

Geographical Variation in the Wing Morphology of the Golden-ringed Dragonfly *Anotogaster sieboldii* (Selys, 1854) (Odonata, Cordulegastridae) Detected by Landmark-based Geometric Morphometrics

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Abstract A previous molecular phylogenetic study showed that *Anotogaster sieboldii* has at least 6 monophyletic groups that are allopatrically distributed in the northern area of Asia (Japanese main islands and Korean Peninsula), Amamioshima, Okinawajima, Yaeyama islands, Taiwan and East China. These groups are difficult to distinguish by qualitative genital morphology; however, canonical variate analysis and linear discriminant analysis of the hind wing shape have been used to clearly distinguish them from each other. In the present study, multiple comparisons of the lengths of the abdomen and hind wing showed that these lengths did not differ significantly among the groups. In particular, the variation in the size range, in the lineage from the northern area, widely overlapped that of the lineages from the other areas. In evaluating the morphological differences, the wing shape was found to be more sensitive than other size variables.

Key words: geometric morphometrics, shape, Odonata.

Introduction

The family Cordulegastridae of the order Odonata consists of 40 species belonging to 5 genera (Tsuda, 2000). In the description of Odonata species, the species specificity of the male genital morphology is one of the most important characteristics. However, a considerable number of cordulegastrid species was described based on differences in body markings, because the qualitative genital morphology of this family is often too similar to allow individual species to be distinguished. Furthermore, these descriptions are often not adequate, because they are based on the examination of limited material without a consideration of the variation. Thus, it is necessary to examine larger numbers of specimens to determine the morphological differences for the taxonomic revision of some species or species

groups (Askew, 2004).

Anotogaster sieboldii (Selys, 1854) is a large cordulegastrid dragonfly that is distributed in Insular East Asia, Korean Peninsula and East China (Tsuda, 2000). Previous taxonomic studies of this species have reported some geographic variations, especially in body marking. Ishida *et al.*, (1988) tentatively recognized 3 allopatrically distributed groups in Japan: those of the Japanese main islands, Central Ryukyus (Okinawajima and Amamioshima) and the Yaeyama group (Iriomotejima and Ishigakijima). According to Asahina (1989), the Korean population morphologically resembles the population of Japanese main islands, whereas the Taiwanese population is morphologically similar to the population of the Yaeyama islands (Wang, 2000). Thus, the populations in Insular East Asia could be divided into 3 geographical groups. However, there have

been few taxonomic studies on the populations in East China. Although a re-evaluation of the populations in Ryukyus and East China is necessary (e.g., Asahina, 1969; Wang, 2000), the lack of phylogenetic information and of morphological characteristics that can be used for identification have prevented taxonomists from delimiting the species. Recently, a phylogeographical study of *A. sieboldii* revealed that at least 6 lineages diverged from the late Pliocene to the early Pleistocene (Kiyoshi, 2008). These lineages are allopatrically distributed in the northern area of Asia (including the Japanese main islands, Korean Peninsula and other adjacent islands), Amami-oshima, Okinawajima, the Yaeyama Group, Taiwan, and East China.

Quantitative comparisons of the wings or genitalia of insects by using landmark-based geometric morphometrics have often been effective in detecting differences between sibling species in many taxa (e.g., Aytakin *et al.*, 2007; Baylac and Daufresne, 1996; Baylac, Villemant and Simbolotti, 2003; Dapporto, 2008; Dujardin, Le pont and Baylac, 2003; Villemant, Simbolotti and Kenis, 2007). Although the wing veins are often used as diagnostic characteristics at the genus rank or for higher Odonata, they are rarely used to distinguish closely related species. In the present study, we determined morphological variations among populations of *A. sieboldii* by comparing the shape of the male hind wings by using landmark-based geometric morphometric methods. We then compared the morphological variations among populations on the basis of the molecular phylogenetic results.

