distance model in MEGA 5.0 (Table 1). All positions containing gaps were deleted from the dataset using the "complete deletion" option, and the vertebrate mitochondrial code was used throughout. Neighbor-joining (NJ) (Saitou and Nei, 1987) trees of K2P distances were created to provide a graphic representation of the pattern of divergence among species. Node support was assessed using the bootstrap method (Felsenstein, 1985).

RESULTS

Barcoding analysis

Of the 66 sequences from the 31 species, 2 individuals were analyzed per species, on average. None of the sequences had a DNA barcoding gap. All of the bird species had distinct COI sequences, of which none was shared between species. The average nucleotide composition was 28.0% T, 31.2% C, 24.8% A, and 16.0% G. The sum content of A and T exceeded 50%. Two hundred and forty-nine variable sites were identified, of which 237 were parsimoniously informative (i.e., 34.15% of the entire sequence).

Intraspecific genetic distances ranged from 0.0 to 1.6%. COI sequence divergences among congeneric species ranged from 1.6% (*Coturnix japonica* and *Coturnix coturnix*) to 14.2% (*Coturnix chinensis* and *Coturnix coturnix*) when all of the species examined were included in the analysis. The average difference in the COI sequence between species from each genus (7.2%) was 24 times higher compared to the average difference within a given species (0.3%). The genetic distance among the 18 genera ranged from 9.0% (*Lophura* and *Crossoptilon*, *Lophura*, and *Chrysolophus*) to 20.2% (*Perdix* and *Arborophila*) (Table 2).

Table 2. Genetic distance among the 18 genera belonging to the Phasianidae family.

	1*	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2*	0.137																
3	0.150	0.124															
4	0.142	0.138	0.126														
5	0.178	0.180	0.163	0.165													
6	0.141	0.147	0.16	0.150	0.171												
7	0.163	0.16	0.165	0.181	0.202	0.174											
8	0.144	0.148	0.133	0.128	0.171	0.16	0.164										
9	0.131	0.152	0.153	0.145	0.161	0.151	0.173	0.141									
10	0.144	0.159	0.163	0.168	0.169	0.181	0.186	0.174	0.169								
11	0.119	0.146	0.158	0.123	0.151	0.147	0.173	0.143	0.141	0.159							
12	0.110	0.149	0.152	0.127	0.171	0.160	0.175	0.136	0.129	0.132	0.127						
13	0.139	0.151	0.134	0.111	0.164	0.156	0.160	0.133	0.159	0.152	0.139	0.153					
14	0.132	0.161	0.166	0.131	0.160	0.165	0.192	0.159	0.158	0.166	0.121	0.149	0.144				
15	0.146	0.171	0.172	0.149	0.169	0.168	0.189	0.180	0.158	0.166	0.130	0.166	0.152	0.090			
16	0.134	0.150	0.142	0.152	0.156	0.156	0.178	0.156	0.158	0.158	0.125	0.139	0.146	0.117	0.117		
17	0.154	0.170	0.175	0.171	0.168	0.173	0.199	0.168	0.181	0.179	0.147	0.161	0.139	0.110	0.132	0.129	
18	0.144	0.181	0.161	0.157	0.146	0.172	0.186	0.174	0.171	0.181	0.126	0.163	0.159	0.090	0.109	0.127	0.110

*Genera: 1 = *Tetraophasis*; 2 = *Tetaogallus*; 3 = *Alectoris*; 4 = *Francolinus*; 5 = *Perdix*; 6 = *Coturnix*; 7 = *Arborophila*; 8 = *Bambusicola*; 9 = *Ithaginis*; 10 = *Tragopan*; 11 = *Pucrasia*; 12 = *Lophophorus*; 13 = *Gallus*; 14 = *Lophura*; 15 = *Crossoptilon*; 16 = *Syrmaticus*; 17 = *Phasianus*; 18 = *Chrysolophus*.

Phylogenetic relationships

The species could be discriminated by their distinct clusters in the phylogenetic tree (Fig-

ure 1). The phylogenetic tree showed low intraspecific and large interspecific divergences with strong bootstrap support (Figure 1). All the 18 genera were grouped into 2 clearly divergent clades (A and B, Figure 1). The genetic distance between A and B was 16.3% based on K2P model.

