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Slovak Raptor Journal 2018, 12: 47–54. DOI: 10.2478/srj-2018-0005

Sex identification comparison of barn owls (*Tyto alba javanica*) using morphological features and molecular-based methods

Porovnanie identifikácie pohlaví plamienky driemavej (*Tyto alba javanica*) pomocou morfologických znakov a molekulárnych metód

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Abstract: Sexing of barn owls, *Tyto alba javanica*, using morphological traits has not been accurate enough due to ambiguous sexual dimorphism between sexes. This has been one of the major problems for the management of barn owls worldwide, especially for translocation and captive-breeding programs. In order to increase the success rate of sexing the barn owl, we compared the results of a molecular sexing method to six morphological traits for sexing the owls: the shape and colour of the facial disc, the colour of the throat area, the tail plumage, the colour of their tarsus, the back plumage, and the frequency of spotting on the chest and underside of the wings. The result of our comparison showed that sex identification using morphological traits had an accuracy of only 72.7%. Three of our samples were identified as females using morphological traits, but molecular sexing determined that these samples were males. We also used our results to determine the best morphological traits for sexing barn owls, and concluded that the best traits for morphological sexing are the frequency of spotting on the chest and underparts of barn owls (accuracy of 81.8%), as well as colour of the owls' facial disc and throat area (accuracy of 63.6%).

Abstrakt: Určovanie pohlaví plamienky driemavej (*Tyto alba javanica*) podľa morfologických znakov nebýva dostatočne presné kvôli slabému pohlavnému dimorfizmu. Toto je jeden z hlavných problémov pre manažment druhu na celom svete, najmä pre presun jedincov a chov druhu v zajatí. S cieľom zvýšiť úspešnosť určovania pohlaví plamienky sme porovnali výsledky molekulárneho určenia pohlaví s určením pomocou šiestich morfologických znakov: tvar a farba tvárového závoja, farba hrdla, operenie chvosta, farba tarzusu, chrbtové operenie a frekvencia škvŕn na hrudi a na spodnej časti krídel. Výsledky nášho porovnania ukázali, že identifikácia pohlaví za pomoci morfologických znakov bola presná len v 72,7 % prípadov. Tri naše vzorky boli pomocou morfologických znakov identifikované ako samice, avšak molekulárne analýzy ukázali, že to boli samce. Naše výsledky sme použili aj na určenie najspoľahlivejších znakov na určenie pohlavia – zistili sme, že najlepšími morfologickými znakmi sú škvrnitosť na hrudi a pod krídlami plamienok (presnosť 81,8 %) a tiež aj farba tvárového závoja a hrdla (presnosť 63,3 %).

Key words: barn owls, *Tyto alba javanica*, morphology, sex identification

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Acknowledgements: We would like to extend our gratitude to Felda Global Ventures (R&D) for providing the samples, as well as the necessary facilities during our sampling period. We also thank Universiti Sains Malaysia for financially supporting the project through Research University Grant 1001/Pbiologi/811270. None of the authors have any conflicts of interest to declare.

Introduction

Sex identification of birds is vital in studies of ecology, behaviour and breeding strategies of propagation programs (Longmire et al. 1993, Helander et al. 2007, Garcia et al. 2009). In birds, the absence of juvenile sexual dimorphism often makes it difficult and sometimes impossible to determine a chick's sex on the basis of external morphology. A similar problem exists for fully-grown individuals of many bird species, as most species are sexually monomorphic. Before the introduc-

tion of modern molecular sexing techniques (Dubiec & Zagalska-Neubauer 2006), the sex of avian species was identified on the basis of behavioural observations (e.g. Elliot 1978, Archawaranon 2004), presence of a brooding patch (e.g. Prus & Schmutz 1987), examination of gonads by laparotomy or laparoscopy (e.g. Archawaranon 1988, 2004), examination of sex chromosomes (e.g. Shields et al. 1982, Prus & Schmutz 1987, Archawaranon 2004), and differences in morphometric traits. Early sex identification using morphology focused on differences in plumage colour, body weight and shape of the beak (Jones et al. 1984). Anatomical structure examinations of the cloaca, pelvis, head, wing, body size and tail length were also used to differentiate sexes among birds (Archawaranon 2004).