Materials and Methods

Species were identified on the basis of specific external morphological characteristics and the structure of the male genitalia (Fraser, 1929; Fraser, 1936; Ishida *et al.*, 1988). The localities of samples used in this study are shown in Fig. 1. The right hind wings of 103 adult male individuals were analyzed morphologically. 3 samples from the Okinawajima population were bor-

rowed from the Okinawa Prefectural Museum, Okinawa (OPM-IS-7062, OPM-IS-7452 and OPM-IS-7453), and the rest are deposited in the National Museum of Nature and Science, Tokyo (NSMT-I-Od3001~NSMT-I-Od3100). Two-dimensional (2-D) images of the right hind wings were captured using a digital scanner (GT9800F, EPSON) with the following settings: 24-bit color, 800 dpi, same scale as specimen and as a reflected image. The 2-D coordinates of 15 homologous landmarks (Fig. 2) were recorded using the software COO version 37 (Dujardin, 2007).

The coordinates of the entire sample were superimposed using the Generalized Procrustes Analysis (GPA) algorithm, and the sum of the squared distance between the homologous landmarks of each object and a reference configuration was iteratively minimized by translations and rigid rotations (Rohlf and Slice, 1990; Goodall, 1995) to obtain the shape coordinates. Canonical variate analyses (CVA) were conducted to determine the shape characteristics that best distinguished the groups of specimens from each other by using these coordinates. In this analysis, the shape coordinate of the population of Zhejiang (East China) was excluded because of an insufficient number of specimens ($n = 2$). The coordinates of these 2 samples were converted using the CVA canonical coefficients for the other shape coordinates. These analyses were performed using MorphoJ version 1.00j (Klingenberg, 2008) and R statistical language, version 2.6.0 (R Development Core Team, 2007).

For the linear discriminant analysis (LDA), the dimensionality of the data was reduced to stabilize the statistical analyses (Baylac *et al.*, 2003; Baylac and Friess, 2005). Subsets of the principal components, rather than the whole set of Procrustes residuals, from a principal component analysis of the covariance matrix of the Procrustes residuals (PCA) were used for LDA. The number of components retained was selected in order to maximize the leave-one-out cross-validated classification results (Baylac and Friess, 2005).

