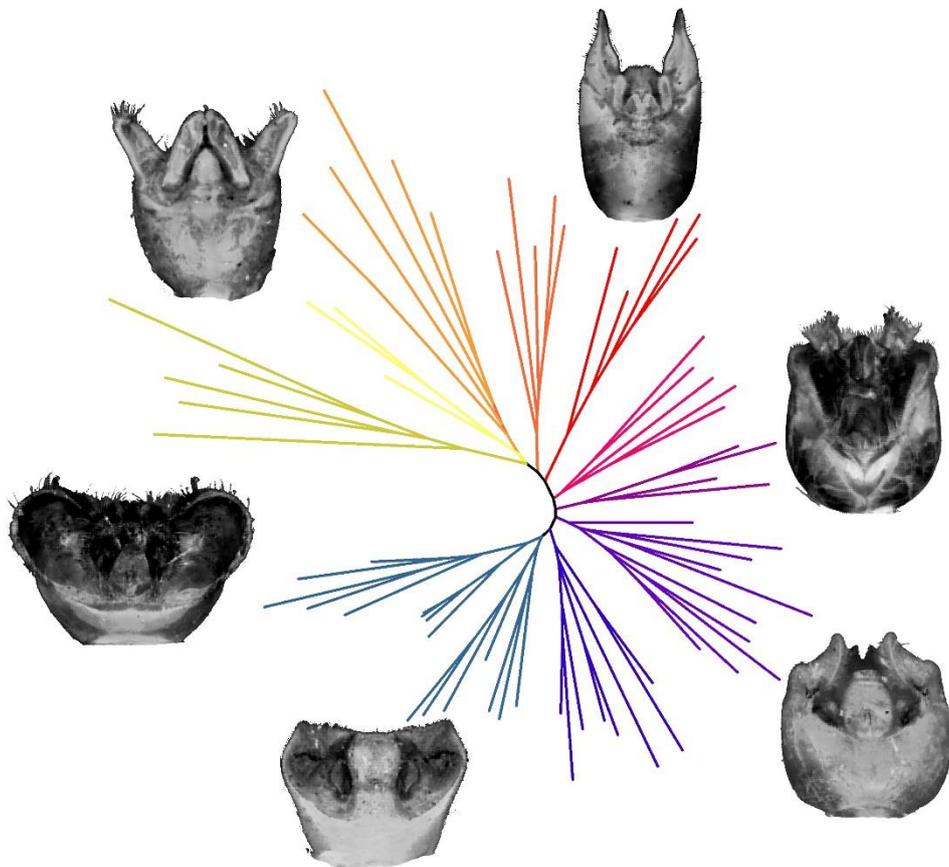


BRUNO C GENEVCIOUS

Padrões e Processos de Evolução Genital em Pentatomidae:
Pentatominae (Insecta, Hemiptera)



UNIVERSIDADE DE SÃO PAULO
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BRUNO C GENEVCIUS

Padrões e Processos de Evolução Genital em Pentatomidae: Pentatominae (Insecta,
Hemiptera)

Patterns and Processes of Genital Evolution in Pentatomidae: Pentatominae (Insecta,
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“Welcome to the wonderfully twisted realm of sexual organs. This is a world where penises may double as weapons during violent combat, or as lassos to snag a mate - often against her will. It’s a world where semen can exert powerful mind control over a female, and vaginas can act to deliberately help or hinder the age-old race of sperm to egg.”

Colin Barras (The Twisted World of Sexual Organs, 2014, BBC)

Resumo

Genitálias de machos tendem a apresentar maior elaboração e taxas de evolução mais altas que as de fêmeas, fenômeno cujos mecanismos associados ainda são pouco compreendidos. Nesta tese buscamos explorar tais mecanismos através da compreensão sobre os papéis funcionais e do desenvolvimento por trás da diversificação genital em Pentatomidae. No primeiro capítulo, investigamos o funcionamento das peças genitais externas em machos e fêmeas de espécies de diferentes linhagens, identificando grupos de estruturas que funcionam de forma mais integradas entre si. Mostramos que existem marcas funcionais únicas a algumas linhagens, mas por outro lado algumas características são conservadas e se repetem em todas as espécies. No segundo capítulo, inferimos uma filogenia para Pentatomidae usando dados moleculares e morfológicos, com enfoque na subfamília mais diversa e confusa taxonomicamente, Pentatominae. Identificamos diversas linhagens propostas na literatura com grande suporte, corroboramos a monofilia de Pentatomidae e a polifilia de Pentatominae. No terceiro capítulo, testamos a hipótese de que diferentes componentes genitais em fêmeas possuem maior restrição evolutiva por serem mais integradas durante o desenvolvimento, em comparação aos machos. Corroboramos esta hipótese, e sugerimos que as genitálias dos machos são evolutivamente moldadas pela forma como funcionam na cópula, enquanto a diversificação nas fêmeas é restringida por sua origem ontogenética.

Palavras-chave: DNA, Hemiptera, integração fenotípica, modularidade, seleção sexual

Abstract

Male genitalia tend to show greater elaboration and evolve faster in relation to females, a phenomenon whose underlying mechanisms are still poorly understood. In this thesis, we explore these mechanisms seeking to understand the functional and developmental roles in the diversification of genitalia in Pentatomidae. In the first chapter, we investigated the function of the external genital parts in males and females from different lineages, with the goal of identifying structures that work in a more integrated fashion. We showed that each species have unique functional signals, but some characteristics are more conserved and invariable. In the second chapter, we inferred a phylogeny to the Pentatomidae using morphological and molecular data, focusing on the most diverse and taxonomically confusing subfamily, Pentatominae. We recognized several lineages proposed on the literature with strong support, corroborate the monophyly of Pentatomidae and the paraphyly of Pentatominae. In the third chapter, we test the hypothesis that different genital components in females are more evolutionarily constrained for being more integrated during the development, comparing to males. We corroborated this hypothesis and suggest that male genitalia are evolutionarily shaped by their functional roles in copula, while the diversification in female genitalia is more constrained due to its ontogenetic origin.

Key words: DNA, Hemiptera, modularity, phenotypic integration, sexual selection

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Introdução Geral

Padrões e processos de evolução genital

Genitálias são amplamente reconhecidas como as mais divergentes das estruturas morfológicas (Simmons 2014), especialmente as masculinas. Tal padrão se repete na maior parte dos grupos animais de fertilização interna, sendo um dos grandes enigmas ainda não resolvidos em biologia evolutiva (Simmons 2014; Langerhans et al. 2016). A grande variação interespecífica tornam as genitálias estruturas muito utilizadas para responder questões de diversas naturezas no estudo de animais, em especial os insetos. Genitálias são classicamente utilizadas na taxonomia, provavelmente as estruturas mais importantes no reconhecimento e delimitação de espécies (Mutanen et al. 2006). Também são fundamentais para estudos em sistemática, uma vez que fornecem grande quantidade de informação morfológica e possibilitam o reconhecimento de linhagens em diversos níveis (Song e Bucheli 2010). Biólogos evolutivos têm se interessado cada vez mais por essas estruturas. Altas taxas de diversificação, grande complexidade anatômico-funcional e sua importância direta no sexo permitiram que genitálias se tornassem modelos para diversas questões evolutivas modernas como seleção sexual (Simmons 2014), especiação (Knowles et al. 2016), plasticidade fenotípica (Firman et al. 2018), biomecânica (Burns e Shultz 2015), entre outros. O crescimento de estudos evolutivos usando genitálias como modelos é notável (Fig. 1).

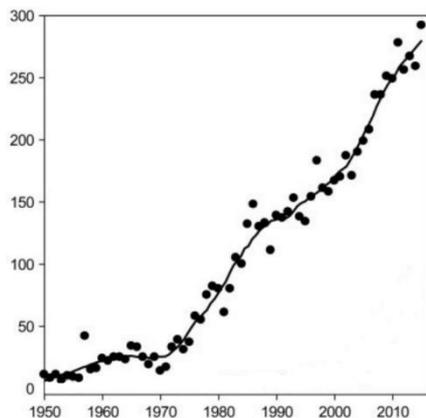


Figura 1. Crescimento de estudos em evolução genital nos últimos 60 anos (adaptado de Langerhans et al. 2016).

As primeiras explicações para os padrões tão peculiares exibidos pelas genitálias datam de Dufour (1848). Em suas próprias palavras, “o armamento copulatório é um órgão, ou melhor, um instrumento engenhosamente complicado, destinado a adaptar-se às partes sexuais externas à fêmea para a conclusão da cópula; é a garantia da preservação dos padrões, a salvaguarda da legitimidade das espécies”. Esta ideia é a base da hipótese “chave-fechadura”, que propõe que genitálias de machos e fêmeas devem divergir e se coadaptar para garantir a espécie-especificidade da cópula e evitar os supostos custos da formação de híbridos (Masly 2012). Apesar de teoricamente intuitivamente, tal hipótese foi frequentemente refutada por estudos empíricos que mostram que diferenças genitais entre espécies irmãs possuem pouca relevância em evitar hibridização (Masly 2012; Simmons 2014; Brennan e Prum 2015). Mais recentemente, estabeleceu-se um consenso na literatura de que seleção sexual representa o principal mecanismo de divergência genital. A primeira evidência foi o trabalho comparativo de Arnqvist (1998) que demonstrou que linhagens de insetos poligâmicos apresentam maiores taxas de diversificação genital em comparação a linhagens monogâmicas. Atualmente, estudos evolutivos têm se concentrado em testar a relevância de diferentes mecanismos de seleção sexual (e.g. escolha críptica da fêmea, conflito intersexual, competição espermática, etc).

Genitálias frequentemente exibem alto grau de complexidade, tanto anatômico como funcional. Complexidade anatômica se refere à presença de diversos subcomponentes

com alto grau de elaboração. Presume-se que tal complexidade resulta da diversidade funcional das genitálias, onde diferentes componentes com funções variadas podem sofrer ação diferentes pressões seletivas. O estudo de Werner e Simmons (2008) ilustra bem este fenômeno: genitálias de machos do besouro escarabeídeo *Ontophagus taurus* possuem três escleritos que participam diretamente na transferência de espermas e um esclerito morfológicamente similar que estabiliza a mecânica da cópula (Werner e Simmons 2008). Este estudo foi dos poucos que mostrou como genitálias complexas compostas por diferentes componentes funcionam na cópula, mas de forma parcial. Por exemplo, o papel das genitálias femininas no acoplamento genital não foi abordado. A ausência de estruturas femininas em estudos de genitália como um todo tem sido apontada como um viés recorrente que impede uma compreensão mais completa da dinâmica evolutiva dessas estruturas (Ah-King et al. 2014).

Genitálias de machos tendem a apresentar maior complexidade que as das fêmeas e geralmente evoluem em taxas mais altas (Genevcius et al. 2017). Por muito tempo atribuiu-se a este fenômeno um suposto papel predominante dos machos na cópula, concluindo-se que genitálias masculinas sofreriam ação de seleção divergente em maior intensidade. No entanto, há evidências de que genitálias de fêmeas possuem papel importante do ponto de vista funcional, tanto durante a cópula – e.g. na interação mecânica com as genitálias dos machos - quanto pós-cópula, e.g. na seleção de espermas, oviposição, etc (Ah-King et al. 2014; Anderson e Langerhans 2015). Desta forma, as razões pelas quais as genitálias masculinas apresentam maior elaboração e evoluem mais rapidamente necessitam de uma explicação alternativa à predominância dos machos na cópula. Uma possível explicação para este fenômeno é que genitálias de machos e fêmeas possuem diferentes mecanismos que atuam durante o desenvolvimento do indivíduo. Se diferentes componentes genitais no macho são determinados sob a regulação de caminhos genéticos mais diversos durante o desenvolvimento, seus componentes apresentariam maior liberdade para evoluírem independentemente uns dos outros.

A tendência de estruturas a evoluírem de forma compartimentalizada, ou seja, em módulos independentes em algum aspecto, é denominada modularidade (Klingenberg 2014). Por outro lado, esta hipótese prediz que os diferentes componentes funcionais das fêmeas são regulados por uma maior quantidade de genes em comum, ou seja, possuem restrições durante o desenvolvimento. A ligação entre função copulatória e desenvolvimento genital ainda é pouco conhecida, e será objeto de estudo desta tese.

Pentatomídeos como modelos para estudos de evolução genital

Os percevejos da família Pentatomidae (Insecta, Hemiptera) representam um modelo interessante para responder questões sobre evolução genital. Primeiramente, a família é extremamente diversa (~ 5000 espécies), o que permite testar hipóteses macro-evolutivas com robustez. Segundo, machos e fêmeas possuem genitálias complexas, compostas de múltiplas partes que supostamente apresentam funções diversas. Terceiro, possuem genitálias externas bem definidas e esclerotizadas, permitindo sua avaliação e mensuração com precisão, mesmo utilizando indivíduos preservados à seco. Por fim mas não menos importante, machos e fêmeas apresentam grande variabilidade interespecífica e baixa variabilidade intraespecífica (Genevcius et al. 2017).

Como para todos os insetos, as genitálias externas dos pentatomídeos são estruturas rígidas derivadas dos segmentos abdominais oito a dez (Scudder 1959; Schaefer 1977). Nas fêmeas, estes segmentos abdominais formam oito placas mais ou menos achatadas, sendo 4 placas do oitavo segmento, 3 placas do nono e o décimo. Nos machos, o segmento oito perde esclerotização e se torna uma estrutura membranosa que movimenta o segmento nove (Leston 1955; Schaefer 1977); por sua vez, o segmento nove é modificado em forma de uma cápsula (= pigóforo) com alto grau de elaboração, enquanto o décimo segmento origina um par de cláspers

(parâmeros). Até então, pouco se conhece sobre o funcionamento destas estruturas, as hipóteses descritas na literatura nunca foram testadas e se resumem a observações esporádicas de casais em cópula. Dado a relativa complexidade é possível prever que devem existir conjuntos de estruturas em cada sexo que funcionam de forma combinada durante a cópula (Genevcius et al. 2017), assim como conjuntos de estruturas entre os sexos que devem interagir. Nesta tese, estudei estas estruturas sob uma perspectiva funcional e de desenvolvimento, do qual proponho hipóteses sobre como pode ter ocorrido a diversificação dessas genitálias ao longo da evolução dos pentatomídeos. Também foi estabelecida, de forma inédita, uma filogenia para Pentatomidae com base em dados moleculares e morfológicos, para que se possa abordar essas questões em uma perspectiva macro-evolutiva. Abaixo detalho as perguntas, objetivos e abordagens gerais que estruturam a tese.

Estrutura da tese

No primeiro capítulo, buscamos compreender como funcionam as peças genitais externas, masculinas e femininas, em Pentatomidae. Para isso, congelamos indivíduos em cópula para observar diretamente a interação entre as partes genitais. Executamos essas análises de morfologia funcional utilizando cinco espécies representantes de diferentes linhagens de Pentatomidae, o que nos permitiu estabelecer quais interações funcionais parecem mais conservadas e quais parecem mais variáveis dentro da família. Primeiramente, mostramos que as peças genitais interagem entre si para executar a estabilização da cópula. Existem diferenças no mecanismo de acoplamento entre as espécies, mas também é possível identificar algumas similaridades. A observação mais interessante é que os gonocoxitos 8 são as únicas estruturas móveis nas fêmeas, sendo prensadas entre bordo dorsal do pigóforo e os parâmeros nos machos. Por outro lado, o bordo ventral do pigóforo interage sempre com as placas fixas, apesar das placas exatas poderem variar.

No segundo capítulo inferimos uma filogenia para a família Pentatomidae, focando no grupo mais diverso, a subfamília Pentatominae, utilizando dados molecular e morfológicos. Identificamos diversas linhagens bem suportadas dentro do grupo, suportamos a monofilia de Pentatomidae e de três das subfamílias atualmente reconhecidas; Pentatominae e Podopinae se mostraram polifiléticas. Este trabalho possui implicações para a classificação da família.

No terceiro capítulo testamos a hipótese de que genitálias de fêmeas são menos complexas e evoluem mais lentamente (Genevcius et al. 2017) por apresentarem restrições durante o desenvolvimento. Para isso, investigamos o padrão de modularidade evolutiva nas genitálias externas de machos e fêmeas num contexto coevolutivo. Mostramos que em machos, os componentes que interagem funcionalmente durante a cópula evoluem de forma mais dependente entre si. Nas fêmeas, os componentes que derivam de uma única estrutura dos imaturos é que evoluem como módulos. Corroboramos a hipótese inicial e sugerimos que as genitálias dos machos se diversificam sob ação de um processo seletivo que garante a integridade funcional de seus componentes durante cópula, enquanto a diversificação nas fêmeas é restringida por sua origem ontogenética.

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CAPÍTULO 1

Strong functional integration among multiple parts of the complex male and female genitalia of stink bugs

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(ANEXO 1)

ABSTRACT

Genitalia are among the most studied phenotypes because they exhibit high anatomical diversity, experience fast evolutionary rates, and may be shaped by several evolutionary mechanisms. A key element to uncover the mechanisms behind such impressive diversity is their copulatory function. This topic has been overlooked, especially concerning structures not directly involved in sperm transfer and reception. Here we conduct a hypothesis driven experimental study to elucidate the operation of various external genital parts in five species of stink bugs with differing levels of phylogenetic relatedness. These insects are unique in that male and female genitalia are externally well developed, rigid, and composed by multiple components. In contrast with their anatomical complexity and diversity, we show that genital structures work jointly to perform a single function of mechanical stabilization during copula. However, distinct lineages have evolved alternative strategies to clasp different parts of the opposite sex. In spite of a high functional correspondence between male and female traits, the overall pattern of our data do not clearly support an intersexual coevolutionary scenario. We propose that the extraordinary male genital diversity in the family is likely a result of a process of natural selection enhancing morphological accommodation, but we consider alternative mechanisms.

Key words: coevolution, *Edessa*, *Euschistus*, functional morphology, *Mormidea*, *Podisus*, sexual selection, sperm competition

INTRODUCTION

Extraordinarily divergent genitalia are ubiquitous across animal taxa with internal fertilization. The evolutionary forces behind this trend have sparked heated debate over the last decades, but most models of natural and sexual selection proposed have been at least partially supported (Hosken & Stockley, 2004; Masly, 2012; Brennan & Prum, 2015; Firman et al., 2017). Distinguishing among these models in a particular group can be challenging because genitalia may exhibit similar patterns of differentiation and coevolution under different pressures. Thus, uncovering the origins of genital diversification is paramount to discern among alternative evolutionary mechanisms. In this sense, a key question is how different genital parts engage during copula and how morphology relates to function (Jagadeeshan & Singh, 2006; Simmons, 2014; Wulff & Lehman, 2016). In the taurus scarab beetle (*Ontophagus taurus*), two distinct functionalities have been described to four male genitalic sclerites: three sclerites act directly in sperm transfer comprising an integrated unit, while the other acts as a holdfast structure (Werner & Simmons, 2008). Such findings are crucial to explain how different parts are able to influence paternity or stabilize the genitalia in copula (Werner & Simmons, 2008), illustrating the importance of studies on functional morphology to detect sources of selection. The scarcity of studies on functional morphology of genitalia have been repeatedly pointed as a key obstacle that hinder progress on this research field (Simmons, 2014; Brennan & Prum, 2015). Although functional integration between male and female is usually thought as a major source of coevolution, evidence for such correlation is yet limited. In fact, the most comprehensive study dealing with this subject has found weak evidence to such correlation (Richmond, Park & Henry, 2016).

