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Insect Systematics & Evolution 40 (2009) 159–170

brill.nl/ise

Species-specificity of male genitalia is characterized by shape, size, and complexity

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While species-specificity of male genitalia is a well-documented pattern among insects which can be explained by sexual selection, there are a number of species that appear to lack species-specific male genitalia despite the presence of stimulation of female genitalia by male genitalia and remating by females which are the conditions for the sexual selection by cryptic female choice. Such contradiction to the general pattern is found in the species belonging to the grasshopper genus *Schistocerca* whose male genitalia are known to be very similar across species and not useful taxonomically. In this study shapes and sizes of two functionally different genital structures of four *Schistocera* species were examined using geometric morphometic analyses. Both shape and size fail as a species-specific character when examined individually because there were extensive overlaps of both variables among species. However, when both variables were examined simultaneously, distinct species-specific clusters were recovered in each genital structure, as well as two structures combined. This finding suggests that the male genitalia of *Schistocerca* should be considered species-specific because the combination of shape and size of both genital structures is unique for each species even if the individual feature might be similar between species. The idea of species-specificity in insect systematics when applied to male genitalia, therefore, needs to be re-examined and should be applied to the shape and size and the composite nature of the structure.

Introduction

Species-specificity of male genitalia is a well-documented pattern in nature (Eberhard 1985) which is especially prevalent among insects. In many insects whose external morphology is similar, differences in male genitalia are often the only reliable species-diagnostic characters. Naturally, male genitalia occupy a special place in insect systematics, and their taxonomic value has shown to be enormous in major lineages of insects

DOI 10.1163/187631209X424571

(Sharp & Muir 1912; Kennedy 1919; Eyer 1924; Peck 1937; Snodgrass 1937; Zumpt & Heinz 1950; Tuxen 1970; Eades 2000). In his landmark book, Eberhard (1985) argued for a reinterpretation of the function of male genitalia. If the main function of male genitalia is simply to transfer sperm, there is no reason for elaborate morphology, but if females can judge the quality of males based on genital shape or performance, then male genitalia should be under a selective pressure. Male genitalia are thus an internal courtship device which is strongly influenced by sexual selection although there is an ongoing debate on which mechanism is responsible for genital divergence (Eberhard & Cordero 2003; Arnqvist 2004; Eberhard 2004; Hosken & Stockley 2004).

All grasshoppers belonging to the family Acrididae (Orthoptera) display direct copulation during which male genitalia contact female counterpart and multiple mating by both sexes. These conditions are prerequisites for sexual selection by cryptic female choice on male genitalia (Eberhard 1985) and therefore rapid and divergent genital evolution should be expected among grasshoppers. Indeed, many taxonomic studies have demonstrated the usefulness of male genitalia in diagnosing species in Acrididae (Hubbell 1932; Otte 2002). Many of the species with the demonstrated taxonomic utility of male genitalia belong to the subfamily Catantopinae, Cyrtacanthacridinae, and Melanoplinae, which often exhibit coercive mating followed by prolonged copulation without any apparent pre-mating courtship behavior (Otte 1970). In the context of sexual selection by cryptic female choice (Eberhard 1985, 1994), it is possible to postulate that there is a correlation between high selective pressure on male genitalia as an internal courtship device and high interspecific genital diversity among these groups. On the other hand, most species belonging to Acridinae and Oedipodinae display a species-specific combination of visual and acoustic pre-mating courtship behaviors (Otte 1970) and taxonomists have noted that they typically have uniform male genitalia across species (Dirsh 1956; Barnum 1959; Otte 1984). If the female choice is mostly based on non-genital male characteristics, then the male genitalia would not diverge rapidly because they would be effectively sheltered from sexual selection (Eberhard 1985). The duration of actual copulation in Acridinae and Oedipodinae is comparatively shorter than the species with species-specific male genitalia (Otte 1970), suggesting a minor role of copulatory courtship. This correlation, however, has not been empirically tested, and exceptions do exist (Eberhard 1985).

