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Gender Identification of *Himantopus Himantopus* Using PCR-Based Method

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Abstract The black-winged stilt (*Himantopus himantopus*: Recurvirostridae) has a wide distribution in nature. In Thailand, there are residents and migratory birds. The adult birds have very long pink legs and black wings. Crown and hindneck patterns vary from white to dusky-grey. In general, the gender can be identified by crown and hindneck color, white in female and black in male. However, in breeding season (April - June) of resident bird at Bung Boraphet where the largest freshwater swamp in central Thailand, most of their crown and hindneck are white. So, this species are not clearly sexually dimorphic and the color of their crown and hindneck are not directly correlated with sex. In this work, a molecular approach was used to assess differences in coloration of feathers in relation to gender. DNA was amplified from the *chromo-helicase-DNA-binding* (*CHD*) gene that located on both Z (*CHD-Z*) and W (*CHD-W*) chromosomes. Using FTA®card and genomic DNA which extracted from a small volume of blood samples were used in this experiment. The resulting PCR products from 2550F/2718R primer showed fragments on a conventional agarose gel electrophoresis with size differences ranging from 150 bp between the two ZW alleles. Males were identified by the presence of a single band about 650 bp (*CHD-Z*) and females were identified by the presence of a second additional fragment length of approximately 500 bp (*CHD-W*). For the resident bird included 32 white and 2 black head, female: male were 181 :6 which 2 black heads are male. On the other hand, the migratory bird included 32 white head, 12 black hindneck, 43 grey head and 5 grey crown. The migratory bird includes 37 female and 46 male. Our results showed clear evidence for a sex- related decrease in the color on the heads of black-winged stilt.

Keywords: *Himantopus himantopus* ,sex identification, *chromo-helicase-DNA binding* (*CHD*) gene

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Introduction

The black-winged stilt (*Himantopus himantopus*: Recurvirostridae) has a wide distribution in nature. The adult birds have white body, needle-like blackish bill, black wings and very long pinkish-red legs. According to gender, black-winged stilt can be identified by crown and hindneck pattern color. Male are typically all white and sometimes can have some variable grey or black while female are browner and may show grey and black (Lekagul and Round, 1991). Brumfield (2010) stated that this species is usually name based on its crown and hindneck patterns and colors. For example, the white-headed stilt/ pied stilt (*Himantopus leucocephalus*) which reside in Australia but also found in Borneo, Java and the Philippines.

In Thailand, black-winged stilt were divided into 2 groups (1) resident birds: non-migratory populations and (2) migratory (visitor) birds. There are listed as protected animal according to Wildlife Preservation and Protection Act B.E. 2535 (1992). The migratory birds migrate to Thailand during November and January. They mostly are found in swamp and wetlands where food resources are available such as Bung Boraphet; the largest freshwater swamp in central Thailand. The resident birds are also resided and breed in the wetland area. The breeding season of resident birds generally starts from April to June. However, the morphological of resident birds are mostly white crown and hindneck pattern from observation. Therefore, this species are not clear sexual dimorphism and the pattern color of crown and hindneck are not directly correlated with their gender. The gender are an important understanding behavior, social structure, breeding system, mechanisms and patterns of migration and estimating extinction risk.

The birds used traditional methods for sex identification (Cerit and Avanus, 2006; Morinha *et al*., 2012) such as sexually dimorphism, acoustic sexing, laparoscopy, cloacal examination, steroid sexing and cytogenetic analysis. These methods are slow, expensive and harmful in some cases. So, the molecular techniques for birds sexing were developed. Most species of birds can be identified based on *CHD* gene; *chromo-helicase-DNA-binding* located on sex chromosomes (Griffith *et al.*, 1998). Male birds are homogametic sex which has two Z sex chromosomes. On the other hand, female birds are heterogametic sex and have Z and W sex chromosomes that containing *CHD-Z* and *CHD-W*, respectively (Watson *et al.*, 2004). Nowadays, molecular technique is a more reliable method for identifying the sex of birds which are monomorphic. However, this technique has not been in black-winged stilt. Therefore, the main aims of this research are to assess differences in coloration of feathers in relation to gender of black-winged stilt. Including, examining

genetics relationship of crown and hindneck colors and its species by using molecular technique.