Insects are probably the most representative organisms in studies on genital evolution. Assessments of their genital functionalities have revealed peculiar and unique modes

of operation such as traumatic insemination (Tatarnic, Cassis & Hochuli, 2006; Kamimura, Tee & Lee, 2016), mating plugs (Baer, Morgan & Schmid-Hempel, 2001; Seidelmann, 2015), sonorous genitalia (Sueur, Mackie & Windmill, 2011) and female penises (Yoshizawa et al., 2014). Three major biases may be identified in studies with insect genitalia. First, thorough investigations covering both functional and evolutionary aspects have been mostly conducted with a few model groups such as flies (e.g. Eberhard & Ramirez, 2004), beetles (e.g. Hotzy et al., 2012) and water striders (e.g. Fairbairn et al., 2003). Second, the historical male bias that characterizes the research on genital evolution as a whole (Ah-King, Barron & Herberstein, 2014) also seems to apply to insects. Third, given the growing acceptance of sexual selection as a preponderant mechanism, studies examining structures associated to sperm transfer and sensory communication are increasingly predominant in relation to those examining structures with secondary sexual functions. However, recent studies provide unequivocal evidence that male and female genitalia may be shaped by alternative processes other than the traditional cryptic female choice and sexual antagonistic coevolution (e.g. Wojcieszek et al., 2012; House et al. 2013; Anderson & Langerhans, 2015; Varcholová et al., 2016). This raises the question of whether these mechanisms of sexual selection are indeed overwhelmingly prevalent as usually thought, especially considering our elusive knowledge on the function and diversity of genitalia in numerous understudied groups.

Among insects, stink bugs (Hemiptera, Pentatomidae) stand out for particularities in male and female genitalia. Both sexes exhibit highly complex and well-developed internal and external genital parts (Sharp, 1890; Marks, 1951) with presumable diverse functionalities (Genevcius, Caetano & Schwertner, 2017). While a couple of studies with pentatomids have linked their intromittent genitals to a complex system of sperm selectivity, transfer, regulation and storage (Adams, 2001; Stacconi & Romani, 2011), the function of non-intromittent external parts in copula remains virtually unknown. The non-intromittent part of the male organ

(=pygophore, male external genitalia herein) is characterized by extraordinary diversity and species-specificity, being consistently the most decisive characteristics in taxonomic studies and showing strong phylogenetic structure at different levels (e.g. Grazia, Schuh & Wheeler, 2008; Ferrari, Schwertner & Grazia, 2010; Genevcius, Grazia & Schwertner, 2012). The structure comprise a capsule and associated structures which can take the form of folds, projections and hooks, originated from a series of modifications and fusions between the 9th and 10th abdominal segments (Bonhag & Wick, 1953; Schaefer, 1977). The female external genitalia is composed by various flattened plates which cover the genital opening, derived from the 8th, 9th and 10th segments (Scudder, 1959). A recent study has found an evolutionary correlation between the pygophore and a pair of female plates, but the functional significance of this trend remains to be investigated (Genevcius et al. 2017). Although our knowledge on how these structures operate is vague, their remarkable diversity and species specificity suggest an important sexual and evolutionary role in the family which has never been scrutinized.

In this study we examined the role of the genital parts that presumably interact externally during copula in Pentatomidae. Given the particularities of their external genitalia, the group offers an interesting model to study the interplay between genitalia function, complexity and evolution in structures disassociated to sperm transfer. We reviewed the literature and compiled a series of testable hypotheses of functional mechanics in the group (Table 1). We performed mating trials for five species showing varying degrees of phylogenetic relatedness and conducted a series of detailed morphological observations to address the following questions: 1) How do the external parts of the male and female genitalia interact with one another during copula? 2) Do the modes of operation vary across species of different lineages of the family? Our results revealed an entangled mechanism of functional integration in which several parts of the genitalia operate in a cooperative fashion to provide stabilization during copula. Furthermore, we show significant among-species variation in the attachment

mechanism, suggesting distinct evolutionary strategies to clasp the opposite sex exhibited by different lineages. We discuss how our data adequate to the functional hypotheses, the evolutionary implications of the genital interactions observed and possible underlying mechanisms.

MATERIAL AND METHODS

Morphology and terminology of genital parts

The terminology used to refer to the male genital components in Heteroptera has been historically inconsistent. Schaefer (1977) compiled and discussed the contrasting classification in Pentatomomorpha (which includes Pentatomidae and related families), proposing a unified terminology. Here we followed his terminology with a few additions of other recent studies (e.g. Genevcius et al., 2012).

The male genitalia is roughly a tube-like sclerotized capsule (=pygophore) with associated structures (e.g. a pair of claspers) and an internal phallus. Although some authors refrain to use the terms “external” and “internal” genitalia, we designate as internal genitalia the movable intromittent parts that penetrate the female internal tract, while the capsule itself, the parameres and the 10th segment are considered as external. The pygophore can be divided into a dorsal and a ventral wall. Since it remains twisted in 180° inside the male’s body while in rest position, the ventral and the dorsal sides are opposite to the body’s plans (Schaefer, 1977). All structures can be seen in dorsal view, including the posterior extremity of the ventral wall, denominated ventral rim (Fig. 1).

We follow Grazia et al. (2008) to the female parts, which compiled the nomenclature and reviewed homology statements. The morphology of the female genitalia is relatively simpler, comprising a series of soft tubes and chambers (the internal genitalia)

covered by various sclerotized plates (the external genitalia). The opening of the female internal tract gets covered by the larger genital plates, the gonocoxites 8 (Fig. 1E-F). The terminology of all genital parts and respective abbreviations used in this work are described in Figure 1 and Table 2.

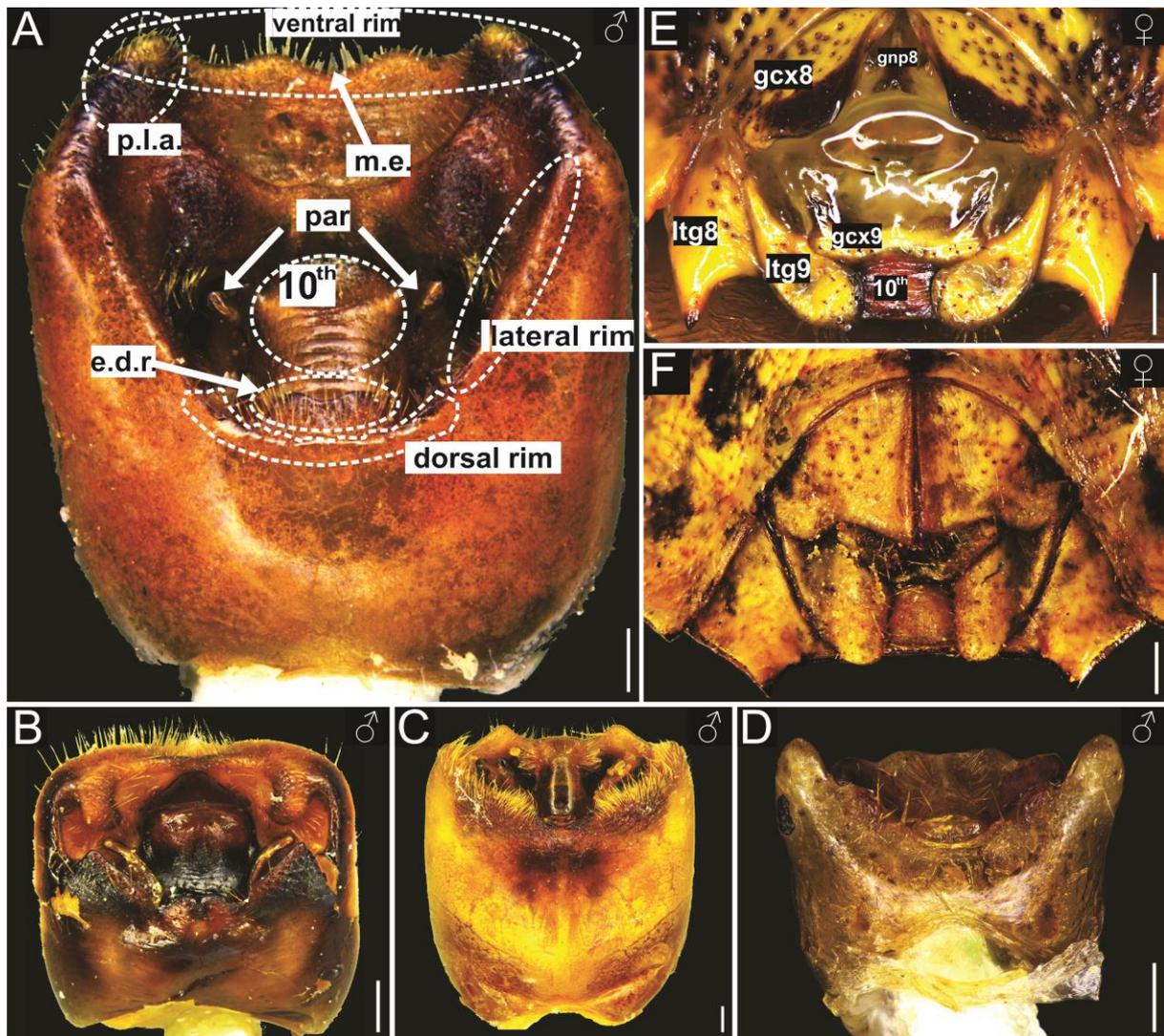


Figure 1. Male (A-D) and female (E-F) external genitalia of the studied genera, with terminology and abbreviations indicated. Female genitalia is represented with the internal tract exposed (E) and unexposed (F). Female parts: 10th (tenth segment), gnx8 (gonocoxite 8), gnx9 (gonocoxite 9), gnp8 (gonapophysis 8), ltg8 (laterotergite 8), ltg9 (laterotergite 9); male parts: 10th (tenth segment), e.d.r. (extension of dorsal rim), m.e. (median excavation), p.l.a. (posterolateral angles). A and F = *Euschistus heros*, B and E = *Mormidea v-luteum*, C = *Edessa mediatubunda*, D = *Podisus nigrispinus*. Scale bar is 0.25 mm.

Species choice, collection and rearing

We investigated the functional morphology of male and female external genitalia in five species of Pentatomidae in a hypothesis driven approach (Table 1). To examine whether the general system of attachment between the genitalia vary within the family, we chose species with different levels of relatedness. Even though a complete phylogeny of the family does not exist, different phylogenetic studies support the recognition of different groups within Pentatomidae (Gapud, 1991; Bistolas et al. 2014; Banho, 2016; Wu et al. 2016) with certain congruence with the current taxonomic classification in tribes and subfamilies (Rider et al., in press). The five species studied herein represent three of the four major and most diverse lineages of Neotropical pentatomids (i.e. Asopinae, Discocephalinae, Edessinae and Pentatominae).

We selected two species from the same genus, *Mormidea v-luteum* (Lichtenstein) and *Mormidea maculata* (Dallas), and a third species from the same tribe as the *Mormidea*, *Euschistus heros* (Fabricius). The three species belong to the group of the Neotropical Carpocorini (subfamily Pentatominae). The fourth and the fifth species belong to other subfamilies: *Podisus nigrispinus* (Dallas) (Asopinae) and *Edessa meditabunda* (Fabricius) (Edessinae). We manually collected specimens in the municipality of Diadema, São Paulo, Brazil (-23.7204, -46.6276), and maintained them in laboratory inside plastic cages of 2L. Males were reared separately from females prior to the experiments using the following conditions to all species: $26 \pm 2^{\circ}\text{C}$, $70 \pm 10\%$ RH and photophase of 14 L: 10 D. Individuals of *E. heros* and *E. meditabunda* were fed on bean pods (*Phaseolus vulgaris*) and peanut seeds (*Arachis hypogaea*), *M. v-luteum* and *M. maculata* on branches of *Brachiaria* sp. and *P. nigrispinus* on larvae of *Tenebrio molitor*.

Table 1. Hypotheses of functional morphology of the external genitalia compiled from literature with reference to the taxon to which each hypothesis has been proposed. Column “structure” refers to the terminology used here, while the original terminology is highlighted within the hypothesis quote. Column “support” denotes whether the hypothesis was supported herein.

Structure	Taxon	Functional hypothesis	Ref	Support
Parameres	Pentatomidae	H1. “The functions of the claspers [...] to assist in separating the genital sclerites of the female, and to assist as clasping organs during copulation.”	[1]	Corroborated
Parameres	Hemiptera	H2. “Also, it appears [...] that the parameres do operate to some extent in keeping apart the gonapophyses which hide the female gonopore...”	[2]	Rejected
Parameres	<i>Piezodorus lituratus</i> (Pentatomidae)	H3. “During copulation in Pentatominae the male gonopods are pressed against the outside of the 2nd valvifers of the female”	[3]	Rejected
Ventral rim of pygophore	Geocorisae [Terrestrial Heteropterans]	H4. “...the infolded portion of the ventral rim , [...] presumably share the function of holding and guiding the aedeagus during copulation.”	[4]	Partially rejected
Ventral rim of pygophore	Geocorisae [Terrestrial Heteropterans]	H5. “These structures [the infolded portion of the ventral rim] appear to have limited functional significance, because they are usually immovable and not provided with muscles; they may provide tactile clues to the female and/or provide support to the various movable structures during copulation.”	[4]	Partially rejected
Pygophore	Pentatomidae	H6. “The aesthetic aspect of the arrangement [of the genital chamber] in many of the higher species, [...], is very remarkable, but I do not think there is at present evidence that would justify us in attaching any special biological importance to it.”	[5]	Partially rejected

[1] Baker (1931); [2] Singh-Pruthi (1925), [3] Leston (1955), [4] Schaefer (1977), [5] Sharp (1890)

Experimental approach

We randomly formed couples which were maintained in separate cages during the mating trials. The number of couples observed per species (n) varied from 3 to 12 (*E. heros* = 12, *E. meditabunda* = 3, *M. v-luteum* = 10, *M. maculata* = 8, *P. nigrispinus* = 3). All observations were consistent showing no differences among pairs of the same species. Mating pairs were frozen in copula in a -20 °C freezer. Because pentatomids commonly tend to copulate for several

hours (McLain, 1980; Rodrigues et al. 2009), we were able wait several minutes after copula had started to guarantee that genitalia were properly coupled. After 20 minutes in the freezer, mating pairs were pinned and promptly analyzed in a stereomicroscope Leica MZ205C. Photographs were taken firstly of the attached genitalia and secondly after slight manipulations, using a Leica DFC450 and the Leica Application Suite software with Z-stacking acquisition.

Table 2. Abbreviations of the genital parts used in text and figures.

Abbreviation	Structure
Female	
10 th	Tenth segment
gcx8	Gonocoxite eight
gcx9	Gonocoxite nine
gnp8	Gonapophysis 8
ltg8	Laterotergite 8
ltg9	Laterotergite 9
Male	
10 th	Tenth segment
e.d.r	Extension of dorsal rim
m.e.	Median excavation
p.l.a.	Posterolateral angle
Par	Paramere
Pyg	Pygophore
s.p.	Superior process

RESULTS

The arrangement between male and female genitalia from a dorsal view of the pygophore was similar in all species. Left and right gcx8 were the only mobile structures of the female genitalia. They touch the dorsal side of the pygophore and are pressed against the lateral rim (Fig. 2) by the parameres internally (Fig. 3). This connection apparently comprises the tightest point of attachment between the two genitalia. In *P. nigrispinus*, the gcx8 are also grasped externally by the superior processes (= genital plates of some authors). The parameres and the superior processes function as tweezers to keep the gcx8 opened (Fig. 3). The opening angle of the gcx8 differed slightly among species. In *M. v-luteum* and *E. meditabunda*, the gcx8 remain virtually parallel to the male's body plan (Fig. 2C-D) whereas the angle is around 45° in the remaining species (Fig. 2A-B). In all five species the connection between genitalia is probably mediated by several sensory setae mostly concentrated on the e.d.r. and p.l.a. of males and on the internal angles of the gcx8 of females (Fig. 3).

The ventral rim of the pygophore makes direct contact with the female plates in all species except *P. nigrispinus*. However, we found three different modes of accommodation between these two traits, each mode corresponding to one genus. In *E. heros*, the ventral rim of the pygophore is pronouncedly differentiated to engage with the female plates (Fig. 4A); the posterolateral angles fit between the ltg8 and ltg9 while the sinuosity of the ventral rim matches the ltg9 and 10th (Fig. 4A). In the *Mormidea*, the ventral rim of the pygophore is less modified showing only a simple v-shaped median excavation (Fig. 1B); the m.e. fits the gcx9, whereas the ltg8, ltg9 and 10th segment remain untouched by the pygophore (Fig. 4B-C). In *E. meditabunda*, the p.l.a. of the pygophore make contact with the outer side of the ltg8 (Fig. 4D). In such species both the ltg9 and 10th lie in the median excavation of the pygophore (Fig. 4D), and the 10th segment is untouched by the ventral rim. We could not visualize whether the gcx9

engages with a specific portion of the male genitalia in *E. heros* and *E. meditabunda* because it was covered by the pygophore ventrally and by the gcx8 dorsally. In *P. nigrispinus*, the ventral rim of the pygophore is not well developed and does not engage with any of the female plates. In this species, the attachment between the genitalia is mediated exclusively by the parameres, lateral rim, and superior processes (Fig. 3A).

