Biological Journal of the Linnean Society, 2008, **94**, 289–301. With 8 figures

Mosaic pattern of genital divergence in three populations of *Schistocerca lineata* Scudder, 1899 (Orthoptera: Acrididae: Cyrtacanthacridinae)

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Received 26 April 2007; accepted for publication 19 July 2007

Sexual selection theory predicts that genital structures in isolated populations are likely to diverge, but male genitalia are often species-specific, which led to the idea that male genitalia are relatively invariable within species. Previous allometric studies collectively suggested that male genitalia are intraspecifically invariable in size compared with external body parts. We investigated whether male genitalia are invariable in shape in three populations of a grasshopper *Schistocerca lineata* Scudder, 1899, using two independent methods of geometric morphometric analyses. Specifically, we focused on the idea that male genitalia are complex structures consisting of many functionally different components, and studied how these individual parts diverge among three populations. Individual components of male genitalia show different population-level divergence, resulting in the mosaic pattern of genital divergence. Individual components diverge independently from each other. Body size is positively correlated with genitalia size, but is significantly correlated with the shape of only one of the three genital structures we measured. Thus, different components of male genitalia may be influenced by different evolutionary processes. This study is the first to show that components of complex genitalia evolve separately within a species. *Biological Journal of the Linnean Society*, 2008, **94**, 289–301.

ADDITIONAL KEYWORDS: geometric morphometric analysis – intraspecific genital variation.

INTRODUCTION

Sexual selection theory predicts that genital structures in isolated populations are likely to diverge, and thus a certain level of intraspecific genital variability is expected (Eberhard, 1985; Hosken & Stockley, 2004). At the same time, many insects have species-specific male genitalia, which would imply that there exists one invariable genital form for each species. Eberhard (1985) attributed this contradiction to taxonomists being more interested in typifying different species, to permit their identification, than in documenting the species' ranges of variability, and concluded that genitalia are generally neither invariant nor so variable within a species. Based on an analysis of intraspecific genital allometry of 20 species of insects and spiders, Eberhard *et al*. (1998) found that male genitalia had consistently lower allometric values than external body parts, and suggested that sexual selection is responsible for the relatively uniform size of genitalia, which is now called the 'one-size-fits-all hypothesis'. Although this idea has been challenged in terms of analytical procedures (Green, 1999) and inference (Bertin & Fairbairn, 2007), numerous allometric studies do support the pattern that the size of male genitalia is relatively invariable intraspecifically (Eberhard *et al*., 1998; Palestrini, Rolando & Laiolo, 2000; Schmitz, Reinhold & Wagner, 2000; Bernstein & Bernstein, 2002; Ohno *et al*., 2003). However, several empirical studies show that intraspecific genital variation in shape may be a widespread phenomenon across insects (Garnier *et al*., 2005; Mutanen & Kaitala, 2006; Polihronakis, 2006). Intraspecific variation can arise from post-emergence development of internal structures (*ontogenetic variation*, Kevan & Lee, 1974; Song, 2004a), may be affected by environmental factors such as day length or temperature (*phenotypic plasticity*, Müller,

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1957 in Shapiro, 1978; Vitalievna, 1995; Andrade, Hatadani & Klaczko, 2005), may be naturally polymorphic (*genital polymorphism*, Johnson, 1995; Huber & Pérez González, 2001), or may vary according to geographical populations (*geographical variation*, Hribar, 1994; Pires *et al*., 1998; Cordero Rivera *et al*., 2004; Garnier *et al*., 2005). Therefore, intraspecific genital variation deserves more investigation.

One aspect of genital evolution that is not often addressed is the complex nature of genitalia. Male genitalia are complex structures consisting of several functionally different parts (Huber, 1996; Huber, 2004; Song, 2004a). For example, a phallic complex of a male grasshopper consists of three main parts, ectophallus, epiphallus, and endophallus, and within each part, several functionally distinct structures also exist (Dirsh, 1956; Dirsh, 1973; Amédégnato, 1976; Eades, 2000). Huber (2004) demonstrated in a pholcid spider that the left and right palpi are under different selective pressure because of functional segregation, leading to morphological differentiation. Therefore, if functionally separate structures are available independently for selection, we would expect to observe different divergence patterns for the individual components.

The bird grasshopper *Schistocerca lineata* Scudder, 1899 (Orthoptera: Acrididae: Cyrtacanthacridinae) is one of the most widespread and most polymorphic grasshoppers in North America. It exists as several ecologically divergent populations, which are often divergent in size and colour (Song, 2004b), but which are united by species-specific male genitalia (Hubbell, 1960; Song, 2004b). Mating behaviour of this species, such as coercive mating, prolonged copulatory courtship, and multiple mating (Otte, 1970), suggest that sexual selection by female choice may be responsible for genital divergence (Eberhard, 1985). There is no known species-specific female defensive structure that would be expected under sexually antagonistic co-evolution (Arnqvist & Rowe, 2002; Eberhard, 2004). In this study, we examine the intraspecific variation in two internal genital structures and one external genital structure in *S. lineata* among three allopatric populations. One of the internal structures, the basal eminence of the cingulum (Fig. 1A), physically contacts the female counterpart during copulation. This structure is covered with sensillae, suggesting it has a sensory or stimulatory function during courtship. The other internal structure measured was the lophus of the epiphallus (Fig. 1B), which hooks onto the base of the female subgenital plate during copulation (Randell, 1963), suggesting that it has a grasping function. Male cerci (Fig. 1C), external genital structures, are lightly pressed into the female abdomen during copulation, suggesting that they have possible grasping, sensory, or stimulatory functions. Specifically, we study the shape variation of these structures using two independent methods of geometric morphometric analyses. Geometric morphometric analysis has revolutionized the study of shape (Bookstein, 1991; Rohlf & Marcus, 1993; Adams, Rohlf & Slice, 2004; Zelditch *et al*., 2004). It has also been applied to the study of genital variation in insects (Liu *et al*., 1996; Arnqvist, 1998; Monti, Baylac & Lalanne-Cassou, 2001; Garnier *et al*., 2005; Mutanen & Kaitala, 2006; Polihronakis, 2006). We test the hypothesis that functionally different components of genitalia are under separate evolutionary pressures, resulting in different population-level divergence for each structure. We also compare the correlation between body size and genitalia size and shape to test whether the divergence in body size has any effect on the divergence of genitalia.