While this general pattern is common within Acrididae, there are some lineages that do not follow the pattern. A bird-grasshopper genus *Schistocerca* Stål (Cyrtacanthacridinae) is such an example. Their mating behavior is similar to Melanoplinae (Otte 1970), which would lead to a prediction that male genitalia should diverge rapidly among species under cryptic female choice. However, taxonomists have noted that the differences in male genitalia among *Schistocerca* species are not as great as other cyrtacanthacridine or melanopline grasshoppers (Dirsh 1974; Song 2004a). Although a certain level of species-specific differences does exist in *Schistocerca* (Hubbell 1960; Song 2004a), it is difficult to diagnose species identify solely based on male genitalia.

Why do male genitalia of *Schistocerca* exhibit an exception to the general pattern? To answer this question, it first needs to be clarified what species-specificity actually means.

Typical biology textbooks define species-specificity as restriction of a characteristic or response to the members of one species. Taxonomists tend to focus on species-specificity in terms of shape, but I would argue that species-specificity must encompass all aspects of phenotypic variation, including shape, size, and complexity. If two species have similarly shaped male genitalia, but of consistently different sizes, they should qualify as having species-specific genitalia. Because male genitalia are a composite structure consisting of functionally different components (Huber 2004; Song & Wenzel 2008), any unique combination of different components should also qualify as having species-specificity, whether one particular part is identical between species or not. In this context, it is difficult to simply dismiss the male genitalia of *Schistocerca* as an exception to the general pattern.

In this study, I re-examine the meaning of species-specificity when the term is applied to male genitalia. Using landmark-based geometric morphometric analyses, I study size and shape variation in the male genitalia of four *Schistocerca* species of varying degrees of phylogenetic relationships. I specifically examine two functionally different genital structures in the phallic complex in detail: the basal eminence of cingulum, which physically contacts female counterpart during copulation and is covered with sensillae (Song 2004b), and the lophus of epiphallus, which hooks onto the base of female subgenital plate during copulation (Randell 1963). By studying the size, shape, and composite nature of male genitalia in conjunction with the phylogenetic relationships, I test whether or not male genitalia of *Schistocerca* are indeed species-specific.

Materials and methods

Taxon sampling and image preparation

To compare whether the same pattern of genital evolution can be observed across different species, I examined four *Schistocerca* species of varying degrees of phylogenetic relationships (Table 1). At the lowest level, I included three populations of ecologically divergent *S. lineata* Scudder, one of the most widespread and most polymorphic species in North America (Song 2004a; Song & Wenzel 2008). At the next level, I studied *S. obscura* (Fabricius) which belongs to the same species complex as *S. lineata*, and *S. ceratiola* Hubbell and Walker, which shares a common ancestor with both *S. lineata* and *S. obscura* (Song 2004a). Finally, I examined *S. americana* (Drury), which belongs to a different clade within *Schistocerca* from the rest of the species included in this study (Song 2004c). These four species represent divergent lineages within the genus, but all display a similar mating behavior.

Specimens of *S. lineata* and *S. obscura* were transported alive in cages from the field and reared to maturity in a temperature-controlled room located in the Ohio State University Biological Sciences Greenhouse. Rearing conditions are described in Song & Wenzel (2008). Specimens of *S. ceratiola* and *S. americana* were collected and killed in a freezer at –80°C. Additional specimens of *S. americana* (originally collected from Gainesville, Florida) were kindly supplied by S. Behmer (Texas A&M University).

Table 1. Collecting information of the species used in this study

Species	Collecting locality and date
Schistocerca lineata (OK)	USA: Oklahoma: Comanche Co. Fort Sill Military Reservation, West Range
	(N 34°39.517' W 098°33.054') July 18-19, 2004
Schistocerca lineata (CO)	USA: Colorado: Bent Co. John Martin Reservoir SWA
	(N 38°05.554' W 103°03.256') July 15-16, 2004
Schistocerca lineata (KS)	USA: Kansas: Bourbon Co. Hollister Wildlife Area
	(N 37°45.521' W 094°51.033') July 22, 2004
Schistocerca obscura	USA: Kansas: Bourbon Co. 5 mi. SW Fort Scott, off Rt. 7 and Eagle Rd.
	(N 37°43.941' W 094°45.377') July 21, 2004
Schistocerca ceratiola	USA: Florida: Putnam Co. Katherine Ordway Preserve
	(N 29°41.541' W 081°58.657') August 7, 2002
*Schistocerca americana**	USA: Florida: Levy Co. Off SR. 121
	(N 29°25.908' W 082°24.060') August 8, 2002
	USA: Florida: Alachua Co. Gainesville, Jct. NW 122 St. and SR 26
	(N 29° 39.457' W082° 28.308') August 3, 2002

*Four specimens of *S. americana* came from a colony that was established using specimens originally collected in Florida, but the exact collecting information is unknown.