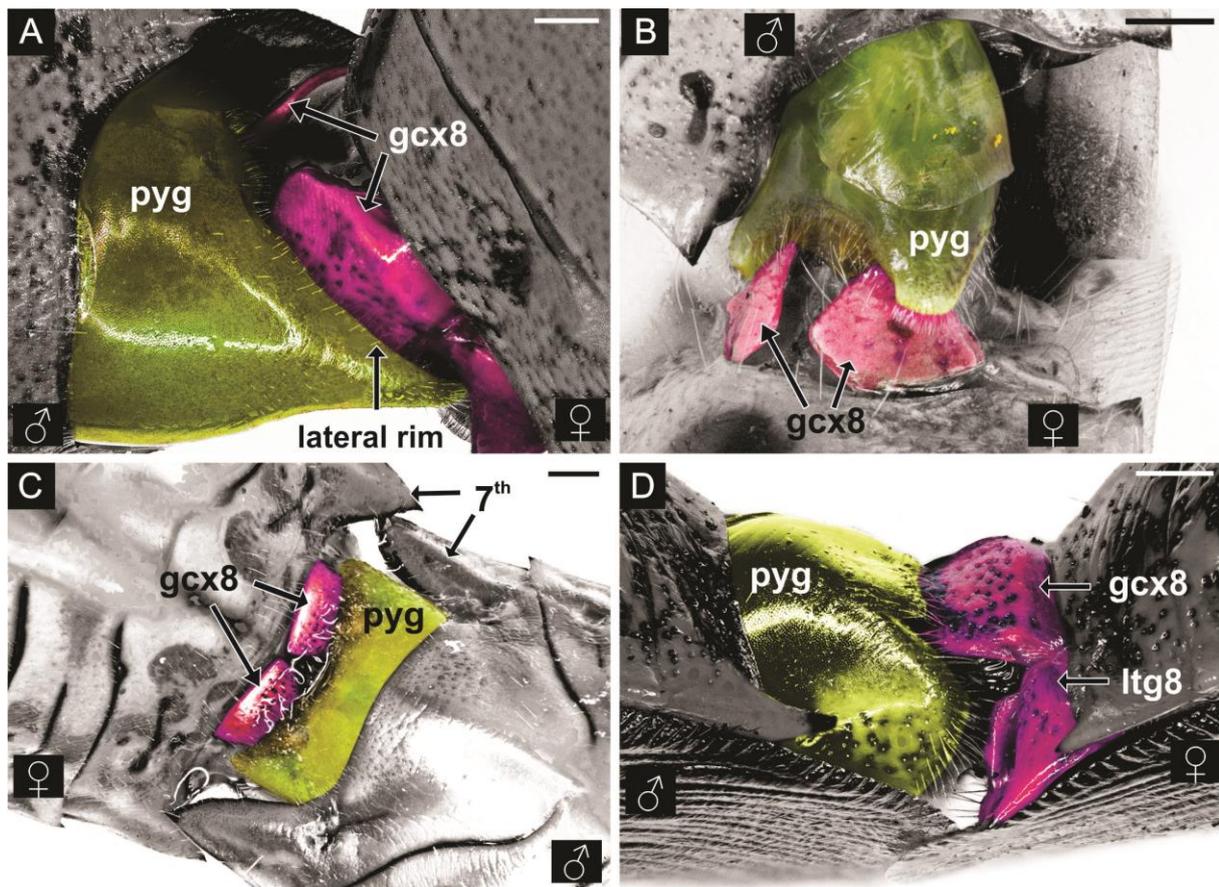


Figure 2. Attached genitalia after 20 minutes in copula, dorso-lateral perspective of the pygophore. Male traits are highlighted in green and female traits in pink. A = *E. heros*, B = *P. nigrispinus*, C = *E. meditabunda*, D = *M. v-luteum*. Scale bar is 0.4 mm.

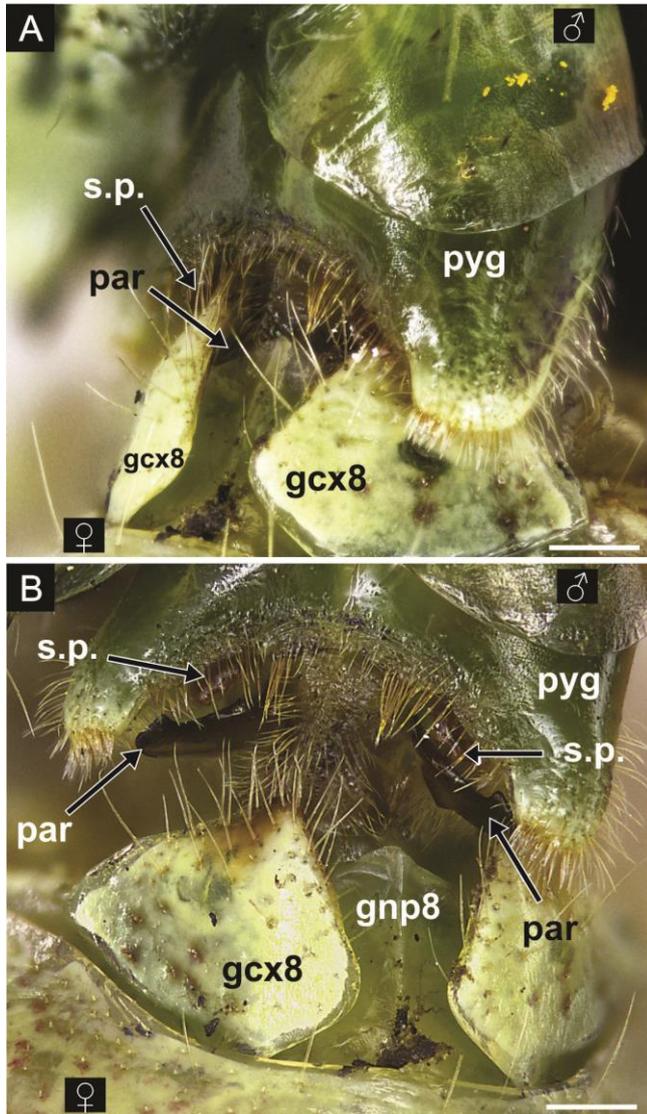


Figure 3. Genitalia of *P. nigrispinus* during copula (A) and after a slight manipulation, with the structures hidden by the gcx8 indicated (B). Scale bar is 0.2 mm.

In the Carpacorini (i.e. *E. heros*, *M. v-luteum* and *M. maculata*), the e.d.r. of the pygophore is well developed and bifurcated (Fig. 1A-B). This structure is used to accommodate the gnp8 (Fig. 5A), which is covered by the gcx8 while in rest position (Fig. 1E-F). In these three species, the bifurcation of the e.d.r. fits thoroughly the median longitudinal elevation of the gnp8 (Fig. 5A). In *P. nigrispinus* and *E. meditabunda*, the e.d.r. is vestigial and do not participate in the connection with the gnp8 (Fig. 3A). We could also visualize the interaction between some anatomical parts that were not focus of our study but can be relevant to interpret mechanisms of evolution (see discussion section). In *E. meditabunda*, the last pre-genital abdominal segment (i.e. the 7th segment) is strongly extended and thickened. The male

projections of the 7th segment anchor on the inner side of the female projections (Fig. 2C). Such anchoring may be important to avoid the rotation of the individuals in copula. After slight manipulation to decouple the mating pairs, we could visualize the intromittent male genitalia (=phallus) inflated inside the female tract (Fig. 5B). While the external parts could be easily untied, this internal connection was much tighter.

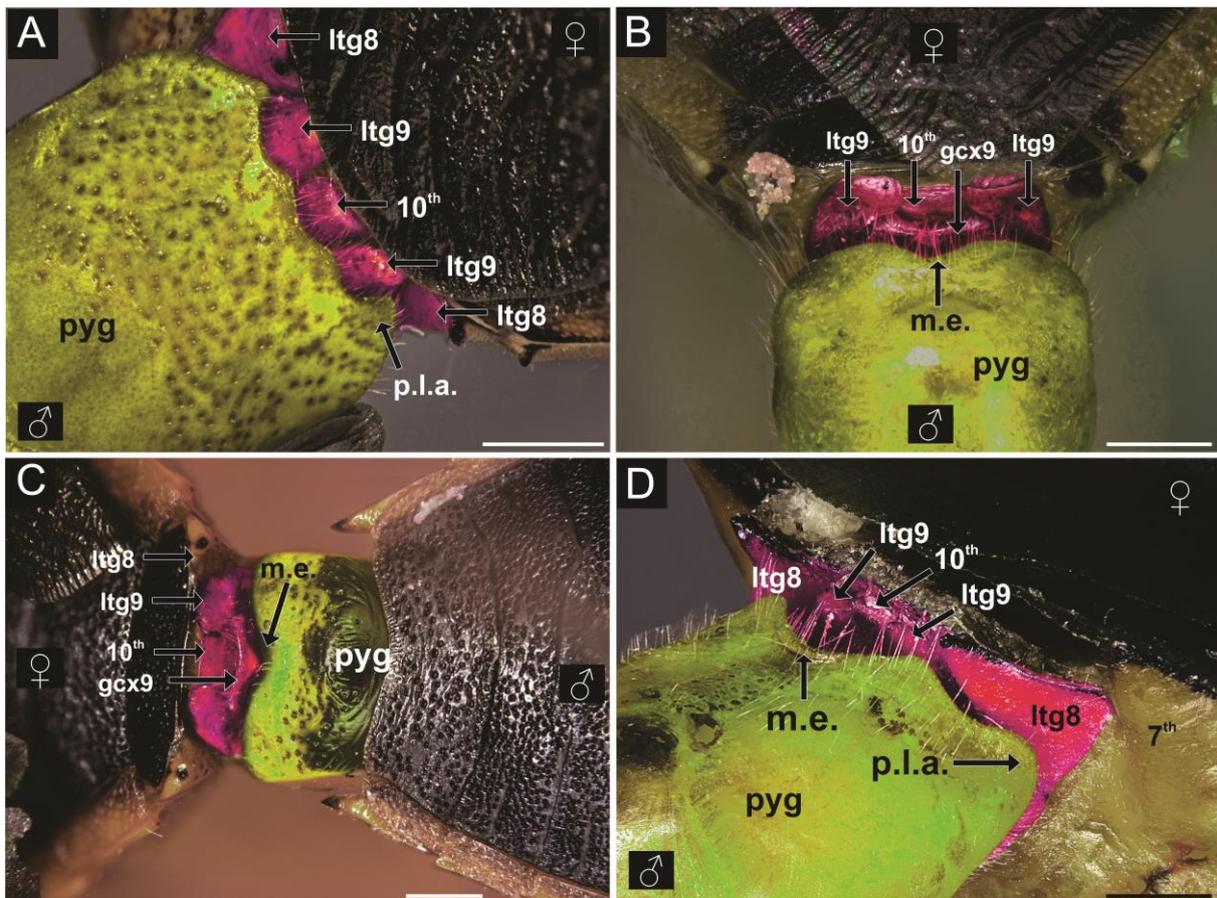


Figure 4. Attachment between the ventral rim of the pygophore (green) and the female plates (pink) from ventral (A-C) and ventro-lateral (D) perspective of the pygophore. A = *E. heros*, B = *M. maculata*, C = *M. v-luteum*, D = *E. meditabunda*. Scale bar is 0.5 mm.

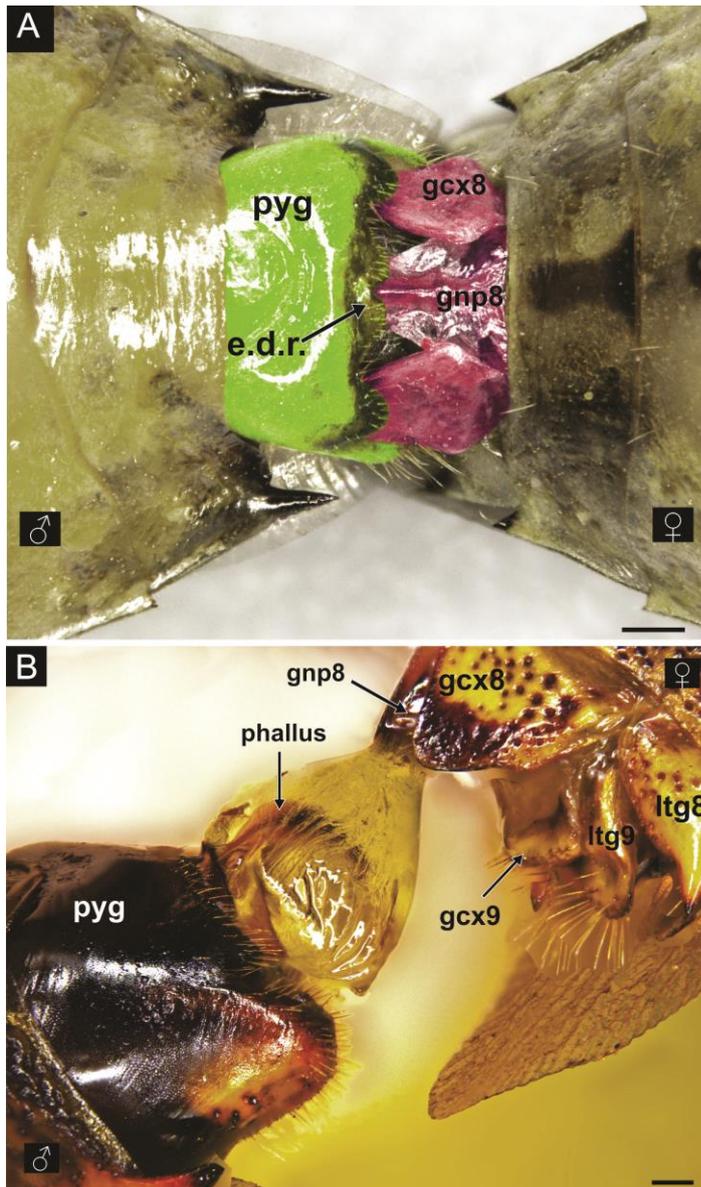


Figure 5. Genitalia of *M. maculata* in copula (A) illustrating the connection between the e.d.r. of the pygophore (green) and the female gnp8 and gcx8 (pink); connection of the internal genitalia of *M. v-luteum* exposed after manipulation (B). Scale bar is 0.3 mm.

In summary, both the dorsal rim of the pygophore and the parameres work jointly to support and keep the gcx8 opened in all species (Figs. 2 and 3). Also similar to all species (except *P. nigrispinus*) was the perpendicular connection between the pygophore and the female genitalia in which the female plates accommodate the ventral rim of the pygophore. However, each genus exhibited a different pattern with respect to which plates engage with the curvatures of the ventral rim and in which portion of the ventral rim the plates get supported (Fig. 4). In *E. heros*, the ventral rim touches all the unmovable plates; in the *Mormidea*, only the gcx9 interacts with the ventral rim; in *E. meditabunda*, the ltrg9 and the 10th lie in the median excavation and

the p.l.a. touch the outer side of the ltg8; in *P. nigrispinus*, the ventral rim does not touch the female genitalia at all. Furthermore, the Carpocorini (i.e. *Euschistus* and *Mormidea*) showed an additional point of stabilization, between the e.d.r. and the gnp8.

DISCUSSION

Our study revealed a unique pattern of strong functional integration among multiple parts of male and female external genitalia. Several male parts, mostly located in the dorsal face, accommodate one or more parts of the female external genitalia. Some of such male structures are apparently modified and specialized to this function. Interestingly, the mechanism of attachment between the genitalia varied among the lineages once certain homologous parts of the male genitalia in different species engage with different parts of the female genitalia. Below we discuss how our data fit the functional hypotheses derived from literature, the evolutionary trends of the genitalia, and the probable underlying mechanisms.

Hypotheses of functional morphology

Sharp (1890) suggested that the pygophore does not participate directly in the copulatory process and it should instead function to protect the internal parts (H6; Table 1). Although it is not possible to discard this “protective hypothesis” with our data, we uncovered an important role of accommodation of the female parts by the pygophore, rejecting his hypothesis at least partially. The most explicit fastening structure of the male genitalia was the ventral rim of the pygophore, which fits either the ltg8, ltg9 and the 10th segment or the gcx9. The ventral rim has apparently evolved to retract in its parts that touch the female plates. Since the female genitalia is being pushed towards the outside by the parameres, such fit between the ventral rim and the female plates probably helps to avoid the male capsule do slide laterally. Particularly in *P.*

nigrispinus, where the ventral rim does not participate in the genital attachment, the superior processes appear to perform this function. These results are to a certain extent in disagreement with Schaefer's (1977) hypotheses that the ventral rim has limited functionalities and should mainly support the internal parts of the male genitalia (H4 and H5). Although the ventral rim per se is clearly engaged with the female genitalia externally, it is possible that certain structures derived from the ventral rim (e.g. the cup-like sclerite) interact with the internal parts during and after intromission. Unfortunately, we were not able to visualize the operation of the internal parts because they were completely covered by the male capsule and the female plates.

We showed that the parameres operate in holding the female gcx8 opened to provide access of the phallus to the internal female genitalia. This result is in line with Baker's (1931) hypothesis (H1) and with the operation mode observed in true bugs of other families (e.g. Moreno-García & Cordero, 2008). However, the parameres keep the gcx8 separate by pressing their inner surface, contrary to Leston's (1955) hypothesis which suggest contact with the outer surface of the gcx8 (H3). Our results also refute Singh-Pruthi's (1925) hypothesis (H2) by showing that the female gonapophyses 8 are supported by the e.d.r. of the pygophore and not by the parameres. In summary, we fully rejected H2 and H3, partially rejected H4, H5 and H6 and corroborated H1.

Functional integration and genital evolution

Anatomically diverse genitalia are usually thought to be also diverse in function (Huber, 2004; Song & Wenzel, 2008), implying that distinct selective pressures should operate within a single genitalia (Rowe & Arnvist, 2012). This has been shown true even to structures that are physically connected (Song & Wenzel, 2008). In contrast with this general view, we show that the multiple components of the Pentatomidae external genitalia are integrated to function exclusively as anchoring structures. The ventral rim of the pygophore is the most obvious

example since it interacts with at least three of the five female external parts in most species (i.e. Itg8, Itg9 and 10th). The female gcx8 are analogous and show a similar level of integration, interacting simultaneously with the lateral rim, e.d.r and the parameres. These results indicate that the external genitalia of the Pentatomidae comprise a system of strong level of functional integration, which means that their parts are prone to vary in a combined and coordinated manner. Accordingly, we suggest that virtually all external genital parts studied here should be directly or indirectly integrated to each other to some degree, a process similar to the one shown in a dung beetle (House & Simmons, 2005; Werner & Simmons, 2008).

In systems as such, it is intuitive to predict that changes in one component would entail changes in another to maintain the coordination integrity among the parts (Klingenberg, 2014). For instance, as the gcx8 are supported on one side by the parameres and by the lateral rim on the other, some level of evolutionary correlation among these three traits would be expected. Nevertheless, our data is limited in supporting an intersexual coevolutionary process between the genitalia. While various anchoring parts of the male genitalia are morphologically peculiar and species specific, the female plates were relatively more constant among the species we studied. For example, the e.d.r. of *E. heros*, *M. v-luteum* and *M. maculata* are differentiated to grasp the female gnp8, but the gnp8 are mostly invariable among all species we analyzed. Several other structures of the male genitalia seem much more diverse among species than the female plates such as the parameres, the 10th segment and the ventral rim, what is consistently observed across the taxonomic literature (e.g. Ferrari et al, 2010; Genevcus et al., 2012). These observations suggest that a probable process of selection enhancing the mechanical fitness of the genitalia should be acting essentially or predominantly over male genitalia, while female genitalia should be subjected to a weaker selective pressure (Genevcus et al, 2017). Alternatively, female genitalia may be constrained due to other processes such as intersexual

differences in gene expression and regulation during the developmental process (Aspiras et al. 2011).