MATERIAL AND METHODS

Collecting and rearing

Three ecologically distinct populations of *S. lineata* were located using data from Song (2004b). The first population was collected from Colorado (Bent Co., John Martin Reservoir SWA, 38°05.554′N, 103°03.256′W) on July 15 and 16, 2004. The second population was collected from Oklahoma (Comanche Co., Fort Sill Military Reservation, West Range, 34°39.517′N 098°33.054′W) on July 18–19, 2004. The third population was collected from Kansas (Bourbon Co., Hollister Wildlife Area, 37°45.521′N, 094°51.033′W) on July 22, 2004. Habitat structures, host plant use, and nymphal behaviour (if the insects were young) were documented (Table 1). Insects were collected along with the host plants as far as possible.

Insects were transported alive in cages. During transportation, they were fed host plants supplemented with Romaine lettuce and wheat bran. Insects were reared in a temperature-controlled room located in the Ohio State University Biological Sciences Greenhouse. The rearing room was kept at 29–32 °C during the day and 23–26.5 °C during the night, with a day : night regime of 14-h day : 10-h night. Each population was kept separately in 38-cm × 38-cm × 63.5-cm cages. All grasshoppers were kept separate by sex until maturation. For the Colorado (CO) and Kansas (KS) populations, host plants collected from the original locality were used as the main food, supplemented with Romaine lettuce and wheat bran. The Oklahoma (OK) population was fed Romaine lettuce and wheat bran daily.

Preparation of specimens

By September 2004, all grasshoppers were sexually mature. The final size at maturity did not appear to

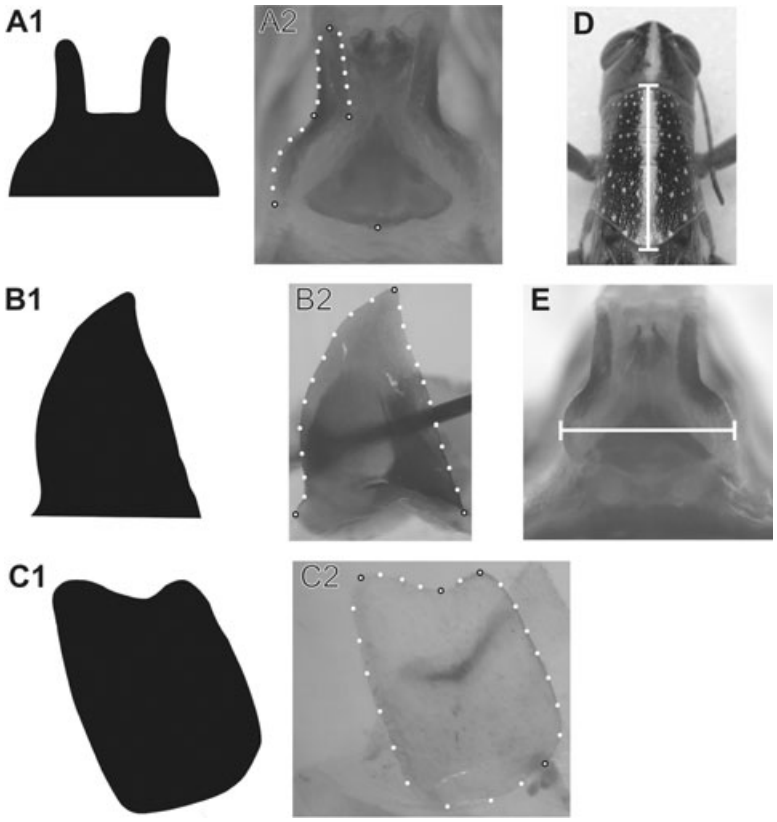

Figure 1. Images used in the geometric morphometric analysis and the analysis of size. A1, A2, basal eminence of the cingulum; B1, B2, lophus of the epiphallus; C1, C2, the male cercus; D, dorsal view of the pronotum; E, dorsal view of the basal eminence of the cingulum. For the outline-based analysis, the images were trimmed and converted to black-and-white bitmap format (A1, B1, and C1). For the landmark-based analysis, the raw images were used to digitize the shape (A2, B2, and C2). Black circles with white centres represent Type-1 and -2 landmarks, and solid white circles represent Type-3 sliding semilandmarks.

Table 1. Ecological characteristics and adult coloration of the three study populations of *Schistocerca lineata*

Population	Collected as	Host plants	Habitat	Adult colour
Colorado (CO) (15–16 July 2004)	Nymphs	*Tamarix* sp.	Dry and sandy habitat	Olive-green with yellow dorsal stripe and red hind tibiae
Oklahoma (OK) (18–19 July 2004)	Adults	Unknown	Short grass mixed habitat	Sandy-yellow with black lateral stripes and yellow dorsal stripe and black hind tibiae
Kansas (KS) (22 July 2004)	Nymphs	*Rhus* sp.	Tall grass prairie habitat	Dark-brown with yellow dorsal stripe and black hind tibiae

be affected by rearing conditions, as it was comparable with the size of museum specimens. Individual grasshoppers were placed in a Falcon tube singly with an identification label, and were killed in a –80 °C freezer. In order to study the variation of male genitalia and male cerci, a total of 59 male specimens from all populations (CO = 13; KS = 20; OK = 26) was dissected. From a whole specimen, the terminal portion of the abdomen was dissected by cutting through the membrane between the eighth and ninth abdominal segments. The dissected part contained epiproct, male cerci, subgenital plate, and the entire phallic complex. Each dissected specimen was given a unique identification number associated with the whole specimen. It was then placed in weak KOH solution for about 4 h to dissolve muscle. The struc-

ture was further dissected into three parts: tergal plate containing epiproct and both cerci, subgenital plate, and the phallic complex. They were preserved in glycerol in glass vials.

Preparation of images

Because the phallic complex is a three-dimensional structure that is difficult to position, extra care was taken to position specimens in the same manner. Each structure was securely braced with minuten pins on a Petri dish, which was about half-filled with 70% ethanol. Digital photographs of the right lophus of the epiphallus and the dorsal portion of the zygoma and rami (basal eminence) were taken with each structure in the plane of the image. Specimens of epiproct and cerci were placed on a silicon-bottomed Petri dish, and a glass microslide was placed over the specimens and braced with insect pins, so that the specimens were firmly pressed and flattened. A white paper was placed under the Petri dish to provide a uniform background. Digital photographs of the correctly positioned specimens were taken using a Nikon Coolpix 990 camera mounted on a Wild microscope.

