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Effectiveness of Morphological Sex Determination in the East Asian Barn Swallow (*Hirundo rustica gutturalis*) on Spring Migration

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Hyun-Young Nam, Seung-Yeon Lee, Sook-Young Cho, Chang-Yong Choi, Se-Young Park, Gi-Chang Bing, Chang-uk Park, Seul-Gi Seo, and Yang-Mo Kim (2018) Information on sexual dimorphism helps explain a species' evolution in sexual selection and conservation issues such as sex-specific response in environmental changes. The Barn Swallow (*Hirundo rustica*) is a well-known sexually dimorphic species in which males have longer tail streamers than females. However, when compared with the European-African or American subspecies, little information is available on morphological sex determination in the East Asian subspecies *H. r. gutturalis*, especially outside the breeding season, when opportunities for molecular sexing are often limited and morphological clues may be the only ones available for sex determination. We collected morphological data on *H. r. gutturalis* during the northbound spring migration at a stopover site off the Korean coast. Two of eight measured variables - streamer length (the difference in length between the 5th and 6th rectrices; T6-T5) and length of the white patch on the outermost tail (6th rectrix; T6) - were selected as the best predictors for sex determination by stepwise discriminant analysis. Quadratic discriminant functions based on these variables showed that 92.4-93.5% of females and 82.9% of males were correctly classified. Our results provide baseline information that will benefit more accurate sex determination of the East Asian Barn Swallows, especially during the early months of a calendar year in non-breeding and stopover areas.

Key words: Barn Swallow, Discriminant function analysis, *Hirundo rustica gutturalis*, Sex determination, Sexual dimorphism.

BACKGROUND

The extent of sexual dimorphism in a population provides information that helps explain the population's evolutionary characteristics, such as sexual selection (Darwin 1871; Andersson 1994), mating system (Emlen and Oring 1977; Lande and Arnold 1985), behavioral adaptation (Ketterson and Nolan 1983; Kissner et al. 2003), and conservation issues related to unfavorable population dynamics resulting from a skewed sex ratio (Donald 2007; Saino et al. 2013a) and sex-differential distribution (Cristol et al. 1999).

The Barn Swallow (*Hirundo rustica*) is a

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sexually dimorphic species in which adult males have longer tail streamers than adult females, and these tail streamers have been regarded as a sexually selected character in some populations (Møller 1994). However, in addition to greater variation in streamer length in males (Neuman et al. 2007), there is considerable overlap in streamer lengths between the sexes (Svensson 1992; Pyle 1997), suggesting that a large proportion of swallows may not be correctly sexed based solely on tail streamer length (Duijns et al. 2011). The tail streamers, the white patches on the tail feathers, and various colored plumage ornaments (including melanin coloration on the throat, forehead and ventral regions) are all known to contribute to sexual dimorphism in Barn Swallows (Duijns et al. 2011; Vortman et al. 2011; Saino et al. 2013b) and are correlated with their reproductive success (Safran and McGraw 2004; Hasegawa et al. 2010; Vortman et al. 2011; Hasegawa et al. 2014a b). As a consequence, many studies of this species have attempted to determine sex using size and color measurements of multiple feather tracts. These efforts have been focused especially on *H. r. rustica* across Europe, West Asia, Africa, and South Asia (Bańbura 1986; Hermosell et al. 2007; Duijns et al. 2011) and *H. r. erythrogaster* in North and South America (Samuel 1971; Patterson 1981; Neuman et al. 2007), but no study has been made in East Asian populations. Given that different geographical and environmental factors affect variation in naturally- and/or sexually-selected traits in this species (Møller 1995; Møller et al. 2006; Neuman et al. 2007; Vortman et al. 2011; Hasegawa and Arai 2013; Matyjasiak et al. 2013; Saino et al. 2013b; Scordato and Safran 2014; Pap et al. 2015; Romano et al. 2017), degree of sexual dimorphism and the effectiveness of sex determination in the East Asian population may differ from that in other, better studied subspecies.