Although we found no explicit evidence of coevolution, it should be noted that our approach only allows for examination of qualitative variation exhibited by the genitalia. Thus, we cannot rule out the hypothesis that female genitalia may evolve in response to male genitalia in a small scale, detectable only by approaches that take continuous variation into account. This hypothesis is somewhat in line with a recent study with stink bugs which shows lesser changes in female genitalia compared to fast evolving male genitalia in a coevolutionary scenario (Genevcius et al., 2017). Because rates of genitalia change have rarely been quantified to males and females simultaneously, similar scenarios with other groups are unknown and we are not able to speculate about its prevalence across animals. We believe various structures of the Pentatomidae genitalia are candidate to be tested for coevolution using continuous data: the margins of the gcx8 and the curvatures of the dorsal rim of the pygophore, the shape of the parameres and the concavity of the gcx8, the length of the spines of the 7th segment, among others. Future fine-scale studies will allow one to test whether different levels of functional integration exhibited by different lineages are good predictors of evolutionary correlation.

Convergence and evolutionary trends of the Pentatomidae genitalia

The overall taxonomic literature of stink bugs documents high levels of pygophore species-specificity. However, if pygophore conformation has fitness consequences and female plates are more evolutionarily conserved, one would expect the repeated evolution of certain male shapes across different lineages. Within *Euschistus*, the biconvex ventral rim of the pygophore in several species are similar to the observed to *E. heros*, for instance in *E. atrox*, *E. acutus*, *E. cornutus*, *E. emorei*, *E. irroratus*, *E. nicaraguensis*, *E. schaffneri* and *E. stali* (Rolston, 1974; Bunde, Grazia & Mendonça-Junior, 2006). As at least four of these species belong to well

separated lineages (Weiler et al., 2016; Bianchi et al., 2017), we may presume at least four episodes of convergent evolution within this genus. By briefly analyzing the taxonomic literature, we found five other species belonging to other tribes and subfamilies that exhibit similar ventral rims: *Acledra* spp. (Faúndez, Rider & Carvajal, 2014), *Cahara incisura* (Fan & Liu, 2013), *Braunus sciocorinus* (Barão et al., 2016), *Edessa puravida* (Fernandes et al., 2015), *Mecocephala bonariensis* (Schwertner, Grazia & Fernandes, 2002). This series of potential convergences reinforce that shape changes of the pygophore in the parts that touch the female plates (and vice versa) are advantageous strategies to perform an effective genital coupling in Pentatomidae.

Interestingly, our analyses revealed that pygophores of different species have evolved in distinct directions to achieve morphological stability in copula. This idea is supported by the fact that certain male parts in different species engage with different parts of the females. For instance, the ventral rim of the pygophore engages with the female ltg8, ltg9 and 10th in *E. heros*, with the gcx9 in the *Mormidea* spp, with the ltg8 in *E. meditabunda* and does not engage with the female genitalia at all in *P. nigrispinus*. We observed certain particularities in the modes of interaction among male and female parts to the four genera studied here, despite the fact that female genitalia are relatively similar in these species. This raises the intriguing question of how many other modes of morphological correspondence exist within Pentatomidae. We believe the extraordinary diversity of pygophores and the existence of peculiar female plates across several lineages of Pentatomidae (Schuh & Slater, 1995; Rider et al., in press) suggest the existence of a high diversity of alternative mechanisms of genital coupling.

Another intriguing implication of our results concerns the use of genital characters in phylogenetic analyses of pentatomids. We hypothesize that several genital structures of the Pentatomidae, if not all, may be more phylogenetically dependent among each other than

previously thought due to a mechanism favoring their morpho-functional integrity. This raises the question of whether using disproportional amounts of genital characteristics in phylogenetic reconstructions may result in strongly genitalia-biased phylogenies that rely on few dependent evolutionary processes. We are not arguing that genital characters should be rejected a priori, especially considering their proven phylogenetic usefulness in insect systematics (Song & Bucheli, 2010). However, since character independence is basically a presumption of the majority of phylogenetic methods (O’Keefe & Wagner, 2001), this issue should be considered with caution.

Which evolutionary mechanisms are most likely?

The majority of studies on sexual behavior of pentatomids report long copulations, sometimes spanning several days. Such mechanism of prolonged copulation seems to be controlled by the male to avoid male-male competition for mates and thereby avoid sperm competition (McLain, 1980; Wang & Millar, 1997). The mechanism employed by males to hold the females is unknown to date, but our results shed some light on this topic. We showed that the external structures of the genitalia are not associated to sperm transfer/storage and thereby should not influence paternity because they interact externally and work as anchoring structures. By manipulating the genitalia to investigate the tightest points of attachment between the individuals, we observed that the external connection was relatively fragile and could be easily untied. On the other hand, the attachment between the inflated phallus and the female internal tract was much stronger (Fig. 5B), indicating that such internal connection might be the determinant mechanism to avoid female access to other males. It should be considered the possibility that while individuals were alive, the parameres could be boosted by muscles to hold the females and the external connection could be actually stronger than we observed with recently dead specimens. However, a functional study with other terrestrial true bug with

relatively similar genitalia suggest a passive mode of operation of the male parts coupled with a cooperative movement of the female plates (Moreno-García & Cordero, 2008). While it remains to be tested whether the inflated phallus has a role in physically displacing rival sperm, our study suggest that they participate at least indirectly in the avoidance of sperm competition by holding females and preventing them from subsequent copulations.

Our results coupled with other experiments with true bugs suggest that both sperm transfer/storage and female holding are performed by interactions of the internal genitalia (Moreno-García & Cordero, 2008; Stacconi & Romani, 2011; Genevcius et al., 2017). Accordingly, the external traits are probably disassociated to any function that may directly influence paternity and intersexual conflict for the control of mating. The apparent absence of male-female coevolution and damaged genitalia in museum collections, as well as the passive mating behaviours exhibited by pentatomids (e.g. Wang & Millar 1997) provide additional support for this hypothesis. Therefore, we believe our data are more indicative of a scenario of natural selection to the external genitalia, which could happen essentially via pure morphological accommodation or species specificity reinforcement (Brennan & Prum, 2015). Since different studies with pentatomids report viable copulation between species with differentiated external genitalia (Foot & Strobell, 1914; Kiritani et al., 1963; Bianchi et al., in press), we believe selection favoring the interlocking effectiveness of genitalia rather than species reinforcement is more plausible (Richmond et al., 2016). However, because we do not know whether and how the external genitalia may interact with the internal parts, an additional aspect should be considered. If the pygophore is used to provide support to the movable internal structures as hypothesized by Schaefer (1977), the morphological diversity exhibited by the external genitalia may have arisen also as a by-product of sexual selection acting on the shape of internal parts. Because most of these mechanisms are not mutually exclusive, discerning

among them will be possible through an examination of the internal and external parts using histological and micro-computed tomography techniques.

CONCLUSIONS

Our study revealed an interesting interlocking genital system in which male and female external structures are functionally integrated to stabilize the genitalia during mating. Furthermore, species from different lineages have evolved to engage with distinct parts of the opposite sex. Because female genitalia is greatly more constant than male genitalia when comparing species, the processes that lead to such morphological stability are certainly more directed to the male parts. Literature data and our observations indicate neither intersexual conflict nor a direct participation of the external parts in sperm transfer and storage. Accordingly, we believe the genital traits we studied here are more prone to a process of natural selection, most likely enhancing the morphological accommodation rather than species reinforcement. To discern among mechanisms of evolution, further studies should attempt to determine why selection should favor a stable and strong coupling, which may be either cooperative or conflicting. Fine-scale analyses using histology and micro ct-scan techniques will make possible to investigate the functioning of the internal parts and to test whether these parts are functionally integrated to the external genitalia.

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CAPÍTULO 2

Phylogeny of the stink bug subfamily Pentatominae (Hemiptera, Pentatomidae) based on molecular markers and morphology

ABSTRACT

The Pentatomidae (stink bugs) stands out within true bugs as the third most diverse family, with nearly 5,000 species. They exhibit a plethora of morphological and behavioral peculiarities that make the group ideal models to approach broad macro-evolutionary questions and to test hypotheses on longstanding morphological puzzles. However, comparative studies as such are hampered by the lack of a phylogenetic hypothesis to the family. Here, we conduct a series of phylogenetic analyses including a comprehensive sample of in-group and out-group taxa, focusing on the “catch-all” subfamily Pentatominae. We used three molecular markers and 73 morphological characters, analyzed in combination and separately, aiming to: i) test the monophyly of Pentatomidae; ii) identify evolutionary lineages within the family; iii) test the hypothesis that Pentatominae is not monophyletic. Our analyses were overall robust, corroborating the monophyly of Pentatomidae and the polyphyly of Pentatominae. Furthermore, several groups proposed in the taxonomic literature were supported as monophyletic, including some of the recognized subfamilies of Pentatomidae and tribes within Pentatominae. We show that the combination of morphological and molecular characters provided results with strongest support levels. A few specific relationships were poorly resolved, and we briefly discuss the reasons and future directions to improve our results.

Key words: classification, Heteroptera, mitochondrial, phylogenetic, systematics

INTRODUCTION

True bugs (Hemiptera, Heteroptera) comprise the most diverse lineage of insects with incomplete metamorphosis (the ‘hemimetabola’), encompassing over 42,000 species divided into 90 families (Schuh and Slater 1995). Heteropterans are found worldwide occupying nearly all biomes. Their enormous diversity of habitats, feeding habits, behavioral repertoires and life-history strategies make the group one of the ecologically and morphologically most diverse lineages of insects (Weirauch et al. 2018). Higher systematics within Heteroptera has experienced considerable progress recently, and the relationships between and within the five recognized infraorders are currently well supported, especially by molecular data (Tian et al. 2011; Li et al. 2012; Weirauch et al. 2018). On the other hand, phylogenetic relationships at deeper nodes of the Heteroptera tree of life have been greatly overlooked (Weirauch and Schuh 2011). Relatively few families have been studied in a phylogenetic perspective, what has certainly hampered our capability of testing hypotheses about their evolution and biogeography.

The Pentatomidae (stink bugs) stands out within Heteroptera as the fourth most diverse family, with 4,949 species and 940 genera (Grazia et al. 2015). Several species are economically important for being pests of food crops and agents in biological control programs (Schaefer and Panizzi 2000). Pentatomids are distributed in all terrestrial biomes except Antarctica, but are more diverse in the Neotropics. They exhibit a plethora of morphological and behavioral peculiarities that make the group ideal models to approach broad macro-evolutionary questions and to test hypotheses on longstanding morphological puzzles. Examples include aposematism, exaggerated sexual traits, parental care, strong adaptations to predation, miniaturization, mimicry, camouflage, etc (Fig. 1). In special, male external genitalia (= pygophore) exhibit striking variation and disproportional levels of elaboration in comparison to other related lineages of true bugs (Genevcius et al. 2017). The evolutionary origins of these

features and the underlying mechanisms are still an unexplored topic in the family, deserving more attention. However, to advance in these fields and prior to establishing the stink bugs as model organisms for evolutionary studies, a crucial first step is establishing a phylogeny and an objective classification to the family.

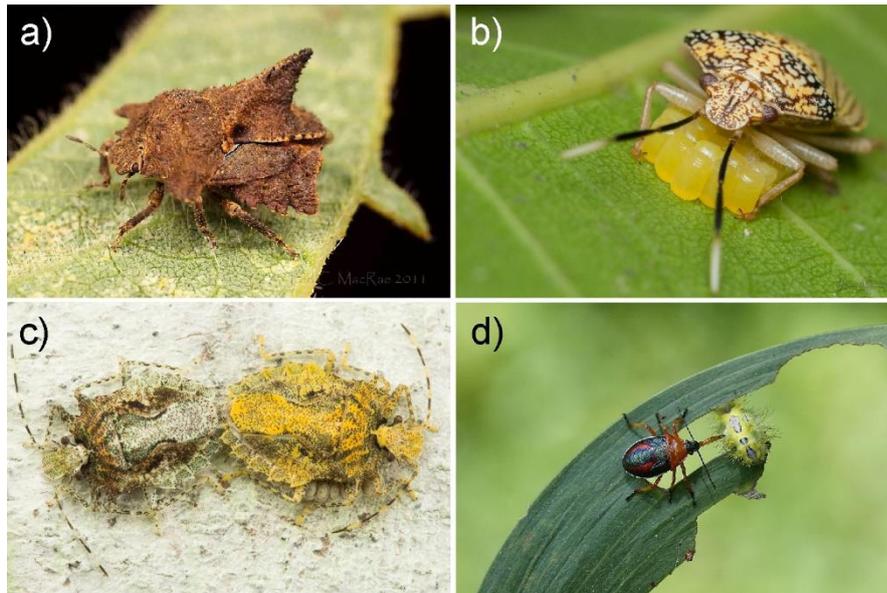


Figure 1. Diversity of pentatomid morphology and behaviors: the exaggerated scutellum in *Cyrtocoris* (a), maternal care in *Antiteuchus* (b), camouflage and dimorphism in *Cloriplatus* (c) and the predaceous habit in Asopinae (d).

Pentatomidae has been indicated as monophyletic (Grazia et al. 2008; Wu et al. 2016), although no study has explicitly tested its monophyly incorporating satisfactory in-group sample. The relationships within the family have been widely ignored. Current classification is essentially based on morphological similarity and tree-based studies without phylogenetic rigor (Rider et al 2018). Ten subfamilies are currently recognized. The subfamilies Serbaninae and Stirosterninae are the smallest – monotypic – subfamilies, occurring in the Oriental and Amazonia, respectively. The subfamily Aphyllinae (3 spp.) are endemic to Australia, while Cyrtocorinae (11 spp.), Discocephalinae (315 spp.) and Edessinae (300 spp.) and are exclusively Neotropical, and Phyllocephalinae (22 spp.) occurs in the Afrotropical, Oriental and Palearctic regions. Lastly, Asopinae (357 spp.), Pentatominae (>3000 spp.) and Podopinae (255 spp.) are distributed worldwide. Almost all subfamilies are easily recognized by unique

morphological features and are hypothesized as monophyletic, based on these same characters (Rider et al. 2018). Examples are the strongly modified feeding apparatus in the Asopinae (Parveen et al. 2015) adapted to predatory habits and the overwhelmingly broad scutellum in the Podopinae (Gapon 2008). The only group whose monophyly is widely questioned is Pentatominae (Rider et al. 2018). This subfamily is a “catch-all” group, without any unique morphological characteristics, and it is mainly composed of all taxa that do not fit the other groups. The subfamily Pentatominae is by far the most diverse, comprising 42 tribes whose compositions are debated.

Here, we conduct a series of phylogenetic analyses with Pentatomidae including a comprehensive sample of in-group and out-group taxa, focusing on the “catch-all” subfamily Pentatominae. By analyzing morphological and molecular data simultaneously, we have three aims. Our first goal is to test the monophyly of Pentatomidae using a good range of other families of Pentatomoidea as out-groups. Second, we sought to identify evolutionary lineages within Pentatomidae, producing a backbone phylogeny to test further evolutionary hypotheses. Finally, we aim to test the hypothesis that Pentatominae is not a monophyletic group and should therefore comprise separate lineages more closely related to other subfamilies.

MATERIAL AND METHODS

Molecular procedures

We extracted genomic DNA from thoracic musculature of ethanol-preserved specimens through incisions in the ventral right portion of the pronotum. Extractions were conducted using the QIAGEN DNeasy blood and tissue kit following the manufacturer’s protocol, extending Proteinase K to twelve hours.

Three molecular markers were amplified via PCR, comprising two mitochondrial genes (16S rDNA and COI) and one nuclear ribosomal gene (28S D3-D5 rDNA). PCR reactions were composed of: 25 μ l of total volume, 14.2 μ l of ddH₂O, 1 μ l of dNTP (2.5 mM), 2.5 μ l of buffer (10 \times), 3.0 μ l of MgCl₂ (25 mM), 1 μ l of each primer (10 μ M), and 0.3 μ l of platinum Taq polymerase (2.5 Units) (Invitrogen, USA). PCR programs were the same to all markers, except the annealing temperature (Table 1): 94°C for 1 min, 35 cycles of 94°C 30 s, 45–55°C 30 s (see Table 1 for each marker), 72°C 1 min, and 72°C for 10 min. Amplification results were visualized through gel electrophoresis (1% gel agarose) with the SyberSafe gel staining and UV illuminator. Purification and Sanger (BigDye) DNA sequencing were conducted by Macrogen, Inc (Seoul, South Korea).

Table 1. Primers and annealing temperatures (T) used to each molecular marker.

	Annealing T	Primers	Reference
COI	50°C	R: GGAACAGGATGAACAGTTTACCCTCC F: TCTGAATATCGTCGAGGTATTC	Simon et al., 1994
16S	55°C	R: CGCCTGTTTAACAAAAACAT F: CCGGTTGAACTCAGATCA	Simon et al., 1994
28S	48°C	R: GGGACCCGTCTTGAAACAC F: TTACACACTCCTTAGCGGAT	Hillis and Dixon, 1991

Sequence treatment and alignment

Raw sequences were concatenated and edited in Geneious R8 (Biomatters Ltd.). Preliminary alignments were conducted in MEGA v.7 (Kumar et al. 2016) to check for sequence qualities and visualize the overall sequence divergence. Each marker was then aligned with MAFFT (Kato and Standley 2013), comparing results with different algorithms (i.e. E-INS-i, G-INS-i, L-INS-I and Q-INS-i) and checking each alignment manually. Individual alignments of all

molecular markers were concatenated into a single molecular dataset for further analyses, comprising a dataset of 1747 nucleotides.

Morphological data

Morphological characters were obtained from literature (Campos and Grazia 2006; Grazia et al. 2008; Garbelotto et al. 2013; Greve et al. 2013) and observation of specimens in a stereo microscope. Dataset consisted of 73 characters, including binary and multistate ones (Table 2). Terminology follows recent studies with pentatomids.

Phylogenetic analyses

Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were conducted in IQ-Tree v 1.4.1. (Nguyen et al. 2015) with both datasets (molecular and morphological) analyzed separately and combined. Terminal taxa and characters available to each taxon are listed in Table 3. To these analyses, we used the substitution model automatically selected by the software according to the bayesian information criterion (BIC). Our analyses allowed different independent models to each molecular marker (e.g. JC, HKY, GTR, etc) and to the morphological partition (ordered or MK). Branch support was assessed with 1000 replicates of ultrafast bootstrap, considering strong support as >90%, 90-70% as moderate support, and <70% weak support.