In order to measure the body size, we photographed the dorsal view of the pronotum for a subset of the sample (CO = 11; KS = 12; OK = 12). Individual insects were braced with insect pins so that the dorsal portion of the specimen was in the plane of the image. For all structures, each specimen was photographed twice, and the images in better focus were used for the analysis.

Geometric morphometric analysis

We performed two types of geometric morphometric analyses to study the shape variations of the basal eminence of the cingulum, the lophus of the epiphallus, and the male cercus among three populations of *S. lineata*. The first method was an elliptic Fourier analysis (EFA), which is a mathematical way of reducing complex curves into their component spatial frequencies (McLellan & Endler, 1998). The EFA is based on elliptic Fourier descriptors (EFDs) that can delineate any type of shape with a closed two-dimensional contour (outline), which can then be analysed statistically (Kuhl & Giardina, 1982). The EFA has been used to analyse complex shapes, including insect male genitalia (Liu *et al*., 1996; Arnqvist, 1998; Polihronakis, 2006). The second method was a landmark-based analysis, which is based on two-dimensional coordinates of biologically definable landmarks (Bookstein, 1991). Initial sets of landmark coordinates are mathematically treated to remove nonshape variations, such as position, orientation, and scale, and the resulting shape variables can be used statistically to compare samples (Adams *et al*., 2004). We used these two methods as independent tests and replicates of each other.

For the EFA, we used the software package SHAPE v1.2 (Iwata & Ukai, 2002). As the software required black and white bitmap images, the raw images were formatted accordingly in PHOTOSHOP CS. The images were first converted into chain code, a coding system describing geometrical information (Freeman, 1974), from which the normalized EFDs were calculated with a procedure based on the point on the contour farthest from the centroid, and manually aligned so that each shape was positioned the same way. The contour shape was described in the first 30 harmonics of Fourier coefficients. A principal component analysis was performed on the resulting normalized EFD coefficients.

For the landmark-based analysis, we used the software in the TPS series (freely available at http://life.bio.sunysb.edu/morph). The initial input files were created using tpsUtil (Rohlf, 2006a), based on raw images without any modification. We quantified the shape of each structure as a set of two-dimensional shape coordinates using tpsDig2 (Rohlf, 2006b). As genital structures do not usually have many distinct homologous landmarks (Type-1 landmarks), we utilized both Type-1 and -2 landmarks, along with sliding semilandmarks (Type-3 landmarks) (Bookstein, 1991; Adams *et al*., 2004). Specifically, five Type-1 and -2 landmarks (LMs) and 16 Type-3 semilandmarks (SLMs) were selected for the basal eminence of the cingulum, three LMs and 20 SLMs were selected for the epiphallus, and four LMs and 20 SLMs were selected for the male cercus (Fig 1). For the basal eminence of the cingulum, we digitized the left half of the structure because the shape was symmetrical. Relative warp scores for each specimen based on landmark data were calculated, and a principal component analysis based on the resulting partial warp scores was performed for each genital structure using tpsRelW (Rohlf, 2006c).

Analysis of size

The longitudinal length of the pronotum (Fig. 1D) was known to be an excellent indicator of grasshopper body size (Hubbell, 1960), and was thus used to represent body size. The width of the basal eminence (Fig. 1E) was chosen as an indicator of genitalia size. These structures were measured from digital images using the image analysis software ABLE IMAGE ANALYSER™ (v2.1; Mu Laboratories). To assess the measurement error, each dimension was measured three times. To study the correlation between body size and the size of male genitalia, a linear regression analysis was performed on log-transformed measure-

Table 2. Principal components (PC) and relative warp scores (RW) resulting from principal component analyses of the coefficients of elliptic Fourier descriptors and partial warps, respectively

	Basal eminence			Epiphallus			Male cercus		
	E.V.	%	Cum.%	E.V.	%	Cum.%	E.V.	%	Cum.%
PC1	0.06274	59.93	59.93	0.02252	64.73	64.73	0.01098	74.08	74.08
PC2	0.02180	20.83	80.76	0.00709	20.36	85.09	0.00168	11.36	85.44
PC3	0.01098	10.49	91.25	0.00244	7.01	92.10	0.00100	6.72	92.16
PC4	0.00362	3.46	94.71	0.00094	2.70	94.80	0.00044	2.98	95.14
PC5	0.00133	1.27	95.98	0.00052	1.51	96.31	0.00020	1.33	96.47
PC6	0.00093	0.89	96.87	0.00036	1.04	97.35	0.00016	1.08	97.55
	S.V.	%	Cum.%	S.V.	%	Cum.%	S.V.	%	Cum.%
RW1	0.34802	36.34	36.34	0.17984	37.50	37.50	0.23725	50.40	50.40
RW2	0.27161	22.13	58.47	0.11796	16.13	53.63	0.12578	14.17	64.57
RW3	0.20238	12.29	70.76	0.10051	11.71	65.35	0.09721	8.46	73.03
RW4	0.16844	8.51	79.27	0.09828	11.20	76.55	0.08091	5.86	78.89
RW5	0.14098	5.96	85.23	0.07530	6.57	83.12	0.07038	4.44	83.33
RW6	0.11791	4.17	89.40	0.05523	3.54	86.66	0.06458	3.73	87.06
RW7	0.10904	3.57	92.97	0.05192	3.13	89.79	0.05777	2.99	90.05
RW8	0.08759	2.30	95.27	0.04318	2.16	91.95	0.04964	2.21	92.25
RW9	0.06811	1.39	96.67	0.03971	1.83	93.78	0.04499	1.81	94.07
RW10	0.05093	0.78	97.44	0.03636	1.53	95.31	0.03646	1.19	95.26

For the elliptic Fourier analysis (EFA), all reported PCs are shown, and for the landmark-based analysis, the first ten RWs are shown. (E.V. = eigenvalue; S.V. = singular value).

ments. To study the size variation among different populations, the analysis of variance was performed.

