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Identification of the Subspecies and Gender of Barn Swallow (*Hirundo rustica*)

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The Barn swallow (*Hirundo rustica* Linnaeus) is one of the most abundant birds and widely distributed. They have long outer tail feathers and white spots across the outer end of the upper tail. They are weak evidence of sexually dimorphic meaning both sexes are identical. There are six currently recognized subspecies: *Hirundo rustica rustica*, *H. r. gutturalis*, *H. r. tytleri*, *H. r. transitiva*, *H. r. savignii* and *H. r. erythrogaster*. In Thailand, barn swallows are migratory birds that the appearance of both genders is similar and there is little information on there subspecies. Therefore, the objective of this study was to assess the gender and subspecies of barn swallows. Sixty samples were collected using mist net method from Nan province for gender identification. Based on *chromo-helicase- DNA-binding protein* (*CHD*) gene, the resulting PCR products from P2/P8 primers revealed one band in male and two bands in female birds with DNA fragments of different sizes clearly about 50 base pairs. The white spot length on the outermost tail feather was significantly related to genders: males (> 21.25 mm, n=25) and females (≤ 21.25 mm, n=28). However, seven samples were not identified (11.67%). For subspecies identification, we analysed 15 informative partial *Nicotinamide adenine dinucleotide dehydrogenase subunit* (*ND2*) sequences compared with five subspecies sequences from GenBank. The sampling can be divided into at least three subspecies. Barn swallows which migrate to Thailand have genetic diversity.

Keywords: *Chromo-helicase DNA binding* (*CHD*), Barn swallows, *Nicotinamide adenine dinucleotide dehydrogenase subunit* (*ND2*), Gender identification

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Introduction

Barn swallows (*Hirundo rustica*) are small birds that are widely distributed swallow species in the world. These birds are divided into 6 subspecies corresponding to geography and differing only the coloration of the underparts. *Hirundo rustica rustica* which breeds in northern Eurasia, Africa and west Asia; *H. r. gutturalis* from southern and eastern Asian; *H. r. tytleri* from eastern Russia and may also reach Australia; *H. r. transitiva* from eastern of Mediterranean; *H. r. savignii* from the Egyptian delta region and *H. r. erythrogaster* from northern America, Alaska, Canada, and central Mexico (Turner and Rose, 1989; Zink *et al*., 2006). In addition, the researcher commonly used molecular markers for assessing genetic diversity in Hirundinidae (Sheldon and Winkler, 1993; Sheldon *et al*., 2005). Furthermore, using six regions of mitochondrial and nuclear DNA that can divide 6 subspecies of barn swallow into two groups: Asia-American and European-Middle Eastern (Dor *et al*., 2010). However, barn swallows are migratory birds which migrate to Thailand in winter. At least three subspecies such as *H. r. tytlerii*, *H. r. gutturalis* and *H. r. manchurica* were previously reported in Thailand but their distribution was not established.

Furthermore, barn swallows are sexually monomorphic, differences in the sexes are usually less distinct during migration. In many birds's species, a polymerase chain reaction (PCR) technique, targeting a chromo *helicase DNA binding protein* (*CHD*) gene, is used to determine gender (Griffiths *et al*., 1998; Cerit and Avanus, 2006; Morinha *et al*., 2012; Poeaim *et al*., 2014). Like the barn swallow, molecular sexing has been used to build discriminant functions to morphological data, population structure and behavior (Saino *et al*., 2002; Boncoraglio *et al*., 2008). In previous reports, the lengths of outermost tail feathers, the fork length and the white spot on the outermost tail feather are often used to distinguish between males and females in barn swallows (Samuel, 1971; Hermosell *et al*., 2007; Duijns *et al*., 2011). Therefore, the objectives of this study were to evaluate the subspecies of Barn swallow using *Nicotinamide adenine dinucleotide dehydrogenase* subnit (*ND2*) gene sequence as well as to determine the correlation between the white spot size on the outermost tail feathers and gender.

Materials and methods

Sample collection and DNA extraction

Barn swallows were trapped by the mist nets method, when they migrate to Thailand (November-January). The barn swallows were captured and

identified to species based on morphological characters by the staff of the Wildlife Research Division, Department of National Parks, Wildlife and Plant Conservation, Thailand. For gender identification, the sixty samples were collected from Pua district, Nan province (NBS) in November 2015. After being trapped, the bird measurements were made such as: wing, bill, head, tar, tail, weight, fat content as well as the length (mm), width (mm) and area (mm^2) of white spots on outermost tail feather (Figure 1). Additionally, the blood or the feather was collected for DNA extraction. For subspecies evaluation, the eight blood samples were sampling from Nan province (NBS) and seven samples from Si Lom road, Bangkok (BS) in January 2016. DNA extraction was as described in Malaitad *et al*., (2015).