Bayesian analyses of the concatenated dataset (morphology + DNA) were run in Mr. Bayes v. 3.2. (Ronquist et al. 2011) through the CIPRES web cluster (<http://www.phylo.org>). Again, each molecular marker and the morphological data were treated as separate and unlinked partitions. Best-fit substitution models were those selected by IQ-Tree and, when unavailable in Mr. Bayes, the most similar model available was used. To the morphological partition, a stochastic discrete unordered model was used. Two runs with four

chains each were used, with 30 million generations, sampling every 2,000 generation, and with default priors. Convergence between the Markov Chains was determined as the point when the average standard deviation of split frequencies reached values below 0.01, and 25% of MCMC samples were discarded as burn-in. Branch support was assessed via posterior probabilities.

Table 2. List of morphological characters and respective states.

01.	Body shape: (0) not depressed; (1) body somewhat depressed and broadly ovate; (2) ventrally depressed (dorsally convex).
02.	Surface of body, punctuation: (0) regular punctuation; (1) rare punctures, quite shiny; (2) slightly irregular, with well-marked punctures.
03.	Body coloration, predominantly: (0) brown, dark, black, etc; (1) green in life, and retaining green coloration after death; (2) yellowish-green in life, usually fading to yellow after death; (3) quite colorful, ranging to yellows, reds, and oranges to metallic blues and greens.
04.	Dorsal surface of head, punctuation: (0) punctuation regularly distributed; (1) nearly impunctate, with oblique transverse ridges or wrinkles; (2) nearly impunctate, without oblique transverse ridges or wrinkles.
05.	Head, margin before eyes: (0) concave; (1) straight; (2) convex.
06.	Length of juga: (0) longer than tylus; (1) subequal or shorter than tylus.
07.	Apex of juga beyond tylus: (0) straight forward; (1) curving towards apex of tylus.
08.	Apex of juga, shape: (0) truncated; (1) pointed; (2) rounded.
09.	Juga, process of apex as an acute process: (0) absent; (1) present.
10.	Maxillary plates with (1+1) acute process: (0) absent; (1) present.
11.	Distance of labial base from buccular anterior margin: (0) distinctly remote from anterior limit of bucculae; (1) slightly to moderately closer to anterior limit of bucculae; (2) strongly and closely associated with anterior limit of bucculae.
12.	Posterior angles of the bucculae: (0) not projected or truncated, evanescent; (1) truncated; (2) lobed.
13.	Distance of labral base from labial base: (0) distinctly remote; (1) slightly to moderate closer, but not contiguous; (2) distinctly contiguous.
14.	Labial development: (0) slender; (1) distinctly incrassate, robust.
15.	Shape of bucculae: (0) straight; (1) flap-like.
16.	Anterior angle of buccula: (0) evanescent; (1) truncate; (2) with an acute process.
17.	First rostral segment: (0) shorter or subequal than buccula; (1) longer than buccula.
18.	Antenniferous tubercle ventral lobe: (0) absent; (1) present.
19.	Length of the first antennal segment related to head apex: (0) exceeding head apex for most of segment; (1) slightly exceeding head apex; (2) short, not exceeding head apex.
20.	Anterior margins of pronotum reflexed: (0) absent; (1) present.
21.	Length of scutellum: (0) short, not or slightly surpassing posterior margin of metathorax; (1) reaching or surpassing connexivum apical angles of 3rd abdominal segment; (2) extending beyond abdominal segment V but not exceeding VI; (3) long, almost attaining apex of abdomen, but not covering most of the abdomen; (4) well developed, covering mostly of abdominal dorsum.
22.	Length of frena: (0) long, attaining or distinctly surpassing middle of scutellum; (1) short, not surpassing middle of scutellum; (2) obsolete or absent.

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23. Antero-lateral margins of pronotum: (0) anterior half crenulated; (1) entirely crenulated; (2) not crenulated.
 24. Development of the humeral angles: (0) produced into spine; (1) triangular, but not produced into spine; (1) rounded.
 25. Posterior margin of pronotum: (0) concave; (1) straight.
 26. Ostiolar ruga, length: (0) long, reaching lateral third of metapleura; (1) auricular, short, not surpassing half of the metapleura; (2) median size, reaching the mid length of metapleura; (3) reduced.
 27. Ostiolar peritreme, development: (0) with sides and apex distinct; (1) distally effaced; (0) absent.
 28. Prosternum sulcus, development: (0) without sulcus to moderately sulcate; (1) deeply sulcate.
 29. Prosternum carina, development: (0) absent or moderately carinate; (1) strongly carinate.
 30. Metasternum process, presence: (0) absent; (1) present.
 31. Metasternum process, development: (0) never projected over mesosternum; (1) projected over the mesosternum.
 32. Mesosternum carina, development and shape: (0) plateau shaped, anteriorly produced; (1) keel shaped, well developed; (2) weakly developed, same height the entire length; (3) weakly developed, but apical third projected anteriorly; (4) absent.
 33. Metasternum raised: (0) present; (1) absent.
 34. Femur, dorsal apical projection: (0) absent; (1) present, acute; (2) present, wide;
 35. Abdominal spine: (0) absent; (1) present, reaching hind coxae; (2) present, reaching middle coxae; (3) present, reaching fore coxae; (4) present, just a bump.
 36. Abdominal venter: (0) flat with a longitudinal sulcus; (1) with a longitudinal keel; (2) not flat, convex.
 37. Anterior margin of urosternite VII, in males: (0) not strongly extended anteriorly; (1) strongly extended anteriorly.
 38. Development of the female external genitalia: (0) all plates well developed and external; (1) basal plates (gc VIII) reduced; (2) all plates are reduced and appear to be recessed into female urosternite VII.
 39. Laterotergites IX, length: (0) surpassing the band uniting laterotergites VIII; (1) not surpassing.
 40. Laterotergites IX, apex shape: (0) rounded/angulated; (1) acute.
 41. Laterotergites VIII, apex process: (0) present; (1) absent.
 42. Gonapophyses VIII: (0) well developed, first rami distinct; (1) gonapophyses VIII and first rami lost.
 43. Gonocoxites VIII: (0) with a distinct apparent median fusion line; (1) completely fused with gonapophyses IX.
 44. Laterotergites IX: (0) contiguous, partially or totally covering segment X; (1) separate, with segment X between them; (2) totally fused, with segment X concealed.
 45. Gonapophyses IX: (0) moderately sclerotized to membranous, second rami present; (1) reduced, fused to gonocoxites IX, second rami lost.
 46. Second valvulae development: (0) well developed, second rami distinct; (1) largely membranous, second rami thinly sclerotized; (2) reduced to a pair of small membranous flaps.
 47. Gonangulum development: (0) well developed, sclerotized; (1) membranous, obsolete; (2) absent.
 48. Ring sclerites beside spermathecal base (= Chitinlipsen): (0) one; (1) two.
 49. Secondary thickening of gonapophysis IX (Schwertner 2005): (0) small; (1) large, occupying more than half of the STG.
 50. Sclerites on spermathecal base: (0) spermathecal base largely membranous; (1) with an elongate, grooved sclerite; (2) with a pair of antero-posteriorly oriented sclerites.
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51. Spermathecal bulb constricted in the middle: (0) absent; (1) present, apex rounded; (2) present, apex acuminate.
 52. Development of ductus receptaculi: (0) dilated or not, but not invaginated; (1) dilated and invaginated, three distinct walls, distal part open; (2) dilated and invaginated, three distinct walls, distal part closed.
 53. Triangulin development [= membranous to sclerotized structure joining the gonocoxites VIII or the gonapophyses VIII]: (0) absent, intergonocoxal membrane between g8 pleated; (1) absent, intergonocoxal membrane between g8 smooth; (2) present, sclerotized as structure joining GC VIII, G VIII and rami 1 lost.
 54. Pars communis, development: (0) areas surrounding orificium receptaculi largely membranous; (1) areas surrounding orificium receptaculi with elongate, grooved sclerite; (2) areas surrounding orificium receptaculi with a pair of sclerites.
 55. Anterior thickening of vaginal intima: (0) present; (1) absent.
 56. Capsula seminalis, processes: (0) absent; (1) present, two or more elongated process anteriorly directed; (2) present, one apical arc shape process; (3) present, one apical finger-like process.
 57. Sternite VIII in males, development: (0) apparent externally, not or partially covered by segment VII; (1) concealed by segment VII.
 58. Tergite VIII in males, sclerotization: (0) sclerotized; (1) membranous.
 59. Pygophore, superior processes, presence: (0) absent; (1) present.
 60. Hypandrium, presence and shape: (0) absent; (1) present, as 1+1 broad and long inflated expansions, surpassing the ventral rim; (2) present, 1+1 flat, long expansions, surpassing ventral rim; (3) present, as 1+1 short expansions, not surpassing the ventral rim.
 61. Median projection: (0) inconspicuous or absent; (1) development, apically; (2) development; laterally.
 62. Segment X apical processes: (0) absent; (1) present, anteriorly directed; (2) present, posteriorly directed.
 63. Paramere: (0) nearly cylindrical, without big processes; (1) laminar, with wide processes; (2) inconspicuous.
 64. Paramere, connection to the ventral wall: (0) absent; (1) present.
 65. Paramere, basal process: (0) absent; (1) present.
 66. Phallosome, sclerotization: (0) slightly to moderately sclerotized; (1) relatively flexible; (2) thickly sclerotized, rigid.
 67. Theca, shape: (0) short and broad; (1) long and cylindrical.
 68. Thecal shield: (0) absent; (1) present.
 69. Conjunctiva, development (Gapud 1991): (0) absent; (1) present, conspicuous; (2) present, reduced.
 70. Phallus, titillators, presence: (0) absent; (1) present.
 71. Conjunctiva, lappet process, presence: (0) absent; (1) present.
 72. Vesica length in relation to the phallosome: (0) shorter than phallosome; (1) subequal to phallosome; (2) longer than phallosome.
 73. Vesica, basal process, presence: (0) absent; (1) present.
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Table 3. Terminal taxa and respective character sampling (x = available; - = unavailable). Dark gray are species out of Pentatomidae and light gray species out of Pentatominae. * are species considered 'unplaced'.

	16S	28S	COI	Morph.
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<i>Acrosternum millierei</i>	X	X	X	X
<i>Aelia acuminata</i>	X	X	X	X
<i>Agonoscelis nubila</i>	X	X	X	X
<i>Amaurochrous dubium</i>	X	X	X	X
<i>Antiteuchus mixtus</i>	X	X	X	X
<i>Arocera sp</i>	X	-	X	X
<i>Arvelius albopunctatus</i>	X	X	X	X
<i>Banasa albopicata</i>	X	X	X	X
<i>Brachystethus geniculatus</i>	X	X	X	X
<i>Brochymena quadripsutulata</i>	X	X	X	X
<i>Brontocoris sp</i>	X	X	X	X
<i>Caonabo pseudocylax</i>	X	X	X	X
<i>Carpocoris purpureipennis</i>	X	X	X	X
<i>Chinavia brasicola</i>	X	X	-	X
<i>Chinavia obstinata</i>	X	X	X	X
<i>Chinavia runaspis</i>	X	X	X	X
<i>Chlorochroa ligata</i>	X	X	X	X
<i>Chlorocoris complanatus</i>	X	X	X	X
<i>Chloropepla sp</i>	X	X	-	X
<i>Cyclopelta sp</i>	X	X	X	-
<i>Dichelops furcatus</i>	X	X	X	X
<i>Dinocoris gibbus</i>	X	X	X	X
<i>Diploxys punctata</i>	-	X	X	X
<i>Dolycoris baccarum</i>	X	X	X	X
<i>Dryptocephala spinosa</i>	X	X	X	X
<i>Edessa meditabunda</i>	X	X	X	X
<i>Edessa rufomarginata</i>	X	X	X	X
<i>Elasmostethus sp</i>	X	X	X	X
<i>Erthesina fullo</i>	X	X	X	X
<i>Eurydema sp</i>	X	X	X	X
<i>Euschistus heros</i>	X	X	X	X
<i>Eusthenes sp</i>	X	X	X	-
<i>Eysarcoris ventralis</i>	X	X	X	X
<i>Fecelia nigridentis</i>	X	X	-	X
<i>Graphosoma lineatum</i>	X	X	X	X
<i>Halyomorpha halys</i>	X	X	X	X
<i>Herrichela sp</i>	X	X	-	X
<i>Loxa deducta</i>	X	X	X	X
<i>Macropygium reticulare</i>	X	X	X	X
<i>Marghita similima</i>	X	X	X	X
<i>Mayrinia curvidens</i>	X	X	-	X
<i>Mormidea vluteum</i>	X	X	X	X
<i>Myota aerea</i>	X	X	X	X
<i>Neotibialis sp</i>	X	X	X	X
<i>Neotiglossa undata</i>	X	X	X	X
<i>Nezara viridula</i>	X	X	X	X
<i>Odmalea sp</i>	X	X	X	X
<i>Oebalus poecilus</i>	X	X	X	X

<i>Oechalia schellenbergi</i>	X	X	X	X
<i>Pachycoris torridus</i>	X	X	X	X
<i>Parantestia sp</i>	X	X	X	X
<i>Patanius vittatus</i>	X	X	X	X
<i>Pellaea stictica*</i>	X	X	X	X
<i>Pentatoma rufipes</i>	X	X	X	X
<i>Phloea sp</i>	X	X	X	-
<i>Phoecia sp</i>	X	X	X	X
<i>Picromerus bidens</i>	X	X	X	X
<i>Platycoris sp</i>	X	X	X	X
<i>Podisus nigrispinus</i>	X	X	X	X
<i>Poecilometis sp</i>	X	X	X	X
<i>Raphigaster nebulosa</i>	X	X	X	X
<i>Runibia perspicua</i>	X	X	-	X
<i>Scotinophora sp</i>	X	X	X	X
<i>Serdia concolor</i>	X	X	X	X
<i>Tholosanus sp</i>	X	X	X	X
<i>Thoreyella brasiliensis</i>	X	X	-	X
<i>Thyanta perditor*</i>	X	X	X	X

RESULTS

Preliminary analyses with different alignment algorithms were extremely similar, resulting in identical trees. Thus, all results are reported using the G-INS-I algorithm.

Analyzing the morphological dataset in isolation resulted in a poorly resolved tree (Fig. 2). Three subfamilies were recovered as monophyletic: Asopinae, Discocephalinae and Edessinae. Podopinae was paraphyletic, as the members of the tribes Graphosomatini (*Graphosoma lineatum*) and Podopini (*Amaurochrous* and *Scotinophora*) were not closely related. It is not possible to conclude about the monophyly of Pentatominae with the morphological data only, due to a lack of resolution. Although there is no indication that the subfamily is monophyletic, its monophyly cannot also be rejected because no species of Pentatominae is related to any other subfamily with good support. Some of the tribes of Pentatominae were monophyletic: Aelini, Catacanthini, Chlorocorini, Halyini, Nezarini and Procliticini.

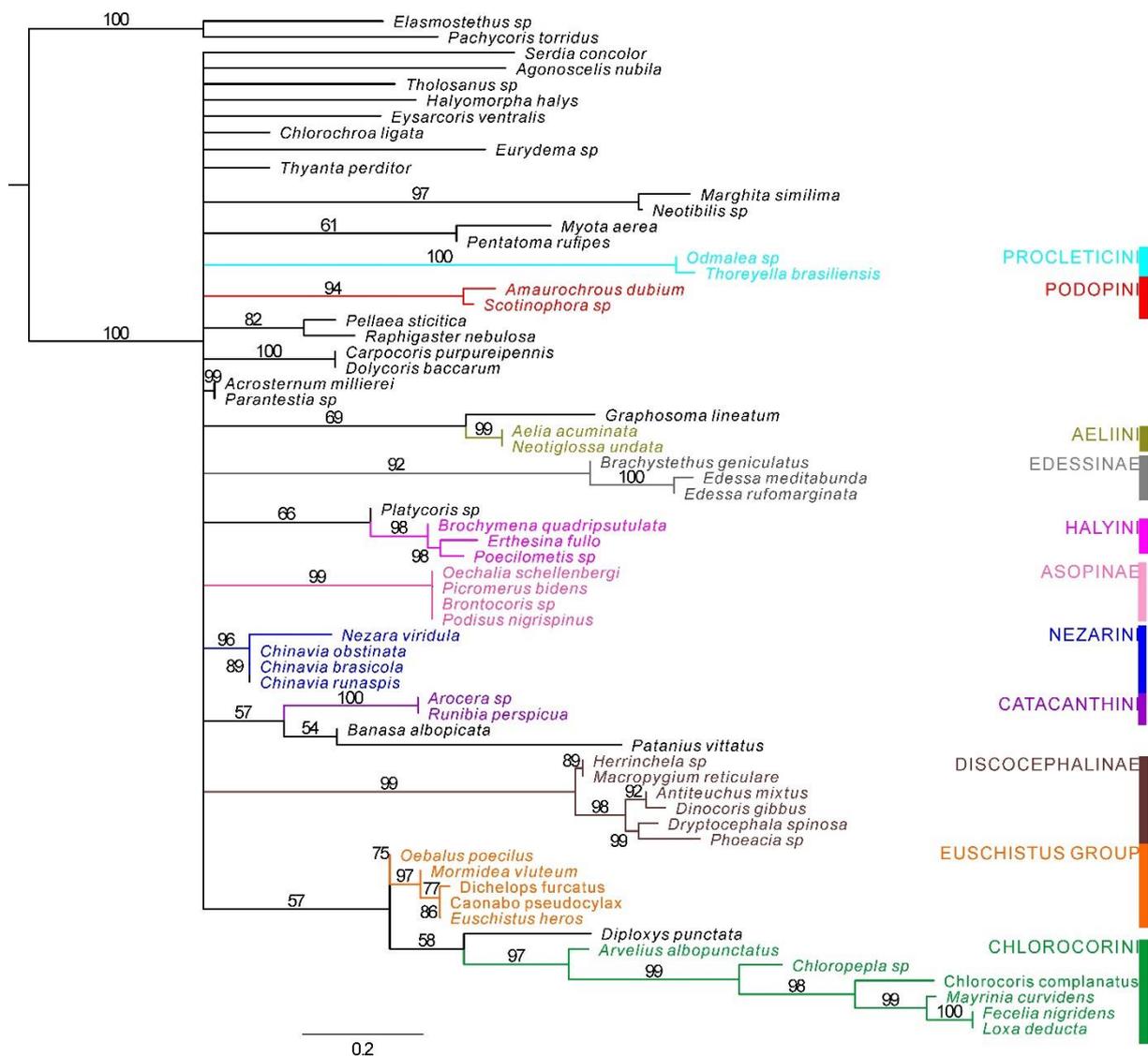


Figure 2. Phylogenetic tree from morphological data analyzed using maximum likelihood. Clades with bootstrap below 50 are collapsed.