The barn owl (*Tyto alba*) has a worldwide distribution (Taylor 1994) and shows distinct variation in its melanin-based plumage traits, i.e. reddish colouration and black feather spots. Plumage is often the most obvious way to tell the difference between male and female barn owls, because males often have bright and whiter plumage, while females generally have more drab and dull-coloured plumage (Marti 1990, Hill 1993, Taylor 1994, Roulin 1999, Roulin et al. 2008, Van den Brink et al. 2011). The abdomen of the males is pale pink with flaky skin, while females have an incubation patch on their abdomen (Marti 1990, Hamid et al. 2010). Chest and throat feathers (Hamid et al. 2010), flecking on their underparts (Taylor 1994, Roulin et al. 2008, Hamid et al. 2010), wing markings (Taylor 1994), and facial discs (Marti 1990, Hamid et al. 2010) are also traits that have been used to identify the sex of barn owls.

Previous studies show that there is too much overlap for there to be any significant difference in morphometric measurements between male and female barn owls, other than weight (Marti 1990, Taylor 1994). In terms of weight, females tend to be heavier than males, especially during the breeding season, and this seems to be true for most subspecies. To list a few examples, males of the North American subspecies *Tyto alba pratincola* generally weigh between 440 to 514 g while females range from 510 to 630 g (Marti 1992), whereas the males of one of the European subspecies *Tyto alba alba* weigh from 320 to 340 and the females from 350 to 400 g (Taylor 1994). Lee (1998) reported that for the Southeast Asian subspecies *Tyto alba javanica* males weigh between 450 to 510 g while females weigh between 500 to 700 g. De Jong (2011) reported that the width of the upper part of the dark band on the first primary feather was an accurate sexing tool, while other studies report that morphometric measurements of wing chord, tail length, and length and width of the foot have substantially less accuracy in sexing barn owls (Marti 1990, Taylor 1994).

We investigated the accuracy of sexing barn owls based on morphological traits by comparing the results of that method with sexing using molecular techniques. Our focus was the Southeast Asian barn owl subspecies, *Tyto alba javanica*. Sex identification of barn owls is important for more successful breeding programs in captivity. Knowledge of the sex of barn owls also plays a vital part in introduction programs of the species for biological control programs and conservation programs.

Materials and methods

Barn owl sampling

We randomly sampled a total of 11 barn owls. The owls were harvested from nest-boxes in oil palm plantations in Jengka 24, Bandar Jengka, Pahang, Malaysia (3° 46' N, 102° 26' E) then transferred to the aviary located on the Main Campus of Universiti Sains Malaysia (USM), Penang, Malaysia (5° 21' N, 100° 18' E). The owls harvested were non-breeding adult owls, and they were harvested during the non-breeding season. The owls were allowed to acclimatize to their new environment for about a week before sampling was done.

Pictures of selected characteristics were taken for sex identification purposes. Photos were taken of i) the facial disc, ii) the chest and throat area, iii) the ventral (underside) view with wings extended, iv) the dorsal (topside) view with wings extended, v) the nape, vi) the dorsal view of their tail, and vii) the tarsus of the owls. These morphological characteristics were then analysed to identify the sex of the individual barn owls according to the distinctions used in several other studies.

Blood was then collected from the owls for molecular sexing. Owls had their heads covered with a breathable cloth material to keep them calm during blood and feather collection. The procedure for bloodletting followed that of Salim et al. (2014). The owls were restrained and positioned in a dorsal recumbency position before blood sampling. The wing of the sample was then gently fully extended and the medial aspect of the humeral area was swiped with alcohol. A small amount of blood was collected from the brachial vein of each owl using a 25 gauge needle and a 3 ml syringe. After blood was taken, the sampling site was disinfected with 75%

alcohol. The amount of blood taken ranged between 0.2 to 1.0 ml per owl. The blood was then placed in tubes containing EDTA to prevent coagulation. The tubes of blood were temporarily stored in an icebox then transported to the lab, where they were stored in a -20°C freezer.