Gender identification

For sexing, P2/P8 primers (Griffiths *et al*., 1998) targeting the *CHD* gene were used in this study, PCR reactions and conditions were as reported by Malaitad *et al*., (2015). To determine the correlation between the white spot size on the outermost tail feathers and gender, the data were analyzed by data mining approach. In this paper, we used RapidMiner Studio version 7.1 to predict correlation between gender and white spot size on outermost tail feather: length, width and area. A decision tree algorithm was used for data classification. First, decision tree model was created from the data, followed by testing the efficacy of the model, then the new data prediction. Finally, an automated decision tree approach to predicting white spot size was compare with molecular sexing.

Subspecies identification

The *ND2* region is located in mitochondrial DNA was amplified using METB and TRPC primers (Dor *et al*., 2010). Amplification was performed in 25 µl total volume that contained 100 ng DNA template, 2.5 µl of 10X standard *Taq* reaction buffer, 0.2 µl of *Taq* DNA polymerase, 4 µl of 1.25 mM dNTPs, 2 µl of 50 mM $MgCl_2$, 1µl of 20 µM each primer and adjusted by 13.3 µl of nuclease free water. The PCR condition was an ininitial denaturing step at 95 °C for 4.5 min, 35 cycles of 95 °C for 1 min, 62 °C for 1 min, and 72 °C for 2 min, and final extension step at 72 °C for 4.5 min.

After that, PCR products were examined by agarose electrophoresis method on 1% agarose gel in 1X TBE buffer. PCR products were sequenced at Bioneer (Korea). Our sequences were compared for relationship with six subspecies of barn swallows which were taken from the GenBank DNA

database. The phylogenetic tree was constructed based on neighbor-joining (NJ) analysis using Phylip program version 3.6.

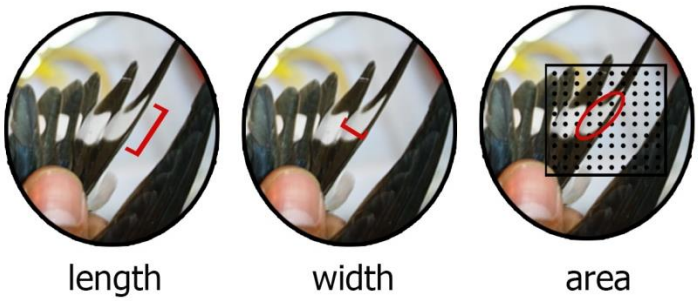

Figure 1 There are 3 scales of measurement, the length (mm), width (mm) and the area (mm^2) of the white spot on the outermost tail feather

Results

Generally, sexes in barn swallows are similar in morphology with the exception of the length of the outermost tail feathers which are significantly longer than in males. Therefore, the researcher used this morphomethic for sexing in the field. In Thailand, barn swallows are migratory birds which have breakage at the tips of the outer tail feather and tail moult, sexing becomes unreliable or even downright false. In this study, differences in the white spot size on the outermost tail feathers in Barn swallows were investigated using the length, width and the area for sexing. For molecular sexing, P2/P8 primers were used and determined with the white spot size on the outermost tail feathers, the data were presented with RapidMiner Studio version 7.1 (Figure 2).

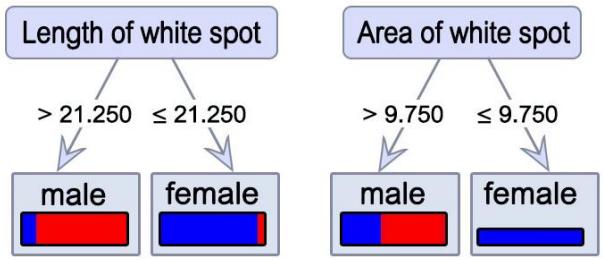

Figure2 The decision tree with Fisher's exact test showed white spot length and white spot area on the outermost tail feather for sexing identification (Blue color = female, Red color = male)

As results, P2/P8 primers were clearly differed between *CHD*-Z and *CHD*-W allele by agarose gel electrophoresis analysis. Sexing identification was attempted in 60 samples which consisted of 27 males and 33 females. Our data have the range of length, width and area of the white spot on the outermost tail feather are 10.50-35.00 mm, 2.50-7.00 mm and 4.50-21.00 mm^2, respectively. From the decision tree with Fisher's exact test, the length of the white spot on the outermost tail feather had positive correlation with gender. The white spot length less than or equal to 21.25 mm for female and greater than to 21.25 mm for male.

