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Short Communication

Isolation of Mitochondrial Control Region for White-nest Swiftlets (*Aerodramus fuciphagus*) Using Primer Walking Techniques

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ABSTRACT

This paper reports on a novel DNA sequence located at the mitochondrial control region (D-loop) of the white-nest swiftlet (*Aerodramus fuciphagus*). This hypervariable control region sequence is potentially useful for studying genetic relationships among the white-nest swiftlet populations. The isolation of the control region involves a primer walking technique, which is simple, fast and cost-effective. In this study, the variability of the control region was assessed and discussed.

Keywords: Aerodramus fuciphagus, control region, Mitochondrial DNA, primer walking

INTRODUCTION

The most commonly used DNA markers in the molecular studies of swiftlets are cytochrome *b* of mitochondrial DNA (mtDNA; Lee *et al*., 1996; Thomassen *et al*., 2003; Price *et al*., 2004; Thomessen *et al*., 2005; Aowphol *et al*., 2008) and NADH dehydrogenase sub-unit 2 of mtDNA (NADH-2; Price *et al*., 2004; Thomassen *et al*., 2005; Aowphol *et al*., 2008). In particular, nuclear 12S and beta-fibrinogen intron regions were sequenced by Thomassen *et al*. (2005), whereas a microsatellite genotyping method was established by Aowphol *et al*. (2008). Notably, most of these markers were not specially developed for resolving the relationships of the swiftlets at lower taxonomic-levels. A non-coding region

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in the mtDNA is, therefore, expected to provide more informative characters in examining the phylogenetic relationships among the swiftlet populations.

One of the most variable regions in the mtDNA genome is the control region, also known as D-loop (Rahman *et al*., 2010). The control region of avian mtDNA contains three domains based on the distribution of the variable nucleotide positions and the differential nucleotide frequencies of parts of the control region (Quinn & Wilson, 1993). It was reported that Domains I and III were more variable compared to Domain II, as the average substitution rates for Domain I, Domain II and Domain III were 16%, 2.7%, 18.6%, respectively (Delport *et al*., 2002). There could also be a big difference in the substitution rate between the first half and second half of Domain I, for example, 2% and 20% were reported for the first and second half of Domain I (Randi & Lucchini, 1998). Sbisà *et al*. (1997) and Randi and Lucchini (1998) suggested the adoption of the following nomenclature for the three D-loop domains: the extended termination-associated sequence (ETAS) of Domain I, the central conserved domain of Domain II, and the conserved sequence blocks (CSB) of Domain III.

As there has been no mtDNA control region sequence reported for the white-nest swiftlet or its related species, this study aims to acquire the DNA sequence of this region using the white-nest swiftlets. This study also intends to develop a primer walking strategy for sequencing a DNA region with no prior information. Primer walking is a rapid and simple strategy developed for obtaining the sequences of large DNA fragments using the DNA cloning method (Strauss *et al*., 1986). This strategy is then widely used with several modifications customised for different circumstances (Kieleczawa *et al*., 1992; Kotler *et al*., 1994; Lodhi & McCombie, 1996; Gromek & Kaczorowski, 2005; Cairns *et al*., 2009).

MATERIALS AND METHODS

Primer Walking

The total DNA of the white-nest swiftlet embryo was extracted using the Promega DNA Extraction Kit following the manufacturer's instructions. The avian universal primers for the mtDNA region spanning the NADH6 to the control region, Thr (L) and H1251 (Desjardins & Morais, 1990) were used in the first step of primer walking. The sequence of the light strand of the 2 kb polymerase chain reaction (PCR) product was determined up to 500 bp from the 5' end. From this sequence, the second forward primer (L453) was designed. A primer pair of L453 and H1251 was used to amplify the DNA sample to give a 1.5 kb PCR product. This process of primer design, PCR and sequencing was continued until the whole control region was sequenced. Primers L12 and H12 were designed to amplify the range of 'partial ND6–$tRNA^{Glu}$–partial control region' for the phylogenetic analysis. All the primers, represented by 1 – 7 (Table 1) and their position in the mitochondrial genome are shown in Fig.1. Polymerase chain reaction (PCR) was run using a Perkin

TABLE 1
The primers used to design the primers of control region in this study.