The correlation between body size and shape variable was analysed using multivariate regression analysis implemented in tpsRegr (Rohlf, 2005). For each structure, the landmark data sample size was concatenated to match the sample size of the length of pronotum.

RESULTS

Elliptic Fourier analysis

For each structure, a total of 30 harmonics was analysed, resulting in 120 normalized EFD coefficients. Independent shape characteristics for the basal eminence of the cingulum, the lophus of the epiphallus, and male cercus were identified by principal component analysis of these 120 coefficients. By default, SHAPE reported only the effective principal components, measured by the proportion that is larger than 1 over the number of analysed coefficients, which were six. These six principal components (PCs) cumulatively accounted for more than 96% of the total variation (Table 2), and we used the first three PCs to describe the shape variation. In all three structures, PC1 contributed the highest percentage, and was a good measure of the width-to-length ratio based on the reconstructed contours (Fig. 2). For the basal eminence of the cingulum, PC2 measured the shape of the apical projection and the constriction of the basal eminence, and PC3 measured the bulbousness of the basal eminence (Fig. 2A). For the epiphallus, PC2 was a good measure of the curvature of the apex of the lophus, and PC3 measured the shape of the base and the apex of the lophus (Fig. 2B). For the male cercus, PC2 measured the shape of the bilobedness and PC3 measured the curvature of the base (Fig. 2C).

In order to compare the shape variation of each genital structure among three populations, we plotted the least-squares means of PC1 of each population against that of PC2 and PC3 (Fig. 3). In all of the measured structures, all three populations overlapped on the PC1 axis, and thus we focused on describing shape variations explained by the PC2 and PC3 axes. When PC1 of the basal eminence of the cingulum was plotted against PC2 (Fig. 3A), the OK population was clearly delineated as a distinct entity, indicating that the shape of the apical projection and the constriction of the basal eminence in the OK population was significantly different from those of the CO and KS populations. Similarly, when PC1 of the basal eminence of the cingulum was plotted against PC3 (Fig. 3B), the OK population was again delineated, indicating that the OK population was different from the other two populations in the bulbousness of the basal eminence. When PC1 of the epiphallus was plotted against PC2 (Fig. 3C), the CO population was distinctly delineated, indicating that

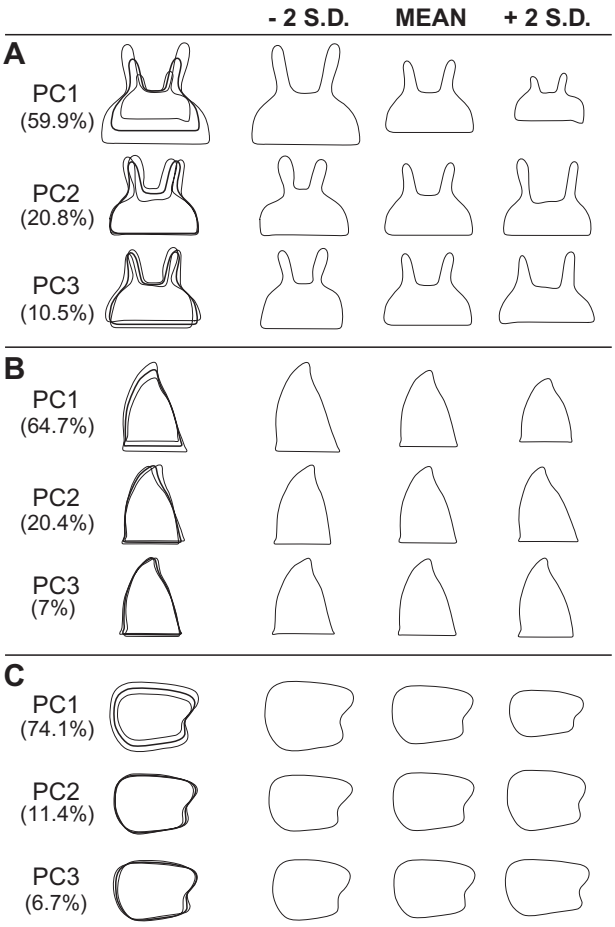

Figure 2. Reconstructed contours of shape variation described by the first three principal components from the elliptic Fourier analysis (EFA). The percentages indicate how much each principal component explains the shape variation. The thick line in the far-left column indicates the mean shape. A, basal eminence of the cingulum; B, lophus of the epiphallus; C, male cercus.

the curvature of the apex of the lophus of the CO population was significantly different from both those of the OK and KS populations. When PC1 of the epiphallus was plotted against PC3 (Fig. 3D), the KS population was delineated, suggesting that its shape of the base and apex of the lophus was different from those of the OK and CO populations. When PC1 of the male cercus was plotted against PC2 (Fig. 3E), the CO population was clearly delineated, indicating that its shape of bilobedness was significantly different from those of the the other two populations. When PC1 of the male cercus was plotted against PC3 (Fig. 3F), however, the three populations overlapped, indicating that there was no statistical difference in the curvature of the base among the three populations.

Landmark-based analysis

The principal component analysis on partial warp scores (relative warps analysis) was performed with an option to minimize the distance between the adjusted position and the corresponding point in the consensus for sliding SLM points. The analysis resulted in 38 relative warps (RWs) for the basal eminence of the cingulum, 42 RWs for the epiphallus, and 44 RWs for the male cercus. Of these, we used the first three RWs in describing the shape variation, which collectively explained more than 65% of the overall variation (Table 2). We used tpsRelW (Rohlf, 2006b) to visualize the shape variation of each structure along each relative warp axis, because the program has a feature to visualize deformations of the consensus configuration corresponding to a point in the space spanned by a particular pair of RWs. For the basal eminence of the cingulum, RW1 (36.34%) was a good measure of the shape and angle of the apical valves of the cingulum, RW2 (22.13%) measured the bulbousness of the basal eminence, and RW3 (12.29%) measured the constriction of the apical valves of the cingulum. For the epiphallus, RW1 (37.5%) was a good measure of the width-to-length ratio, RW2 (16.13%) measured the curvature of the apex of the lophus, and RW3 (11.71%) measured the shape and curvature of the inner side of the lophus. For the male cercus, RW1 (50.4%) was a good measure of the width-to-length ratio, RW2 (14.17%) measured the shape of bilobedness, and RW3 (8.46%) also measured the shape of bilobedness, especially of the upper lobe.