Permission for this study was granted by the Malaysia Ministry of Natural Resources and Environment and the Malaysia Department of Wildlife and National Parks (Permit Number: NRE 600-2/2/21 Jld. 4 (12). The study protocol was approved by the Animal Ethics Committee of Universiti Sains Malaysia (Approval Number: USM/Animal Ethics Approval/2015/(96)/(629)).

Molecular sexing

DNA was extracted from the owl blood using a Qiagen DNeasy® Blood and Tissue Kit. The standard protocol provided in the kit manual was followed for extracting the genomic DNA. The concentrate obtained from each sample was then stored in a -20°C freezer.

The DNA was amplified using Polymerase Chain Reaction (PCR). The PCR components consisted of 14µl of nuclease-free water, 2µl of 10x *Taq* Buffer (prepared with 3M Tris-HCl pH 8.8, 1M $(NH_4)_2SO_4$, 1M $MgCl_2$, 0.01% Tween-20 and autoclaved distilled water), 1 µl of dNTP, 1 µl of primers, 1 µl of *Taq* polymerase and 1 µl of genomic DNA extracted from the blood. The primers used were 2718R/2550F (Fridolfsson & Ellegren 1999) and the primer sequence was as follows: 2718R (5'- ATT GAA ATG ATC CAG TGC TTG -3') and 2550F (5'- GTT ACT GAT TCG TCT ACG AGA -3') (Fridolfsson & Ellegren 1999).

The PCR cycle carried out was an initial single cycle at 95°C for 2 minutes, then 40 cycles of (i) 95°C for 30 seconds, (ii) 55°C for 30 seconds, and (iii) 72°C for 40 seconds. This was then followed by a single cycle at 72°C for 3 minutes. The PCR products were loaded and run on 3.0% agarose gel. The gels were then run at 90 V for 70 minutes in 1.0X TAE buffer solution. Pictures of the gels were then taken using GelDoc and were analysed for bands. Two bands indicated that the sample was female while a single band indicated the sample was male (Fridolfsson & Ellegren 1999).

Sexing using morphological traits

Six morphological traits were used to sex the owls; i) the shape and colour of the facial disc, ii) the colour of the throat area (an area about 3 cm wide below the facial disc), iii) the colour of the tail, iv) the colour of the tarsus, v) the plumage of the back (dorsal surface) of the owls and vi) the frequency of spotting on the chest and underside (ventral surface) of the owls.

Based on reports and observations by other researchers, female barn owls have a wider, round-shaped facial disc with brown smudges (Marti 1990, Hamid et al. 2010), light brown or buff throat area (The Barn Owl Trust 1989), dark brown tail with dark grey or black barring (The Barn Owl Trust 1989, Hamid et al. 2010), lighter pale pink tarsus (Hamid et al. 2010), darker colouring on their back with fewer but more intense grey patches (Colvin 1984, The Barn Owl Trust 1989, Marti 1990, Hamid et al. 2010), and more pronounced spotting on their underparts (The Barn Owl Trust 1989, Taylor 1994, Roulin et al. 2008, Hamid et al. 2010).

On the other hand, male barn owls have a facial disc which is longer and whiter in colour (Marti 1990, Hamid et al. 2010), an almost always white throat area (The Barn Owl Trust 1989), a paler tail where light grey barring may be absent (The Barn Owl Trust 1989, Hamid et al. 2010), darker tarsus (Hamid et al. 2010), light brown or brighter back plumage with more grey patches (The Barn Owl Trust 1989, Hamid et al. 2010), and less spotting on their underparts (The Barn Owl Trust 1989, Taylor 1994, Roulin et al. 2008, Hamid et al. 2010).

