Molecular sexing and stable isotope analyses reveal incomplete sexual dimorphism and potential breeding range of Siberian Rubythroats *Luscinia calliope* captured in Taiwan

GUO-JING WENG, HUI-SHAN LIN, YUAN-HSUN SUN & BRUNO A. WALTHER

The Siberian Rubythroat *Luscinia calliope* breeds widely across Siberia and several other north Asian countries, migrating to overwinter to the south, including Taiwan. Its presence in Taiwan presented a unique opportunity to investigate questions which could not be reliably answered before the development of modern molecular techniques. We used molecular sexing techniques to determine whether there is overlap between the sexes in the measurements of colour and morphometric traits. We also used stable hydrogen isotope analysis to determine the potential breeding areas of these individuals. We found consistent morphometric and colour differences between males and females, but with so much variation that no single measurement could be used to sex individuals and, as already suspected, neither size nor colour of the red throat-patch or the bordering sub-malar stripes are reliable field marks for sexing Siberian Rubythroats. However, a combination of wing length and red throat-patch area when entered into a logistic regression correctly identified all males and females. This result could not be repeated when we used a discriminant function with cross-validation, which is currently the standard procedure for sex discrimination. The stable hydrogen isotope analysis showed that individuals captured in Taiwan potentially originate from large parts of its known breeding range, but mostly from southern areas. This suggests a 'leap-frog' migration by this species. Furthermore, larger individuals originated from higher latitudes, in accordance with Bergmann's rule. Modern molecular techniques thus shed interesting new insights into the morphology and ecology of Siberian Rubythroats.

INTRODUCTION

The Siberian Rubythroat *Luscinia calliope* breeds from the central Ural Mountains across Siberia to the coast of Far East Russia, northern Japan, northern Korea, central China and parts of Mongolia, and overwinters in South and South-East Asia, the south-east seaboard of China, Taiwan and the Philippines (Glutz von Blotzheim 1988, Pagenkopf 2003, Collar 2005, Brazil 2009). In Taiwan, Siberian Rubythroats are passage migrants or stay to overwinter (Wang *et al.* 1991); ringing data collected by the Chinese Wild Bird Federation (CWBF) between 2000–2007 showed that the species arrives in Taiwan in October and returns north by early May.

The presence of Siberian Rubythroats in Taiwan presented us with a unique opportunity to investigate some questions which could not be reliably answered before the arrival of modern molecular techniques: (1) are Siberian Rubythroats consistently dimorphic, or is there overlap in the measurements of colour and morphometric traits between the sexes? (2) where are the breeding areas of Siberian Rubythroats which overwinter in Taiwan?

Due to the rapid advance of reliable molecular techniques (Griffiths *et al.* 1998, Fridolfsson & Ellegren 1999), sexing of birds has moved from using colour, morphometric or behavioural differences to more reliable DNA techniques (e.g. Jodice *et al.* 2000, Redman *et al.* 2002). Before these techniques were available, the main colour difference between Siberian Rubythroat sexes was described as the metallic ruby-red chin and throat of the male compared with the white chin and throat of the female (Kennedy *et al.* 2000, Severinghaus *et al.* 2010), although most field guides and handbooks noted that some females had a yellow-tinged buff (Collar 2005), pinkish or even reddish throat (Glutz von Blotzheim 1988, Barthel 1996, Grimmett *et al.* 1998, Lee *et al.* 2000, MacKinnon & Phillipps 2000, Bergmann 2001, Robson 2008, Brazil 2009). However, it is unclear from these sources how reliable the sexing of individuals was; most are field guides, while others (Glutz von Blotzheim 1988, Barthel 1996, Collar 2005) cite original research but their sources all pre-date the use of DNA sexing techniques.

Likewise, Pagenkopf (2003) documented that 28% of breeding females in central Siberia had obvious red colouration on their throats, and then cited Svensson (1992) which claimed that 80% of females showed such colouration. While Svensson (1992) determined the sex using body size, brood-patch and the shape of the cloacal region, Pagenkopf (2003) did not describe how he verified the sexes, but claimed that a better diagnostic colour trait than the throat colour is the dark lines bordering the throat-patch (hereafter referred to as 'sub-malar stripes'), supposedly always black in males and brown in females. As a result of these conflicting identification guidelines, there are many records of unsexed Siberian Rubythroats in the literature (e.g. Pagenkopf 2003) and databases (e.g. the CWBF Taiwan bird banding database). Females are, on average, smaller than males (Glutz von Blotzheim 1988, Kennedy *et al.* 2000, Pagenkopf 2003). Pagenkopf (2003) established that females, on average, had less body mass and shorter wings, tails, tarsi and toes (with all differences statistically significant at the $p < 0.001$ level, except for the back toe); this assertion was supported by his review of 16 previous publications (Pagenkopf 2003).

To our knowledge, no previous study had used DNA sexing techniques when we adopted this method to clarify the extent and variation of the supposed colour and morphometric differences between male and female Siberian Rubythroats. Because DNA sexing techniques require invasive blood sampling and extensive laboratory work, we also investigated whether we could develop a statistical method based on morphometric and colour traits to reliably determine the sex of Siberian Rubythroats.