Hirundo rustica gutturalis breeds in parts of Eastern Russia, central and eastern China, Korea, Japan, and Taiwan, and winters in South East Asia and northern parts of Australia (McClure 1974; YIO 2002; Turner 2004). This East Asian subspecies has undergone a rapid decline in numbers over the recent several decades (Deguchi et al. 2012), whereas studied populations of other subspecies have shown variable trends (Turner 2006; Ambrosini et al. 2012; Birdlife International 2017). Although conservation issues affecting Barn Swallows in Asia have arisen locally over the last decade (Hung et al. 2009; WWF-HK 2010; WBSJ 2016), their sex ratio structure - including sex determination - is poorly understood and described outside the breeding areas. The significance of accurate sexing using morphological clues has been neglected because most studies of Barn Swallows have been conducted only on the breeding grounds in Asia where relative sexual differences in a pair are easily detectable and aided by sex-differentiated breeding behaviors (*e.g.* female incubation, male song) and temporarily apparent morphological traits (*e.g.* incubation patch, cloacal protuberance). However, sex determination is challenging at stopover areas where large numbers of individuals from different origins migrate together and when breeding-related morphological and behavioral clues are not available. Sex determination of swallows during the spring migration is more difficult because of the compounding effects of sex and age, whereas in autumn young recruits retaining juvenile feathers are readily identifiable. Furthermore, to the best of our knowledge, there is little reliable information about its feather molting strategy (*e.g.* timing, sequence, extent) and age identification in winter, the spring migration route, and the intermingling of the various populations of *H. r. gutturalis*. Studies of various populations of *H. r. rustica* and *H. r. erythrogaster* have shown that it is difficult to separate young birds (after their first molt) from adults on their wintering grounds or after arriving back on the breeding grounds because both ages usually molt completely in the winter quarter (Jenni and Winkler 1994; Pyle 1997). The Asian subspecies *H. r. gutturalis* has a different molting strategy from the other subspecies (Mano 2009); a higher proportion of *H. r. gutturalis* adults suspend their molt during autumn migration than *H. r. rustica* or *H. r. erythrogaster* do (Mano 2009), but no study has been done on its wintering grounds. These gaps in our knowledge emphasize the need for a detailed morphological description on determining sex in the East Asian subspecies, regardless of the origins of local breeding populations.

This study aimed to provide new information on the morphological characteristics of *gutturalis* Barn Swallows to assess the reliability of morphological sex determination during the early months of a calendar year in non-breeding and stopover areas.

MATERIALS AND METHODS

Study site and capture methods

The study area is on Heuksan-do (34°41'20"N, 125°25'37"E), an island 90 km from the south-western tip of the Korean Peninsula. The island is located on the East Asian Flyway and is a major stopover site visited by migratory birds resting and refueling before or after crossing the Yellow Sea, which is a significant ecological barrier for songbirds (KNPS 2007). From March to May 2013, we used mist nets to capture a total of 176 Barn Swallows at two freshwater marshes and at roosting sites in a nearby village. Among the 176 individuals, we found two individuals unusually retaining juvenile tail feathers with no streamers and excluded them from the dataset. After capture, birds were marked with metal rings on the right tarsi, and their body sizes and colored parts were measured. All birds were released immediately during daytime or kept in a roosting box for release in the morning if captured at night. We followed the institutional procedures and terminology for capture, handling, marking, measurements, feather numbering, and sampling (Nam et al. 2014). Fieldwork was approved and the relevant permits were issued by local governments and authorities.

Molt status and sexing

We found about 19% of the captured birds (33 of 174) had retained old primary feathers (generally the first and second primaries by descendant numbering), indicating that they were presumably adults older than their second calendar year. Among them, females had statistically longer tails than those possibly in their second year (mean ± SD: 75.48 ± 3.79 mm in the possible second year; 78.65 ± 4.47 mm in the possible adults; $df = 90$, $t = -3.22$, $p = 0.002$), suggesting potentially age-related differences in measurements. On the other hand, no such difference in tail length between age groups was found in males (mean ± SD: 87.67 ± 7.49 mm in the possible second years; 90.84 ± 9.92 mm in the possible adults; $df = 80$, $t = -1.29$, $p = 0.201$). There is no confirmed molt information from the wintering grounds of this subspecies, so we could not consider the difference in molting status in order to avoid any possible errors in age determination. Therefore, we pooled all data into one age class (after hatching year: AHY), including all birds that had undergone at least one post-juvenile molt.