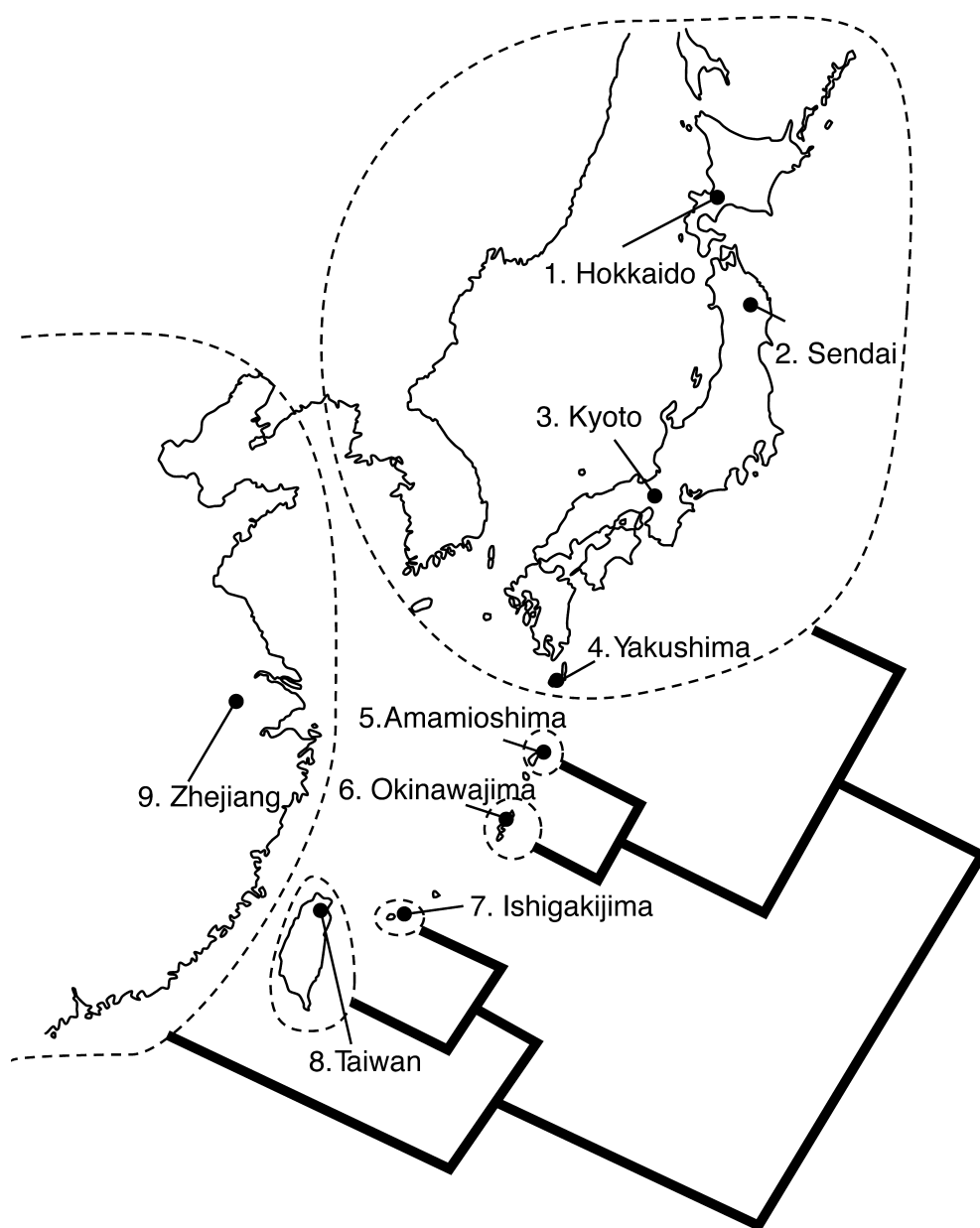


Fig. 1. Localities of the examined samples. — Data for phylogenetic relationships among populations are taken from Kiyoshi (2008). The areas enclosed by the dashed lines indicate the distribution range of the lineages recognized by Kiyoshi (2008).

For the size comparison among populations, the centroid sizes, i.e., the square root of the sum of the squared distances between the center of the object and the landmark for 15 landmarks was calculated from the 2-D images rescaled from a 0.01-mm caliper (Mitsutoyo). The length

of the abdomen was also measured using the caliper and compared among populations by using Tukey-Kramer multiple comparison tests. To describe the relationships between the centroid size of the hind wing and the abdominal length in male individuals, a simple allometric equation

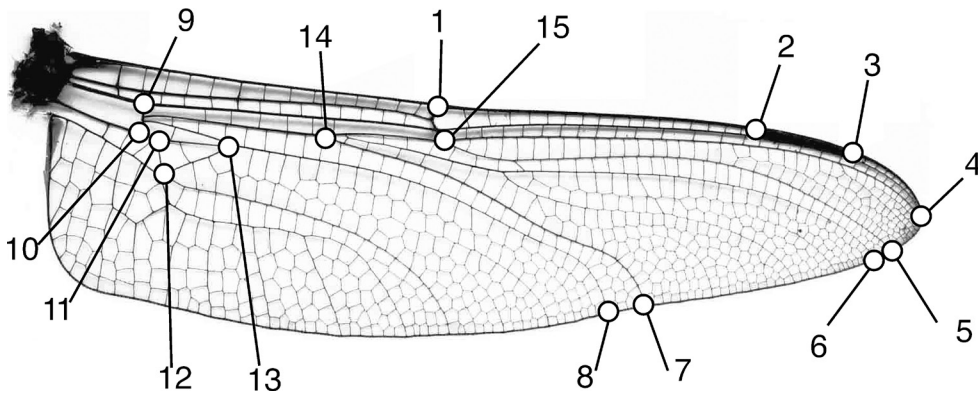


Fig. 2. The 15 landmarks of the male right hind wing used in the geometric morphometrical analyses.