Molecular data alone yielded a slightly better supported tree comparing to the morphological one (Fig. 3). However, fewer groups proposed on the literature were monophyletic; for instance, the only tribes of Pentatominae supported were Aeliini, Nezarini and Procleticini.

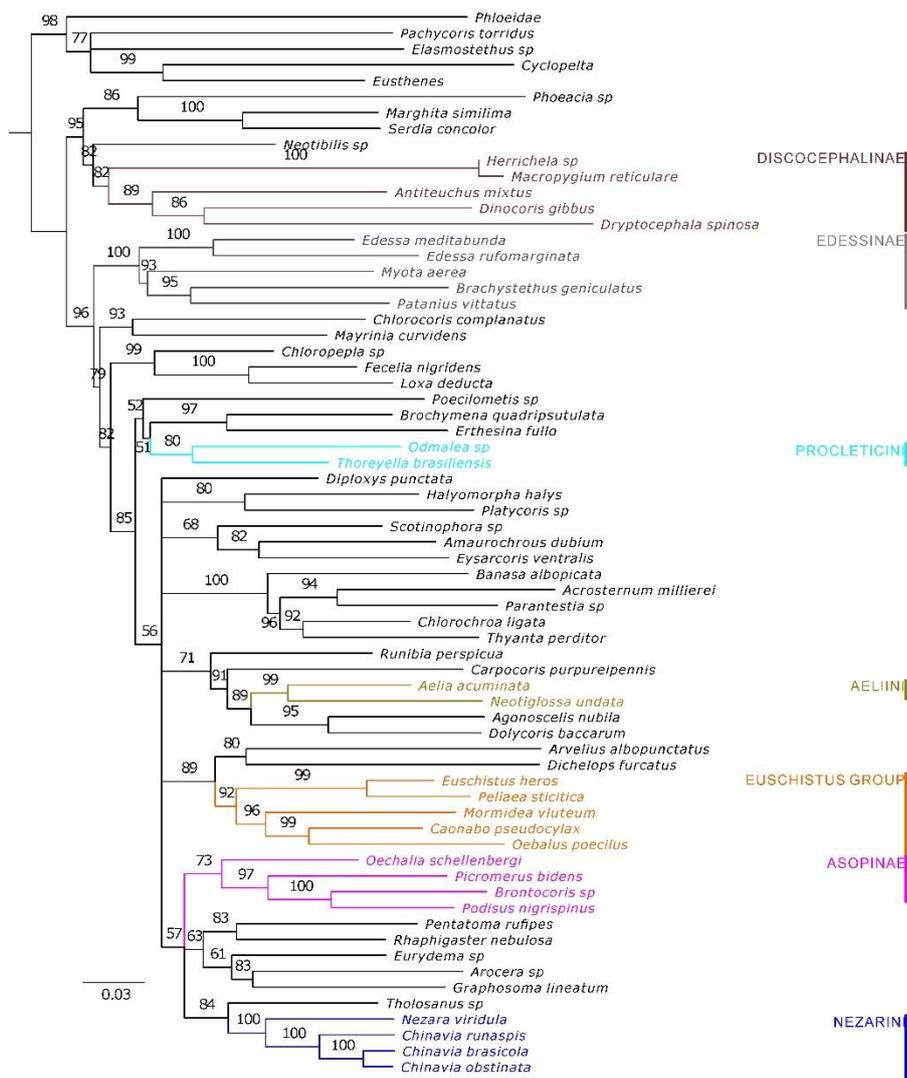


Figure 3. Phylogenetic tree of the molecular dataset analyzed using maximum likelihood. Clades with bootstrap below 50 are collapsed.

The combined analyses resulted in the most well supported trees, with only three clades with very low support (Fig. 4). Results of the Maximum Likelihood (ML) and Bayesian analyses (BA) were overall congruent. As expected in these cases, the Bayesian tree was a less supported version of the fully resolved ML tree, with similar topology but with the least supported clades from the ML collapsed. These analyses strongly suggest the Pentatomidae as monophyletic and Pentatominae as polyphyletic. All tribes and subfamilies supported in the analyses using part of the data (morphology or molecules) are strongly supported in the

combined dataset. Additionally, there was a taxonomic group recovered only in the combined analyses: Antestiini.

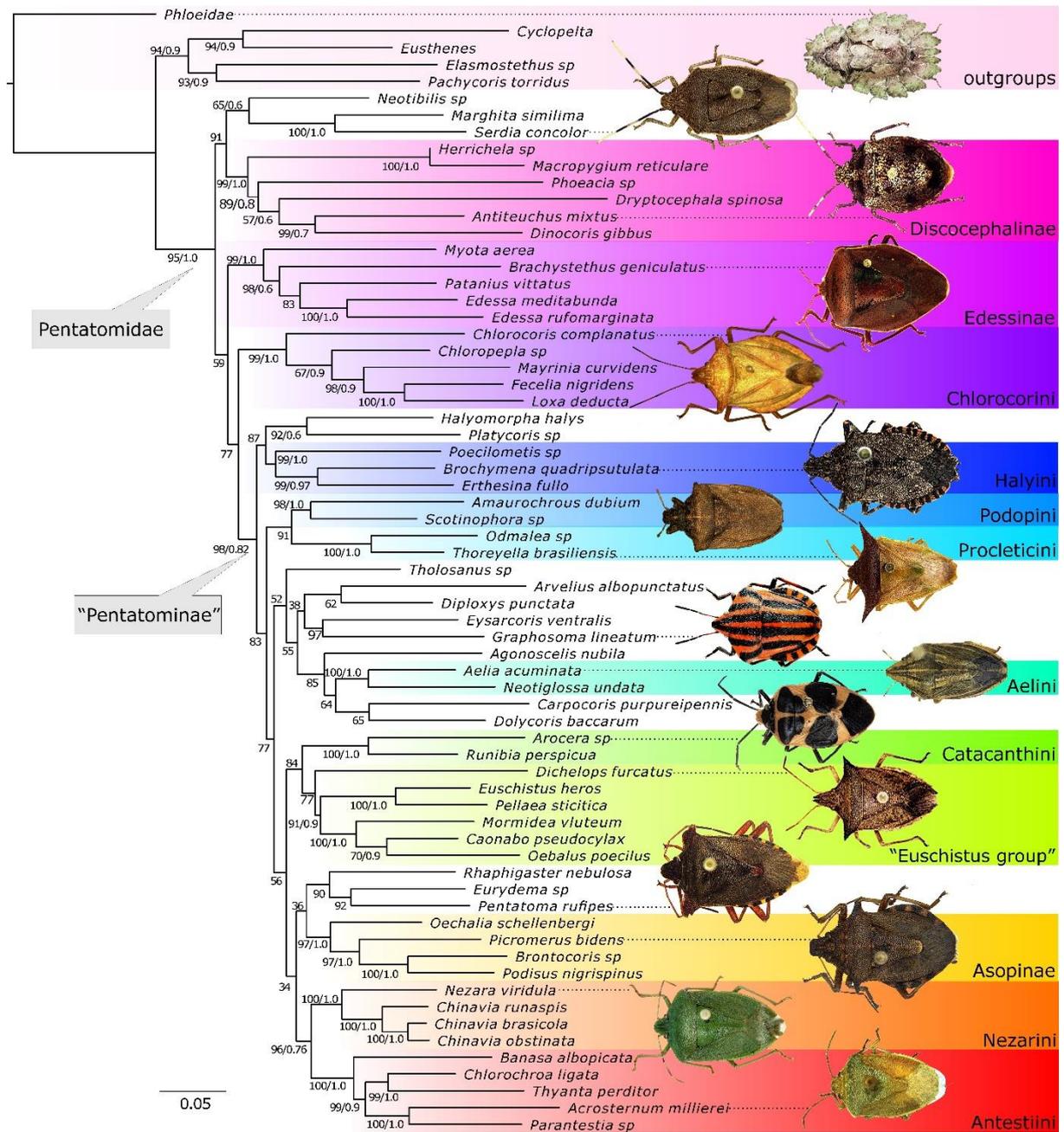


Figure 4. Phylogenetic tree of the combined (DNA + morphology) dataset analyzed using maximum likelihood. Clades also supported in the Bayesian analysis have posterior probabilities displayed (numbers to right in branches).

DISCUSSION

Phylogenetic studies focusing on major groups within Pentatomomorpha and, more broadly, Heteroptera, have included pentatomid representatives, indicating its monophyly (Grazia et al. 2008; Wu et al. 2016; Li et al. 2017). However, the monophyly of Pentatomidae has not been explicitly tested including a broad sample of taxa across the family. Our study represents the first phylogeny with a comprehensive sample of Pentatomidae as in-group taxa, representing main lineages from all regions in the world where these insects occur. The family was unequivocally monophyletic, exhibiting high support levels under morphological, molecular and combined analyses.

Our results from the molecular and combined datasets strongly support the Pentatominae as a polyphyletic group. We corroborate our initial hypothesis that this is a “catch-all” group proposed without biological and evolutionary meanings (Rider et al. 2018). Part of the genera included in the subfamily, namely *Neotibilis*, *Marghita* and *Serdia*, appear as closely related to members of the subfamily Discocephalinae. These three genera, together with the “basal” Discocephalinae and the Edessinae, exhibit marked developments in the metasternum (Barcellos and Grazia 2003). This feature is also shared by several groups of closely related families (Genevcius et al. 2016), suggesting it is a plesiomorphic characteristic within Pentatomidae. At the same time, two other subfamilies of Pentatomidae fall within Pentatominae: the predacious Asopinae, and Podopinae (which was also polyphyletic, represented by two independent lineages, *Amaurochrous* + *Scotinophora* - Podopini and *Graphosoma* - Graphosomatini). It is noteworthy that the Asopinae and the Podopinae have been classified into separate subfamilies for exhibiting unique and remarkable morphological features. Species of Asopinae, for instance, exhibit a series of adaptations in the head and mouthparts related to its predacious habits (Parveen et al. 2015). These peculiar characteristics

led taxonomists to separate these species into a distinct group. However, our results support the hypothesis that the Asopinae and the genera included currently in Podopinae are actually lineages of Pentatominae that evolved highly - but punctual and autapomorphic – specialized modifications. These observations highlight the importance of including multiple-source information in a phylogenetic framework to achieve a stable and evolutionarily meaningful classification.

Our combined analyses yielded the strongest support values in lineages with relative recent split, i.e. within tribes, between genera and apical nodes. On the other hand, lowest values were found at deeper nodes, in the relationships among tribes and subfamilies for example. Alternative explanations may account for these observations. For example, our data may possibly contain high amounts of homoplasy, incongruences between morphology and DNA (or between markers), or a general lack of informative characters. Although these three factors may yield patterns such as the ones we found (Barrett et al. 2014; Heikkilä et al. 2015), we believe the lack of informative characters is more significant here. Our analyses resulted in trees with very long branches in the tips and very short branches at deeper nodes. This seem to suggest a lack enough variation in our data to reflect more ancient splits in the evolutionary history of the Pentatomidae. This is likely a result of the disproportional amount of mitochondrial data in our analyses, as only one nuclear marker was used and mitochondrial markers are more adequate to reflect recent divergence events (Springer et al. 2001; Fisher-reid and Wiens 2011). Delineating morphological characters to resolve such deeper relationships would also be very challenging, given the amount of phenotypic differentiation between tribes and subfamilies (Grazia et al. 2008, 2015) and the subsequent difficulty in proposing homologies. As suggested in other similar cases (Hubert et al. 2014), including additional nuclear data seems the most plausible solution to improve the resolution of our phylogeny at deeper nodes.

It should also be considered that the pentatomids might have experienced rapid radiation in the past, what could ultimately result in few character information left to reflect these ancient divergences. This has been shown particularly true to other groups of insects that undergone greater diversification during the Cretaceous-Tertiary (Whitfield and Kjer 2008; Heikkilä et al. 2015), which seems to be also the case of the pentatomoids (Li et al. 2012, 2017). If this is the case, we believe that only with the implementation of genomic data we will be able to achieve a fully resolved and highly supported tree.

In summary, we show that combining the morphological data with standard nuclear markers can provide interesting insights into the divergence patterns across Pentatomidae. While morphological and DNA data separately produced partially incongruent trees and several clades with low support, their combination may be an interesting solution when neither of the data are extensive. The combination of both types of characters may also be useful to include extremely rare species in the future, to which extracting DNA would be unfeasible (Weirauch and Štys 2014). This would be particularly important for the Pentatomidae, in which three of the ten recognized subfamilies are known only from a few exemplars (i.e. Aphyliinae, Serbaninae and Stirotarsinae). We achieved our main goals, determining with good confidence the monophyly of Pentatomidae, the polyphyly of Pentatominae and the recognition of several other lineages within the family. Our data will foster crucial information to improve pentatomid classification. In special, the organization at the subfamily level will require reformulations to reflect the evolutionary history within the family. Next steps to improve our results are already being conducted in our laboratory, including a larger sample of species (e.g. from Phyllocephalinae, Cyrtocorinae and the Australian groups) and the inclusion of three additional nuclear markers (28S D1, Deformed and Histone H3).

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CAPÍTULO 3

Developmental and functional roles in the evolution of stink bug genitalia

ABSTRACT

Male genitalia are among the most variable structures across animals with internal fertilization, while the equivalent structures in females are considered more conserved. Yet, little is known about the mechanisms underlying these intersexual differences. One possible explanation is that male genital diversification is mostly determined by functional demands. Male genitals would be composed of different subunits, representing targets of independent selective pressures that have more degrees of freedom to change. Alternatively, female genitals may be more evolutionarily constrained if their components are more integrated due to developmental limitations. Here, we determine the roles of functional morphology and development in shaping the evolution of male and female genitalia of pentatomids in a coevolutionary framework. We quantify the variation in both structures using geometric morphometrics and test between different models of modularity that represent two alternative scenarios. The first is a scenario where traits originated from a single structure in immatures evolve as semi-independent modules (developmental modularity). The second is a scenario where functionally integrated components evolve jointly and independently of the other components (functional modularity). We show that male and female genitalia, in spite of coevolving and being functionally complementary, are shaped by different factors. While male genital evolution is shaped exclusively by functional demands, female genitalia is mostly determined by developmental constraints. We provide novel quantitative evidence of a persistent selective process favoring a mosaic modular architecture of genitalia in broad evolutionary scales. We also propose that functional diversity, in the absence of any type of constraints, may be a great facilitator of genital diversification.

Key words: biomechanics, coevolution, reproduction, selection

INTRODUCTION

Male genitalia are acknowledged as the most variable and complex structures across animals with internal fertilization (Arnqvist 1997; Langerhans et al. 2016). On the other hand, the equivalent structures in females are considered more conserved (Ah-King et al. 2014). In spite of these differences, intersexual genital coevolution have been shown to a variety of vertebrate and invertebrate taxa (Brennan and Prum 2015). Male and female genitalia may coevolve even when the sexes exhibit marked disproportional diversification (Genevcius et al. 2017). This phenomenon is still a big puzzle to evolutionary biologists, and the underlying mechanisms are yet poorly understood.

A recurrent explanation is that male traits may exhibit a greater functional diversity in copula (Genevcius et al. 2017). Different components of a single male genitalia may be engaged in different copulatory functions, for instance, stimuli of the female tract (Briceño et al. 2016; Wulff et al. 2017), mate guarding (Moreno-garcia and Cordero 2008), female grasping (Arnqvist et al. 1995), etc. This argument implies that male genitalia should be composed of different subsets of structures (= modules) that may represent targets of independent selective pressures. Subsets that evolve semi-independently tend to have more degrees of freedom to change, providing an explanation to why male genitalia exhibit such an overwhelming diversity compared to somatic and female genital traits. While studies with different organisms support the notion that male genitals are indeed multi-functional structures (Moreno-garcia and Cordero 2008; Werner and Simmons 2008; Genevcius and Schwertner 2017), no study has yet tested the tendency of these functional subunits to evolve semi-independently, especially in a coevolutionary scenario.

The functions exerted by female genitalia in mating may be more diverse than previously thought. Roles of female genitalia have been broadly neglected, essentially because

female genitals are usually more difficult to access, measure and evaluate (Puniamorthy et al. 2010). However, recent studies have shown that female genitals may play active and pivotal roles in copula, regardless of the fact that they are less elaborated (Orr and Brennan 2015; Orbach et al. 2017). These observations put in question the widely held view that male genitals are functionally predominant in copula. Furthermore, they suggest that a frequently assumed lack of functional properties in female genitalia cannot provide a comprehensive and satisfactory explanation to why such structures evolve slower and show less elaboration than males.

An alternative explanation is that female genitalia may be more evolutionarily constrained due to developmental factors. Studies with insects have shown that genital traits develop under the control of hundreds of specific genes, which, in turn, are up and/or down regulated by fewer *Hox* and appendage-patterning genes (Aspiras et al. 2011; Ledón-Rettig et al. 2017). Female genitalia may be more constrained if their different components develop mediated by fewer genes, by fewer types of genes, or a more entangled network of regulatory genes. On the other hand, male genital components would evolve faster if they are regulated by a greater variety of independent developmental pathways. If this is the case, a macro-evolutionary consequence is that these different female components should evolve more dependently of one another. Even if a particular female component experiences directional selection in a functional context, its elaboration would be at some point constrained because changes in this structure could result in changes in another developmentally dependent structure. In other words, female genitalia may be less capable of diverging because they may experience stronger pleiotropic effects during the development. To corroborate this hypothesis, one of the following predictions should be supported. First, female genitalia should not exhibit a pattern of evolutionary modularity, i.e. their components should evolve more dependently of one another comparing to males. Alternatively, female genitalia could evolve in a modular

fashion but each module should comprise components that share the embryonic tissue of origin, and not functional units.

Here we test how the evolution of genitalia in pentatomids is influenced by functional and developmental demands in a comparative and coevolutionary framework. We first show that male and female genitalia coevolve, what may be explained by either their functional or developmental correspondences. Second, we investigate whether developmental or functional processes have been mostly determinant in shaping the diversification of male and female genitalia in macro-evolutionary scale. To answer this question, we test between two models of modularity in each sex. Following a developmental modular model, genital traits derived from the same abdominal segments in immatures should comprise distinct modules that evolve semi-independently. This model represents a scenario where genital evolution is constrained for sharing embryonic origins. Accordingly, the functional modular model describes a scenario where development do not constrain diversification. In this case, different components that act jointly in copula (i.e. share a functional role) represent evolutionary modules.

