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Assessment of genetic diversity and relationships among *Charadriusmongolus* and *Charadriusleschenaultii* using AFLP markers

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*Charadriusmongolus*and*Charadriusleschenaultii*are migratory shorebirds in Thailand during the non-breeding season in which adult plumages are not clearly expressed sexually dimorphic. Eighty-six samples were trapped by cannon net from Trang and SamutSakhon province. To determine the gender, *chromo-helicase-DNA-binding* (*CHD*) genes were amplified with 2550F/2718R primer. Gender identification was attempted in 46 *C. mongolus* and 40 *C. leschenaultii*. For *C. mongolus*, the male gender in SamutSakhon province revealed the number more than in Trang province.Amplified fragment length polymorphisms (AFLP) technique was used to study genetic diversity and relationships among 11 *C. mongolus* and 7*C. leschenaultii*.Forty primers were screened and ten primers were able to amplify DNA fragments. Five primers were produced reproducible fragments with easily recordable bands which were selected for analysis.In total 323 bands were scored, the result showed 193 polymorphic bands (59.75%).Dendrogram constructed with the unweighted pair-group method with arithmetic mean (UPGMA) of genetic relationships based on simple matching coefficients varied from 0.75to 0.92.The cluster analysis was classified the plovers into two major groups which is related to the species. Including, the plovers can be separated by sampling area. Result showed the AFLP markers for assessing genetic relationship among *C. mongolus* and *C. leschenaultii*that corresponded to the geographicdistribution.

Keywords: gender identification, genetic diversity, AFLP markers,*Charadriusleschenaultii*, *Charadriusmongolus*

Introduction

Bird migration is made in a response to change for food availability, habitat, climate change or extreme weather. So, shorebird surveys have been done as a useful tool for understanding bird population, migration routes or flyways and the distribution of migrants as well as the relative abundance by

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habitat type or location. However, these yearly migrations, birds have the potential of disperse pathogens that can be dangerous for human and animal health (Reed etal., 2003).Therefore, to understand the population structure and migration flyways are necessary to predict and prevent human or birds infections.

Shorebirds are members of the order Charadriiformes which is a large and diverse group of bird species. The most species of migratory shorebird are the Scolopacidae and Charadriidae. *Charadrius* is a genus of plovers, a group of wading birds which are found throughout the world. During the breeding season, researchers are able to sexing. However, during the non-breeding season, male and female plovers are similar in appearance (sexually monomorphic). Therefore, sex identification is difficult to observe in timing of migration.The greater sand plover (*Charadriusleschenaultii*) and lesser sand plover (*Charadriusmongolus*) are a small wader in the plover. They are migratory shorebirds in Thailand during the non-breeding season in which greater sand plover is the most similar to lesser sand plover. In addition,both were divided into several distinct subspecies or races, which sometimes overlap each other in morphological appearance, in some biometrics and in geographical distribution. In the greater sand plover have three subspecies: *C. l. columbinus*, *C. l. crassirostris*(*C. w. crassirostris*) and *C. l. leschenaultii*(Hirschfeldet al., 2000) or *C. l. scythicus* (Carlos et al., 2012). For lesser sand plover, they can be divided into two subgroups and five subspecies: *atrifrons*group including the three races *atrifrons*, *pamirensis* and *schaeferi*and *mongolus*group including the two races *mongolus* and *stegmanni*(Hirschfeld et al., 2000).Including, females and non-breeding males have a similar plumage. So, adult plumages are not clearly both sexually dimorphic and species.

Therefore, the aims of research finding were to determine the gender of sexually monomorphic plover using PCR-based molecular sexing technique. Including, examining genetic diversity and relationships among *C. leschenaultii* and *C. mongolus* from two areas in Thailand (Trang and SamutSakhon Province) using amplified fragment length polymorphism (AFLP) markers.