Due to the rapid advance of stable-hydrogen isotope techniques, we can now determine the potential breeding or wintering areas of migratory species, at least to the accuracy of a regional scale (e.g. Hobson 2005, Reichlin *et al.* 2010). Since birds consume water directly or indirectly via food, the stable-hydrogen isotope ratios of their feathers (δD_f) should reflect the δD of the precipitation (δD_p) of the geographic region where an individual grows its feathers (Hobson & Wassenaar 1997). So far, only a few studies (Rocque *et al.* 2006, 2009, Chang *et al.* 2008, Pérez *et al.* 2010) have used this technique in East Asia, and none for the Siberian Rubythroat. In the case of some species, linkage between breeding

and wintering areas was established through ringing recoveries, satellite tracking or morphological studies (e.g. using different races). However, Siberian Rubythroats have very low ringing recovery rates, are too small to carry transmitters, and show little, if any, racial variation (Glutz von Blotzheim 1988, Barthel 1996, Pagenkopf 2003, Collar 2005). Therefore, we used the stable-hydrogen isotope technique to determine the potential breeding areas of Siberian Rubythroats captured in Taiwan.

METHODS

Capture and handling of individuals

Siberian Rubythroats were sampled during winter 2007/2008. Between 6 November and 14 December 2007, birds were examined in a cage-bird trade shop in Neipu township, Pingtung county, south-west Taiwan; these had been captured by using playback to lure them into mist-nets set along the banks of the Donggang River and its tributaries (22.633°N 120.617°E). We arranged to be immediately informed when birds were captured and, consequently, individuals were sampled, measured, banded and released within 3 days or less of their arrival at the shop. Between 21 January and 27 February 2008, birds were trapped using the same technique around the headwaters of the Nioujiaowan stream (22.633–22.650°N 120.417–120.583°E) upstream of the Donggang River. Essentially, both sets of birds were captured in the same area using the same method but by two different groups of people.

We assumed that the measured colour and morphometric traits and δD_f values did not change during captivity. However, we did exclude body weight from our analyses. Blood and feather samples along with colour and morphometric measurements were taken from each individual when possible. However, a few individuals developed signs of stress (e.g. open bills and fast breathing) during handling and were released immediately, hence the unequal sample sizes in some of our analyses.

Molecular sexing

A few drops of blood were collected from one brachial vein of each individual using half-inch needles and capillaries and then stored in 99% ethanol until isolation of genomic DNA. We isolated total genomic DNA using traditional chloroform/isoamyl alcohol (24:1) extraction and then identified the sex of each individual with the 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') primer set (Fridolfsson & Ellegren 1999). We performed PCRs on iCycler thermal cyclers (Bio-Rad) in 10-μL reaction volumes containing about 0.5 ng of genomic DNA, 0.1 mM of each dNTP, 1 × PCR buffer (BioScience), 0.1 U of *Taq* DNA polymerase (BioScience), 0.5 mM of MgCl$_2$, and 0.2 μM of each primer. The PCR conditions

were 95°C for 180 s, followed by 35 cycles of 95°C for 30 s, 46°C for 40 s, and 72°C for 100 s. The final extension was at 72°C for 300 s. PCR products were separated in 1.2% agarose gels and visualised by ethidium bromide staining. To minimise risk of error, the entire procedure was repeated independently by two different laboratory workers.

Age determination

Each individual was classified as subadult (first-winter) or adult. Subadults have incompletely ossified skulls (Jenni & Winkler 1994) and/or light-brown tips on the greater coverts 1–7, and tertials which contrast with the dark brown of the remainder of the feathers (Glutz von Blotzheim 1988, Barthel 1996, Collar 2005). However, subadults may lose the light-brown tips through abrasion (Glutz von Blotzheim 1988) and/or may have completely ossified skulls. Therefore, individuals with either light-brown tips or incompletely ossified skulls were classified as subadults; all others were classified as adults. To ensure consistency, all age classification, colour and morphometric measurements were conducted by H-SL.

Colour and morphometric traits

All morphometric measurements (Table 1) were taken according to instructions detailed in banding manuals of the Taiwan Bird Banding Center (1989) and were measured to the nearest 0.1 mm, except for the area of the red throat-patch which was determined by counting the red-filled 2.5×2.5 mm grid squares in photographs of each individual (Plate 1). When grid squares were only partially red, each was classified visually using ten categories (red coverage 1–10%, 11–20% etc). Any 'orange' areas were scored as red because the 'orange' is the result of a different viewing angle or a change in ambient light. Although several authors suggested that the sub-malar stripes are black in males and more grey-brown in females

Plate 1. Variation of red throat-patches and sub-malar stripes of Siberian Rubythroats. (A) A typical male with dark red throat-patch and black sub-malar stripes. Clear plastic with a grid size of 2.5×2.5 mm was placed over the throat to estimate the area covered by red colour. (B) An atypical female showing significant red throat-patch and strong sub-malar stripes. (C) A typical female with a faint reddish throat-patch and faint light-brown sub-malar stripes. A sub-malar stripe in B and the throat-patch in C were manually framed (thin yellow lines) with the software ImageJ to measure their grey values.
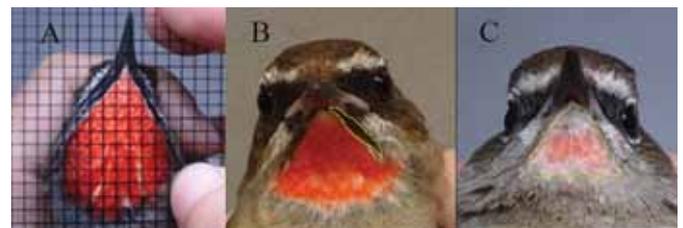
Table 1. Colour and morpholometric traits of Siberian Rubythroats. Two-tailed Mann-Whitney U tests were used to compare differences between males and females.