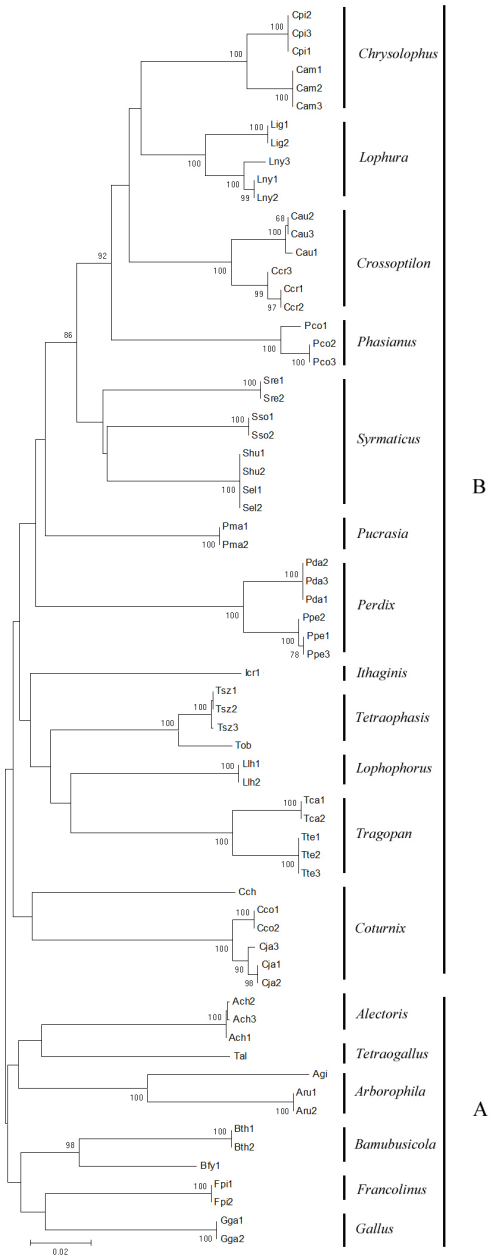

Figure1. Phylogenetic trees of eighteen genera of phasianidae constructed from mtDNA COI sequence. Numbers (in internodes) represent bootstrap values (≥80%) from 10,000 replications. The codes were shown in Table 1.

Clade A contained 6 genera (*Alectoris*, *Tetraogallus*, *Arborophila*, *Bambusicola*, *Francolinus*, and *Gallus*). Analysis of COI genes supported the inclusion of *Alectoris*, *Tetraogallus*, and *Arborophila* in a subclade. *Bambusicola*, *Francolinus*, and *Gallus* formed the other subclade (Figure 1).

Clade B contained the other 12 genera, which were separated into 3 subclades. Members of *Coturnix* formed one subclade. NJ analysis grouped *Ithaginis*, *Tetraophasis*, *Lophophorus,* and *Tragopan* into another subclade. The third subclade contained the other 7 genera (Figure 1).

DISCUSSION

Many preceding studies of birds have already ascertained the reliability of COI barcodes. These studies have confirmed a clear gap (the so-called barcoding gap) between intra- and interspecific K2P distance distributions (Breman et al., 2013). Hebert et al. (2004) suggested a "10x rule," which is a sequence threshold of 10 times the mean intraspecific variation for the group under study, to screen for splits referred to as putative species. However, Park et al. (2011) found that the CO1 sequence difference between closely related species (5.0%) was 25 times higher compared to within species differences (0.2%). This result was similar to the value obtained in the present study and by Yoo et al. (2006). In comparison, Scandinavian birds produced much higher differences, of up to 33 times (Johnsen et al., 2010). The rate of COI gene evolution is subject to variation in different clades of birds (Pereira and Baker, 2006). Therefore, it might be inappropriate to suggest a universal distance criterion for different species. In any case, distance-based DNA barcoding seems to provide sufficient information to identify and delineate a large majority of bird species, including those belonging to the Phasianidae family, through pairwise comparisons (Yoo et al., 2006; Kerr et al., 2007; Tavares and Baker, 2008; Aliabadian et al., 2009; Johnsen et al., 2010; Cai et al., 2010; Breman et al., 2013). These preceding studies also indicate that the COI barcode facilitated the separation of even the most closely related species.

The results of the present study clearly demonstrated the discriminatory power of COI barcodes for the identification of Phasianidae species. Phylogenetic analysis separated the different Phasianidae species into distinct branches, with high bootstrap support. None of the species shared sequences or had overlapping clusters with another species.

Phasianidae are a well-studied group of birds; however, some aspects of their evolutionary relationships remain unclear or ambiguous. Here, we provide the first phylogenetic analysis for Phasianidae using the COI gene. The phylogenetic tree grouped all the 18 genera into 2 divergent clades (Figure 1). In clade B (Figure 1), the taxonomic status of *Pucrasia*, *Perdix*, and *Coturnix* remained unresolved. Members of these 3 genera are generally monochromatic, with no highly dimorphic or ornamented species, and are considered to be partridges (Cheng, 1978). This feature contrasts with species in the other genera of clade A, most of which have males with a high degree of ornamentation, and are considered to be pheasants. Kimball et al. (1999) suggested that the terms pheasant and partridge should only be used to include suites of related behavioral and morphological traits, rather than implying anything about the evolutionary history of Phasianidae birds.

COI gene analysis well supported the inclusion of *Chrysolophus*, *Lophura*, *Crossoptilon*, *Phasianus*, and *Syrmaticus* in a single lineage (Figure 1). These genera exhibit a high

degree of ornamentation, with males having elongated tails and crests. Behaviorally, males are often polygynous, and do not tend to participate in parental care. Members of the 5 genera have been shown to be typical pheasants (Cheng, 1978). Molecular data were consistent with the morphology and behavior observed for these genera. *Chrysolophus* has been frequently grouped with *Phasianus* in many previously published studies (Kimball and Braun, 2008), which contradict the results presented here. This may be due to different genetic markers. Another, hybridization is widespread in Galliformes birds. *Chrysolophus* can hybridize with *Lophura* and *Phasianus* (McCarthy, 2006). Hybridization presents challenges to the reconstruction of phylogenies (Grant and Grant, 1992). NJ analysis grouped *Tragopan* with 3 other genera (*Ithaginis*, *Tetraophasis*, and *Lophophorus*) to form a single subclade, which was consistent with the results proposed by the mtDNA control-region gene (Huang et al., 2009).

In conclusion, DNA barcoding is an effective molecular tool for species identification and phylogenetic inference. Since this technique is based on molecular-level variation, it offers greater accuracy and authenticity compared to the more subjective plumage-based phylogeny of birds (Arif et al., 2011).

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