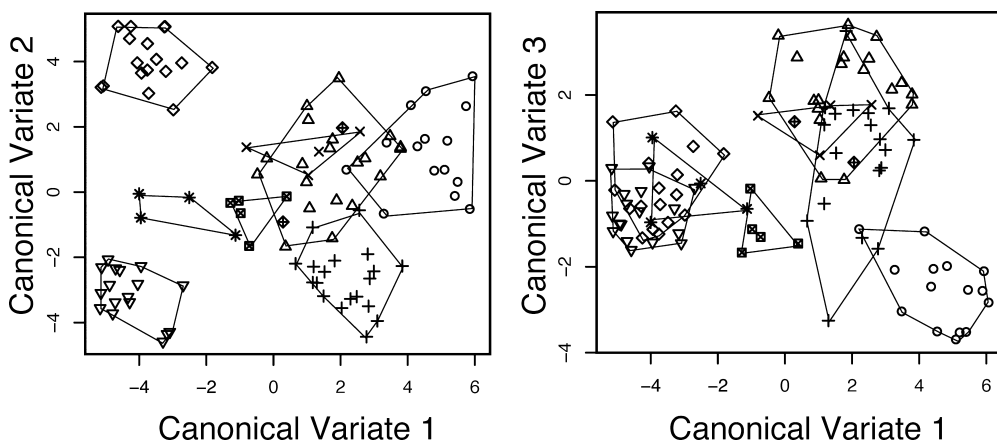


Fig. 3. The scatter plots of the first 2 canonical axes obtained by canonical variate analyses. — Left: first 2 canonical variates. Right: first and third canonical variates. Circle, Hokkaido ($n = 15$); triangle, Sendai ($n = 20$); plus, Kyoto ($n = 19$); cross, Yakushima ($n = 4$); diamond, Amamioshima ($n = 17$); asterisk, Okinawa-jima ($n = 4$); downward triangle, Ishigakijima ($n = 17$); square with cross, Taiwan ($n = 5$); diamond with plus, East China.

($\log (\text{Centroid size of hind wing}) = a * \log (\text{abdominal length}) + b$) was used, where a is the slope of the linear regression and b is its elevation. Multiple regression equations of populations were created by analysis of covariance (ANCOVA) in R statistical software, version 2.6.0.

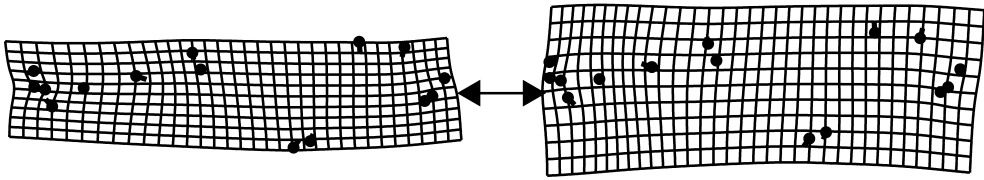
Results

Shape differences of the hind wing. CVA was applied to the Procrustes coordinates extracted from the hind wings of 101 specimens; samples from East China were not evaluated (Fig. 3). The

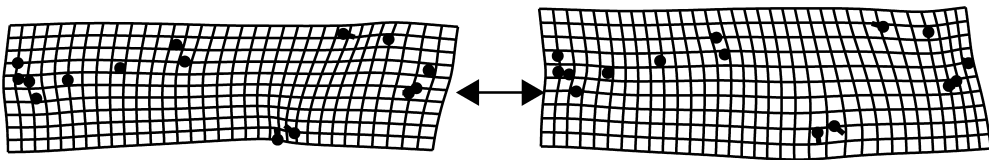
shape coordinates of the East Chinese samples were converted using the canonical coefficients of the CVA. The CVA scores of the northern-area samples from the 4 populations (Hokkaido, Sendai, Kyoto and Yakushima) were found to overlap.