Materials and methods

Sample collection: shorebirds were captured during the non-breeding season between November 2014 to January 2015 by using cannonnets which have the potential to capture a larger number of birds at once. The plovers were captured and identified into species based on morphological appearance by the staff of the Wildlife Research Division, Department of National Parks, Wildlife and Plant Conservation, Thailand. The plover used in this study were gathered

from Kantang River Estuary, Kantang District, Trang Province (sample code: CT), South of Thailand and Bang YaPhraek Sub-district, MueangSamutSakhon District, SamutSakhon Province (sample code: CS), Central of Thailand.

The feather quill contained soft tissue or blood were collected from individual plover and used for DNA extraction using 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. Some plover, blood was collected directly onto Whatman FTA® cards (GE Healthcare, UK) and purified with FTA Purification Reagent.

Sex determination: molecular sexing was run according to the procedure described byPoeaim et al., (2014). Briefly, samples were sexed using2550F/2718R primer (Fridolfsson and Ellegren, 1999) targeting two different size of *CHD*-W and *CHD*-Z.The reaction mix was added to the sample tube containing the purified and immobilized DNA or 100 ng genomic DNA.Polymerase chain reaction (PCR) amplifications were carried out in 25 µL reaction volume containing 12.5 µL of 2 X Taq master mix (Vivantis, Malaysia) and 1 µL of each 20 µM primer and adjust volume with nuclease free water.The thermal profile for amplification was 95°C for 3 min, and followed by 35 cycles of 95°C for 45 sec, 50°C for 45 sec and 72°C for 45 sec and finished by a final extension of 72°C for 10 min using the EppendorfMastercycler EP Gradient S.PCR productswere separated by1.5% agarose gel (Vivantis, Malaysia) electrophoresiswith 50 bp DNA Ladder (New England Biolabs, USA) that used as size markers. The gel was visualized under UV light after ethidium bromide staining and photographed.

The AFLP analysis was carried out according to Vos et al. (1995) with minor modifications. Briefly, 100 ng of genomic DNA was double digested with 1 FDU/µLeach of *Eco*RI and *Tru*IIrestriction enzymes and incubated overnight at 37°C in a total volume of 20 µL. Double stranded *Eco*RI and *Mse*I adapters (5 and 25 µM, respectively) that specific to each site were ligated to the digested DNA fragments using 1 U of T4 DNA ligase in ligation buffer (1X T4 DNA ligase buffer) and incubated at 22°C for 1 hr. Pre-selective amplification of ligated DNA (diluted 10-fold) was performed with primers complementary to the *Eco*RI and *Mse*I adapters with one selective nucleotide (*Eco*RI-A and *Mse*I-C) at the 3' end. It was performed in the EppendorfMastercycler EP Gradient S: an initial step of 2 min at 94°C, 25 cycles of 94°C for 30 sec, 56°C for 60 sec and 72°C for 60 sec, and a final step of 10 min at 72°C. For selective amplification, 5 µL of a 10-fold diluted pre-amplification mixture was amplified in the same thermocycler as pre-amplification consisting an initial step of 2 min at 94°C, of 12 cycles of 30 sec

at 94°C, 30 sec at 65°C to 56°C (with a decreasing of 0.7°C each cycle), and 60 sec at 72°C, then by 24 cycles of 30 sec at 94°C, 30 sec at 56°C, and 60 sec at 72°C using *Eco*RI and *Mse*I with three selective nucleotides (*Eco*RI-ANN/*Mse*I-CNN). The pre-selective nucleotide primers, adaptor sequences and forty primer combinations were assessed in Table 1.