Similarly to the EFA analysis, we plotted the least-squares means of RW1 of each population against that of RW2 and RW3, to compare the shape variation of each structure (Figs 4–6). We used tpsRelW (Rohlf, 2006b) to visualize the mean shape of each structure for each population so that we could exactly describe how shape differed among populations. When RW1 of the basal eminence of the cingulum was plotted against RW2 (Fig. 4A), the OK population was clearly different from the other two populations. The visualization showed that the OK population was different from the others in the shape of the apical valves of the cingulum. When RW1 of the basal eminence of the cingulum was plotted against RW3 (Fig. 4B), three populations were all distinctly delineated without overlapping. When RW1 of the epiphallus was plotted against RW2 (Fig. 5A), the three populations were all distinctly delineated without overlapping. The same result applied when RW1 was plotted against RW3 (Fig. 5B). This indicated that the shape of the epiphallus in each population was unique. When RW1 of the male cercus was plotted against RW2 (Fig. 6A), the CO population was distinctly delineated from the

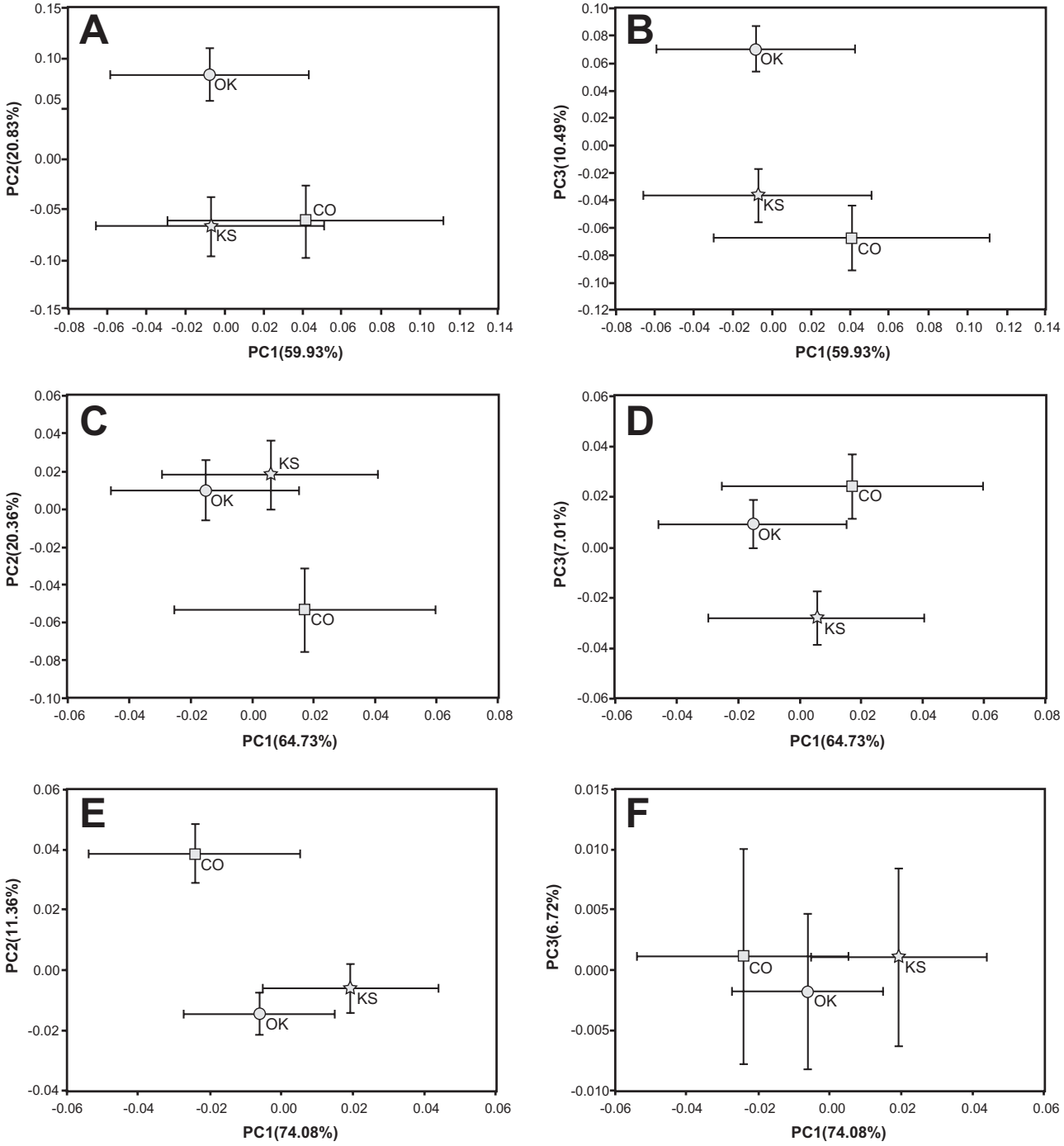

Figure 3. Population-level divergence in individual genital structures analysed by principal component (PC) analysis of the coefficients of the elliptic Fourier descriptors. These plots are based on least-squares means and standard errors of PC1, PC2, and PC3 for each structure. A, B, basal eminence of the cingulum; C, D, lophus of the epiphallus; E, F, male cercus.