We used a molecular technique (Griffiths et al. 1998) to determine the sex of sampled individuals. We collected 1-2 body feathers before releasing the birds and stored the feathers in the -24°C refrigerator until the fieldwork was finished. DNA was extracted from the quill roots of body feathers using a commercial extraction kit (DNeasy Blood & Tissue Kit, Qiagen, Germany) and two chromobox-helicase-DNA-binding genes (CHD-W and CHD-Z) were amplified in the polymerase chain reaction using P2 and P8 primers (Griffiths et al. 1998).

Morphological traits

Many size- and color-related characteristics have been measured previously to assess sexual dimorphism in the Barn Swallow (Kose and Møller 1999; Perrier et al. 2002; Safran and McGraw 2004; Hasegawa et al. 2014b), but our list of measurements only included measurements those can be swiftly and conveniently collected and processed for sex determination in the field.

We measured eight size- and color-related morphometric variables from captured swallows: wing length, bill length, forehead patch length, and five tail characters. We used flat wing rulers to measure the maximum wing length to 0.1 mm, and also measured bill length (total culmen length from the bill tip to the anterior edge of the skull) to 0.01 mm using digital vernier calipers (M500, Mitutoyo Inc., Japan). The outermost tail length (T6) was measured from the base of the tail to the end of the longest feather. We also measured the difference in the lengths between the outermost and its next tail feathers (T6-T5: streamer length) and the difference in the lengths between the outermost and innermost tail feathers (T6-T1: fork depth). In addition to measuring the lengths of the other rectrices to 0.1 mm using thin metal rulers, we measured streamer width by measuring the width of T6 at the distal tip of the overlaid T5 to 0.01 mm with calipers. Two color-related patches were also measured to 0.01 mm with vernier calipers: 1) the length of the dark-red forehead patch from the anterior edge of skull (Borras et al. 2000) to the patch's posterior edge measured in the midline of the head and 2) the length of the white tail patch on T6 (Duijns et al. 2011).

Statistical analysis

Female bill size did not fit a normal distribution (Shapiro-Wilk's test: $W = 0.96$, $p = 0.008$), so we used the Mann-Whitney *U* test to compare the difference in bill size by sex. The *t*-test was used for all other measurements. The Satterthwaite method was used for those variables (T6, T6-T5, and T6-T1) that did not meet the assumption of the equality of variance (SAS Institute 2011). We calculated the sexual dimorphism index (SDI) for each variable as Log_{10} (mean male size) - Log_{10} (mean female size) (Møller 1994). To compare the degree of dimorphism among the characteristics, we also listed the effect size as Cohen's d = (mean of males - mean of females) / pooled standard deviation (Cohen 1988).

Stepwise discriminant function analysis (DFA) was conducted to select the best parameters for separating the sexes. We used quadratic DFA because the selected parameters did not meet variances of homogeneity by sex (Bartlett's test; $\chi^2 = 49.43$, $df = 3$, $p < 0.001$; Morrison 1976). In order to estimate the proportion of correctly classified individuals by quadratic DFA, we used resubstitution and jackknife crossvalidation (leave-one-out) methods (Dechaume-Montcharmont et al. 2011). We also performed canonical discriminant analysis to calculate the discriminant score of each individual (Bavoux et al. 2006; Dmitrenok et al. 2007). To compare the correct classification rate among the variables used in previous studies, we calculated canonical functions of individual and combinations of tail characteristics. All statistical procedures were performed using SAS 9.3 software (SAS Institute 2011).

RESULTS

Molecular sexing confirmed that our samples consisted of 92 females and 82 males; the sex ratio did not differ significantly from 1:1 (Fisher's Exact Test: $p = 0.495$). Males were bigger in wing, tail, and color related measurements than females, but the differences tended to be greater in tail characters (Table 1).

Streamer length (T6-T5) and white patch were selected from stepwise DFA procedures (Table 2) as the morphological traits best discriminating females and males. Frequency distribution of each variable and the distribution of the two variables on a contour map with isopleths of posterior probability were shown in figures 1 and 2, respectively.