Table 1 The primers and adaptor sequences used in this study

Primer/adaptor name		**Sequences (5'-3')**
Adaptors	*Eco*RI adaptor	5'-CTC GTA GAC TGC GTA CC-3' 3'-CAT CTG ACG CAT GGT TAA-5'
	*Mse*I adaptor	5'-GAC GAT GAG TCC TGA G-3' 3'-TA CTC AGG ACT CAT-5'
Preselective primers	*Eco*RI-A	5'-GAC TGC GTA CCA ATT CA-3'
	*Mse*I-C	5'-GAT GAG TCC TGA GTA AC-3'
Selective primers	*Eco*RI-AAC	5'-GAC TGC GTA CCA ATT CAA C-3'
	*Eco*RI-AAG	5'-GAC TGC GTA CCA ATT CAA G-3'
	*Eco*RI-ACA	5'-GAC TGC GTA CCA ATT CAC A-3'
	*Eco*RI-ACC	5'-GAC TGC GTA CCA ATT CAC C-3'
	*Eco*RI-ACG	5'-GAC TGC GTA CCA ATT CAC G-3'
	*Eco*RI-ACT	5'-GAC TGC GTA CCA ATT CAC T-3'
	*Eco*RI-AGA	5'-GAC TGC GTA CCA ATT CAG A-3'
	*Eco*RI-AGC	5'-GAC TGC GTA CCA ATT CAG C-3'
	*Eco*RI-AGG	5'-GAC TGC GTA CCA ATT CAG G-3'
	*Eco*RI-AGT	5'-GAC TGC GTA CCA ATT CAG T-3'
	*Mse*I-CAA	5'-GAT GAG TCC TGA GTA ACA A-3'
	*Mse*I-CAC	5'-GAT GAG TCC TGA GTA ACA C-3'
	*Mse*I-CAG	5'-GAT GAG TCC TGA GTA ACA G-3'
	*Mse*I-CAT	5'-GAT GAG TCC TGA GTA ACA T-3'

PCR samples were denaturated by adding an equal volume of formamide buffer (98% formamide (v/v), 10 mM EDTA, pH 8.0, 0.05% bromo-phenol blue (w/v), and 0.05% xylene cyanol (w/v)), heating for 3 min at 98°C and chilled on ice. PCR products were visualized on a 6% denaturing polyacrylamide gel about 3 hr in a sequencing gel electrophoresis apparatus (Bio-Rad Sequi-Gen GT) containing 7 M urea in 1XTBE buffer. The DNA bands were visualized by silver staining. Only bright, clearly distinguishable bands between 50 and 700 bp were recorded for analysis.

Data scoring and analysis of AFLP: clear and well resolved bands of the plover sampleswere compared with each other.DNA fragments were scored as a binary character (1 for presence and 0 for absence) from each primer. Coefficients of genetic similarity (GS) between the individuals were obtained using the simple matching coefficient (Rohlf, 2000) and constructed a dendrogram based on genetic similarity matrix using the UPGMA (unweighted

pair group mean average) method by NTSys Version 2.0e program. Including, principle coordinate analysis (PCA) among all individuals was analyzed using SPSS software.

Results and discussion

Sex determination have been identified in some plover such as Kentish plover (*C. alexandrines*) (Szekely et al., 2004; Küpper et al., 2009), Crab plovers (*Dromasardeola*) (De Marchi et al., 2012). *Charadrius* spp. or plover are migratory shorebirds in Thailand during the non-breeding season in which adult plumages are not clearly shown sexually dimorphic. So, determining the gender of sexually monomorphic plover by molecular sexing techniques is considered to be more reliable than morphometric analysis which can be influenced by age and season. In a previous study that normally used three common primer pairs, including P2/P8 (Griffiths et al., 1998), 1237L/1272H (Kahn et al., 1998) and 2550F/2718R (Fridolfsson and Ellegren, 1999) to determine six *Charadrius* spp. gender (Poeaim et al., 2014). The 2550F/2718R primers were shown clearly different between male and female birds by fragments on an agarose gel electrophoresis. In this study, gender identification was attempted in 86 plovers asshown in Table 2.Fortygreater sand plover (*C. leschenaultii*)were consisted of 23 males and 17 females whichshows the male to female sex ratio about 1: 1.35.Forty-six *C. mongolus* were consisted of 24 males and 22 females which the male fromSamutSakhon province showed the number more than from Trang province (M: F = 3.8: 1). However, the female from Trang province showed the number more than from SamutSakhon province (M: F = 1: 3.4).