Morphometric traits	Males						Females						U-value	p-value
	n	Min	Max	Med	Mean	s.d.	n	Min	Max	Med	Mean	s.d.		
Red throat-patch area (mm²)	39	202.5	413.1	276.9	283.5	45.1	17	0	272.5	70.6	116.8	105.8	193	<0.0001
Head length (mm)	39	36.2	39.8	38.2	38.2	0.8	16	35.9	38.9	37.2	37.3	0.9	272.5	0.001
Bill length (mm)	37	11.5	14.3	13.3	13.2	0.7	16	12.2	14.0	13.0	12.9	0.5	331	0.050
Body length (mm)	39	141.0	159.0	151.0	149.9	4.3	16	135.0	152.0	146.5	145.5	5.0	294	0.004
Tarsus length (mm)	39	29.3	32.8	30.8	30.9	0.9	17	28.6	30.9	29.5	29.7	0.7	255	<0.0001
Wing length (mm)	39	74.5	83.5	79.0	78.8	2	17	72.0	77.0	75.0	75.1	1.3	198	<0.0001
Flattened wing length (mm)	39	75.5	86.5	81.0	80.5	2.2	17	75.0	79.5	77.0	77.0	1.3	212.5	<0.0001
Tail length (mm)	39	58.0	68.0	62.5	62.4	2.7	16	55.0	63.5	60.0	59.7	2.3	276.5	0.002

(see Introduction), we noticed that the darkness of the sub-malar stripes correlated with the darkness of the throat-patches, regardless of sex. In order to quantify the darkness of each throat-patch and sub-malar stripe, a front-view photo of each individual was taken. Grey values of the throat-patch and sub-malar stripe were measured under the red, green and blue (RGB) mode using the software ImageJ (Schneider *et al.* 2012). The grey value measures the light intensity at each pixel with black as the weakest (grey value = 0) and white at the strongest (grey value = 255) intensity. The areas of each throat-patch and each sub-malar stripe to be measured were manually framed using the Freehand Selection tool in ImageJ (Plate 1). For each throat-patch, the entire area was used, including any whitish areas. For the sub-malar stripes, only one was randomly chosen and measured, as we assumed that the light intensity of the two stripes was essentially equal.

Stable-hydrogen isotope analyses

We combined maps (Glutz von Blotzheim 1988, Grimmett *et al.* 1998, Collar 2005) of the breeding and wintering areas of Siberian Rubythroat to create a shape-file of maximum breeding and wintering areas using ArcGIS 9.2 software. To interpret the geographic locations of the breeding area of Siberian Rubythroats correctly, we needed to ensure that the collected feather samples were actually grown in the breeding region and not later during migration or wintering. We used three approaches to evaluate the reliability of the feather samples.

First, we considered the published moult chronology of Siberian Rubythroats to sample the appropriate feathers from which to analyse δD_f. After the breeding season but before migration, adult Siberian Rubythroats go through a complete annual moult between August and September, while subadults moult the feathers of the head, body, lesser coverts, medium coverts and parts of the greater coverts 8–10 between late August and late September (Glutz von Blotzheim 1988, Svensson 1992, Cramp 1998). Therefore, for adults and subadults, the δD_f values of the rectrices should reflect the δD_p of the breeding region. We collected the innermost rectrix on the right side (R1) from each individual. In one case, R1 had been lost for an unknown reason. Because the replacement feather growing at R1 could therefore not be used, we collected the first rectrix on the left side of R1. We refer to these feathers as 'breeding feathers'.

Second, it is well established that subadults grow all of their feathers on the breeding grounds prior to migration. Therefore, similar mean δD_f values of the breeding feathers for subadults and adults would further indicate that the breeding feathers of adults are also grown in the breeding area and not later during migration. On the same lines of reasoning, the δD_f values of the breeding feathers should exhibit smaller variation if they were grown in the breeding area and larger variation if they were grown along the migration route. We thus compared the mean and variation of δD_f values between subadults and adults.

Third, we collected feathers from nine individuals showing signs of recent feather growth. We refer to these feathers as 'wintering feathers'. If any of our breeding feathers were actually grown during migration, we would expect to observe δD_f values for at least some individuals which would lie somewhere between the mean δD_f value of the breeding and wintering feathers. If the arguments made above all favour the hypothesis that all breeding feathers were indeed grown on the breeding grounds, we could then be confident that we could use the δD_f values of our breeding feathers to interpret the geographic range where Siberian Rubythroats breed.

To determine the distribution of δD_p values within the maximum breeding and overwintering areas, we downloaded the global mean δD_p map of the growing season from http://www.waterisotopes.org (Bowen *et al.* 2005); the resolution of its grid squares was 20×20 geographic minutes. This growing-season δD_p map (Figure 3) shows clear and roughly latitudinal δD_p bands within the maximum breeding range, without any trend from coast to inland, which suggests that heavier (and thus less negative) δD_p values are roughly associated with lower latitudes. Consequently, the δD_f values of the breeding feathers should also reflect this latitudinal patterns if the conditions above hold.

The mean δD_p value for Pingtung county from November to the following March was -20‰ during 2005 and 2007 (C.-H. Wang *in litt.* 2013) while the mean δD_f value of our wintering feathers was -37‰, which is 17‰ more depleted than the δD_p value (see Results). This difference of -17‰ was used as the discrimination factor to transform the growing-season δD_p map to the map of expected δD_f values (Figure 4).