On the first canonical axis, the scores of the northern-area samples were separate from those of 3 other populations (Amamioshima, Okinawa-jima and Ishigakijima). The scores of the samples from the latter 3 populations clearly overlapped. The scores of the Taiwanese and East Chinese samples also overlapped. Shape defor-

(a) CV1



(b) CV2



(c) CV3

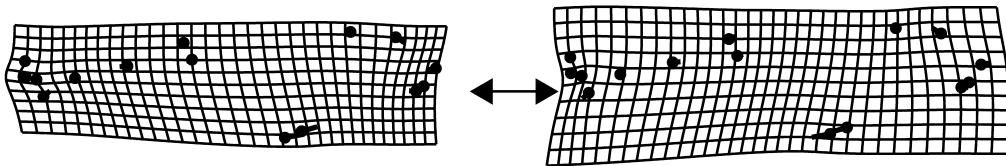


Fig. 4. Patterns of shape change along each canonical variate axis (CVs) represented by the thin plate spline function. — (a) CV1; (b) CV2; (c) CV3. Each deformation is exaggerated by 20 times. The images on the left and right correspond to the negative and positive direction of each axis, respectively.

mation corresponding to the negative value of this axis is mainly caused by the relative elongation in the horizontal direction (Fig. 4a).

For the second canonical axis, the scores of 3 populations (Amamioshima, Okinawajima and Ishigakijima) could be discriminated from each other clearly, but those of the populations from northern-area, East China and Taiwan were found to overlap. The shape deformation corresponding to the negative value of this axis corresponds to the relative narrowing of the half of the wing distal to the wing base (Fig. 4b).

The third axis divided the scores of the samples from Taiwan and East China, but the scores of the samples from these regions still overlapped with those of other populations. In all of the first 3 axes, the scores of the Taiwan population overlapped those of other populations, although the overlapping areas along the first and third axes were very small. For the East Chinese

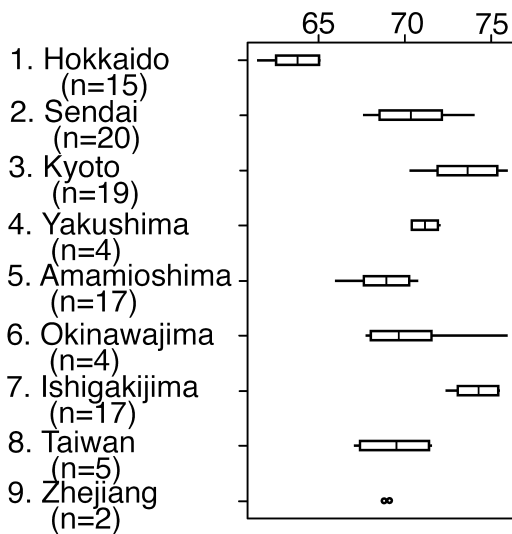
population, all of the first 3 scores overlapped those of the northern-area populations. The limited number of specimens available might have caused an insufficient pooling of their between-group variance in the CVA. Therefore, the result of the CVA approximately classified the populations into 5 clusters: (1) northern-area and Zhejiang, (2) Amamioshima, (3) Okinawajima, (4) Ishigakijima and (5) Taiwan.

An LDA for all pairs of populations (103 individuals), applied to the first 10 principal components out of 26 from the PCA (94.0% of the variance), showed that 16 individuals were misclassified (15.5%; Table 1); however, 12 cases of misclassification occurred among the populations of the northern area of Asia. Thus, if this manner of misclassification was not accounted for, only 4 cases were wrongly classified (3.9%). In all, 31 individuals (29.8%) remained wrongly classified after leave-one-out

Table 1. Assignment by the linear discriminant analysis between populations.

Population	Hokkaido	Sendai	Kyoto	Yakushima	Amamioshima	Okinawajima	Ishigakijima	Taiwan	Zhejiang
Hokkaido	14	1	0	0	0	0	0	0	0
Sendai	0	16	3	1	0	0	0	1	0
Kyoto	0	1	17	0	0	1	0	0	0
Yakushima	0	0	0	4	0	0	0	0	0
Amamioshima	0	0	0	0	17	0	0	0	0
Okinawajima	0	0	0	0	0	3	0	1	0
Ishigakijima	0	0	0	0	0	0	17	0	0
Taiwan	0	0	0	0	0	0	0	5	0
Zhejiang	0	0	0	0	0	0	0	0	2