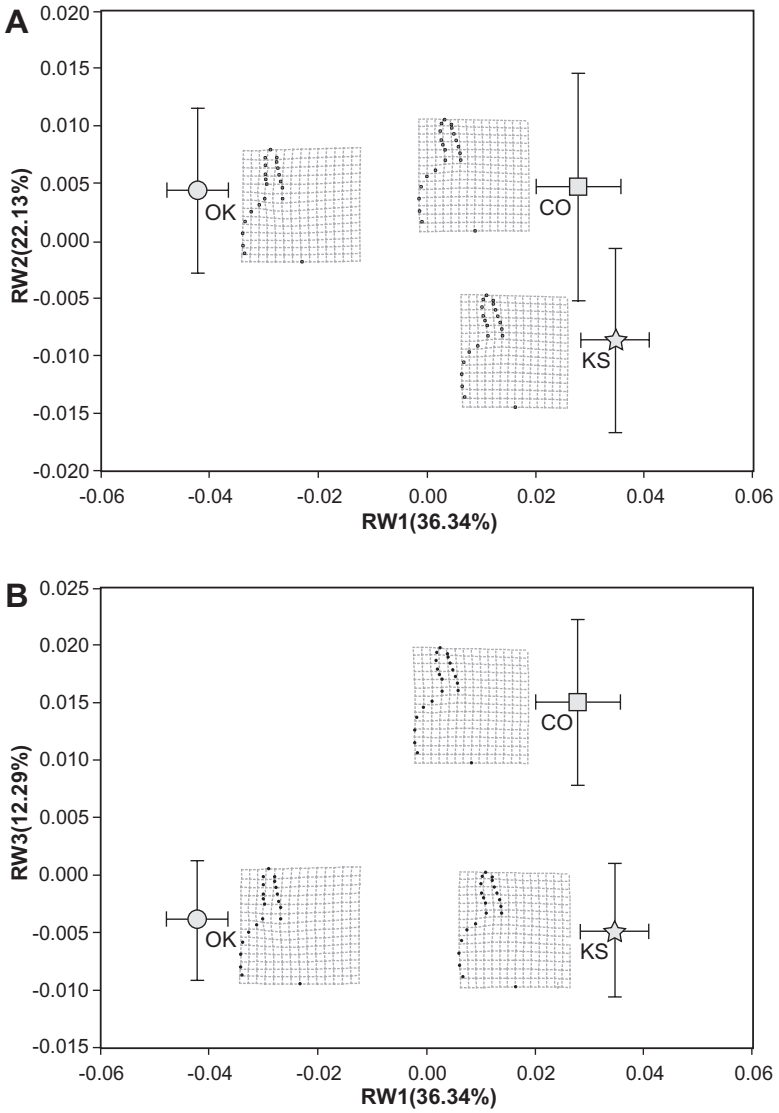

Figure 4. Population-level divergence in the basal eminence of the cingulum analysed by relative warp (RW) analysis based on landmark data. These plots are based on least-squares means and standard errors of RW1, RW2, and RW3. Each landmark figure represents the reconstructed mean shape for each population, visualized in tpsRelW based on least-squares means. A, RW1 plotted against RW2; B, RW1 plotted against RW3.

other two, suggesting that its shape of bilobedness was different from the KS and OK populations. When RW1 of the male cercus was plotted against RW3 (Fig. 6B), a similar pattern was observed, although the KS and OK populations overlapped less on the RW3 axis.

Analysis of size

The average measurement error for the pronotum was 0.153% (range 0–0.742%), and was 0.329% (range 0–1.492%) for the genitalia. Because the measurement error was very low, we used the mean value of each dimension for further analyses. The length of the pronotum, representing body size, was significantly different by population (Fig. 7; ANOVA, $N = 35$; $F = 35.83$; $P < 0.0001$). When compared against one another, individuals from OK were the largest, followed by individuals from CO and KS (Tukey's pairwise comparison). Genitalia size, measured by the width of the basal eminence, was also significantly different according to population, resulting in a pattern similar to that found in body size (Fig. 7; ANOVA, $N = 35$; $F = 16.05$; $P < 0.0001$). Males from OK had significantly larger genitalia than those from CO and KS. The CO genitalia were larger than the KS genitalia, although there was no statistical sig-

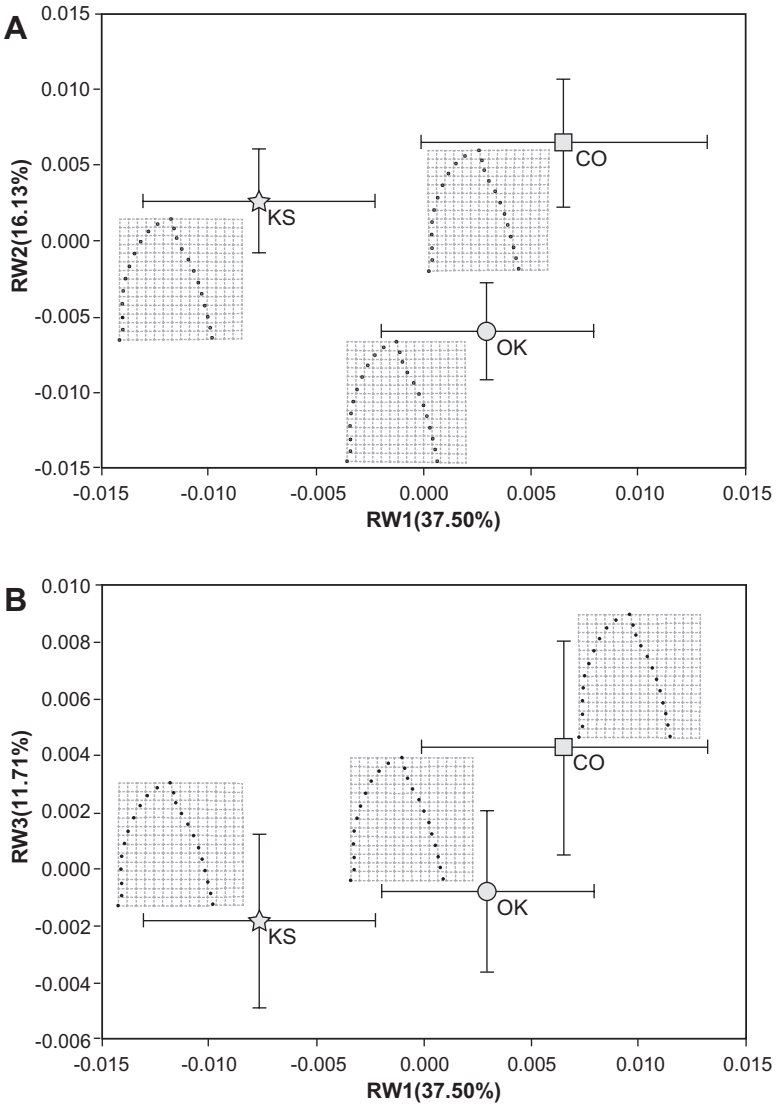

Figure 5. Population-level divergence in the lophus of the epiphallus analysed by relative warp (RW) analysis based on landmark data. These plots are based on least-squares means and standard errors of RW1, RW2, and RW3. Each landmark figure represents the reconstructed mean shape for each population, visualized in tpsRelW based on least-squares means. A, RW1 plotted against RW2; B, RW1 plotted against RW3.

nificance between the two populations. A linear regression analysis found a positive correlation between body size and genitalia size (Pearson correlation coefficient = 0.638).