Initially 40 primers were screened; ten primers were able to amplify DNA fragments. Five primers (*Eco*RI-AAG/*Mse*I-CGA, *Eco*RI-AAG/*Mse*I-CGT, *Eco*RI-AAC/*Mse*I-CGT, *Eco*RI-AAG/*Mse*I-CGC and *Eco*RI-AGA/*Mse*I-CGG) were produced reproducible fragments with easily recordable bands which were selected for analysis. The number of bands per primer ranged from 45 (*Eco*RI-AGA/*Mse*I-CGG) to 92 (*Eco*RI-AAG/*Mse*I-CGT) with an average 64.6. In total 193 polymorphic bands were scored which *Eco*RI-AAG/*Mse*I-CGT primer gave the highest number of polymorphic fragments (Table 3).

Table 2 Gender identification of lesser sand plover and greater sand plover from Trang andSamutSakhon province

Species	Province	Number of sample		Totals
		Male	Female	
Lesser Sand Plover (*C. mongolus*)	Trang	5	17	22
	SamutSakhon	19	5	24
	Totals	24	22	46
Greater Sand Plover (*C. leschenaultii*)	Trang	14	13	27
	SamutSakhon	9	4	13
	Totals	23	17	40

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Table3 Primer codes, number of tatol bands and polymorphism detected by the use of five AFLP primers in 18 plover samples

Primer codes	Number of total bands	Number of polymorphic bands	Polymorphism (%)
*Eco*RI-AAG/*Mse*I-CGA	63	43	68.25
*Eco*RI-AAG/*Mse*I-CGT	92	60	65.22
*Eco*RI-AAC/*Mse*I-CGT	72	40	55.56
*Eco*RI-AAG/*Mse*I-CGC	51	31	60.78
*Eco*RI-AGA/*Mse*I-CGG	45	19	42.22
Total	323	193	59.75

The simple matching coefficient among 18 plovers based on the AFLP fragments was used to construct a dendrogram (Figure 1) by UPGMA analysis. The value of similarity coefficient ranged from 0.75-0.92 which indicated that genetic variation of plover wasmoderate level. The cluster analysis was classified the samples into two major groups which related to species.The first major group (I) consisted of the 7 greater sand plovers which could be subdivided into 2 subgroups (IA and IB). These subgroups were separated from each other which related to the geographic distribution. The one group was come from Kantang District, Trang Province (CT13, CT15, CT17, CT21 and CT24) and the other was come from MueangSamutSakhon District, SamutSakhon Province (CS24 and CS28). The other major groups (II) consisted of the 11 lesser sand plovers which could be subdivided into 3 subgroups (IIA, IIB and IIC). The IIB group was come from Kantang District, Trang Province (CT43,CT45, CT46 and CT47) and IICwas come from MueangSamutSakhon District, SamutSakhon Province (CS29, CS34, CS35, CS36, CS39 and CS40). Considered of CT27 or IIAfrom Trang Province was separated branch from each other in this group which is grouping by two-dimensional principal coordinates analysis in Figure 2.