According to the results of quadratic DFAs with the above two variables, we acquired correct classification rates of 93.5% for females and 82.9% for males using the resubstitution method. The correct classification rate based on the jackknife crossvalidation method was 92.4% for females and 82.9% for males (Table 3). However, DFA using a single or combined tail characters also had high classification rates similar to that of the best canonical function using streamer length and white patch together (Table 3).

Table 1. Difference in measurements of the East Asian Barn Swallow *Hirundo rustica gutturalis* by sex. Sexual size dimorphism was calculated as Log_{10} (mean male size)-Log_{10} (mean female size) (Møller 1994). Effect sizes were presented to compare relative differences in the size dimorphism among measurements with different size scales. Effect size was calculated as Cohen's d effect size: (mean of males - mean of females)/pooled standard deviation (Cohen 1988)

	Female			Male			t ($U^{\S}$)	Sexual dimorphism index (SDI)	Effect size (Cohen's d)
	N	Mean ± standard deviation (SD)	Range	n	Mean ± standard deviation (SD)	Range			
Wing	92	115.5 ± 3.3	106.4 - 124.8	82	116.4 ± 3.2	108.6 - 123.9	-2.01*	0.0034	0.2787
Bill	92	12.26 ± 0.77	10.15 - 13.83	82	12.24 ± 0.72	9.98 - 14.19	3587.00$^{\mathrm{NS}\S}$	-0.0007	-0.0278
T6	92	76.2 ± 4.2	66.8 - 90.1	82	88.1 ± 7.9	69.3 - 116.1	-12.30***	0.0630	1.9801
T6-T5	92	20.4 ± 3.0	14.2 - 28.2	82	31.3 ± 6.5	16.0 - 58.1	-13.93***	0.1859	2.3440
T6-T1	92	32.7 ± 4.1	24.3 - 43.6	82	45.8 ± 7.7	26.8 - 77.9	-13.89***	0.1463	2.2423
Streamer width	75	3.05 ± 0.40	1.71 - 3.97	68	2.84 ± 0.46	2.09 - 3.88	2.87**	-0.0304	-0.4783
Forehead patch length	92	6.53 ± 1.25	3.14 - 9.47	82	7.18 ± 1.12	4.32 - 10.26	-3.60**	0.0998	0.5500
White patch length	92	16.80 ± 3.86	7.47 - 26.81	82	22.62 ± 4.54	10.77 - 33.23	-9.15***	0.1291	1.3869

*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, $^{\mathrm{NS}}$: not significant. §Bill length in female did not satisfy the normality and, therefore, the difference was tested using the Mann-Whitney *U* statistic.