Materials and methods

Sample collection

Thirty-four adult black-winged stilts were trapped by spring trapping method during breeding season (April-June) and ninety-two of migratory birds were trapped by cannon netting method during migration season (November-January) at Bueng Boraphet, Thailand. After being trapped, the bird measurements were made such as: wing, bill, head, tar, tail, weight and fat content. Secondly a small volume of blood sample was collected onto FTA®card (GE Healthcare, UK) by a puncture from the toe vein of the birds. Then photograph of the birds were taken especially their crown and hindneck pattern color. After all these processes the birds were released back to nature.

Purification of DNA onto FTA® Card

Blood samples in FTA®card were punched on dried blood sample (approximately 2 mm diameter). Place each disc in a PCR amplification tube and washed two times with 125 µl FTA Purification Reagent (GE Healthcare, UK). Each time the sample was mixed by pipetting up and down several times and incubates at 65 ℃ for 10 min after which the liquid was removed. After that, 125 µl of 0.1 mM TE buffer was added and incubated at room temperature for 10 minutes. TE buffer was discarded and repeated the TE buffer step again. Finally the disk in PCR tube was dried at 65 ℃ for 10 minutes.

PCR amplification and sequencing

For gender identification, the PCR reactions were used 3 primer sets, including P2/P8 primers (Griffiths *et al.*, 1998), 1237L/1272H primers)Kahn *et al.*, 1998 (and 2550F/2718R primers) Fridolfsson and Ellegren, 1999(. Amplification was performed in 25 µl total volume that contained 12.5 µl of 2X *Taq* Master mix (Vivantis), 1 µl of 20 µM from each primer set and 10.5 µl of nuclease free water. The conditions for PCR amplification conditions were a initial denaturing step at 95 ℃ for 5 min, 35 cycles of 95 ℃ for 45 sec, 50 ℃ for 45 sec, and 72 ℃ for 45 sec, and final extension at 72 ℃ for 5 min. PCR products were electrophoresed through 1.5% agarose gel in 1X TBE buffer comparing with 50 base pairs DNA ladder (Vivantis) and stained with ethidium bromide. Then, PCR products were purified by GF-1 AmbiClean Kit (Gel &

PCR) from Vivantis. In addition, PCR products were sequenced by 1st BASE (Malaysia). Sequences were edited and analyzed by Bioedit and MEGA 6.

Results and discussion

Three primer sets (including P2/P8, 1237L/1272H and 2550F/2718R) were used for gender identification of *CHD* gene of black-winged stilt. The results were shown that P2/P8 primers are not specific for this bird and 1237L/1272H primers could not identify the gender (Fig.1). Nevertheless, 2550F/2718R primers were shown clearly different between male and female birds by fragments on a conventional agarose gel electrophoresis. The size differences ranged from 150 base pairs between the two ZW alleles. Fridolfsson and Ellegren (1999) show that the resulting PCR products from 2550F/2718R primers showed fragments on a conventional agarose gel electrophoresis with size differences ranging from 150 base pairs between *CHD-Z* and *CHD-W* allele. Male birds amplified a single band of approximately 650 base pairs. However, female birds amplified two bands 500 base pairs (*CHD-W*) and 650 base pairs (*CHD-Z*). Therefore, 2550F/2718R primers were used to identify the gender with all samples. Vucicevic *et al.* (2012) reported that the use of 2550F/2718R primers also gave good results in 50 species; most of them are in the same class as black-winged stilt. At the same time, P2/P8 primers were successful used for sexing in Eurasian Oystercatchers (*Haematopus ostralegus*) which is in Charadriiformes as same as black-winged stilt (Watson *et al.*, 2004). In these studies, the female chicken (*Gallus gallus domesticus*) was used as a positive control (Fig. 2: the last lane). However, some samples (7.1%) could not amplify (Fig. 2: lane 2-3). This failure may be from FTA®card purification step. In order to ensure success, FTA®card should be washed away all cell debris.