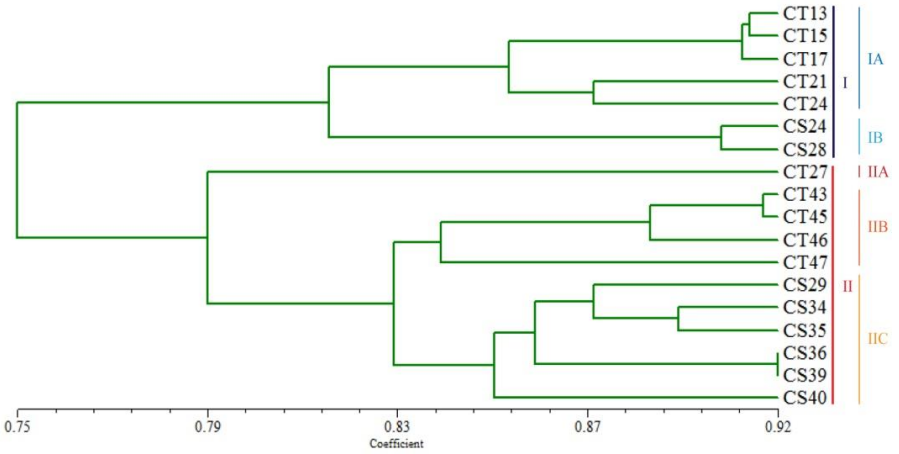

Figure1.Dendrogram constructed with the unweighted pair-group method with arithmetic mean (UPGMA) of genetic relationships among 18 samples of plovers using five primer combinations based on simple matching coefficients from AFLP marker

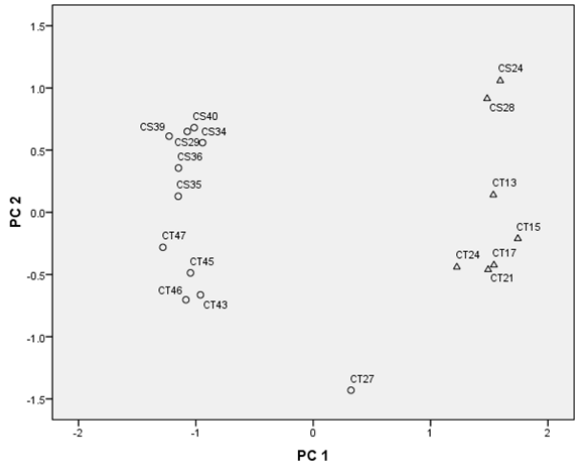

Figure 2 Two-dimensional Principal coordinates analysis among 11 *Charadriusmongolus*(○) and7*Charadriusleschenaultii*(△) based on AFLP markers

In previous study, *CHD* gene and ATPase subunit 6/8 gene were used to identify species in plovers that the ambiguity identified in the field. Phylogenetic tree of the ATPase 6/8 gene (826 bp) can be divided into three clades including lesser sand plover (*C. mongolus*), greater sand plover (*C. leschenaultii*) and *C. alexandrinus* complex plover. The *C. alexandrines* can be subdivided into Kentish plover (*C. alexandrinus*), white-faced plover (*C. alexandrinusdealbatus*) and Malaysian plover (*C. peronii*). However, lesser sand plover or greater sand ploverhas failed to detect geneticdifferentiation

between the subspeciesor subgroup from those sequences.Genetic diversity is commonly measured by genetic distance or genetic similarity, both of which imply that there are either differences or similarities at the genetic level. From AFLP, the genetic similarity of 18 plovers was observed ranging from 0.75-0.92 with an average value of 0.85, indicating moderate level of genetic diversity.

However, in CT27was collected from TrangProvience showed a deep distance from the other lesser sand plovers. From data, it is suggested that the sample was the *atrifrons* group which could be solved by *cytochrome c oxidase* I (*COI*) sequence. Because of in this region of the mitochondrial gene, *COI* might serve as a DNA barcode for the identification of animal species (Gibsonand Baker, 2012).Moreover, Hebert et al., (2004) reported that the effectiveness of a *COI* barcode in discriminating bird species, average of nucleotides differences between and within species, 7.93% and 0.43%, respectively. However, Juliaet al., (2013)retrieved sequences for atotal of ten markers; including eight mitochondrial and twonuclear markers for shown thephylogenetic position of *Charadriusobscurus*, the geographic distribution between northern and southern populations and the level of genetic differentiation between the two subspecies *C. o. obscurus* and *C. o. aquilonius*. As results, the AFLP markers are promised efficacy in assessing genetic relationship among *C. mongolus* and *C. leschenaultii* that corresponded to the geographic distributionand the level of genetic differentiation inlesser sand plover.However, the study also found that when a small number of the plover were analyzed.