No.	Primer name	Primer sequence (5' – 3')	Forward / Reverse
1.	Thr(L)	TTG TAA CAA GGA CAT TTG GTT TCT	Forward
2.	H1251	TCT TGG CAT CTT CAG TGC CRT GC	Reverse
3.	L453	CAA CGA CAC AAA GGA GAG GC	Forward
4.	L103	CAT AAG AGT TTC CAC TTG GC	Forward
5.	H238	AAA TGC CGC GAT TAC GGG TG	Reverse
6.	L12	AAC CAA CCA CCC CAT AGT AA	Forward
7.	H12	GAG ATA GCG GCA TAC CTA GC	Reverse

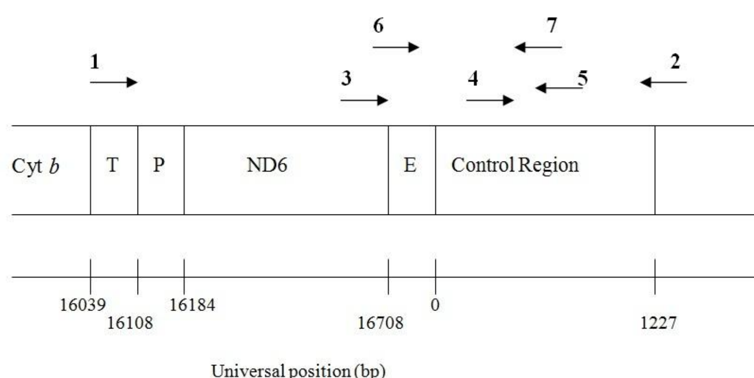

Fig.1: The universal positions of the primers. The numbers indicate the primer listed in Table 2.3. The arrows indicate the direction of the primers. T refers to $tRNA^{Thr}$, P refers to $tRNA^{Pro}$, E refers to $tRNA^{Glu}$

Elmer GeneAmp 9600 Thermocycler with the programme set at 2 min at 95.0°C; 30 cycles of 30 a at 94.0°C, 45 s at annealing temperature, 1 min at 72.0°C; 5 min at 72.0 °C; hold at 4.0°C. Annealing temperatures ranged from 55.0°C to 62.0°C. The PCR reaction mixture contained 1.5 mM $MgCl_2$, 0.5 µM forward and reverse primers each, 0.2 mM of dNTPs, 1× PCR buffer and ~10 ng of DNA samples. PCR products were purified using the Promega PCR Clean-up System kits following the instructions by the manufacturer. The purified PCR products were sent to the commercial laboratories [FirstBase Laboratories Sdn. Bhd. and Century Science Equipment (Sarawak) Sdn. Bhd.] for direct sequencing. All the DNA sequences obtained were deposited into Genbank (Accession number: JF269187–JF269235).

The partial control region sequences of 35 individuals were then obtained using Primers L12 and H12 (Table 1). These individuals were collected from the swiftlet houses of five localities: 10 from Endau-Rompin (02° 40−48' N; 103° 29−36' E), nine from Kuantan (03° 49' N; 103° 19' E), seven from the West Coast of Peninsular Malaysia (Perak and Selangor, i.e., 03° 46' − 04° 13' N; 100° 41−59' E), six from Sumatra

(03° 35' N; 98° 40' E) and three from Sibu (02° 18' N; 111° 49' E).

Data Analysis

The DNA sequences were trimmed to readable bases on both ends of the strands. In most cases, the scoring of the bases started by the light-strand complementing the light-strand towards the centre. The mitochondrial control region sequence of the closest related species thus far reported was that of the *Apus apus* (Apodiformes, the swift family) (GenBank accession no.: NC008540; Slack *et al*., 2009). This sequence was aligned with one of the DNA sequences obtained for the white-nest swiftlets in the present study.

To assess the variability of the control region, the sequences of all individuals were aligned using the Clustal X version 1.81 (Thompson *et al*., 1997) and adjusted manually using Bioedit (Hall, 1999) whenever necessary. Indels were coded following the Simple Indel Coding method (Simmons & Ochoterena, 2000) using the FastGap1.1 programme (Borchsenius, 2009). Maximum parsimonious (MP) analysis was conducted using PAUP4.0 (Swofford, 2002) and the phylogenetic tree was rooted with *Apus apus* (GenBank accession no. as mentioned above), *Alectura lathami* (GenBank accession no.: NC007227; Slack *et al*., 2005) and *Anser albifrons* (GenBank accession no.: AF363031; Slack *et al*., 2003). Bootstrap analysis was run for 1000 replicates.

RESULTS AND DISCUSSION

In the present study, the primer walking technique (Strauss *et al*., 1986) was modified to design the species-specific PCR primers for the mtDNA region without prior knowledge. This method is rapid and simple as it involves only repeated steps of PCR, direct sequencing and primer design. It is especially suitable for the organellar genomes, for instance, mtDNA, because organellar genomes are circular and relatively smaller in size compared to the nuclear genome.