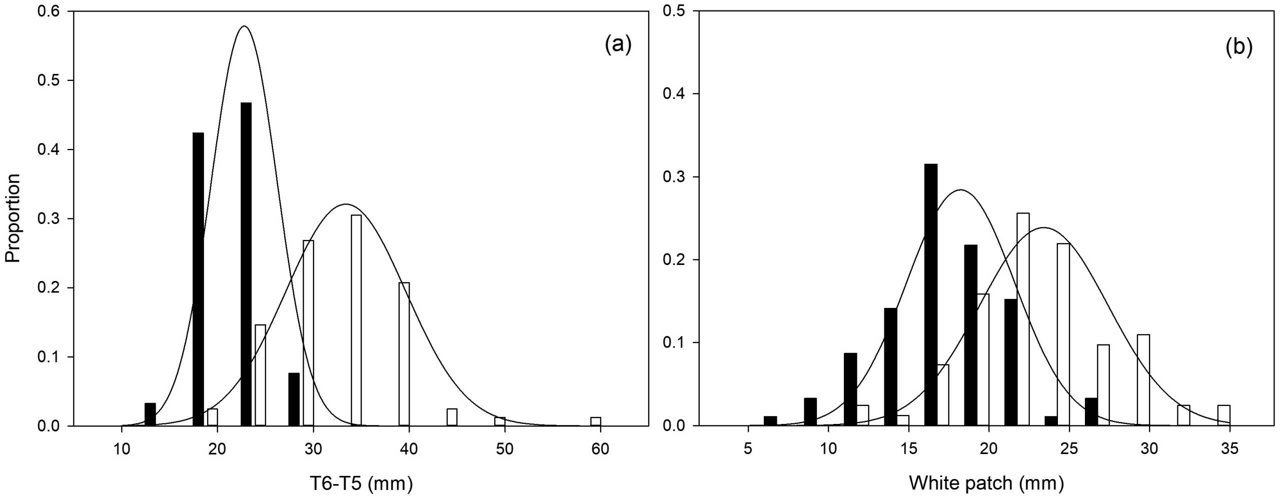

Fig. 1. Frequency distribution of (a) streamer lengths (T6-T5) and (b) white patch lengths selected by stepwise discriminant function analysis for the sex determination of the East Asian Barn Swallow *Hirundo rustica gutturalis*. Black columns denote females and white columns denote males; curved lines are the expected normal distributions of measurements reconstructed for each sex.

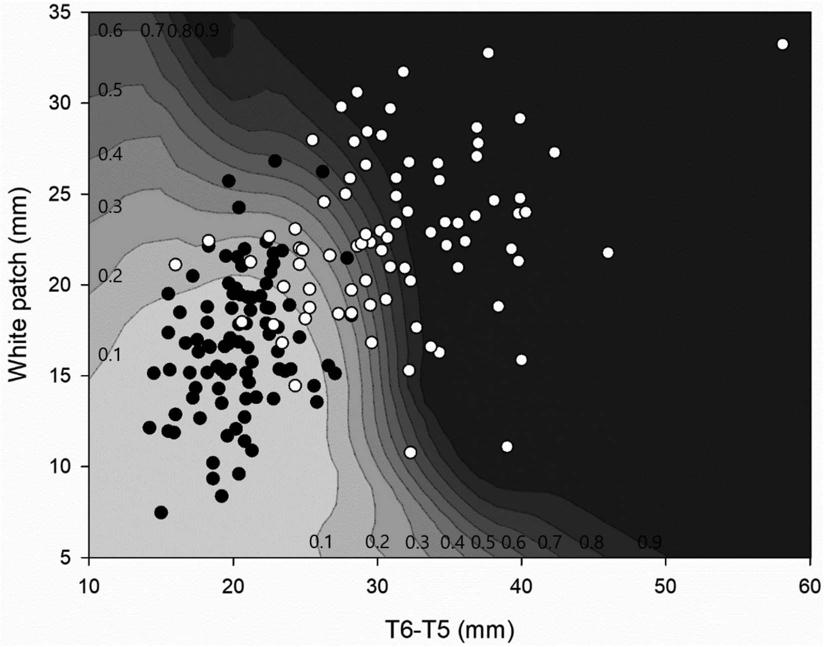

Fig. 2. Distribution of the streamer length (T6-T5) and white tail patch length of the East Asian Barn Swallow *Hirundo rustica gutturalis*. Black circles denote females and white circles denote males. Posterior probability of being male, calculated from the discriminant function, is presented as a contour map; isopleths of probability are marked from 0.1 to 0.9.

Table 2. Two selected variables from eight morphological measurements of the East Asian Barn Swallow *Hirundo rustica gutturalis* by stepwise discriminant function analysis. Significance level for the function was adjusted to 0.15

Step	Entered variables	Partial R-square	*F*-value	*p* > F	Wilks' λ	*p* < λ	Average squared canonical correlation (ASCC)	*p* > ASCC
1	T6-T5	0.537	163.24	< 0.001	0.463	< 0.001	0.537	< 0.001
2	White patch	0.075	11.35	0.001	0.429	< 0.001	0.571	< 0.001

We extracted one canonical component from canonical DFA (canonical correlation = 0.76, $F_{2,171} = 119.24$, $p < 0.001$) and developed a canonical discriminant score (D) as follows:

$$D = -6.013 + 0.168 \times (T6\text{-}T5) + 0.088 \times (\text{white patch}),$$

where the probability of being female is higher than that of being male when $D < 0$ and vice versa. Mean canonical scores for both sexes were significantly different (Satterthwaite *t*-test: $df = 121.79$, $t = -15.01$, $p < 0.001$), with a mean score of -1.11 for females (SD = 0.69, CV = 0.62, range: -2.84-0.70) and 1.24 for males (SD = 1.26, CV = 1.02, range: -1.47-6.67) (Fig. 3).