For owls with morphological traits of both sexes, the characteristic used to determine the sex of the bird using morphology was the frequency of spotting and the plumage of the bird, as these traits are genetic (Roulin & Jensen 2015).

Results

Molecular sexing

PCR runs with 2718R/2550F primers resulted in single bands approximately 600 bp in size and any second bands present were approximately 1000 bp in size. Single bands indicated the samples were male and double bands indicated a female sample (Fridolfsson & Ellegren 1999). Thus samples 1, 4, 8, 9 and 10 were males while the remaining samples were females.

Morphological sexing

Two (18.2%) of the samples were sexed as male using morphological characteristics (Fig. 1), while the remaining nine (81.8%) samples were identified as females using morphological traits (Fig. 2). None of the morphological traits indicating one sex were present exclusively in any of the owls.

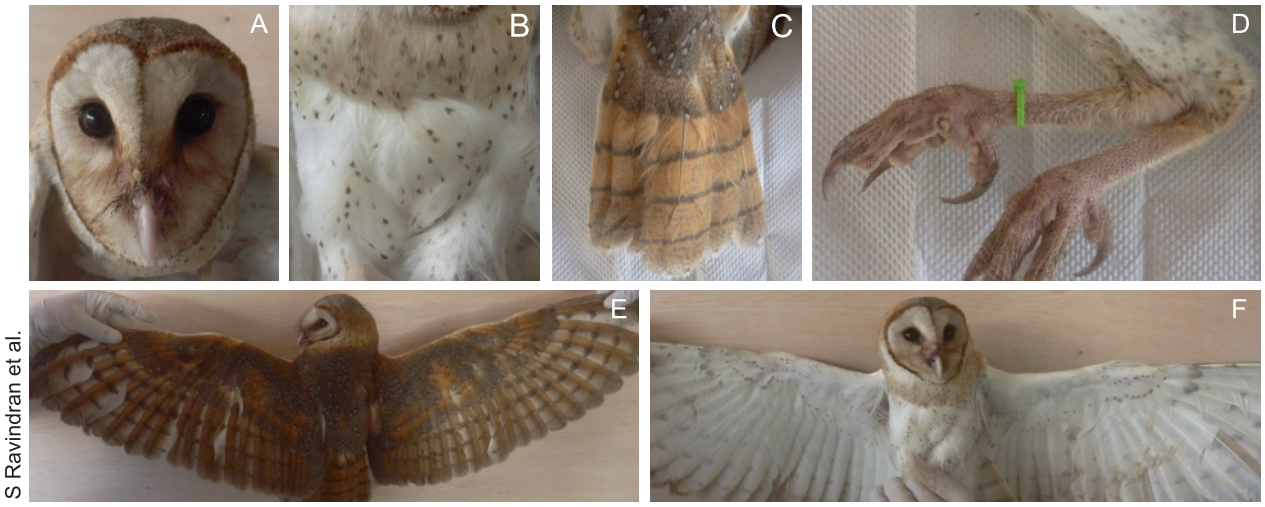

Fig. 1. Morphological traits identifying a male barn owl (Sample 1). (A) Facial disc is long and oval-shaped, as well as white in colour with brown smudges. (B) Throat area is white with light brown patches and there is less spotting on chest area. (C) Tail is bright brown in colour with black bands across it. (D) Tarsus is pale pink in colour. (E) Back plumage is bright brown in colour with frequent grey patches. (F) Less spotting on the underparts.

Obr. 1. Morfologické znaky identifikujúce samca plamienky (vzorka 1). (A) Tvárový závoj je dlhý a oválneho tvaru, bielej farby a s hnedým nádychom. (B) Oblasť hrdla je biela so svetlohnedými plochami a je menej škvrnitá. (C) Chvost je jasnohnedý s čiernymi priečnymi pruhmi. (D) Tarzus je bledoružový. (E) Chrbtové operenie je jasnohnedé s častými sivými plochami. (F) Na spodnej časti krídel je menej škvŕn.

