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Sex identification in barn swallows (*Hirundorustica* Linnaeus) by molecular technique

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Sex-ratio of birds is an important understanding behavior, population structure, patterns of migration and estimating extinction risk. However, it is difficult to understand in some birdsbecause their external features are sexually monomorphic. In Thailand, the barn swallows (*Hirundorustica* Linnaeus) asa migratory bird that the female is similar in appearance to the male. So, the polymerase chain reaction (PCR) technique for determination of the sex were investigated. Birds sexing can be identified which based on*chromo-helicase-DNA-binding*(*CHD*) genelocated on sex chromosomes.Male birds are homogametic sex (ZZ sex chromosomes)while female birds are heterogametic sex (Z and W sex chromosomes). To selection a suitable primer for gender identification,the PCR reactions were used three primer sets, including P2/P8, 1237L/1272H and 2550F/2718R primers.As results, P2/P8 primers wereclearly differed between *CHD*-Z and *CHD*-W allele by agarose gel electrophoresis analysis.Therefore, sexing identification was attempted in 61 samples of *H.rustica* using P2/P8 primers.The sample consisted of 41 males (67.21%) and 17 females (27.87%): however, three samples (4.92%) could not amplify.The results of molecular sexing would also have implications for sex-ratio data ofbarn swallowsin Thailand.

Keywords: Sexing identification, *chromo-helicase DNA binding* (*CHD*), barn swallows

Introduction

The barnswallows (*Hirundorustica* Linnaeus) are migratory bird that is a group of passerines in the family Hirundinidae. They migrate northwards into Thailand after the breeding season. In Bangkok, they are very numerous during peak migration period between November and January. Physical characteristics, the barn swallows are small to medium-sized birds about six inches long. They have a dark blue upper body and head, extending in a line through the eye. Their throat and forehead is brown or dark rusty orange with a paler orange chest and underside. The outer couple tail is the longest with a deeply forked

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tail. Juveniles look similar to adults, but have much shorter tails. Including, these migratory birds are sexually monomorphic, which means that the female is similar in appearance to the male. However, some reports showed that the female's tail is a little less forked and her underparts are a little paler (Brown and Brown, 1999). In bird,colorful and elaborate feather are important traits in mate choice.Like the barn swallows, white tail markings or white spots and the length of the outermost tail feathers are importance and quality for sexual selection (Kose *et al.*, 1999). In particular,the length of the outermost tail feathers used to determine the sex that males had significantly longer outermost tail feathers than females (Smith and Montgomerie, 1991; Hermosell *et al.*, 2007). However, this observation might uncertain because the dual tail is broken from migration or flight. So, several papers showed significant positive correlation between the lengthor area of white spots and gender (Møller *et al.*, 1995; Kose and Møller, 1999). For example, Duijns *et al.* (2011) were found that the length of white spots less than 17.5mm as females and those with a white spot length more than 29.5 mm as males.However, this method is difficult to apply in the field and also take a long time for the measurement. So, the molecular techniques for birds sexing are developed.

Nowadays, birds sexing can be identified which based on *chromo-helicase-DNA-binding* (*CHD*) gene located on sex chromosomes. Male birds are homogametic sex (ZZ sex chromosomes) while female birds are heterogametic sex (Z and W sex chromosomes) (Griffiths and Tiwari, 1995; Ellegren, 1996; Griffiths and Korn, 1997; Ellegren and Sheldon, 1997; Griffith *et al*., 1998). Because of the intron length difference between the *CHD*-Z and *CHD*-W allele, amplicons with a single band are observed in males and two bands in females. However, the universal primers in bird sexing take advantage of size differences in the *CHD*-Z and *CHD*-W allele sush as P2/P8 (Griffiths *et al.*, 1998) 1237L/1272H (Kahn *et al*., 1998) and 2550F/2718R (Fridolfsson and Ellegren, 1999) primer.Those primers has been a commonly used primer set for sex identification in short-toed-eagle (Sacchi *et al*., 2004), black-faced spoonbill (Cheng *et al*., 2006), Eurasian oystercatcher (Watson *et al*., 2004), some plover (Poeaim *et al.*, 2014), black-winged stilt (Siripong *et al.*, 2015). In Thailand, sex-ratio has not been reported in barn swallows. Therefore, the aims of research findings were investigated by the PCR technique for sex identification in barn swallows. Furthermore, the different primers (P2/P8, 1237L/1272H and 2550F/2718R) to select a suitable primer for gender identification were also determined.