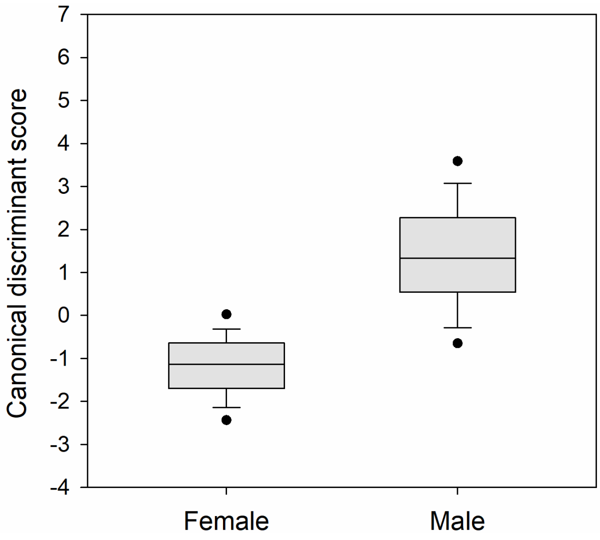

Fig. 3. Canonical discriminant scores of each sex calculated for the sex determination of the East Asian Barn Swallow *Hirundo rustica gutturalis*.

We drew a discriminant score (D) for each individual and fitted a logistic curve from the discriminant scores to assess the posterior possibility of being males (PP) as follow (Fig. 4):

$$PP = 1/\{1 + \exp(-2.7696 \times D)\} \quad (r^2 = 0.9751)$$

DISCUSSION

The population of *Hirundo rustica gutturalis* that migrates through Korea in spring showed morphological sex differences in body and colored-patch sizes. Although males had significantly longer wings than females, there was considerable overlap in wing length between the sexes and the difference was not greater than those of any tail measurements. This indicates that wing length of the East Asian population is not recommended for sex determination in the field. Dark-red forehead patch length also showed considerable overlaps, though it was significantly different between the sexes. The length of dark-red plumage patches on the Barn Swallow's throat are known as one of the sexually selected traits in Japanese populations (Hasegawa and Arai 2013). However, this study did not include the throat patch size (or length) into the variables due to failure in the repeatability of measurement. Future studies on size and color of the dark-red plumage parts including both of forehead and throat is needed to fully understand its role in the sex determination, sexual dimorphism, and its implication as sexually selected traits.

Our study also showed that males had longer

Table 3. Result of six discriminant function analyses (DFA) using the various parts of tail. The best predictor separating the sexes by DFA was the streamer length (T6-T5) associated with the white patch length (see Table 2). The correct classification rate was shown based on the resubstitution and jackknife crossvalidation for both sexes

Variable	Canonical correlation	Canonical function (D)	Correct classification rate (%)			
			Resubstitution		Crossvalidation	
			Female	Male	Female	Male
White patch	0.57 ($F_{1,172} = 83.64^{***}$)	$D = -4.671 + 0.239 \times (\text{white patch})$	79.35	75.61	79.35	75.61
T6	0.69 ($F_{1,172} = 160.29^{***}$)	$D = -13.174 + 0.161 \times (T6)$	93.48	79.27	93.48	79.27
T6-T1	0.74 ($F_{1,172} = 205.94^{***}$)	$D = -6.454 + 0.166 \times (T6\text{-}T1)$	90.22	84.15	90.22	84.15
T6-T5	0.74 ($F_{1,172} = 209.22^{***}$)	$D = -5.111 + 0.200 \times (T6\text{-}T5)$	92.39	82.93	92.39	82.93
T6-T1 + white patch	0.76 ($F_{2,171} = 114.68^{***}$)	$D = -7.007 + 0.139 \times (T6\text{-}T1) + 0.082 \times (\text{white patch})$	92.39	84.15	90.22	84.15
T6-T5 + white patch	0.76 ($F_{2,171} = 119.24^{***}$)	$D = -6.013 + 0.168 \times (T6\text{-}T5) + 0.088 \times (\text{white patch})$	93.48	82.93	92.39	82.93