In order to study the variation of male genitalia, a total of 60 male specimens (*lineata* (OK) =12; *lineata* (CO) = 11; *lineata* (KS) = 12; *obscura* = 10; *ceratiola* = 8; *americana* = 7) were dissected. From a whole specimen, phallic complex was dissected by cutting through the membrane between eighth and ninth abdominal segments. Each dissected specimen was given a unique identification number associated with the whole specimen. It was then placed in a weak KOH solution for about 4 h to dissolve muscle tissues, rinsed in ethanol and preserved in glycerol in glass vials. Because a phallic complex is a three-dimensional structure which 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 epiphallus and the dorsal portion of the zygoma and rami (basal eminence) were taken with each structure in the plane of the image. For all structures, each specimen was photographed twice and the images with a better focus were used for the analysis. Digital photographs of the correctly positioned genital specimens were taken using a Nikon Coolpix 990 mounted on a Wild microscope.

Analysis of size and shape

In order to measure genital size, the centroid size of each structure was calculated using software in TPS series (freely available at http://life.bio.sunysb.edu/morph/).

Initial input files were created using tpsUtil (Rohlf 2006a) based on raw images without any modification. I quantified the shape of each structure as a set of two-dimensional shape coordinates using tpsDig2 (Rohlf 2006b). Because genital structures often do not have many distinct homologous landmarks (Type 1 landmark), I utilized both Type 1 and 2 landmarks with sliding semilandmarks (Type 3 landmark) (Bookstein 1991; Adams et al. 2004). Specifically, 5 Type 1 and 2 landmarks (LMs) and 16 Type 3 semilandmarks (SLMs) were selected for the basal eminence of cingulum (Fig. 1A) and 3 LMs and 20 SLMs for the lophus of epiphallus (Fig. 1B). For the basal eminence of cingulum, we digitized the left half of the structure because the shape was symmetrical. To assess measurement error, five measurements of each dimension were repeated non-consecutively. To study the relationship between shape variables, 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).

Resulting relative warps scores for each structure was plotted against each other to detect species-specific clustering based on shape. The centroid sizes of the basal eminence of cingulum and the lophus of epiphallus were plotted against each other to detect species-specific clustering based on size. The first relative warp scores for each structure were plotted against the centroid sizes to detect the species-specific clustering based on both shape and size.