Fixed discrimination factors have been widely used for assigning migratory species to their breeding area, e.g. Northern Pintails *Anas acuta* breeding in Alaska (Yerkes *et al.* 2008) where water sources are probably as variable as in the breeding area of Siberian Rubythroats. Similarly, Pérez *et al.* (2010) assigned waterbirds moulting in north-central Mongolia to their breeding region using a fixed discrimination factor of -28‰ calculated for North American waterfowl by Clark *et al.* (2006). We also used a discrimination factor but, unlike Pérez *et al.* (2010), we derived it from our own data, specifically -17‰ as explained above.

We thus subtracted 17‰ from each grid square of our growing-season δD_p map (Figure 3) to derive a map of the potential breeding area of Siberian Rubythroats (Figure 4) within the maximum breeding range. We then colour-labelled those grid squares whose value matched the mean δD_f value of our breeding feathers as well as those grid squares which fell (1) within one standard error, (2) within the 95% confidence interval of the mean δD_f value and (3) within the total range of the δD_f of the breeding feathers, to rank each grid square for its likelihood as the location where these feathers were actually grown.

After collection, all feathers were soaked in a 2:1 chloroform:methanol solution to remove surface oil and dirt; then each sample was air-dried and then weighed to 0.5 mg before analysis (Wassenaar & Hobson 2000, Norris *et al.* 2006). The samples were analysed at the Stable Isotope Facility, University of California, Davis. There, feather samples and four keratin standards with known values of non-exchangeable hydrogen (BWB, CFS, CHS and CH1) were stored in a vacuum desiccator to equilibrate the exchangeable hydrogen in feather samples and standards. The Heckatech HT Oxygen Analyzer, interfaced to a continuous-flow Isotope Ratio Mass Spectrometer (IRMS) known as PDZ Europa 20-20 (Sercon Ltd., Cheshire, UK), was used for the analysis during which the keratin standards and the standard VSMOW (Vienna Standard Mean Ocean Water), which has no exchangeable hydrogen with -120‰ (NBS22), were used to adjust for the stable-hydrogen isotope ratios of the feather samples.

Statistical analyses

Since some data relating to colour, morphometric and feather measurements were not normally distributed, the Mann-Whitney U test was used to test for differences between two groups, while the Pearson's correlation coefficient (r) was used to evaluate correlation between two variables. We also used a non-parametric one-sample sign-test to test for a general trend in the direction of correlations between latitude and measures of body size. We used SPSS 12.0 for these analyses.

To choose the optimal model for sex determination of Siberian Rubythroats, we used the logistic regression procedure with the stepwise effect-selection method in SAS 9.0, whereby the binary dependent variable sex was regressed against nine independent variables, namely the seven morphometric traits (Table 1), the red throat-patch area (Table 1) and the grey value (Figure 1). Independent variables were added to the model when $p < 0.05$ and

were excluded from the model when $p > 0.35$. Once some of these independent variables had been selected by this logistic regression model, these same variables were then used in a discriminant analysis for sex determination. We also conducted a cross-validation to evaluate the performance of the discriminant function. The individual to be validated was not included when we calculated the respective discriminant function, and this discriminant function was then used to determine the sex of this omitted individual.

RESULTS

Sex determination using DNA analyses

A total of 56 individuals (8 from mist-nets, 48 from the cage-bird trade shop) were captured. Genetic sexing identified 39 males and 17 females (Table 2), with the results from the two independent laboratory workers agreeing completely.

Table 2. Variation of the colour of the throat-patch among sex and age categories in Siberian Rubythroats. The red and white categories meant that > 95% of the throat-patch was either red or white, and all other individuals fell into the red-and-white category.

| Sex	Age	Red	Red-and-white	White	Total
		Colour of throat-patch		
Male	Adult	11	0	0	11
	Subadult	28	0	0	28
Female	Adult	3	3	1	7
	Subadult	1	6	3	10
Total		43	9	4	56

Morphometric and colour traits

No significant differences in morphometric or colour traits (Table 1) were found between the birds captured by the authors ($n = 8$) and by the cage-bird shop owners ($n = 48$) except for tail length—cage-bird shop birds had marginally longer tails (U = 136.0, $p = 0.04$). We therefore lumped all individuals together. All medians and means of our eight traits were greater for male than for female; moreover, all differences were statistically significant at the $p < 0.05$ level except for bill length (Table 1). However, there was always some overlap: the maximum female measurement was always greater than the minimum male measurement but always smaller than the maximum male measurement. The minimum female measurement was always smaller than the minimum male measurement, except for bill length (Table 1).

Classifying the throat-patch colouration into three types, we observed that 43 of the 56 individuals (76.8%) had red throat-patches (Table 2). All males fell into this category, but also one subadult and three adult females. The nine individuals with red-and-white throats (16.1%) and the four with white throats (7.1%) were all females (Table 2). Thus, if individuals with red patches had been identified exclusively as males and individuals with red-and-white patches or white patches exclusively as females, 7.1% of all individuals and 23.5% of all females would have been incorrectly sexed. Twenty-eight subadult males (100%) but only one subadult female (10%) had red throat-patches (Table 2), indicating that, at least for males, the red throat-patch appeared in their first winter and was not a good indicator of age at this time of year, in accordance with Barthel (1996).