tails (T6, T6-T1, T6-T5) and narrower streamers (streamer width) than females; in particular, the fork depth (T6-T1) and streamer length (T6-T5) were likely to have higher SDIs than the other measured body characteristics. The outermost tail feather, T6, is a commonly measured parameter that has been used solely or with other parameters in various studies on the sex determination of Barn Swallows (Svensson 1992; Pyle 1997; Hermosell et al. 2007; Duijns et al. 2011). However, we concluded that the streamer length or fork depth are better predictors of sex than a single T6 measurement when it is used alone due to its higher *F*-values, SDI scores, and effect sizes (see Table 1).

Some studies of *H. r. rustica* populations have described the white tail patch as a sexual ornament that acts as a handicap rather than a signal amplifier (Kose and Møller 1999; Kose et al. 1999), but it was not found to be related to male viability in *H. r. gutturalis* with shorter tail feathers than other subspecies (Hasegawa et al. 2014b). In our study, the length of the white tail patch was selected as one of the parameters for sex determination; though the white patch length had a lower discrimination power than either T6-T1 or T6, those might cause high multicollinearity with T6-T5, and, thus, they were probably excluded from the selected stepwise discriminant function. However, when used together with the streamer length, the white patch size enhanced the rate of correct sex discrimination up to 92.4% for females and 82.9% for males (jackknife crossvalidation method). In the field, measurement of white patch might not be necessary because the sole measurement of T6-T5 or T6-T1 without white patch also predicted the sex to a similar level. However, we suggest measuring the white patches whenever possible because the patch is an easily measurable and important supportive parameter for sex determination and because it can be measured even in severely abraded or damaged tails, as suggested by Duijns et al. (2011).

The rate of correct sex classification in this study was higher for females than males. A similar pattern has been observed in various breeding populations of the European Barn Swallow (Hermosell et al. 2007). Greater variance of tested variables in males, affecting the lower correct classification rate, may be caused by greater variation in male body size, which leads to greater variation in individual optimum tail length in terms of natural selection (Evans 1998; Bro-Jørgensen et al. 2007) and/or that part of tail length is the result of sexual selection (Møller 1994; Møller et al. 1998; Hedenström and Møller 1999). To what