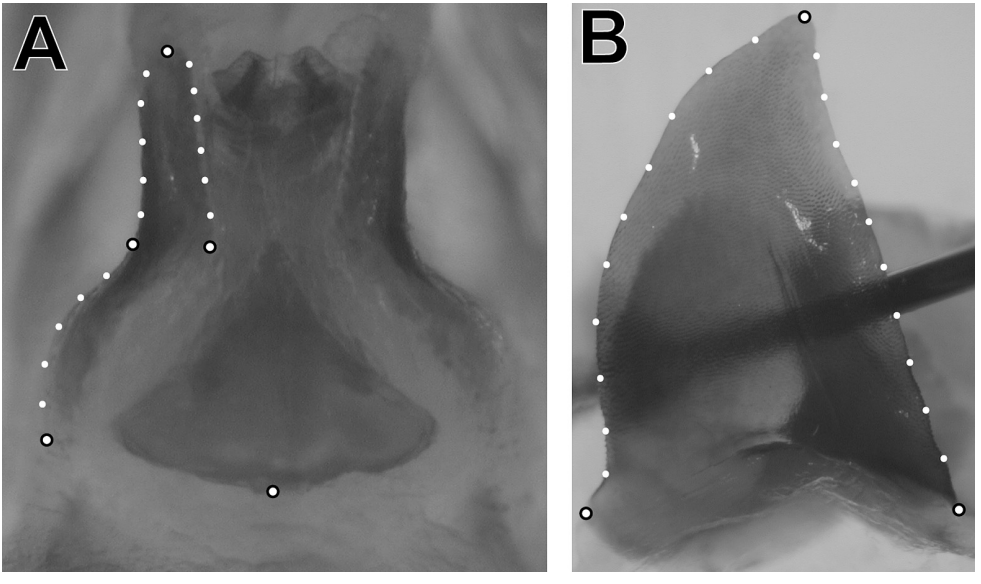

Fig. 1. Structures and dimensions measured in the geometric morphometric analysis. (A) basal eminence of cingulum; (B) lophus of epiphallus. Black circle with white center represents Type 1 and 2 landmarks, and solid white circle represents Type 3 sliding semilandmark.

Results

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 semilandmark points. The analysis resulted in 38 relative warps (RWs) for the basal eminence of cingulum and 42 RWs for epiphallus. Of these, the first three RWs collectively explained more than 70% of the shape variation (Table 2). In order to visualize the shape variation of each structure along each relative warp axis, tpsRelW (Rohlf 2006c) was used. For the basal eminence of cingulum, RW1 (45.29%) was a good measure of the bulbousness of the basal eminence and constriction of apical valves of cingulum, RW2 (20.27%) measured the dorso-ventral curvature of the basal eminence, and RW3 (9.88%) measured the constriction at the base of the apical valves of cingulum. For epiphallus, RW1 (52.22%) was a good measure of the width to length ratio, RW2 (12.13%) measured the curvature of the apex of lophus, and RW3 (8.64%) measured the curvature of at the base of lophus. Because the overall measurement error was relatively low (5.25%) and did not differ much across groups, the original values in the analyses were used. Potential errors due to positioning also appeared to be minor. However, because this study was based on a very small sample size of each species, the inference made here should be considered with caution.

When RW1 of the basal eminence of cingulum was plotted against RW2, there was a distinct division between *S. obscura* and the rest of the species (Fig. 2A). A similar pattern was found when RW1 of the basal eminence of cingulum was plotted against RW3 (Fig. 2B). When RW1 of epiphallus was plotted against RW2, *S. americana* was distinctly separated out, while the rest of the species overlapped widely (Fig. 2C). A similar pattern was found when RW1 was plotted against RW3 (Fig. 2D).

Table 2. Relative warp scores (RW) resulting from principal component analyses of the partial warps

	Basal eminence of cingulum			Lophus of piphallus		
	SV	%	Cum.%	SV	%	Cum.%
RW1	0.510	45.29	45.29	0.299	52.22	52.22
RW2	0.341	20.27	65.56	0.144	12.13	64.35
RW3	0.238	9.88	75.44	0.122	8.64	72.99
RW4	0.194	6.56	82.00	0.112	7.40	80.39
RW5	0.174	5.30	87.30	0.102	6.10	86.49
RW6	0.147	3.78	91.08	0.071	2.97	89.47
RW7	0.116	2.36	93.44	0.063	2.29	91.76
RW8	0.106	1.97	95.41	0.059	2.03	93.79
RW9	0.088	1.36	96.76	0.048	1.37	95.16
RW10	0.059	0.61	97.37	0.044	1.12	96.28

Only the first 10 RWs are shown. SV = singular value.