MATERIAL AND METHODS

Genital traits

External genitalia in pentatomids, defined here as structures of the terminalia that participate directly in copulation, comprise the eighth, ninth and tenth abdominal segments modified to mate (Fig. 1a). These segments are strongly similar and undifferentiated in male and female immatures (Bianchi et al. 2011), and originate the genitalia in later developmental stages. In female stink bugs, these segments originate eight genital plates in adults: two gonocoxites 8 and two laterotergites 8 (comprising the eighth segment), two laterotergites 9 and one

gonocoxite 9 (ninth segment), and a single tenth segment (Fig. 1a). In males, the eight segment suffer a drastic reduction and great loss of sclerotization, originating a ring-like membrane that control the movements of the rest of the genitalia (Schaefer 1977). The ninth segment is the genital capsule, or pygophore, while the tenth segment originates a pair of lateral appendages (= parameres or claspers) and the adult tenth segment itself (Fig. 1a). The abdominal segment of origin was used to delineate the hypotheses of developmental modularity (see below).

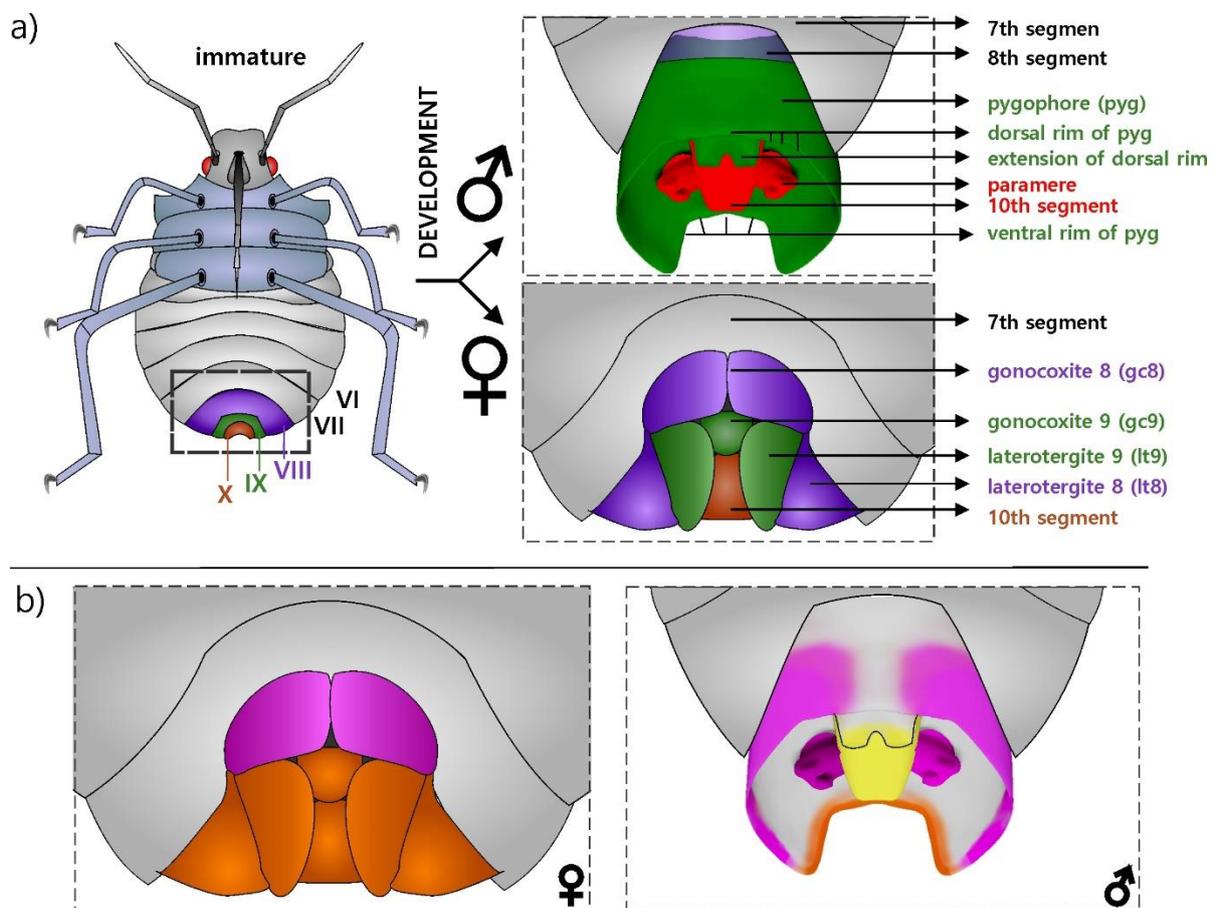


Figure 1. a) Development of male and female genitalia from immatures to the adult; structures in the adult are colored accordingly to the abdominal segment of origin, which will ground the developmental modularity hypotheses. b) male and female genitalia colored accordingly to their functional roles; female gonocoxites 8 (gc8, pink - left) are active movable plates that protect the internal genitalia and are pressed by male parameres and dorsal rim (pink - right); the other plates are fixed and provide anchoring points to the male ventral rim of the pygophore (orange); yellow parts in male are the extension of the dorsal rim and 10th segment, which do not interact directly with female genitalia and provide mechanical support to the intromittent parts.

Genital structures derived from a single abdominal segment have different functional properties (Genevcius and Schwertner 2017). For instance, in females, the eighth segment gives origin to a pair of movable structures (gc8) that protect the bursa copulatrix (internal female tract) and are pressed between the parameres and the dorsal rim of the pygophore in copula (Fig. 1b). This same segment also gives origin to two fixed plates (ltg8) that anchor the ventral rim of the pygophore. The functional interaction among these structures were used to construct the functional modularity models (see Fig. 1b and below).

Species and landmarks

The dataset comprises 52 species spanning all major lineages of Pentatomidae. We did not use all species from the previous chapter because either we had no access to one of the sexes or the external genitalia is reduced and covered by the seventh segment. The following taxa from Chapter 2 were used: *Acrosternum*, *Aelia*, *Agonoscelis*, *Amaurochrous*, *Antiteuchus*, *Arvelius*, *Banasa*, *Brachystethus*, *Brontocoris*, *Caonabo*, *Carpocoris*, *Chinavia brasicola*, *Chinavia obstinata*, *Chinavia obstinata*, *Chlorochroa*, *Chlorocoris*, *Chloropepla*, *Dinocoris*, *Dolycoris*, *Dryptocephala*, *Dyploxis*, *Edessa rufomarginata*, *Erthesina*, *Eurydema*, *Euschistus*, *Eysarcoris*, *Graphosoma*, *Halyomorpha*, *Loxa*, *Marghita*, *Mayrinia*, *Mormidea*, *Myota*, *Neotibialis*, *Neottiglossa*, *Nezara*, *Ochlerini*, *Oebalus*, *Oechalia*, *Macropygium*, *Pachycoris*, *Patanius*, *Pellaea*, *Pentatoma*, *Picromerus*, *Podisus*, *Rhaphigaster*, *Runibia*, *Scotinophora*, *Serdia*, *Tholosanus* and *Thyanta*. Only one specimen per species was used. Although it may seem problematic in principle, we believe this choice is justifiable. Characters of external genitalia are the most putative and reliable to distinguish between species in Pentatomidae. Virtually all taxonomic studies in the family show remarkable differences even between sister species (see Genevcius et al. 2017 and references therein). Our personal experience with species with large samples in museum collection suggests that the within species variation is negligible.

Male pygophores were removed and observed in dorsal view for the analyses, while the female plates were kept in the body and observed in ventral view. Landmarks were obtained from digital photographs taken in a Leica M205 C stereo microscope using the “auto-stack” tool. A total of 35 landmarks/semi-landmarks were used to describe the variation in female genitalia and 34 in males (Fig. 2a).

General morphometric procedures

Landmarks were digitalized in tpsDig v. 2.31 (Rohlf 2008) and then exported to the R environment for further analyses. Effects of size, orientation and translation were corrected using a Procrustes superimposition in the R package geomorph v. 3.0.5 (Adams and Otárola-Castillo 2013). During Procrustes superimposition, we combined both fixed and sliding semi-landmarks; the later were allowed to slide between adjacent landmarks with the algorithm of minimum bending energy.

We conducted a test of phylogenetic signal using the K_{mult} statistic to determine whether genital configurations tend to be more similar among closely related species due to common ancestry, i.e. phylogenetic non-independence (Adams 2014). The maximum likelihood tree from Chapter 2 was used, pruning the taxa not studied here. We also tested for the effect of size on shape variation by conducting a phylogenetic regression of shape data in function of the log-transformed centroid size. To females, we detected non-significant signals of both phylogenetic ($K = 0.5, p = 0.37$) and allometric ($p = 0.7$) effects, thus all further analyses were conducted with the Procrustes coordinates without corrections. To males, we detected significant phylogenetic ($K = 0.70, p = 0.01$) but no allometric ($p = 0.16$) signals. Thus, further analyses were conducted on the evolutionary covariance matrix calculated under a Brownian motion model of evolution rather than on raw Procrustes coordinates (Adams and Felice 2014).

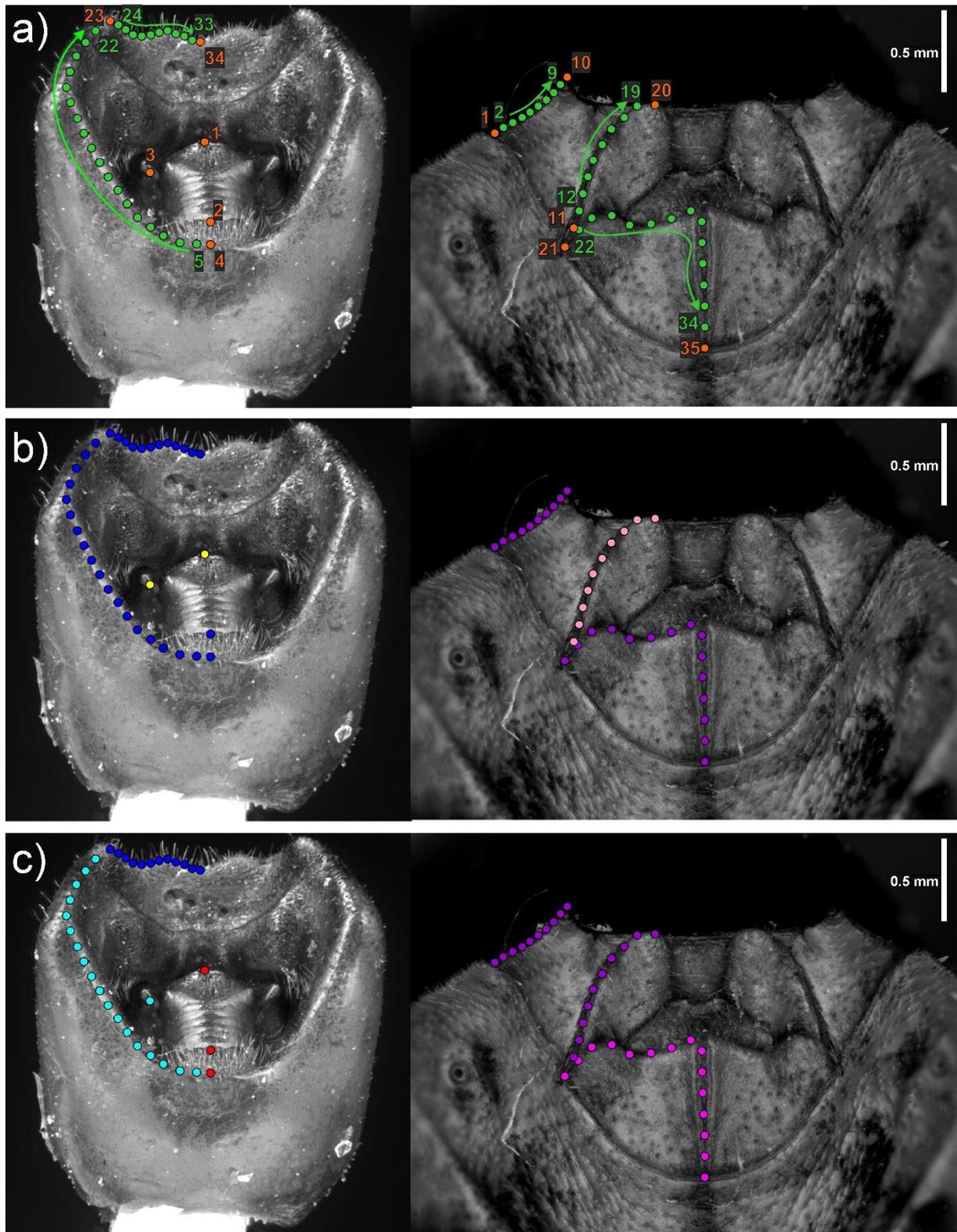


Figure 2. a) Landmarks (orange) and sliding semi-landmarks (green) used to sample the genital variation in males (left) and females (right). b) landmarks colored accordingly to the developmental modularity hypotheses. c) landmarks colored accordingly to the functional modularity hypotheses. See text and Fig. 1 caption for description of the hypotheses.

Male-female coevolution

To test for coevolution, we conducted a two-block partial least squares analysis in geomorph v. 3.0.5 (Adams and Otárola-Castillo 2013). This approach allow testing the degree of morphological integration between two sets of structures (Procrustes coordinates), simultaneously accounting for phylogeny, and under a Brownian motion model of evolution.

Modularity

Genital landmarks and semi-landmarks were tested for modular structure, firstly, using the CR coefficient in geomorph 3.0.5. The CR coefficient is a confirmatory approach, exhibiting appropriate type I error rates and higher statistical power in comparison to other approaches (Adams 2016). To each genitalia, we tested between two modularity models. The first is a *developmental modular model*, where genital components derived from the same abdominal segment in immatures are hypothesized to be different evolutionary subsets (Fig. 2b). This model represents a scenario where genital diversification is constrained by developmental factors. Information about the developmental origins of each genital component was derived from the Pentatomidae and Heteroptera literature (Bonhag and Wick 1953; Leston 1955; Scudder 1959; Genevcus and Schwertner 2017). The second is a *functional modular model*, where genital components that act jointly in copula are hypothesized to be semi-independent modules (Fig. 2c) (Leston 1955; Genevcus and Schwertner 2017).

The CR coefficient estimates the ratio of the overall covariation between modules in comparison to the overall covariation within modules (Adams 2016). The hypothesis defined *a priori* is then tested against a range of different modular configurations derived from random landmark permutations. However, because different pre-defined models are not directly comparable with the CR coefficient, it is possible that both the developmental and functional models show significant modular signal, rejecting the null hypothesis of no modularity. In this

case, to test between the two models, we employed a second test where the fitness of the data to different models is compared in a maximum likelihood framework (Goswami and Finarelli 2016). In this step, we compared the likelihood of the developmental and functional models, besides a null model where there is no modularity. These analyses were done using the R package EMLi (Goswami and Finarelli 2016).

RESULTS

Partial least square analysis revealed significant correlated evolution between male and female genitalia. Overall, most variation in male genitalia is related to three aspects: i) the degree of curvature in the dorsal rim of the pygophore (red structure in Fig. 3); ii) the distance between the paramere apex and the dorsal rim; iii) the distance between the tenth segment and the extension of the dorsal rim (yellow spots in Fig. 3). In species where all these three parameters are exaggerated (lower values in male PLS block; Fig. 3), female genitalia comprises shorter laterotergite 8, longer laterotergite 9, and longer gonocoxite 8.

Female genitalia showed positive signal of modularity according to the CR coefficient and EMLi model tests (Table 1). According to the CR tests, both developmental and functional models, separately, reject the null hypothesis of no modularity. While the EMLi model tests also rejected the null model, they indicate the developmental model as strongly most likely (Table 1).

Only the functional model of males was supported by the CR coefficient, while the developmental model was highly unlikely (Table 1). These results are congruent with the EMLi model tests, which rejects the null model of no modularity and indicates the functional model as highly more likely.

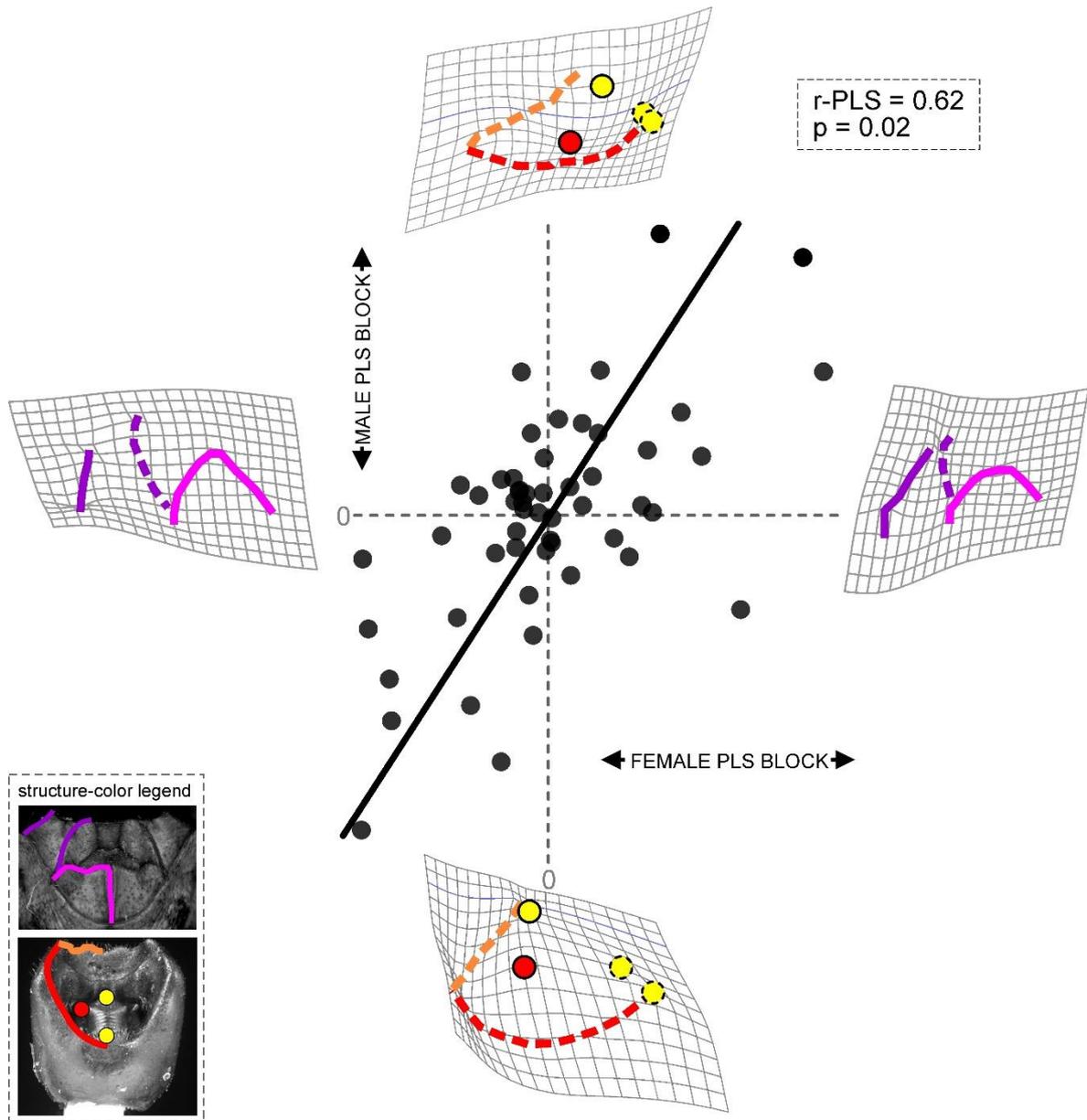


Figure 3. Evolutionary correlation between male and female genitalia from PLS analysis. Functionally correspondent structures are in the same color, while developmentally corresponding structures are in dashed/solid lines.