(a) Centroid size



(b) Abdominal length

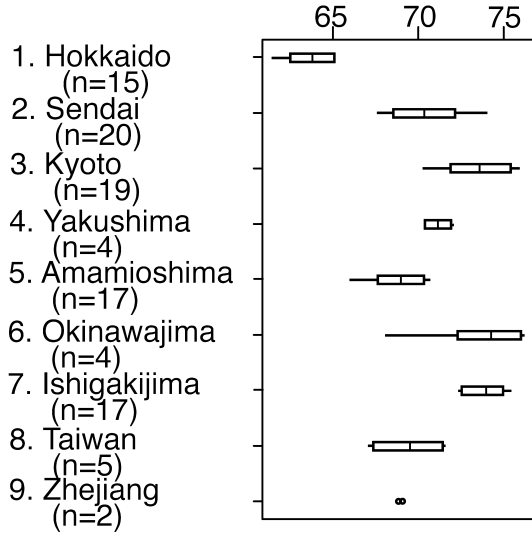


Fig. 5. Centroid sizes of the hind wing (a) and abdominal length (b). — A vertical line, a box and a horizontal bar indicate the mean, standard deviation, and range, respectively. Measurements for Zhejiang population are presented by as absolute values of the open circles because of the small sample size. The numbers denoting locality names correspond to those of Fig. 1.

cross-validation (not shown); however, 19 of these instances occurred in populations of the northern area of Asia. Six population groups corresponding to the lineages in the molecular phylogenetic tree were clearly distinguishable by the LDA: northern area, Amamioshima, Okinawajima, Ishigakijima, Taiwan and Zhejiang.

The Procrustes residuals were size free, but they might be allometrically correlated to size variables. Thus, the correlation between the centroid size of the hind wing and the canonical variate should be evaluated. Fig. 5 illustrates the centroid size of the male right hind wings for

each population. The analysis of variance for the centroid size among 8 populations, except the Zhejiang population, was significant ($d.f. = 8, 94$, $F = 57.90$, $P < 0.05$). Pooled within-group regressions between the logarithms of the centroid size and the CVA score on each axis were used to evaluate the allometric effect on the CVA. The explained variance of each CVA axis was rather small (first axis, 6.4%; second axis, 7.5%; and third axis, 0.6%), which suggests that the size differences had a rather limited influence on the clustering obtained in the CVA.

Size comparisons. First, pairwise comparisons

of the centroid size of the hind wing (Fig. 5a) among 8 populations, except the Zhejiang population, were conducted using the Tukey-Kramer test (Table 2). The variable range of the northern-area population (Hokkaido, Sendai, Kyoto and Yakushima) was wide, and it overlapped that of other populations. The variable range of the Amamioshima population was overlapped by that of the Okinawa population. The centroid size of the Amamioshima population was significantly smaller than that of the Taiwan and Ishigakijima populations without any overlapping range. The Okinawa population had a significantly smaller centroid size than the populations of Ishigakijima and Taiwan did, and it was overlapped by the size of the Ishigakijima population. For the Taiwan and Ishigakijima populations, the variable ranges of the centroid size did not overlap, but the difference between the centroid sizes of these 2 populations was not significant. The centroid size range of the Zhejiang population was overlapped by that of the northern-area, Amamioshima, Taiwan and Okinawajima popu-

lations.

Pairwise comparisons of the abdominal length (Fig. 5b) among 8 populations, except Zhejiang population, were also conducted using the Tukey-Kramer test (Table 3). The variable range of the population of the northern area was wide, and it overlapped that of the other populations. The variable range of the population of Amamioshima was overlapped by that of the sister population in Okinawa. The abdominal length of the Amamioshima population was significantly lesser than that of the Ishigakijima and Taiwan populations; nevertheless, the ranges overlapped between Amamioshima and Taiwan population. The population of Okinawa had a significantly smaller centroid size than did the Ishigakijima population; nevertheless, their variable ranges overlapped. The variable ranges of the Okinawa and Taiwan populations overlapped, and there was thus no significant difference. The variable ranges of the Taiwan and Ishigakijima populations did not overlap, and the difference was significant. The abdominal length of the Zhejiang

Table 2 Multiple comparisons of the centroid size of male right hind wings between populations by the Tukey-Kramer multiple-comparison test.