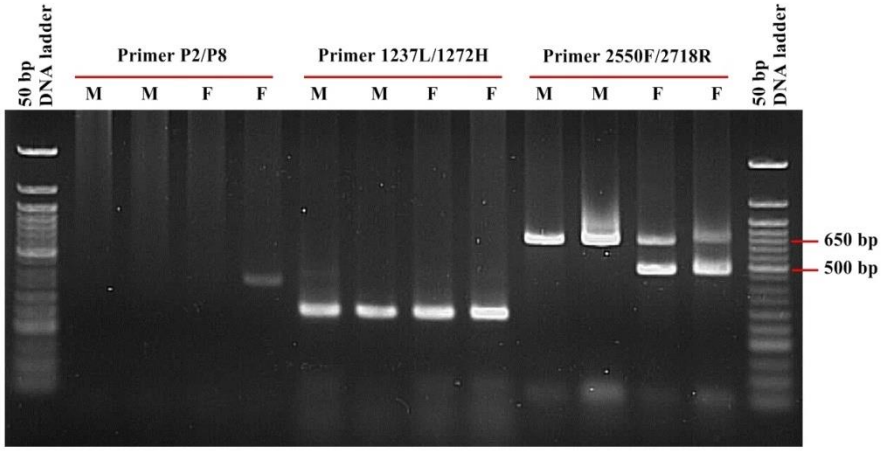

Figure 1. Gender identification of black-winged stilt using 3 primer sets were separated on 1.5% agarose gel. The first and last lane is 50 base pairs DNA ladder. Lane 2-5: samples were amplified by using P2/P8 primers. Lane 6-9: samples were amplified by using 1237L/1272H primers. Lane 10-12: samples were amplified by using 2550F/2718R primers

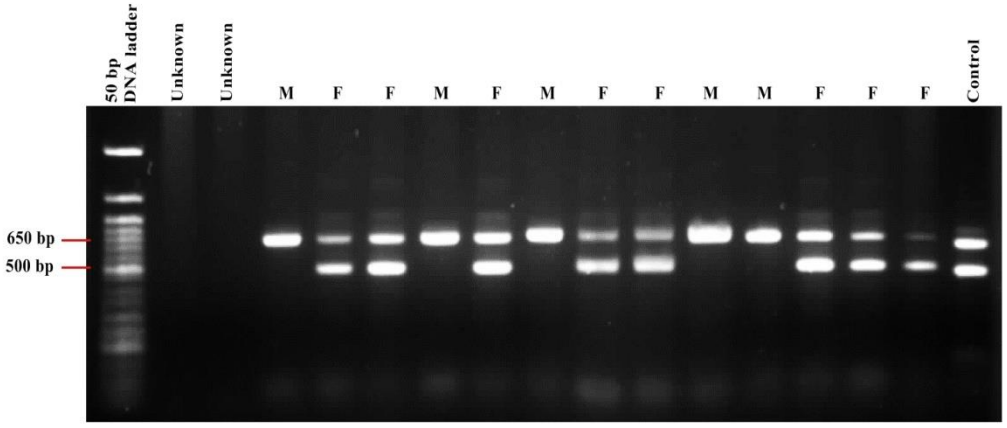

Figure 2. DNA samples were extracted from FTA®card and were amplified by using 2550F/2718R primers. The first lane is 50 base pair DNA ladder, with black-winged stilt samples in lane 2 onwards. Samples with single bane are male and those with two bands are female. The last lane is female chicken which shows two bands pattern as positive control

Table 1. Gender identification of black-winged stilts comparing with their crown/hindneck pattern colors during breeding and migratory season

Season	Crown/hindneck pattern color	Number of sample			Totals	
		Male	Female	Unknown		
Breeding	White	16	16	-	32	
	Black	2	-	-	2	
	Totals	18	16	-	-	34
Migratory	White	14	16	2	32	
	Black	5	6	1	12	
	Grey	22	15	6	43	
	Grey crown	5	-	-	5	
	Totals	46	37	9	-	92

The adult resident and migratory birds were separated into 2 and 4 patterns, respectively (Fig. 3). In breeding season of resident birds, there are white crown/hindneck and black hindneck pattern colors. However, there are (1) white crown/hindneck, (2) black hindneck, (3) grey crown/hindneck and (4) dusky-grey crown in migration season. From 126 samples, resident birds show that female: male were 181 :6 which 2 black hindneck are male. On the other hand, migratory birds were 37 female and 46 male (Table 1). Our results showed clear evidence for a sex- related decrease in the color on the heads of black-winged stilt.