In both sexes the average area of the red throat-patch was larger in adults than in subadults, but these differences were not statistically significant in either sex (male: U = 271.0, $p = 0.11$; female: U = 65.5, $p = 0.81$), which is due to the large overlap in values (Figure 2). The grey value of the throat-patch was positively correlated with the grey value of the sub-malar stripe ($n = 56$, $r =$

0.73, $p < 0.0001$; Figure 1). This relationship was slightly stronger for females ($n = 17$, $r = 0.72$, $p = 0.002$) than for males ($n = 39$, $r = 0.44$, $p = 0.005$). Figure 1 further illustrates that, in general, males have significantly darker throat-patches and sub-malar stripes than females but again with some overlap (U = 668 and 742, $p = 0.001$ and < 0.0001, respectively). Variation of grey values was larger in females than in males for throat-patch (s.d. = 45.2 and 19.9 respectively) and for sub-malar stripe (s.d. = 32.2 and 22.4 respectively) (Figure 1). In both sexes, there was no statistically significant difference for the grey values of the throat-patch or the sub-malar stripe between subadults and adults (Mann-Whitney U tests, all $p > 0.08$; Figure 1).

Figure 1. Relationship between the grey values of the red throat-patch and the grey values of the sub-malar stripe of Siberian Rubythroats (filled circle = adult male, open circle = subadult male, closed triangle = adult female, open triangle = subadult female). Possible grey values range from 0 (black) to 255 (white), whereby the corresponding gradation bars show that lower grey values appear darker in vision. The lower-left and upper-right crosses show the means and standard deviations for males and females, respectively.
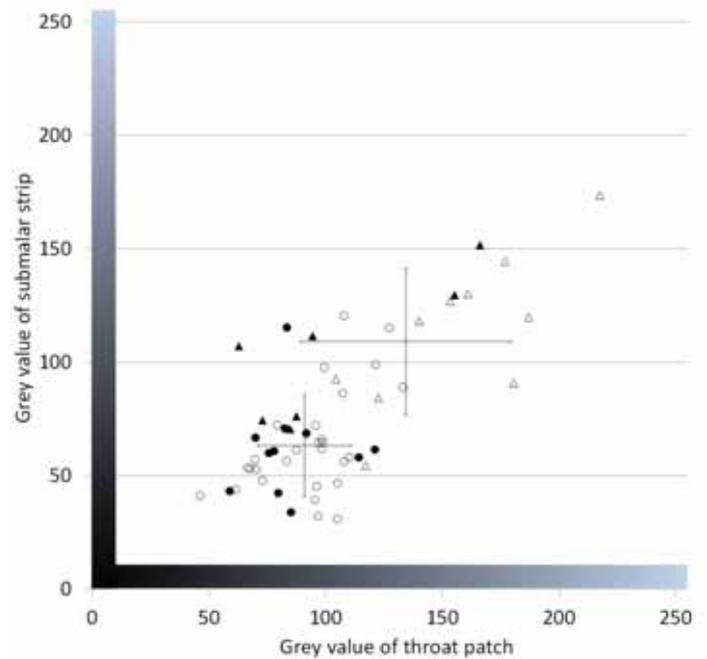
Figure 2. Relationship between the red throat-patch area and the wing length of Siberian Rubythroats (same symbols as in Figure 1). The values of two females overlap at zero red throat-patch area and 75 mm wing length.
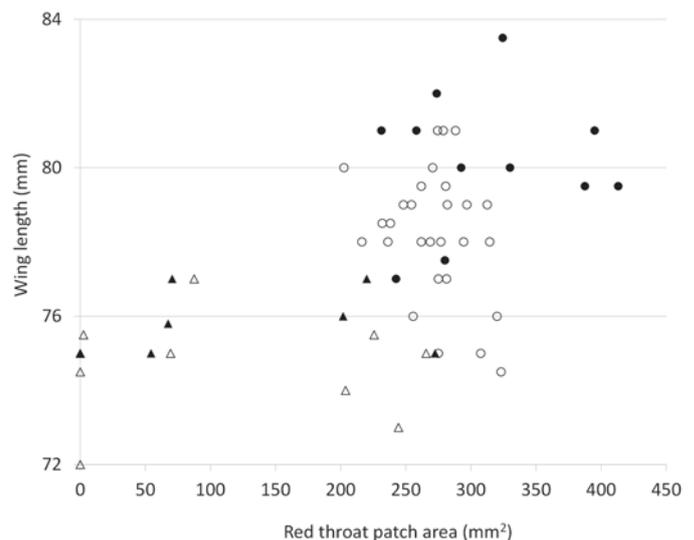
Table 3. An example of one of the possible logistic regression models to distinguish the sexes of Siberian Rubythroats. Because a gap exists between males and females in Figure 2, a maximum likelihood estimate does not exist and therefore the logistic equation is not unique; rather, many different possibilities exist, of which the one below is one example. Negative values always identify males and positive values always identify females correctly (number of observations = 47).

Parameter	F	Estimate	SE	Wald chi-square	*p*-value
Intercept	1	705.60	597.70	1.394	0.24
Red throat-patch area	1	-0.36	0.31	1.425	0.23
Wing length	1	-8.08	6.90	1.372	0.24

The logistic regression identified two independent variables which correlated with the binary dependent variable sex, namely red throat-patch area and wing length (Table 3). This function yielded a 100% accuracy of sex determination because negative values resulting from the application of the logistic regression function always identified males correctly, and positive values always females correctly. Accordingly, plotting red throat-patch area versus wing length shows clearly separated clusters of males and females, although it also shows the considerable overlap between the sexes for both variables when they are considered by themselves (Figure 2).