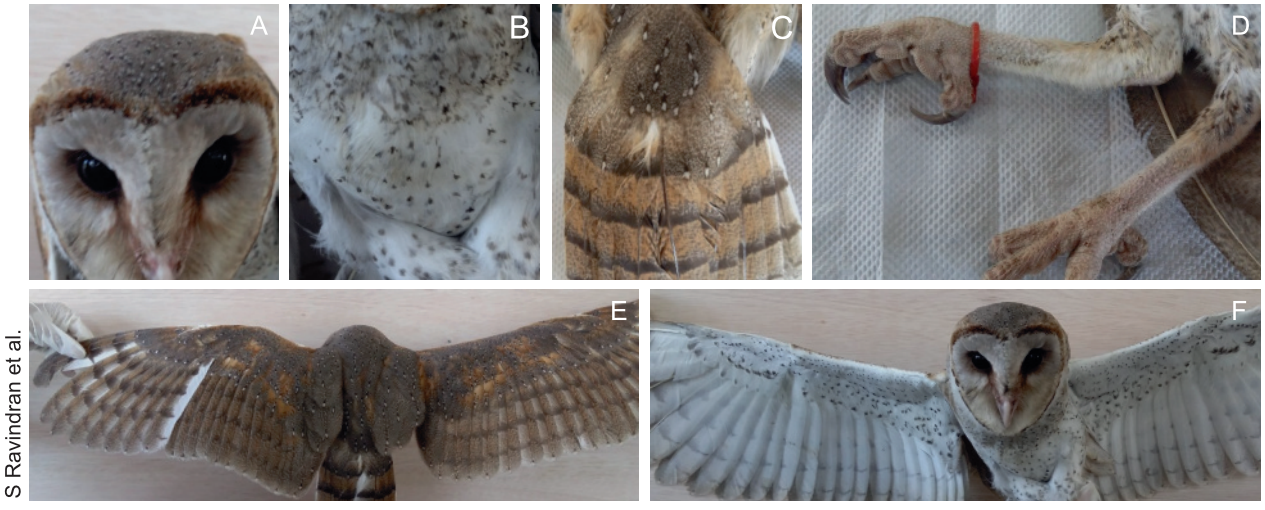

Fig. 2. Morphological traits identifying a female barn owl (Sample 6). (A) Facial disc is wide and round, and pale white in colour with faint grey patches. (B) White throat area with patches of brown and frequent spotting on chest. (C) Tail is light brown in colour with thick black bands. (D) Tarsus is pink-brown in colour. (E) Back plumage is bright brown in colour with numerous grey patches. (F) Frequent, numerous spotting on the underparts.

Obr. 2. Morfologické znaky identifikujúce samicu plamienky (vzorka 1). Tvárový závoj je široký a okrúhly, bledej farby so svetlosivými plochami. (B) Oblasť hrdla je biela s hnedými plochami a hrdlo je početne škvrnité. (C) Chvost je jasnohnedý so širokými čiernymi priečnymi pruhmi. (D) Tarzus je ružovohnedý. (E) Chrbtové operenie je jasnohnedé s častými sivými miestami. (F) Na spodnej časti krídel sú početné škvrny.

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Fig. 3. Male barn owl with female morphological characteristics (Sample 4). (A) Facial disc is wide and round in shape, as well as white in colour with brown patches. (B) White throat area with patches of light brown, with frequent spotting on chest. (C) Tail is bright brown in colour with thick black bands. (D) Tarsus is pink-brown in colour. (E) Back plumage is dull brown in colour with fewer grey patches. (F) Frequent, numerous spotting on the underparts.
Obr. 3. Samec plamienky so samičími morfologickými znakmi (vzorka 4). (A) Tvárový závoj je široký a okrúhly, bledej farby s hnedými plochami. (B) Oblasť hrdla je biela so svetlohnedými plochami a hrdlo je početne škvrnité. (C) Chvost je jasnohnedý so širokými čiernymi priečnymi pruhmi. (D) Tarzus je ružovohnedý. (E) Chrbtové operenie je matne hnedé s nepočetnými sivými miestami. (F) Na spodnej časti krídel sú početné škvrny.