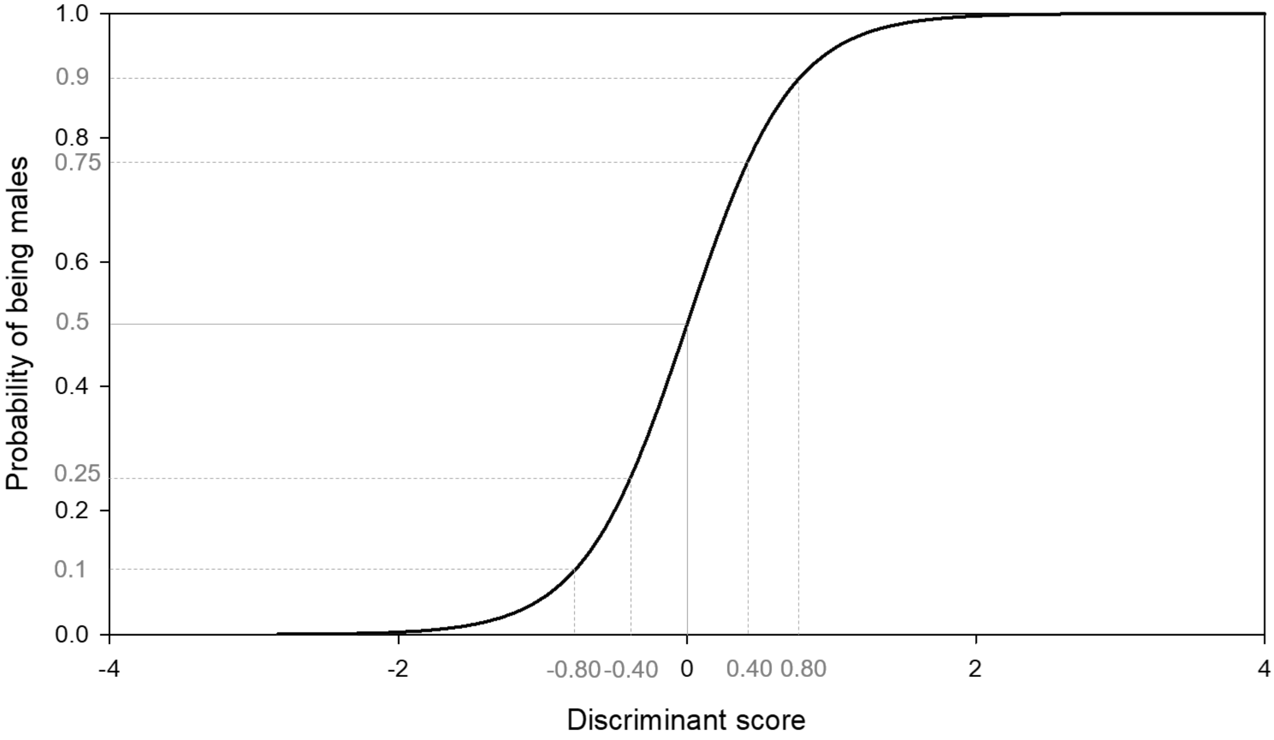

Fig. 4. Probability of being males by discriminant score in the East Asian Barn Swallows *Hirundo rustica gutturalis.*

extent natural selection and sexual selection have contributed to tail length is still an issue for debate and there still no study in the East Asian subspecies. Comparative studies with the *H. r. gutturalis* population, which has shorter tail feathers than *H. r. rustica*, can provide clues revealing the evolution of tail morphology in Barn Swallows (Scordato and Safran 2014; Romano et al. 2017). Weaker discriminant power in males can also be explained that there are differential effects of geographical variation on morphological variation, especially among males (Møller 1995; Møller et al. 2006; Hasegawa and Arai 2013; Pap et al. 2015). Unlike site specific or localized morphological studies with known breeding grounds, we may assume that our samples from a stopover area include birds from several geographic origins, widely ranging from N 34°40' (southern parts of Korea and Japan) to N 52°50' (Amur region of the Russia Far East) (McClure 1974). Therefore, the difference in discrimination power between the sexes in our study population (92.4% for females and 82.9% for males) seems to be larger than that in the European populations (Spanish population: 93.0% for females, 87.1% for males; Danish population: 93.3% for females, 89.8% for males; Hermosell et al. 2007) because these diverse origins may cause the greater variance in the morphological characters of males. Although the presence of birds from a wide geographic range in North East Asia in our dataset may have lowered the power of correct morphological sexual discrimination, our study provides more general information on morphological sexual differences in the subspecies *H. r. gutturalis* rather than information from a specific breeding site.

CONCLUSIONS

We evaluated various morphological factors for sex determination of the *Hirundo rustica gutturalis* population that passes through a stopover area off Korean coast during spring migration in order to address the lack of information about how reliable morphological sex determination is in the East Asian Barn Swallow. In spite of the relatively lower discriminant power than that of European populations, the streamer length (T6-T5) associated with the length of the white patch on the outermost tail (T6) was the best predictor of sex for the East Asian Barn Swallows. The fork depth (T6-T1) or streamer length alone were also good predictors, and the white patch would be a good supportive measurement. Consequently, our results provide information about the accuracy and effectiveness of the morphological sex determination in the *gutturalis* swallows during the early months of a calendar year, when sex determination in the field is often challenging. In addition, the probability curve of being males and females based on a given discriminant score and the six discriminant functions that we produced using several tail-related characteristics would be easily adopted in different situations where feather conditions are not suitable for one discriminant formula.

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Authors' contributions: HY Nam and CY Choi designed the study, performed the statistical analysis, and wrote the manuscript. HY Nam, SY Lee, SY Cho, and SY Park designed the field protocol. HY Nam, SY Lee, SY Cho, SY Park, GC Bing, CU Park, SG Seo, and Y Kim performed the field work.

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Availability of data and materials: All data are presented in the text, tables, and figures.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

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