Materials and methods

Sample collection and DNA extraction

The barn swallows were captured inJanuary 2015 at Si Lom Road, Bangkok, Thailand which this site is a traditional roosting place of barn swallows during migration season. The barn swallows were identified into species based on morphological characters and banded by the staff of the wildlife research division, Department of National Parks, Wildlife and plant Conservation, Thailand.The feather quill contained soft tissue which were collected from individual barn swallow and placed in a 1.5 ml microcentrifuge tube containing 70% ethanol. A 0.2-0.5 cm section was cut from the terminal portion of feather quill, and used for DNA extraction with GF-1 tissue DNA extraction kit (Vivantis, Malaysia). The yield of the extracted DNA was quantified by spectrophotometry and DNA concentration was also checked by agarose electrophoresis method on 1 % agarose gel in 1X TBE buffer.

PCR amplification

In order to select a primer set, molecular sexing was run according to the procedure described in Poeaim *et al*., (2014). In a preliminary test, two examples of barn swallows (BS19 and BS20) were used to amplify with chicken which know sex used as positive control (cock: C1 and hen: C2). For 61 barn swallows using PCR amplification of slightly modified. Briefly, samples were sexed using three universal targeting two different sizes of *CHD*-W and *CHD*-Z alleles. PCR reactions are consisted of 300 ng DNA template, 12.5 µL of 2 X Taq master mixes (Vivantis), 4µL of each 20 pmol/ul primers and adjusted by 1.5 µLnuclaese free water. The conditions for PCR amplification conditions were determined an initial denaturing step at 95 ºC for 5 min, 35 cycles of 95 ºC for 1 min, 50 ºC for 1 min, and 72 ºC for 1 min, and final extension at 72 ºC for 10 min. PCR products were separated by 3% agarose gel (Vivantis) electrophoresis with 50 bp DNA Ladder (New England Biolabs) that used as size markers.

Resultsand Discussions

In this study, the genomic DNA from featherswere isolated that the DNA yield was sufficient in quantity andquality.Although blood is highly recommended for DNA extraction however, the birds become stressand trauma.In order to select a primer set, three primer sets (including P2/P8, 1237L/1272H and

2550F/2718R) were used for gendering identification of CHD gene of barn swallows.The results were shown that 2550F/2718R and 1272H/1273L primers were not suitable for sex identification ofbarn swallows.Although, 2550F/2718R primers would give a difference in length between introns in the *CHD*-Z and *CHD*-W alleles, it seems that the deepness of *CHD*-W bands was not clearly shown. The 1272H/1237L primers were not stable both expressedmultiple bands and not clearly shown the bands between male and female birds.Nevertheless, P2/P8 primers were differently cleared between male and female birds by fragments on anagarose gel electrophoresis. The females had two PCR products of 400 bp (*CHD*-W) and 350 bp (*CHD*-Z) while males shared a single product of 350 bp (*CHD*-Z) (Figure 1). The reduced PCR products about 50 bp, enhanced the relative size contract between the two PCR amplicons with not easy and clear resolution in routine 1% agarose gel. So, a little higher 3% agarose gel was then used, running the gel slower than usual could help resolution and fresh buffer are always useful.In these barn swallows, the size differences ranged from 50 bpbetween the two ZW alleles.In general, the difference in size between *CHD*-Z and *CHD*-W fragments amplified with the P2/P8 primers ranges from 10 to 80 bp. These results were similar to the works of Fridolfsson and Ellegren (1999), Jensen *et al*. (2003) and De Marchi *et al*. (2012). For sexing determination,the P2/P8 primer sets usually used to amplify the CHD genes in barn swallows was also explained by Kleven *et al*. (2006), Boncoraglio *et al*. (2008) and Vortman *et al*. (2011). Therefore, P2/P8 primers were used to identify the gender with all samples.