Population	Hokkaido	Sendai	Kyoto	Yakushima	Amamioshima	Okinawajima	Ishigakijima
Sendai	*						
Kyoto	*	*					
Yakushima	*	ns	ns				
Amamioshima	*	ns	*	ns			
Okinawajima	*	ns	*	ns	ns		
Ishigakijima	*	*	ns	*	*	*	
Taiwan	*	*	ns	ns	*	*	ns

* $P < 0.05$, ns: not significant.

Table 3. Multiple comparisons of abdominal length between populations by the Tukey-Kramer multiple-comparison test.

Population	Hokkaido	Sendai	Kyoto	Yakushima	Amamioshima	Okinawajima	Ishigakijima
Sendai	*						
Kyoto	*	*					
Yakushima	*	ns	*				
Amamioshima	*	ns	*	ns			
Okinawajima	*	ns	*	ns	*		
Ishigakijima	*	*	ns	ns	ns	*	
Taiwan	*	*	ns	ns	*	ns	ns

* $P < 0.05$, ns: not significant.

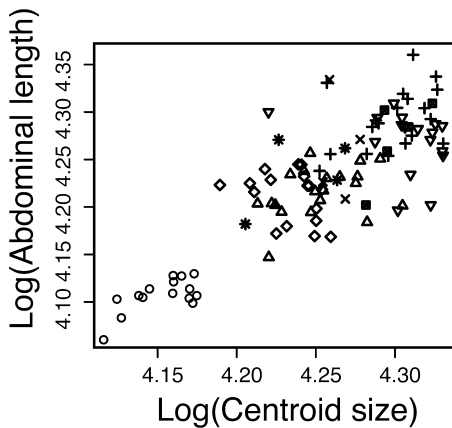


Fig. 6. Scatter plot of the centroid size of the hind wing against the absolute abdominal length. Both variables were log transformed. Circle, Hokkaido ($n = 15$); triangle, Sendai ($n = 20$); plus, Kyoto ($n = 19$); cross, Yakushima ($n = 4$); diamond, Amamioshima ($n = 17$); asterisk, Okinawajima ($n = 4$); downward triangle, Ishigakijima ($n = 17$); square with cross, Taiwan ($n = 5$).

samples was overlapped by that of the populations of the northern-area, Amamioshima and Taiwan but not by that of the Ishigakijima and Taiwan populations. Thus, these 2 variables often overlapped in their distribution.

The regression lines between the logarithms for the centroid size and abdominal length in each population (Fig. 6) were compared using ANCOVA. No significant difference ($P > 0.05$) was detected for the slopes and elevations of the regression lines among the populations. The ratio between the centroid size and abdominal length might not differ greatly among the populations.

Discussion

In the comparative analysis of the wing shape among the populations of *A. sieboldii*, 6 monophyletic groups determined by the previous molecular phylogenetic analysis (Kiyoshi, 2008) were clearly distinguished, but their sizes did not differ greatly. Therefore, an analysis of shape is more sensitive for detecting differences, because even if the size overlaps, the lineages can be differentiated. Moreover, the shapes showed a

remarkable similarity among the samples from the northern area of Asia (Hokkaido, Sendai, Kyoto and Yakushima).

The mitochondrial gene genealogy revealed that *A. sieboldii* consists of at least 6 monophyletic groups that are highly divergent in terms of genetics (northern area, Taiwan, Ishigakijima, Okinawajima, Amamioshima and East China; Fig. 1). These allopatric groups roughly correspond to the morphotypes that have been recognized by qualitative morphological differences in yellow body markings. The shape comparisons clearly showed differences among 5 groups of the East Asian islandss but the size comparisons did not show notable differences. As Mutanen and Pretorius (2007) suggested, geometric morphometrics provides a potent method to search for differences among taxa and serve as a novel method to visualize morphological variations in shape. Morphological comparisons of shape in combined with qualitative characteristics such as body markings in an abundant sample would enhance the understanding of cordulegastrid fauna in the East Asia.

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