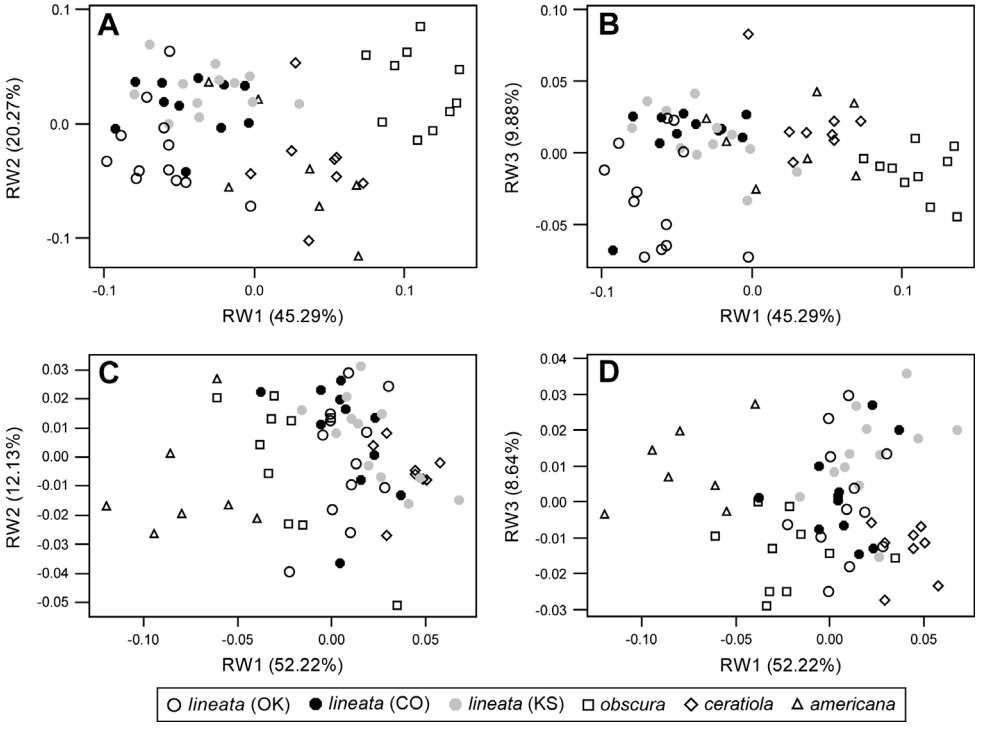

Fig. 2. Scatter plot between shape variables. The first three relative warp (RW) scores were used for plotting. Number in parentheses indicates the percentage of each relative warp score in explaining the shape variation after principal component analysis of partial warp scores. (A) Between RW1 and RW2 of the basal eminence of cingulum; (B) between RW1 and RW3 of the basal eminence of cingulum; (C) between RW1 and RW2 of the lophus of epiphallus; (D) between RW1 and RW3 of the lophus of epiphallus. Each group is indicated by a specific symbol shown in the box.

When RW1 of the basal eminence of cingulum was plotted against RW1 of epiphallus, *S. obscura* and *S. americana* formed distinct clusters, and there was a slight overlap between *S. lineata* and *S. ceratiola* (Fig. 3A).

To examine the general pattern among the size of both genital structures, I plotted the log-transformed measurement of each structure against each other (Fig. 3B). Three populations of *S. lineata* were tightly grouped together, and *S. obscura*, *S. ceratiola*, and *S. americana* each formed a loosely grouped cluster. The size of epiphallus among *S. obscura*, *S. ceratiola* and *S. americana* was comparable, but there was a distinct divergence in the size of the basal eminence of cingulum, with *S. obscura* being the largest, followed by *S. ceratiola* and *S. americana*.

Relationship between size and shape in male genitalia was examined. When the size of basal eminence of cingulum was plotted against RW1 of the same structure, *S. lineata* and *S. obscura* were distinctly grouped, while there was a slight overlap between *S. ceratiola* and *S. americana* (Fig. 3C). When the size of epiphallus was plotted against RW1 of the same structures, all four species were distinctly grouped (Fig. 3D).