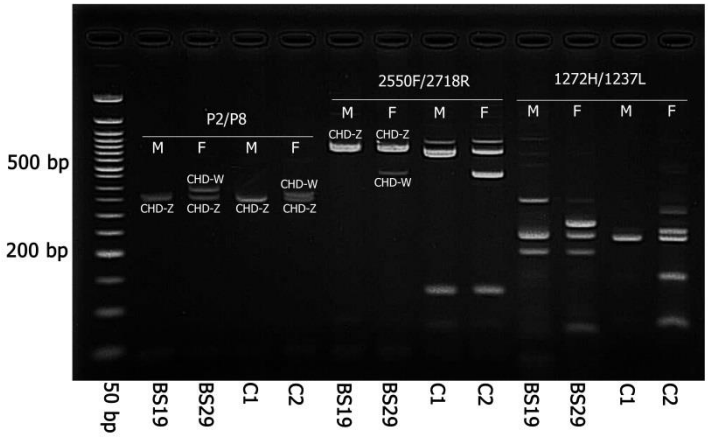

Figure 1. Comparison of PCR products in barn swallows (*Hirundorustica*: BS19 and BS29) and chicken (*Gallus gallus*: C1 and C2) from P2/P8, 1237L/1272Hand 2550F/2718R primers by 3% agarose gel electrophoresis.M = male andF = female

To identify sex in 61 barn swallows, thesuitable condition toamplify DNAwas found which the period of annealing at temperature 50°C by using primer P2/P8. The PCR products are the most shown clearly bands. After 3% gel agarose analysis, imager to check for the presence of bands; female PCR productsappeared as two separated bands (BS53 and BS58) and male PCR productsas a single (*CHD*-Z) band. The samples codes BS10, BS11, BS12, BS13, BS14, BS15, BS54, BS55, BS56, BS57, BS59, BS60, BS61 are male as seen in Figure 2.

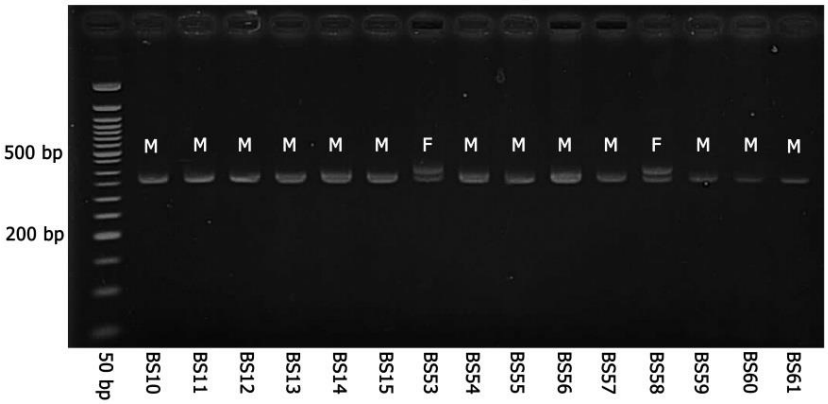

Figure 2. Sex identification in barn swallows (*Hirundorustica*) with P2/P8 primers for *CHD* gene. The PCR products were separated on 3% agarose gel. Female have two band (about 400 and 350bp) and males have one band (350bp) and marker DNA 50bp (lane1) M = male and F = female

Table 1. Expession ofsexing identification of barn swallows

Sexing	Number of samples	Percentage(%)
Male	41	67.21
Female	17	27.87
No band	3	4.92

Sexing identification was attempted to distinquishin 61 samples of *H. rustica* using P2/P8 primers. The samples consisted of 41 males (67.21%) and 17 females (27.87%). However, three samples (4.92%) were failured to amplify or no band (Table 1). Other three sampleswere not identified because there were shown less concentration DNA. For the examples ratio of barn swallows in the period of January 2015 in Thailand, found that the ratios of male and

female were 2.41:1.00 which males were shownmore than females. In a recent experimental study, many researchers reported that brood sizeenlargement had larger negative impact in male than femaleoffspring when compared to brood reduction. Hence, male offspring appear to suffer more from chronic food intake reduction as similar reports from Kleven *et al*. (2006), Boncoraglio *et al*. (2008) and Vortman, *et al.* (2011).

Conclusion

Some bird species are dimorphic, which means there are visible differences in appearance between male and female birds plumage. In Thailand, the period of migration, barn swallows are hard to identify sex by using these external features. So, PCR technique by using *CHD* gene with P2/P8 primers can be used. For analysis, the PCR products were separated on 3% agarose gel. Female showed two bands clearlty as 400 and 350 bp and males showedonly 350 bp band. For 61 barn swallows in January 2015, the ratios of male and female were 2.41:1:00 which males had more than females.The results of molecular sexing would also have implications forunder standing behavior, population structure, patterns of migration and estimating pollution risk.

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