The DNA sequences obtained using Primers L12 and H12 consisted of the 3' end of the NADH region (~40 bp), tRNA-Glu (~73 bp) and a partial control region (~346 bp). The typical strings of Cs at the beginning of the avian mitochondrial control region were also observed in the white-nest swiftlets (represented by the individual KT152; Fig.2). Unlike *Apus apus*, the white-nest swiftlets have three C-strings (Fig.2). The starting point of the control region falls at position 113 following the mtDNA characterisation of the *Apus apus* (Slack *et al*., 2009). The sequence upstream to the starting point is tRNA-Glu and NADH-6. The control region sequence obtained in this study was located in Domain I assuming that the white-nest swiftlet mtDNA did not differ much from the typical avian mtDNA gene arrangement and sizes (Quinn & Wilson, 1993; Quinn. 1997). The control region of the white-nest swiftlet affirms the findings of Randi and Lucchini (1998), that the second half of Domain I had a greater degree of variation (20%) than the

first half of Domain I (2%). The variations occurred in abundance after position 302 (data matrix not shown).

The aligned DNA matrix of the 35 white-nest swiftlet individuals (i.e. without the outgroups) was 350 characters in length, including 341 bases and nine indels. Among the 50 variable characters, 18 were parsimony-informative, that was 5.14%. A comparison with the cytochrome-*b* data obtained in Goh (2007) suggests that the control region of the white-nest swiftlets has a higher variability compared to the cytochrome-*b* (Table 2). Among the white-nest swiftlets sampled in this study, 15 individuals formed a well-supported clade (bootstrap value=84%; Fig.3), indicating that there are at least two distinct lineages among the house-farmed swiftlet populations.

TABLE 2
Comparison of the DNA data variability of control region and cytochrome-*b* sequence among the house-farmed white-nest swiftlets.

	DNA characters	Indel characters	Total characters	Variable characters (%)	Parsimony-informative characters (%)
Cytochrome-*b* (Goh, 2007)	558	0	558	17 (3.05)	6 (1.08)
Control region (present study)	341	9	350	50 (14.29)	18 (5.14)

Fig.2: Characterisation of the mtDNA sequence obtained in this study. *Aerodramus fuciphagus* was represented by the individual 'KT152' and the sequence was aligned with the mtDNA sequence of *Apus apus* (NC008540.1; Slack *et al*., 2009). Dots indicate characters identical with *A. apus* sequence. Letters designate base substitutions. ' – ' indicates gap.

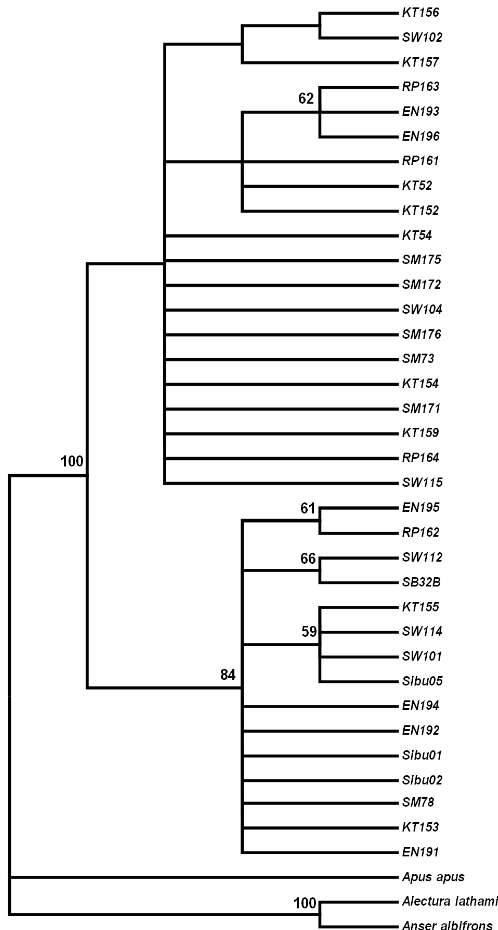

Fig.3: Strict consensus of the 72 most parsimonious trees based on the mitochondrial control region of the white-nest swiftlets. Bootstrap values of >50 % were shown next to the nodes. The prefix in the sample ID indicates the sampling localities (KT=Kuantan, RP=Rompin, EN=Endau, SM=Sumatra, SW=Perak, SB=Selangor, Sibu=Sibu).

CONCLUSION

This study suggests that the control region is a promising DNA marker for resolving the lower-level phylogenetic relationships among the closely related lineages of the swiftlets as well as to understand the genetic structure of the white-nest swiftlet populations. This study does not recommend if the control region is more advantageous over other mtDNA regions (such as cytochrome-*b*), but it provides one more choice of DNA markers which could be incorporated in future studies on the white-nest swiftlets. Primers L12 and H12 were proven to be specific to the white-nest swiftlets. Alternatively, Primers L12 and H1251 could be used if one were to sequence the full length of the control region. However, an additional step (e.g. DNA cloning) may have to be taken because H1251 is less species-specific. A similar technique can be used for developing other mtDNA regions of the white-nest swiftlets or the mtDNA control region primers for other avian groups.

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