Multivariate regression analysis was performed between the shape of each structure and the body size. Because of the small sample size, permutation tests were performed with 1000 random permutations, as implemented in tpsRegr (Rohlf, 2005). There was a significant correlation between the shape of the basal eminence of the cingulum and the body size represented by the length of the pronotum (Fig. 8A; Generalized Goodall F-test, $F = 9.6233$, d.f. = 38, 1254, $P < 0.0001$). No significant correlation against body size was found for either the epiphallus (Fig. 8B; $F = 0.9218$, d.f. = 42, 1302, $P = 0.6151$) or the male cercus (Fig. 8C; $F = 1.4264$, d.f. = 44, 1408, $P = 0.0358$).

DISCUSSION

Our study demonstrates that individual components of genitalia can diverge from each other, and can do so differently in different populations. Two independent shape analyses support this finding. Both analyses agree that the shape of the basal eminence of the cingulum of the OK population was significantly different from those of both the CO and the KS popula-

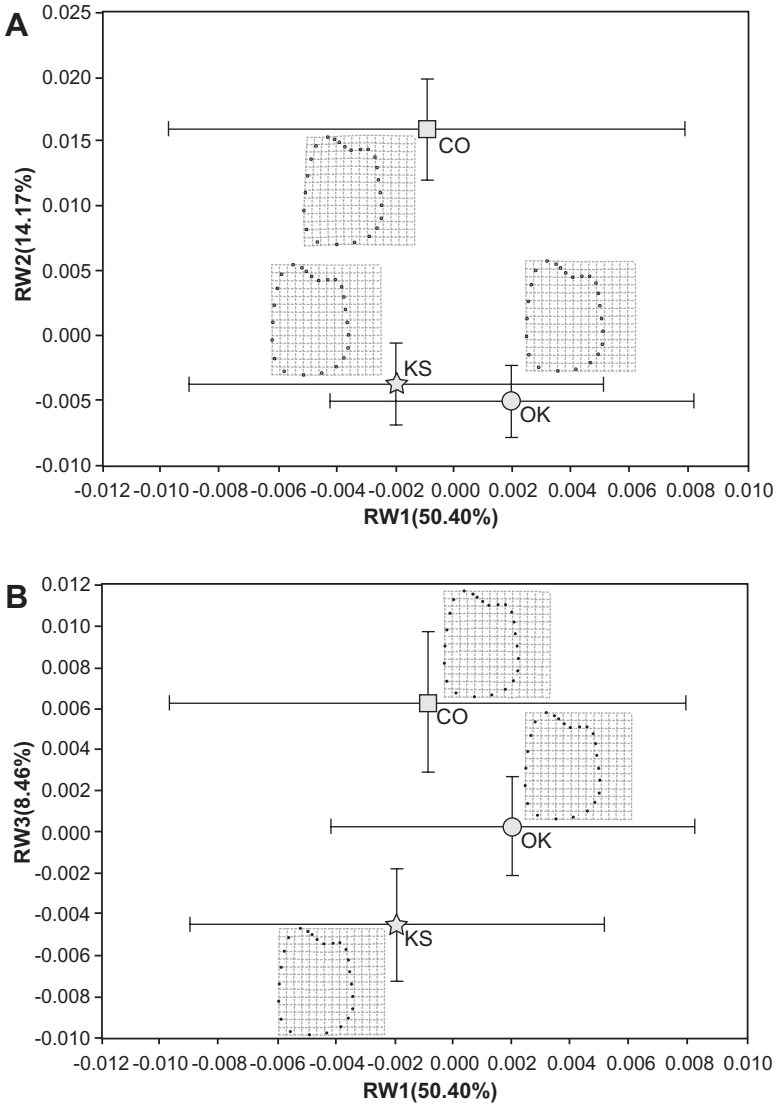

Figure 6. Population-level divergence in the male cercus analysed by relative warp (RW) analysis based on landmark data. These plots are based on least-squares means and standard errors of RW1, RW2, and RW3. Each landmark figure represents the reconstructed mean shape for each population, visualized in tpsRelW based on least-squares means. A, RW1 plotted against RW2; B, RW1 plotted against RW3.

tions, in that the apical valves of the cingulum are longer and straighter, and the bulbousness of the basal eminence was less distinct (Figs 3A, B, 4). The landmark-based analysis further separated out the shape differences between the CO and KS populations (Fig. 4). In analysing the shape of the epiphallus, the two analyses differed in that the landmark-based analysis suggested that the shape of the epiphallus was population specific, and did not overlap between populations (Fig. 5), whereas the EFA suggested that the CO population was different from the other two in terms of the curvature of the apex of the lophus (PC2, Fig. 3C), but also suggested that the KS population was different from the other two in terms of the shape of the base and apex of the lophus (PC3, Fig. 3D). Both analyses agree that the male cercus of the CO population was significantly different from those of both the KS and the OK populations, in that it was wider at the base and that the bilobedness was more distinct (Figs 3E, F, 6). When looking at individual components of genitalia, we find that the basal eminence of the cingulum is similar between CO and KS populations, the epiphallus is population specific, and the male cercus is similar between KS and OK populations. In other words, individual components appear to diverge separately among the three populations, which effectively suggests that there is a 'mosaic' pattern of genital

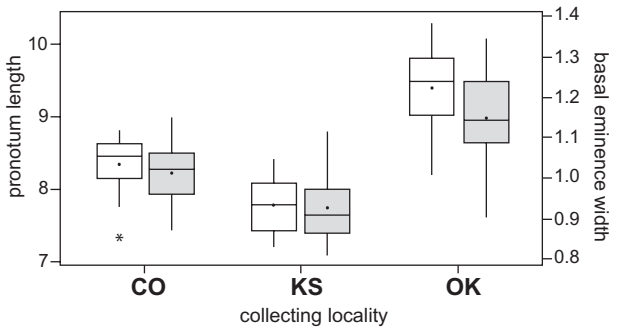

Figure 7. Body size (white) and genitalia size (grey) divergence among the three study populations. Black dots indicate mean values and the asterisk indicates an outlier.