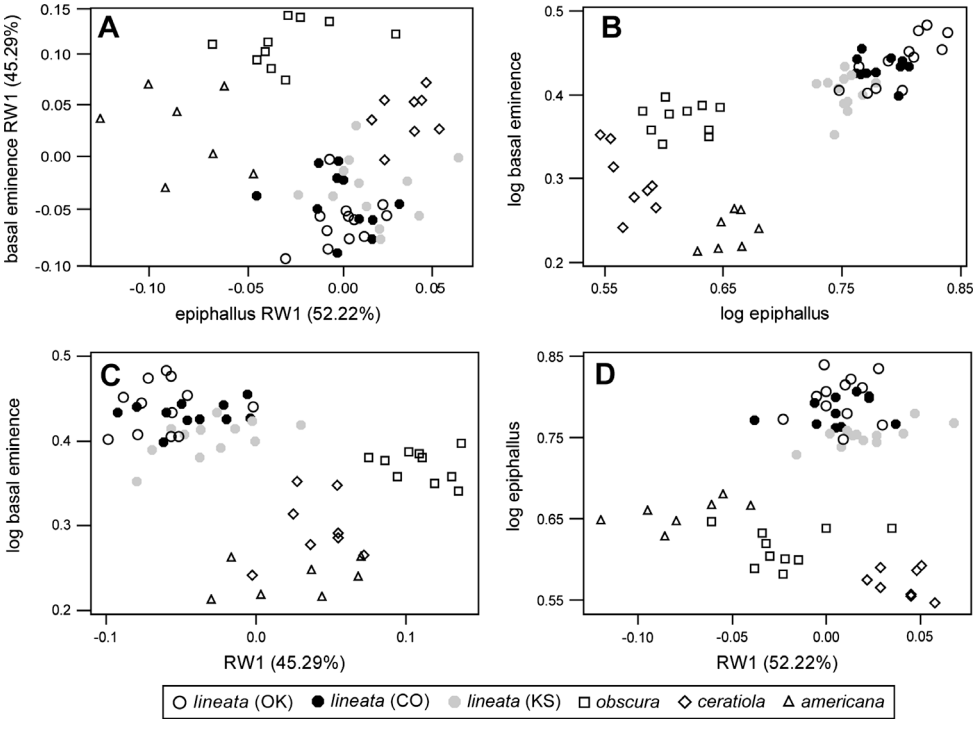

Fig. 3. Scatter plot between size and shape variables. The first RW, which explained the largest amount of variation, was used as a representative of the shape for each structure. Number in parentheses indicates the percentage of each relative warp score in explaining the shape variation after principal component analysis of partial warp scores. Log_{10}-transformed measurements of the centroid sizes of both structures were used. (A) Between RW1 of the basal eminence of cingulum and RW1 of the lophus of epiphallus; (B) between the basal eminence of cingulum centroid size and epiphallus centroid size; (C) between size and shape of the basal eminence of cingulum; (D) between size and shape of the lophus of epiphallus. Each group is indicated by a specific symbol shown in the box.

Discussion

There are a number of species in nature that display a type of mating behavior in which species-specific male genitalia are expected to evolve rapidly under sexual selection, but in fact do not have such specificity (Eberhard 1985). This study examines such an example in a grasshopper genus *Schistocerca* which does not have what taxonomists would define as species-specific male genitalia (Dirsh 1974) based on a comparative study in the framework of geometric morphometric analyses.

The idea of species-specificity is commonly attributed to only the shape of a given morphological structure. Based on the shape alone, male genitalia of *Schistocerca* cannot be considered species-specific. The results from this study show that both genital structures do not form distinct species-specific clusters, but overlap among different species (Fig. 2). This finding based on quantitative measurements concurs with a long-standing view suggested by the previous taxonomists who studied *Schistocerca* (Dirsh

1956; Hubbell 1960; Song 2004a). The size of male genitalia is much less examined by taxonomists as a species-specific character, perhaps because the size in general represents a continuous variable which may vary depending on nutritional intakes during development or environmental factors (Andrade et al. 2005). Song and Wenzel (2008) showed that the size of male genitalia could vary among different populations in a grasshopper species and this pattern was positively correlated with the body size variation. A number of allometric studies have demonstrated that the size of male genital traits varies less than body size within a given population, thus displaying low allometric variation (Eberhard et al. 1998; Palestrini et al. 2000; Schmitz et al. 2000; Bernstein & Bernstein 2002; Ohno et al. 2003). Eberhard et al. (1998) postulated that the low allometric values of male genitalia might be a result of the precise tactile nature of internal courtship in which male genitalia that can stimulate female counterpart of the most typical size would be selected for. Even if the size of male genitalia varies little within a population and varies across populations, it can still be a useful species-specific character if different species have specific genital sizes that do not overlap with each other. However, the present study shows that the size of male genitalia also fails to be a species-specific character because there is a large overlap between species for both structures.