Conclusion

Lesser sand plover (*C. mongolus*) and greater sand plover (*C. leschenaultii*) are migratory shorebirds in Thailand during the non-breeding season in which adult plumages are not clearly shown sexually dimorphic. The molecular approach was used to determine the gender that *CHD* gene was amplified with 2550F/2718R primer. The number of male gender of *C. mongolus*, in SamutSakhon province revealed more than the number in Trangprovince (Sex-ratio; M: F = 3.8: 1). However, the number of female in Trang province showed more than the number in SamutSakhon province (M: F = 1: 3.4).Sex-ratio is an important understanding behavior, social structure, breeding system dynamics, mechanisms and patterns of migration and estimating extinction risk. In genetic diversity from AFLP markers suggests that two subspecies or subgroups of lesser sand plovers would be recognized to identify species with others technique. Genetic diversity of lesser sand plover

and greater sand ploverhas been applied to increase for understanding of the distribution and extent of genetic variation within and between those species. Genetic database can be used to describe genetic diversity of *Charadrius* sp. or other migratory shorebirdwhich would be used to predict and monitor human or birds infection.

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References

Carlos, CJ.,Roselaar, S. and Voisin, JF. (2012). A replacement name for *Charadriusleschenaultii*crassirostris (Severtzov, 1873), a subspecies of Greater Sand Plover. Bulletin of the British Ornithologists' Club 132(1): 63-65.

De Marchi, G., Fasola, M., Chiozzi, G., Bellati, A. and Galeotti, P. (2012).Sex discrimination of Crab Plovers (*Dromasardeola*) by morphometric traits.Waterbirds 35(2): 332-337.

Fridolfsson, AK. andEllegren, H. (1999). A Simple and universal method for molecular sexing of non ratite birds.Journal of Avian Biology 30: 116-121.

Gibson, R. and Baker, A. (2012). Multiple gene sequences resolve phylogenetic relationships in the shorebirdsuborder Scolopaci (Aves: Charadriiformes). Molecular Phylogenetics and Evolution64: 66-72.

Griffiths, R., Double, MC., Orr, K. and Dawson, RJ. (1998). A DNA test to sex most birds. Molecular Ecology 7: 1071-1075.

Hebert, PDN.,Stoeckle, MY., Zemlak, TS. and Francis, CM. (2004). Identification of birds through DNA barcodes.PLoS Biology 2(10): e312.

Hirschfeld, E., Roselaar, CS. and Shirihai, H. (2000).Identification, taxonomy and distribution of greater and lesser sand plovers. British birds 93: 162-189.

Julia, MIB., Michael, M. and Bruce CR. (2013). Phylogenetic position and subspecies divergence of the endangered New Zealand dotterel (*Charadriusobscurus*).PlosOne8: 1-6.

Kahn, N., John JS. and Quinn TW. (1998). Chromosome-specific intron size differences in the avian *CHD* gene provide an efficient method for sex identification in birds. Auk 115: 1074-1078.

Kupper, C., Augustin, J., Kosztolanyi, A., Burke, T., Figuerola, J. and Szekely, T. (2009). Kentish versus snowy plover: phenotypic and genetic analyses of *Charadriusalexandrinus*reveal divergence of Eurasian and American subspecies. Auk 126: 839-852.

Poeaim, S., Chobarporn, N., Eiamampai, K., Nimnuan, S. and Sornsa, T. (2014). Gender identification in some Plover using *CHD* gene. International Journal of Arts and Sciences 07(02): 567-572.

Reed, KD.,Meece, JK., Henkel, JS. andShukla, SK. (2003). Birds, migration and emerging zoonoses: West Nile virus, Lyme disease, influenza A and enteropathogens. Clinical Medicine and Research 1: 5-12.

Rohlf, FJ. (2000). NTSYS-pc.numerical taxonomy and multivariate analysis system version 2.1. New York: Exeter Publications.

Szekely, T., Cuthill, IC.,Yezerinac, S., Griffiths, R. and Kis, J. (2004). Brood sex ratio in the Kentish plover.Behavioral Ecology 15(1), 58-62.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414.