Using red throat-patch area and wing length as independent variables, a quadratic discriminant function was established which misclassified two males and one female in cross-validation, which amounts to an error rate of 5.1% in identifying males and 5.9% in identifying females. The cross-validation failed to identify sexes as perfectly as the logistic regression model because the two misidentified males had relatively short wing lengths (75 mm) while the misidentified female had a relatively long wing length (77 mm). The cross-validation thus showed that male and female Siberian Rubythroats could not always be reliably identified within a discriminant function.

Isotope analysis

The δD_f values of the breeding feathers did not differ significantly between the sex and age categories with an overall mean ± s.d. of -99.9‰ ± 12.0‰ (Table 4; sex comparison: U = 540.0, p = 0.64;

age comparison: U = 527.5, p = 0.44). Indeed, the mean δD_f values of the breeding feathers from adults and subadults were almost identical (Table 4), suggesting that all individuals originated from similar regions (see Methods). Furthermore, if any of the breeding feathers were grown outside the breeding range, we would see a larger s.d. of δD_f for adults than for subadults (see Methods), but adults possessed a smaller s.d. than subadults (Table 4). Finally, our wintering feathers (mean ± s.d.: -37.0‰ ± 7.7‰, range: -45‰ to -23‰) differed substantially from the breeding feathers in terms of mean, s.d., and range of δD_f values (Table 4). If any of the breeding feathers had been grown during migration, we should have found δD_f values somewhere between -99.9 and -37.0‰, as explained in the Methods. These facts thus support the hypothesis that all breeding feathers were indeed grown in the breeding area and can be used to pinpoint the breeding region using the growing-season δD_p map (Figure 3) and our discrimination factor.
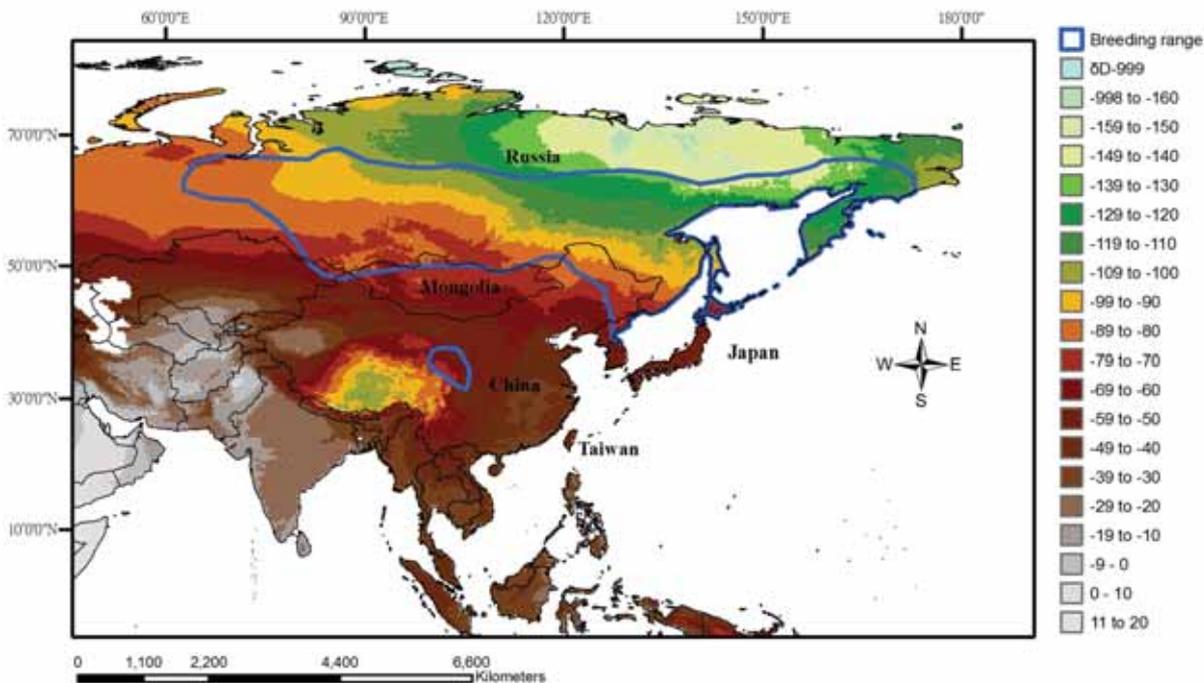
The derived map of the potential breeding areas of Siberian Rubythroats overwintering in Taiwan shows that, except for the most westerly part of the published breeding range, the potential breeding area identified by the most likely grids is located mostly in the southern part of the maximum breeding range (Figure 4).

As estimates of overall body size, we chose five of our seven morphometric traits (Table 1). We randomly excluded one of the two measurements of wing length and excluded bill length because it is not well correlated with overall body size. Correlating these five measurements with the mean δD_f values of the breeding

Table 4. Sample size (*n*), mean ± standard deviation, minimum and maximum δD_f values of the breeding feathers collected from Siberian Rubythroats of different ages and sexes.