Discussion

Consistent with the previous report by Duijns *et al.*, (2010), this result showed the length of the white spot on the outermost tail feather is sexually dimorphic. The 101 adult barn swallows were caught during the non-breeding season in Zambia, the white spot length less than to 17.50 mm as a female and greater than to 29.50 mm as a male with 95% accurate. However, the rang between 17.50 to 29.50 mm was not reported. In this report, sex determination using the white spot length is 88.33% accurate. Only seven samples are error, it could be from juveniles birds. Because spot size increases with age with the total area of spots differing significantly between juveniles and adults, with the latter having larger spots than juveniles. The area of the white spot on the outermost tail feather was significantly related to the tail length of males, adult males had on average a total area larger than adult females (Kose and Mùller, 1999). The area of the white spot on the outermost tail feather was weakly significantly correlated with male, but not in females. It was found from the decision tree that the white spot area less than or equal to 9.75 mm^2 as a female and greater than to 9.75 mm^2 as a male. However, sex determination using the white spot area is 70% accurate which eighteen samples are unreliable. Nevertheless, the females have the white spot area on the outermost tail feather smaller than the male. There was no significant correlation between the width of white spots of the outermost tail feather with gender since the RapidMiner can not make the decision tree.

For the subspecies and genetic diversity of barn swallows were evaluated using *ND2* sequences. The fifteen birds (eight samples from Nan province: NBS and seven samples from Bangkok: BS) were analyzed for the relationship with five subspecies (six of *Hirundo rustica rustica*: ru, only one *H. r. gutturali*: gu, four of *H. r. transitive*: tr, two of *H. r. savignii*: sa and four of *H. r. erythrogaster*: er) in NCBI database. The phylogenetic tree was divided to two groups. Most of them in this study are in the same group which relate with *H. r.*

gutturalis with 93 bootstrap. However, only one sample (BS76) was related with *H. r. erythrogaster* with high bootstrap values (100%) as shown in the figure 3. The bootstrap values can be used for confidence levels for phylogenetic trees (Efron *et al.,* 1996). Moreover, fifteen Barn swallows are probably relative with *H. r. tytlerii* toward having no sequence of *ND2* region in NCBI database. We also found *H. r. rustica*, *H. r. transitiva* and *H. r. savignii* in the same group as well as *H. r. erythrogaster* and *H. r. gutturalis* in the other group. The result was consistent with the result of Dor *et al*., (2010) that divided genus *Hirundo* into Asia-American and European-Middle Eastern with *ND2* and *Cyt-b* sequence. Therefore, *Hirundo rustica* in Thailand may be in *H. r. erythrogaster, H. r. gutturalis* or *H. r. tytlerii*. However, the diversity of *H. rustica* in Thailand could be study in another region in mitochondrial DNA such as *Cytochome C oxidase subunit l (COl)* or *Cytochome b (Cyt-b)* (Sheldon and Winkler, 1993; Hebert *et al*., 2004; Sheldon *et al*., 2005; Dor *et al*., 2010) that will decrease our ability to determine the relationships among them.

Conclusion

The objectives of this study was to determine the correlation between the white spot size: the length, width and the area of the white spot on the outermost tail feather and gender. Sixty samples were collected from Nan province for analyzed. Our finding indicate that the length of the white spot on the outermost tail feather had positively correlation with gender. The white spot length less than or equal to 21.25 mm as a female and greater than to 21.25 mm as a male. Future research, this trait will be applying to sexing of barn swallows in the field. Forthemore, this study shown the diversity of *H. rustica* which may be in *H. r. erythrogaster, H. r. gutturalis* or *H. r. tytlerii*.

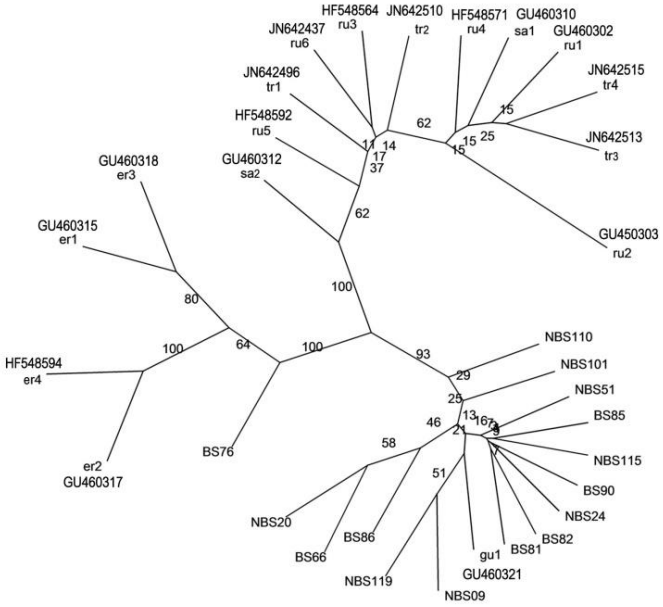

Figure 3 The phylogenetic tree showed the relationship of barn swallows in Thailand (NBS= sampling of Nan province, BS = sampling of Si Lom Bangkok) with five subspecies of Barn swallow.(*Hirundo rustica rustica* = ru, *H. r. gutturali* = gu, *H. r. transitive* = tr, *H. r. savignii* = sa and *H. r. erythrogaster* = er)

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