Comparison between morphological and molecular sexing

Eight out of 11 of our samples had the same sex identification using both morphological traits and molecular sexing. Thus our study indicates that sexing of barn owls (*Tyto alba javanica*) using morphological traits is 72.7% accurate. Barn owl samples 4, 9 and 10, morphologically identified as females, were shown to be male through molecular sexing. These owls had mostly female identifying morphological traits, i.e. a smudged face, brown colouring on their chest and throat area, and frequent spotting on their underparts and chest (Fig. 3).

Accuracy of morphological traits

Each morphological trait was assessed for accuracy by comparing the sex indicated by each trait compared to the sex of the bird confirmed through molecular sexing. From our results, the most accurate morphological trait for sexing of barn owls was frequency of spotting on the chest and underside of the wings (81.8%). The next most accurate traits with 63.6% of accuracy were facial disc colour and colour of throat area. The shape of the facial discs of owls had a sexing accuracy of 45.5%, and tail colour had an accuracy of 36.4%. The trait with the lowest sexing accuracy was the colour of the tarsus (27.3%). Back plumage was the least effective trait for sexing the owls, as almost all birds had the same brown plumage with numerous patches of grey.

Discussion

Six morphological characteristics of barn owls were used to identify the sex of our samples: the shape and colour of their facial discs, the plumage on their back, the colour of their tail, the colour of their tarsus, the colour of the throat area as well as the frequency of spotting on the chest and underside of their wings. We concluded that the plumage of their back, the colour of the tail and colour of the tarsus were not reliable indicators for sexing of Southeast Asian barn owls, *Tyto alba javanica*.

The distinguishing characteristics of the facial discs of barn owls are often used for sex identification (Marti 1990, Hamid et al. 2010). However, in our study this proved to be a less accurate distinguishing feature. The ambiguous shape of some facial discs is hard to discern, and interpretation of it is subjective to the observer. Our comparisons show that the colour of the facial disc has higher accuracy than its shape; however, this feature is again subjective to the observer. Additionally, it is diffi-

cult to discern whether apparent smudges are the owl's natural plumage or smudges of dirt or food residue.

Various studies report that the sex of barn owls can be distinguished based on plumage differences. Male barn owls often have brighter and whiter plumage with more grey patches on their back, while females are reported to have generally drab and dull-coloured plumage (Colvin 1984, Looman et al. 1985, The Barn Owl Trust 1989, Marti 1990, Taylor 1994, Roulin 1999, Roulin et al. 2008, Hamid et al. 2010, van den Brink et al. 2011). However, using morphological traits for sexing can be subject to both spatial and temporal environmental variation (Zwarts et al. 1996). Most of the previous research concentrates on the European and North American subspecies, while the focus of our study, the Southeast Asian subspecies, is known to have extremely dark colouring compared to most other subspecies (Taylor 1994). The male barn owls in our study had dark brown plumage which when seen from a distance looked like the plumage of females. We found the difference in brightness hard to discern, and most samples showed the same frequency of grey patches on their back.

The tail was observed to analyse the colour and the pattern of transverse black bands, while the tarsus was analysed just for colour. We concluded that both of these characters were unreliable indicator traits. Other observations and reports (e.g. The Barn Owl Trust 1989, Hamid et al. 2010) state that males have paler tails with lighter barring while females have darker tails with more pronounced black barring. However, in our study there were no obvious differences between the black bands on the tails of males and females, nor any difference in the brightness of the brown plumage. Hamid et al. (2010) reported that males had darker-coloured tarsus feathers compared to females. We observed similar distinctions in some of our samples, however only a few owls had any clear distinction. This trait is also not a useful tool for sexing owls from a distance.