			δD_f(‰)		
		n	Mean ± s.d.	Minimum	Maximum
Sex	Male	39	-100.9 ± 11.6	-126	-72
	Female	17	-97.6 ± 13.0	-118	-72
Age	Adult	18	-99.2 ± 10.5	-119	-80
	Subadult	38	-100.3 ± 12.8	-126	-72
Total		56	-99.9 ± 12.0	-126	-72

Figure 3. Distribution of the δDp values across the published maximum breeding region (outlined in blue) of the Siberian Rubythroat.
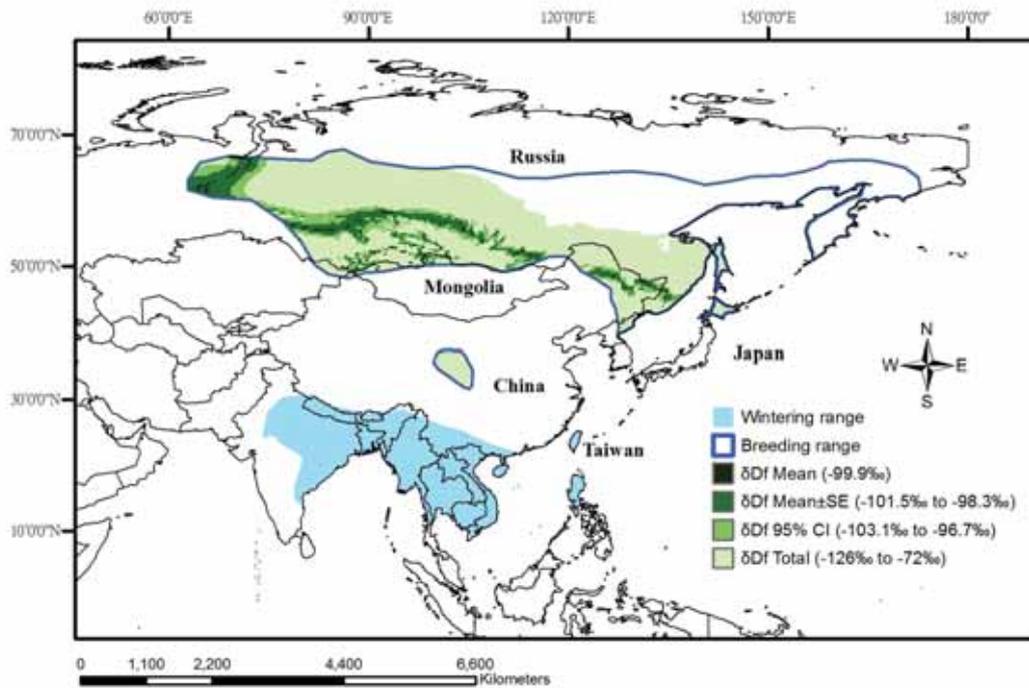
Figure 4. Map of the potential breeding area of Siberian Rubythroats overwintering in Taiwan. The published maximum overwintering and breeding ranges of the Siberian Rubythroat are outlined in light blue and blue, respectively. Only those grid squares within the maximum breeding range which were equal to the mean δD_f value of the breeding feathers (dark green), within one standard error (green), within the 95% confidence interval (light green) around the mean, or within the total range of δD_f values of the breeding feathers (pale green), have been coloured.

Table 5. Correlations between five body size measurements and mean δD_f values of breeding feathers taken from Siberian Rubythroats captured in Taiwan (r = Pearson's correlation coefficient; p = p-value; n = sample size). Eighteen of 20 correlations were negative (one sample sign-test, p = 0.0004), meaning that there was an overall significant trend that larger individuals originated from higher latitudes (which are associated with lower δD_f values), even if only two of the 20 correlations themselves had an associated p-value < 0.05.

		Male	Female	Adult	Subadult
Head length	r	-0.17	0.08	-0.01	-0.17
	p	0.29	0.77	0.96	0.32
	n	39	16	17	38
Body length	r	-0.09	-0.51	0.02	-0.22
	p	0.60	0.04	0.95	0.20
	n	39	16	18	37
Tarsus length	r	-0.18	-0.004	-0.32	-0.12
	p	0.28	0.99	0.20	0.47
	n	39	17	18	38
Flattened wing length	r	-0.38	-0.05	-0.40	- 0.30
	p	0.02	0.85	0.10	0.07
	n	39	17	18	38
Tail length	r	-0.13	-0.44	-0.29	-0.25
	p	0.42	0.09	0.24	0.13
	n	39	16	18	37

feathers, there is a general trend for larger individuals to have lower mean δD_f values (Table 5), which are roughly associated with higher latitudes (Figure 4).

DISCUSSION

Through the use of relatively novel molecular techniques, we were able for the first time to reliably test how male and female Siberian Rubythroats differ in colour and morphometric traits as well as to determine the potential breeding grounds of individuals captured in Taiwan.

Our results demonstrate that neither the size nor the colour of the red throat-patch nor the bordering sub-malar stripes are reliable field marks for sexing Siberian Rubythroats, particularly females.

Whilst males overall have larger and darker throat-patches and darker sub-malar stripes, there is overlap between males and females and therefore scope for misidentification. The same is true for all morphometric traits which cannot be used in isolation to reliably sex individuals. We emphasise that these results are novel because this is the first study based on reliable sexing techniques. Therefore, previous studies (Glutz von Blotzheim 1988, Svensson 1992, Barthel 1996, Cramp 1998, Pagenkopf 2003) which described colour and morphometric differences between the sexes should now be viewed cautiously, as some individuals may have been misidentified if sexing was not based on additional sexing methods, e.g. song, brood-patch or sexual organs.