While the shape and the size fail as a species-specific character when examined individually, an interesting pattern arises when both variables are examined simultaneously in that each species forms a roughly species-specific cluster (Fig. 3C, D). This suggests that there can be divergence in size while maintaining the shape or vice versa, which can result in species-specificity. The shape and the size are not the only variables that can define species-specificity. Because male genitalia are a composite character consisting of several functionally different components (Huber 2004; Song & Wenzel 2008), it is important to consider this complexity as a species-specific character as well. When the shape of the basal eminence of cingulum and that of the lophus of epiphallus are plotted against each other, species-specific clusters are found although there are some minor overlaps between species (Fig. 3A). When the sizes of these two structures are compared against each other, distinct species-specific clusters appear (Fig. 3B). In other words, a taxonomist would be able to correctly identify the male genitalia of each species even if the shape and the size of individual genital components might be very similar among species. For instance, while the shape of the lophus of epiphallus is almost indistinguishable between *S. lineata* and *S. obscura*, the size of the same structure is consistently larger in *S. lineata* and the shape of basal eminence of cingulum is consistently different between the two species. It is, therefore the combination of these variables that defines the species-specificity.

One of the leading hypotheses in genital evolution is sexual selection by cryptic female choice (Eberhard 1985, 2004). Eberhard (1985) argued that the rapid divergence in genital morphology between closely related species is due to the Fisherian runaway selection. In this study, I find that the three populations of *S. lineata,* although adapted to different environments and host plants (Sword & Dopman 1999; Song 2004a; Song & Wenzel 2008), have nearly identical male genitalia when compared with other species. In a previous study, I showed that *S. lineata* exhibited population-specific size and

shape variation in male genitalia (Song & Wenzel 2008). While this pattern is still found in the present study, intraspecific variation is much less than interspecific variation, which suggests that species integrity through male genitalia is maintained within this species. *Schistocerca obscura* is a closely related species to *S. lineata*, both of which belong to the Alutacea Group, a group of six sedentary species found in North America (Song 2004a). Both species are ecologically very similar and often found sympatrically (Song, personal observation). Interestingly, these two species have the most different male genitalia among all four species examined in terms of both shape and size of two genital structures measured (Fig. 2). Although it needs to be tested empirically, this pattern of two closely related species having highly divergent male genitalia may be a result of rapid divergence via runaway selection by cryptic female choice. *Schistocerca ceratiola* is the smallest and most unusual member of the genus because it is monophagous Florida rosemary *Ceratiola ericoides* and nocturnal and endemic to the central Florida (Hubbell & Walker 1928; Smith & Capinera 2005). *Schistocerca americana*, on the other hand, is a generalist species and one of the largest members of the genus, which is widely distributed in the southwestern USA (Capinera et al. 2001). Despite the biological and ecological differences, the basal eminence of cingulum is nearly identical in terms of shape and size between the two species (Fig. 2A, B). The shape of the lophus of epiphallus is however different between the two, with that of *S. ceratiola* nearly identical to *S. lineata* (Fig. 2C, D). The observed pattern suggests that the individual components of the male genitalia in *Schistocerca* are highly homoplasious, which also explains why the previous taxonomists considered the male genitalia of the genus not useful in classification. It is also difficult to rule out a possibility that the similarities in male genitalia among different species may be a result to non-selective processes such as drift.

This study strongly demonstrates that the male genitalia of *Schistocerca* species must be considered species-specific because the combination of the shape and size variations and the differences in individual genital components is unique for each species. In light of this finding, it is possible to speculate in the context of sexual selection that females may choose males based on multiple tactile cues coming from the entire phallic complex, rather than the shape of an individual genital part. The idea of species-specificity, therefore, needs to be approached from a holistic view and the taxonomic utility of male genitalia in the insect groups that used to be considered not useful needs to be re-examined.

Acknowledgements

John Capinera (University of Florida) provided invaluable information on the ecology of the grasshoppers during the fieldwork. The Department of the Army kindly allowed me to conduct a field study in Fort Sill, OK. Spencer Behmer (Texas A&M University) kindly provided specimens. George Keeney of the Ohio State University Biological Sciences Greenhouse provided invaluable help in rearing grasshoppers. Comments by an anonymous reviewer improved the clarity of the manuscript. This research was

partly supported by the NSF Graduate Research Fellowship and OSU Alumni Grants for Graduate Research and Scholarship.

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