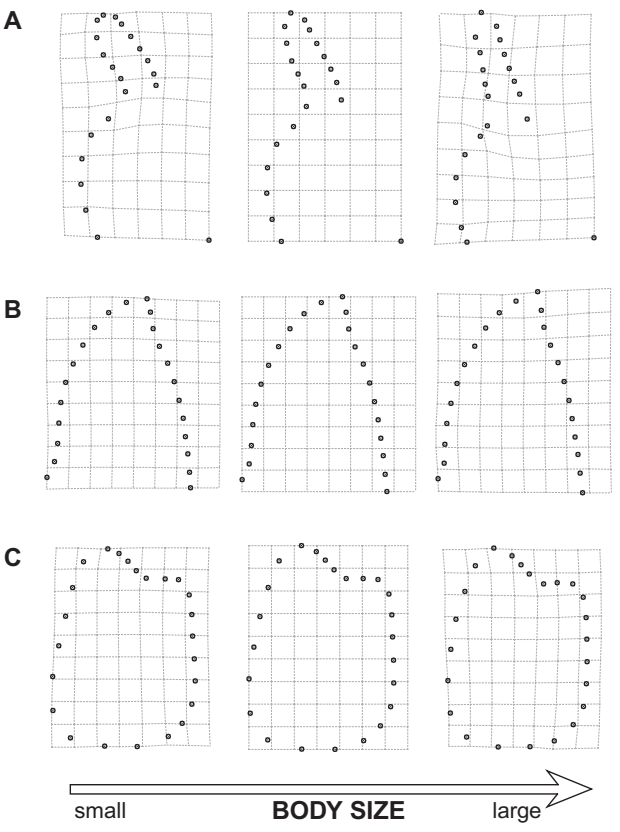

Figure 8. The shape visualization according to the fitted regression line depicting the correlation between body size and genital shape. A, the basal eminence of the cingulum; B, the lophus of the epiphallus; C, the male cercus.

divergence in *S. lineata*. This mosaic pattern can only be only when individual genital structures diverge independently of each other. Perhaps different divergence patterns between internal (basal eminence of the cingulum and the epiphallus) and external (the male cercus) genitalia may be expected because of obvious differences in location. However, we also observed different divergence patterns between internal genital structures. Two internal genitalia structures that we measured are major components of the grasshopper phallic complex, and are physically connected to each other (Dirsh, 1956). Despite this physical dependence, our results show a different divergence pattern for each structure, which suggests that each genital structure diverges separately, independent of its physical location.

The three populations of *S. lineata* that we studied are divergent in body size: with the OK population being the largest, followed by the CO population, and with the KS population being the smallest. Population-level divergence in genitalia size is closely correlated with body size (Fig. 7). Divergence in body size appears to have a considerable impact on genitalia size, which suggests that the observed pattern in genitalia size divergence may be a by-product of body size differentiation resulting from possible physiological linkage between external and internal structures. Previous studies collectively showed that male genitalia, although also sexually selected, have lower allometric values than other body parts (Eberhard *et al*., 1998; Bernstein & Bernstein, 2002; Kawano, 2004; Mutanen, Kaitala & Mönkkönen, 2006). Indeed, male genitalia of *S. lineata* have lower allometric values than other external body parts (H. Song, unpubl. data), which indicates that females do not necessarily evaluate the quality of males based on the size of genitalia.

Because individual components of male genitalia have different population-level shape divergence patterns, as shown above, we would expect a different correlation pattern between body size and genital shape for each structure. Of the three measured structures, the shape of the basal eminence of the cingulum is significantly correlated with body size, in that the apical valves of the cingulum become longer and more parallel with each other as individuals become larger (Fig. 8). Because the basal eminence of the cingulum is sensory during internal courtship, females may evolve to favour a specific shape, which can lead to the population-level divergence. If females could evolve to associate this shape with a specific body size, the observed pattern may be explained. In two other structures, however, no such correlation is found. In *S. lineata*, both the epiphallus and the male cercus are used as grasping organs during the male's coercive mating behaviour (Otte, 1970). Therefore, males may evolve these grasping organs to be less correlated with body size to ensure successful mounting to females of any size. Because sexual selection acts on the function and performance during internal courtship (Eberhard, 1985; Hosken & Stockley, 2004), it is possible for functionally different genital parts to be under separate selective pressures, resulting in the

observed pattern. Although sexual selection is a likely process for the genital divergence in *S. lineata*, an alternative explanation that the population-level divergence in male genitalia may be a result of prolonged allopatry and subsequent morphological differentiations is possible. More studies are needed to distinguish which evolutionary process is responsible, but our study is a strong demonstration that the components of complex male genitalia evolve separately within a species.

Our study adds to a list of growing evidence that male genitalia of many insects are intraspecifically variable in shape (Garnier *et al*., 2005; Mutanen & Kaitala, 2006; Polihronakis, 2006). This phenomenon is troublesome for the taxonomic groups that rely heavily on genital characters to distinguish species such as the grasshopper genus *Melanoplus* Stål, 1873, the 200 or so species of which were described based on male genitalia (Hubbell, 1932). For many insect groups, the shape of male genitalia is often assumed to be stereotyped for a species. Compared with other closely related species, the male genitalia of *S. lineata* are relatively uniform, but we show that there still exists a large level of intraspecific genital variation. This observation suggests that the shape of male genitalia can be just as variable as the external characters, and that their individual components can be influenced by separate evolutionary processes. If the intraspecific genital variation is a widespread phenomenon among insects, the number of species described based on genital morphology may potentially give rise to an unreliable estimate of the actual number of species. Therefore, we argue that genital variation should be studied with the same rigour as is directed to the external traits.

ACKNOWLEDGEMENTS

The US Army kindly allowed us to conduct a field study in Oklahoma Fort Sill. We are grateful to G. Keeney of the Ohio State University Biological Sciences Greenhouse for helping us rear grasshoppers and for the supply of cages. We thank M. Belk for help with geometric morphometric analyses and J. Freudenstein for identifying host plants. Comments by M. Mutanen and two anonymous reviewers significantly improved this manuscript. This study was supported by the NSF Graduate Research Fellowship and OSU Alumni Grants for Graduate Research and Scholarship to HS.

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