While a logistic regression without cross-validation was able to reliably sex individuals, this result could not be repeated with a discriminant function analysis, which is the standard procedure for sex discrimination. Further studies should test whether these results would change when sample sizes are increased or when different populations are sampled.

Previous studies have claimed that fledglings within their first year of life have no or almost no red in their throat-patches, regardless of sex (Glutz von Blotzheim 1988, Bergmann 2001), although Barthel (1996) differed in claiming that subadult males already have red throats during the autumn of their first year while maintaining their light-brown feather tips. Barthel's claim is supported by our findings that both subadult and adult males have red throat-patches, while subadult and adult females may have white, red-and-white or red throat-patches (Table 2).

Bergmann (2001) claimed that the red throat colouration is dependent on nutrition and possibly exposure to sunlight, as captive birds lose most/all of the throat's red colouration after their moult. This effect is well known from other species (Hudson 1994). If age is not an important determining factor for the area and darkness of the red throat-patch, as our results suggest, then perhaps the red throat-patch is a signal which indicates the capability of its bearer to obtain the required carotenoid-containing foods (Olson & Owens 1998, Senar & Escobar 2002, Saks *et al.* 2003, Griggio *et al.* 2007).

Despite the conclusion that the colour and morphometric traits discussed above cannot by themselves (but possibly in combination) reliably sex individuals in every case, our study lends support to previous studies which revealed that, on average, males are larger

and have larger and darker red throat-patches and darker sub-malar stripes. Therefore, besides the obvious colour dimorphism of the throat area, there is indeed also a slight size dimorphism in the species. Owens & Hartley (1998) found that differences in size dimorphism were associated with variation in social mating system and sex difference in parental care, while differences in plumage-colour dimorphism were associated with variation in the frequency of extra-pair paternity. Thus social mating system, parental care and extra-pair paternity could all play a role in the evolution of the Siberian Rubythroat's body size and colours. The closely related Bluethroat *Luscinia svecica* has already been used to investigate the relation between colouration and male and female mate choice, mate guarding and sperm competition (Amundsen *et al.* 1997, Johnsen *et al.* 1998, 2003). In the future, Asian ornithologists should perhaps consider using the Siberian Rubythroat to study these topics.

Since the mean and variation of our δD_f values of the breeding and wintering feathers met the conditions set out in our Methods, we are confident that the breeding feathers were all grown within the breeding area. Furthermore, the s.d. values of our breeding feathers (Table 4) were comparable with those reported from feathers grown at similar latitudes by other bird species examined in Europe (Hobson *et al.* 2004); 25 species, s.d. = 1.0–10.6‰) and North America (Hobson & Wassenaar 1997); 6 species, s.d. = 4.0–11.0‰), indicating that Siberian Rubythroats overwintering in Taiwan originated from a relatively restricted latitudinal range. Based on their studies in North America, Farmer *et al.* (2008) stated that 'two samples that differ by less than 31‰ cannot be confidently said to originate from different latitudes'. The δD_f values of our breeding feathers ranged from -126‰ to -72‰, thus spanning approximately 54‰, which means that our samples should span about 2° of latitude. The relatively small s.d. and range of our δD_f values thus suggest that the actual breeding range of Siberian Rubythroats overwintering in Taiwan could be even more restricted than Figure 4 suggests.

Our map of the potential breeding area of Siberian Rubythroats (Figure 4) suggests that birds overwintering in Taiwan originate only from the southern part of the published breeding range, except for the most westerly regions. Given that Taiwan is located at the most northerly part of the documented overwintering range, this result suggests possible leap-frog migration (Kelly *et al.* 2002, Paxton *et al.* 2007, Reichlin *et al.* 2010), whereby the most northerly breeding populations migrate to the most southerly overwintering regions and thereby literally leap over those populations which migrate the least latitudinal distance from breeding to overwintering regions. Therefore, it would be interesting to repeat this analysis for other overwintering populations within east Asia to reliably establish the linkages between various breeding and overwintering areas.

Given that there is a rough correlation between δD_p and latitude (Figure 3), our results (Table 5) also suggest that larger individuals breed at higher latitudes. Such a correlation is in agreement with Bergmann's rule (Bergmann 1847, Futuyma 2009). However, as discussed above, our Siberian Rubythroats probably originated from within only 2° of latitude. This finding should therefore also be corroborated by further studies of other overwintering populations within east Asia.

Modern molecular techniques, in combination with older techniques such as colour and morphometric measurements, thus shed interesting new insights into the morphology and ecology of Siberian Rubythroats.

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Guo-Jing WENG, Institute of Wildlife Conservation, National Pingtung University of Science and Technology, Pingtung 912, Taiwan. Email: gjw@mail.npust.edu.tw

Hui-Shan LIN, Institute of Wildlife Conservation, National Pingtung University of Science and Technology, Pingtung 912, Taiwan. Email: blackkite588@yahoo.com.tw

Yuan-Hsun SUN, Institute of Wildlife Conservation, National Pingtung University of Science and Technology, Pingtung 912, Taiwan. Email: ysun@mail.npust.edu.tw

Bruno A. WALTHER, Master Program in Global Health and Development, College of Public Health and Nutrition, Taipei Medical University, 250 Wuxing St., Taipei 110, Taiwan. Email: bawalther2009@gmail.com (corresponding author)