Regarding morphological traits, we conclude that the best indicator for sexing *T. alba javanica* is the frequency of spotting on the chest and underparts of the owls, which had a sexing accuracy of 81.8%, and the colour of the throat area, which had a sexing accuracy of 63.6%. Previous studies report that both males and females of *T. alba alba*, *T. alba guttata* (The Barn Owl Trust 1989, Taylor 1994, Roulin et al. 2008) and *T. alba javanica* (Hamid et al. 2010) are white on the underside of their wings, with females having more pronounced flecking in the underparts than males, an observation seen in our samples as well. Female barn owls usually have spots present on their sides sometimes extending across the breast from one side to the other (The Barn Owl Trust 1989). In our samples, some females had spotting even as far down as their leg feathers. The females in our study had a white throat area with brown patches, while some even had brown-feathered throat areas. On the other hand, the males had white throat areas with some featuring just a few light brown patches. This difference in plumage colouration of the throat area was similarly reported for *T. alba alba* (The Barn Owl Trust 1989). The differences in spotting frequency as well as the colour of the throat area between the sexes are easier to discern and make identification faster compared with all the other traits, whose differences are subtle in the Southeast Asian barn owl (*T. alba javanica*).

Molecular sexing of barn owls using primers 2718R/2550F produced bands 600 bp in size, and when a double band was present it was 1000 bp in size. As mentioned above, a single band indicated the sample was male, while a double band indicated a female. Fridolfsson and Ellegren (1999) tested 2718/2550F primers on two Strigiformes owls: *Aegolius funereus* and *Strix nebulosa* and the tested owl species produced band fragments 600–650 bp and 1200 kb in size.

Comparison of the two methods highlights the fact that using morphological characteristics alone to differentiate the sexes of barn owls is not entirely reliable. Only 72.7% of samples were sexed correctly using morphological characteristics, and all the misidentified samples were males which had been identified as females. These males had frequent spotting, and some had smudged throat areas and facial discs, which are female identifying characteristics. Abundant black spots in males have also previously been reported for *T. alba pratincola* (Marti 1992).

Plumage traits of barn owls have been proven to be strongly heritable (Roulin & Jensen 2015). A few studies suggest that aside from being hereditary, these plumage traits could also be affected by climate. Roulin and Randin (2015) suggested that on a continental scale, climatic factors are associated with plumage traits. Roulin (2003) and Roulin and Randin (2016) reported that barn owls in continental Europe and in the British Isles have larger spots in the colder north-east regions and smaller spots in the warmer southern regions. Meanwhile, in North America, Roulin and Randin

(2015) reported barn owls displaying larger black feather spots in regions where ambient temperatures were colder. On the other hand, Roulin et al (2009) reported barn owl taxa located near the equator as displaying larger black spots than taxa located towards the poles. It has also been repeatedly shown that spot size is strongly associated with the ability to resist stressful situations (Roulin & Ducrest 2011). Assuming this hypothesis is true, the male barn owls in our study with more pronounced spottiness could be responding to environmental stresses such as climate or food availability, an aspect which requires further study.

Conclusion

From our results, the best morphological characteristic for sexing of Southeast Asian barn owls, *T. alba javanica*, is the frequency of spotting on their chest and underside of their wings, as well as the colour of their throat area. *T. alba javanica* with more spotting are usually females, while owls with less spotting are usually males. *T. alba javanica* with brown-feathered throat areas are females, while owls with a white throat area are typically males. These traits can be seen from a distance and allow sexing to be easily done in the field. Molecular sexing showed that only 72.7% of identification using morphology was correct, as some owls showing female characteristics were subsequently proven to be males. Hence, when accurate results are needed, the sex of owls should be determined using molecular sexing.

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Received: 1. 10. 2018
Accepted: 15. 12. 2018