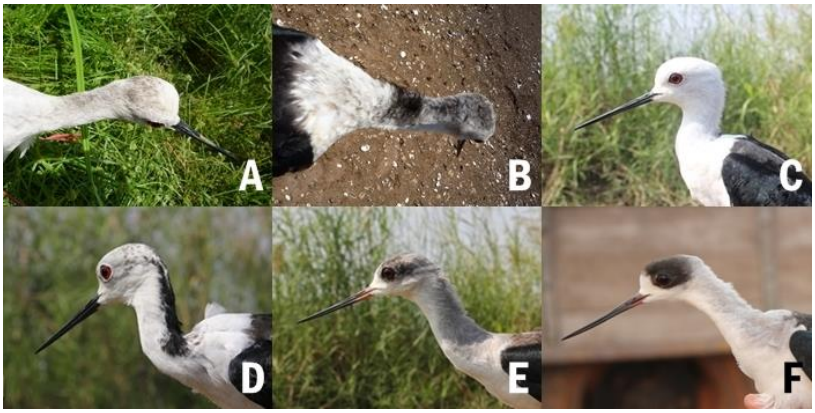

Figure 3. The resident birds (A-B) and migratory birds (C-F) were separated by the crown and hindneck pattern color. A: white crown/hindneck, B: black hindneck, C: white crown/hindneck, D: black hindneck, E: grey crown/hindneck and F: dusky-grey crown

In addition, PCR products from 2550F/2718R primers were analyzed by comparing *CHD-Z* allele from 9 resident and 12 migratory birds (Fig. 4). About 554 base pairs nucleotide sequence were shown 2 regions of single nucleotide polymorphism. The black column on left side presents a transversion mutation which involves exchange of purine (A) and pyrimidine (G). On the other hand, the black column on right side presents a transition mutation which involves bases of purine (A) and purine (T). This result corresponds to Faux *et al.* (2014) which stated that the conserved regions between *CHD-Z* and *CHD-W* of Southern Ocean seabirds contain some base changes.

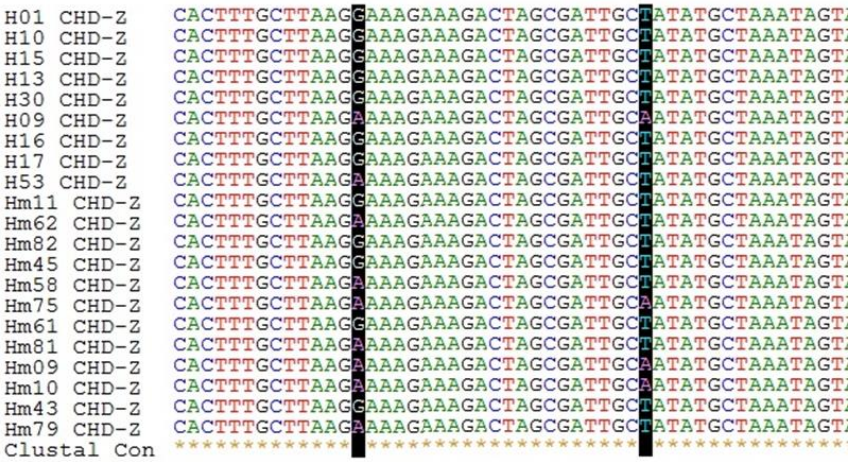

Figure 4. Two regions of single nucleotide polymorphism on sequence of *CHD-Z* allele from 9 resident (H) and 12 migratory birds (Hm)

Conclusion

From 126 samples of black-winged stilt, the PCR of *CHD* gene could be detected 93% of samples for gender identification. Also, the use of FTA®card is successfully to this study and less harmful to the birds. However, the results of the *CHD* were compared with the gender assigned to each bird by crown and hindneck pattern colors, it confirmed that the gender could not be identify by the colors alone. In the future, it would be better if DNA analysis and the biometrics of the birds to be used to compare. Additional, another gene and molecular techniques such as RAPD, SRAP and iPBS are interested for future research studies.

Acknowledgements

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