Table 1. Results of modularity tests. ML = maximum likelihood, K = number of parameters in the model, AICc = small sample size corrected Akaike information criterion.

Model	CR (<i>p value</i>)	ML	K	AICc	Posterior Probability
Female					
No modularity	-	-492.08	2	988.18	<0.001
Developmental	0.648 (0.001)	-180.98	4	370.03	1
Functional	0.639 (0.001)	-296.88	4	601.83	<0.001
Male					
No modularity	-	151.26	2	-298.49	<0.001
Developmental	1.607 (0.980)	170.21	3	-334.38	<0.001
Functional	0.834 (0.036)	278.5	7	-542.79	0.544

DISCUSSION

Our study provide new insights on the structural conformation of stink bug genitalia, and potentially to insects as a whole. We revealed that both male and female genitalia show clear and robust signals of modularity. While modularity has been proven prevalent in other systems like vertebrates cranium (Jojic et al. 2015; Simon and Marroig 2017) and insect wings (Ren et al. 2017; Blanke 2018), to our knowledge, this is the first evidence to genital traits. Our results corroborate theoretical expectations that genitalia should comprise independently evolving sub-structures diverging as a result of functional segregation (Song and Wenzel 2008; Werner and Simmons 2008) and, at least partially, to match developmental demands (Macagno and Moczek 2015). Studies to date had shown that, in different populations of a single species, distinct genital components can diverge in opposite directions (Song and Wenzel 2008; Tatarnic and Cassis 2013). However, studies as such only support the notion that different genital parts may be subjected to differential selective pressures in distinct ecological/geographical contexts. On the other hand, our results provide novel quantitative evidence of a persistent selective process favoring a mosaic modular architecture of genitalia in broad evolutionary scales.

Interestingly, we show that male and female genitalia, in spite of coevolving and being functionally complementary, are shaped by different factors. There is wide evidence that genital traits may be shaped simultaneously by multiple selective processes (Anderson and Langerhans 2015; Frazee and Masly 2015). However, those processes have been with no exception associated to copulatory and post-copulatory functional demands like mate guarding, sperm selection, egg laying, etc. Our study differs from these as we indicate alternative sources of selection, apart from function, in determining the evolution of genitalia. In female genitalia, both function and development may play a role in genital evolution, according to the CR coefficient. However, because the developmental model was strongly most likely when directly comparing the two models, the ontogenetic origin of these structures seems more determinant to the patterns of diversification in females. In contrast, there are no developmental influences over male genitalia. This is surprising to a certain extent because different subsets of the male genitalia like the dorsal rim and the ventral rim, although functionally separate, are adjacent and continuous parts of a single structure (Schaefer 1977; Genevcius and Schwertner 2017). This contrasts with the female genitalia, in which a single evolutionarily module comprises anatomically separate structures (e.g. the gc8 and lt8). These observations support the notion that the evolvability of a particular structure may be less dependent on how morphologically linked this structure is to another, but more to how functionally independent these structures are.

Two-block PLS analysis revealed significant intersexual genital coevolution. Theoretically, male-female coevolution may arise as result of both developmental and functional integrations (Brennan and Prum 2015; Macagno and Moczek 2015). Although it is not possible to discern unequivocally between these two mechanisms with our data, we believe that functional integration between the sexes is much more likely underlying the observed correlation. As we detected weak signals of developmental modularity in male genitalia, it is

very unlikely that overwhelmingly different structures in males and females develop and evolve under strong pleiotropic effects. Therefore, we propose that the coevolutionary dynamics in the stink bug genitalia has happened as follows. Modifications in a functional unit in one sex impose morphological adaptations in the other, guaranteeing the functional properties of genital attachment. Simultaneously, rates of female change are more evolutionarily constrained due to ontogenetic limitations. For instance, more pronounced gc8 are correlated with stronger curvatures in the dorsal rim of the pygophore. Even though these structures are intimately connected in copula, the levels of change and elaboration in the gc8 are limited because changes in this structure would inflict changes in the lt8, which in turn is involved in another functional role. This scenario, taken together with the relatively low rates of evolution of female genitalia in stink bugs, is congruent with several other studies showing that developmental factors are major constraints of morphological diversification (Galis et al. 2018). On the other hand, a lack of developmental constrains in male genitalia may help explain the high levels of change and elaboration exhibited by these structures. This observation is in line the notion that functional diversity, in the absence of any type of constrains, may be a great facilitator of genital diversification. To explicit test this hypothesis, future studies should attempt to quantify changes in the degree of modularity across taxa and correlate it with levels of morphological diversification.

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CONSIDERAÇÕES FINAIS

Ao longo do desenvolvimento deste trabalho, buscamos compreender um dos padrões mais prevalentes em animais com fertilização interna: a diferença nas taxas de evolução e complexidade entre genitálias de machos e fêmeas. Os pentatomídeos apresentam este mesmo padrão e possuem diversas características sexuais, tanto morfológicas quanto comportamentais, que tornam o grupo interessante para explorar estas questões. Estas características são discutidas na introdução geral. Antes de tratar da questão evolutiva diretamente, que corresponde ao capítulo 3 desta tese, foi necessário entender aspectos mais básicos – mas não menos interessantes - sobre estes organismos: o modo como as genitálias externas funcionam e a filogenia do grupo. Tais aspectos foram explorados nos dois primeiros capítulos. Abaixo resumimos o que encontramos de mais interessante ao longo dos três capítulos e concluímos fornecendo algumas direções para trabalhos futuros nos temas abordados.

No primeiro capítulo, observamos indivíduos em cópula e descrevemos como as peças genitais externas de machos e fêmeas interagem. Observamos que existem mecanismos passivos e ativos nesta interação. Também mostramos que alguns aspectos do acoplamento genital podem variar entre diferentes espécies/linhagens, enquanto outros parecem mais conservados. Esta variação nos mecanismos de interação genital nunca havia sido mostrada em outros grupos de insetos, uma vez que morfologia funcional genital raramente se aborda em um contexto comparativo, dada a dificuldade na obtenção, criação e experimentação com diversas espécies. Com este trabalho, estabelecemos aspectos básicos para a compreensão da peculiaridade das genitálias em Pentatomidae e ampla utilização no reconhecimento e proposição de espécies e outros grupos na classificação da família.

No segundo capítulo, inferimos a primeira filogenia para a Pentatomidae com ampla amostragem das principais linhagens reconhecidas atualmente na sua classificação.

Mostramos pela primeira vez que a família é monofilética utilizando grande amostragem de grupos internos e uma boa representatividade das famílias próximas como grupos externos. Suportamos também o reconhecimento de várias das linhagens propostas na literatura, e rejeitamos a monofilia de outras. Apesar de alguns clados da filogenia serem fracamente suportados, conseguimos cumprir os objetivos propostos para uma primeira filogenia de um grupo onde o uso de dados moleculares ainda é superficial. Concluimos que a inclusão de novos táxons e marcadores, em especial nucleares, serão fundamentais para melhorar a resolução e robustez da filogenia e permitir uma classificação estável e baseada na história evolutiva do grupo.

No terceiro e último capítulo, buscamos compreender a correlação entre função e desenvolvimento, e investigamos suas consequências macro-evolutivas. Apesar do crescente interesse de biólogos evolutivos e ecólogos sobre as genitálias, estudos sobre função e desenvolvimento, combinados, ainda são inexistentes. Primeiro mostramos que genitálias de macho e fêmea evoluem de forma modular, ou seja, possuem subunidades que evoluem de forma semi-independente umas das outras. Depois mostramos que genitálias de macho e fêmea evoluem de forma correlacionada. Por fim, apesar de coevolurem, mostramos evidências que a diversificação destas duas estruturas é direcionada por mecanismos diferentes. Enquanto a genitália do macho é determinada pela forma como suas peças interagem (ou deixam de interagir) entre si, as modificações nas genitálias de fêmeas parecem ser limitadas pelo desenvolvimento.

A publicação dos resultados deste projeto terão diversos impactos. De forma geral, esperamos contribuir com estudos sobre “evolução genital”, mostrando como mecanismos funcionais e do desenvolvimento podem impactar na evolução de estruturas que compõe um único sistema. Também mostramos que a integração de abordagens em escala micro e macro-evolutiva pode ser interessante para compreender os processos que determinam a grande

diversificação genetal observada no tempo evolutivo. Acreditamos que as questões mais interessantes a serem estudadas a seguir se referem às bases genéticas do desenvolvimento (*evo-devo*) das genitálias. Quais e quantos genes estão associados ao desenvolvimento genetal? Quais as diferenças genéticas entre macho e fêmea que resultam nos padrões de modularidade observados aqui?

Mais especificamente, avançamos no conhecimento da história evolutiva dos pentatomídeos. Primeiro, podemos ter uma noção sobre porque as estruturas mais importantes taxonomicamente se modificam nas formas que observamos. Segundo, estabelecemos uma filogenia que permitirá compreender como evoluem diversas características interessantes no grupo como hábitos alimentares, cuidado parental, etc. Os próximos passos neste sentido envolverão compreender como funcionam as partes genitais *internas* na família, que possivelmente estão associadas a processos de seleção de parceiros e competição espermática; em relação à filogenia, será prioridade a implementação de novos marcadores e novos táxons para que se atinja um conhecimento mais complexo sobre a diversificação de linhagens no grupo.

ANEXO 1

Artigo publicado

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“Strong functional integration among multiple parts of the complex male and female genitalia of stink bugs”

Strong functional integration among multiple parts of the complex male and female genitalia of stink bugs

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Genitalia are among the most studied phenotypes because they exhibit high anatomical diversity, experience fast evolutionary rates and may be shaped by several evolutionary mechanisms. A key element to uncover the mechanisms behind such impressive diversity is their copulatory function. This topic has been overlooked, especially concerning structures not directly involved in sperm transfer and reception. Here, we conduct a hypothesis-driven experimental study to elucidate the operation of various external genital parts in five species of stink bugs with differing levels of phylogenetic relatedness. These insects are unique because their male and female genitalia are externally well developed, rigid and composed of multiple components. In contrast with their anatomical complexity and diversity, we show that genital structures work jointly to perform a single function of mechanical stabilization during copula. However, distinct lineages have evolved alternative strategies to clasp different parts of the opposite sex. In spite of a high functional correspondence between male and female traits, the overall pattern of our data does not clearly support an intersexual coevolutionary scenario. We propose that the extraordinary male genital diversity in the family is probably a result of a process of natural selection enhancing morphological accommodation, but we consider alternative mechanisms.

ADDITIONAL KEYWORDS: coevolution – *Edessa* – *Euschistus* – functional morphology – *Mormidea* – *Podisus* – sexual selection – sperm competition.

INTRODUCTION

Extraordinarily divergent genitalia are ubiquitous across animal taxa with internal fertilization. The evolutionary forces behind this trend have sparked heated debate over the last decades, but most models of natural and sexual selection proposed have been at least partially supported (Hosken & Stockley, 2004; Masly, 2012; Brennan & Prum, 2015; Firman *et al.*, 2017). Distinguishing among these models in a particular group can be challenging because genitalia may exhibit similar patterns of differentiation and coevolution under different pressures. Thus, uncovering the origins of genital diversification is paramount to discern among alternative evolutionary mechanisms. In this sense, a key question is how different genital parts engage during copula

and how morphology relates to function (Jagadeeshan & Singh, 2006; Simmons, 2014; Wulff & Lehmann, 2016). In the taurus scarab beetle (*Ontophagus taurus*), two distinct functionalities have been described to four male genitalic sclerites: three sclerites act directly in sperm transfer comprising an integrated unit, while the other acts as a holdfast structure (Werner & Simmons, 2008). Such findings are crucial to explain how different parts are able to influence paternity or stabilize the genitalia in copula (Werner & Simmons, 2008), illustrating the importance of studies on functional morphology to detect sources of selection. The scarcity of studies on functional morphology of genitalia has been repeatedly pointed as a key obstacle that hinders the progress on this research field (Simmons, 2014; Brennan & Prum, 2015). Although functional integration between male and female is usually thought as a major source of coevolution, evidence for such correlation is yet limited. In

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fact, the most comprehensive study dealing with this subject has found weak evidence to such correlation (Richmond, Park & Henry, 2016).

Insects are probably the most representative organisms in studies on genital evolution. Assessments of their genital functionalities have revealed peculiar and unique modes of operation such as traumatic insemination (Tatarnic, Cassis & Hochuli, 2006; Kamimura, Tee & Lee, 2016), mating plugs (Baer, Morgan & Schmid-Hempel, 2001; Seidelmann, 2015), sonorous genitalia (Sueur, Mackie & Windmill, 2011) and female penises (Yoshizawa *et al.*, 2014). Three major biases may be identified in studies with insect genitalia. First, thorough investigations covering both functional and evolutionary aspects have been mostly conducted with a few model groups such as flies (e.g. Eberhard & Ramirez, 2004), beetles (e.g. Hotzy *et al.*, 2012) and water striders (e.g. Fairbairn *et al.*, 2003). Second, the historical male bias that characterizes the research on genital evolution as a whole (Ah-King, Barron & Herberstein, 2014) also seems to apply to insects. Third, given the growing acceptance of sexual selection as a preponderant mechanism, studies examining structures associated to sperm transfer and sensory communication are increasingly predominant in relation to those examining structures with secondary sexual functions. However, recent studies provide unequivocal evidence that male and female genitalia may be shaped by alternative processes other than the traditional cryptic female choice and sexual antagonistic coevolution (e.g. Wojcieszek *et al.*, 2012; House *et al.*, 2013; Anderson & Langerhans, 2015; Varcholová *et al.*, 2016). This raises the question of whether these mechanisms of sexual selection are indeed overwhelmingly prevalent as usually thought, especially considering our elusive knowledge on the function and diversity of genitalia in numerous understudied groups.

Among insects, stink bugs (Hemiptera: Pentatomidae) stand out for particularities in male and female genitalia. Both sexes exhibit highly complex and well-developed internal and external genital parts (Sharp, 1890; Marks, 1951) with presumable diverse functionalities (Genevicius, Caetano & Schwertner, 2017). While a couple of studies with pentatomids have linked their intromittent genitals to a complex system of sperm selectivity, transfer, regulation and storage (Adams, 2001; Stacconi & Romani, 2011), the function of non-intromittent external parts in copula remains virtually unknown. The non-intromittent part of the male organ (=pygophore, male external genitalia herein) is characterized by extraordinary diversity and species specificity, being consistently the most decisive characteristics in taxonomic studies and showing strong phylogenetic structure at different levels (e.g. Grazia, Schuh & Wheeler, 2008;

Ferrari, Schwertner & Grazia, 2010; Genevicius, Grazia & Schwertner, 2012). The structure comprises a capsule and associated structures that can take the form of folds, projections and hooks, originated from a series of modifications and fusions between the ninth and tenth abdominal segments (Bonhag & Wick, 1953; Schaefer, 1977). The female external genitalia is composed of various flattened plates that cover the genital opening, derived from the eighth, ninth and tenth segments (Scudder, 1959). A recent study has found an evolutionary correlation between the pygophore and a pair of female plates, but the functional significance of this trend remains to be investigated (Genevicius *et al.*, 2017). Although our knowledge on how these structures operate is vague, their remarkable diversity and species specificity suggest an important sexual and evolutionary role in the family which has never been scrutinized.

In this study, we examined the role of the genital parts that presumably interact externally during copula in Pentatomidae. Given the morphology of the external genitalia of its members, the group offers an interesting model to study the interplay between genitalia function, complexity and evolution in structures disassociated to sperm transfer. We reviewed the literature and compiled a series of testable hypotheses of functional mechanics in the group (Table 1). We performed mating trials for five species showing varying degrees of phylogenetic relatedness and conducted a series of detailed morphological observations to address the following questions: (1) How do the external parts of the male and female genitalia interact with one another during copula? (2) Do the modes of operation vary across species of different lineages of the family? Our results revealed an entangled mechanism of functional integration in which several parts of the genitalia operate in a cooperative fashion to provide stabilization during copula. Furthermore, we show significant among-species variation in the attachment mechanism, suggesting distinct evolutionary strategies to clasp the opposite sex exhibited by different lineages. We discuss how our data adequate to the functional hypotheses, the evolutionary implications of the genital interactions observed and possible underlying mechanisms.

MATERIAL AND METHODS

MORPHOLOGY AND TERMINOLOGY OF GENITAL PARTS

The terminology used to refer to the male genital components in Heteroptera has been historically inconsistent. Schaefer (1977) compiled and discussed the contrasting classification in Pentatomomorpha (which includes Pentatomidae and related families), proposing a unified terminology. Here, we followed his

Table 1. Hypotheses of functional morphology of the external genitalia compiled from literature with reference to the taxon to which each hypothesis has been proposed

Structure	Taxon	Functional hypothesis	Reference	Support
Parameres	Pentatomidae	H1. 'The functions of the claspers [...] to assist in separating the genital sclerites of the female, and to assist as clasping organs during copulation'.	Baker (1931)	Corroborated
Parameres	Hemiptera	H2. 'Also, it appears [...] that the parameres do operate to some extent in keeping apart the gonapophyses which hide the female gonopore...'	Singh-Pruthi (1925)	Rejected
Parameres	<i>Piezodorus lituratus</i> (Pentatomidae)	H3. 'During copulation in Pentatominae the male gonopods are pressed against the outside of the 2nd valvifers of the female'	Leston (1955)	Rejected
Ventral rim of pygophore	Geocorisae (Terrestrial Heteropterans)	H4. '...the infolded portion of the ventral rim , [...] presumably share the function of holding and guiding the aedeagus during copulation.'	Schaefer (1977)	Partially rejected
Ventral rim of pygophore	Geocorisae (Terrestrial Heteropterans)	H5. 'These structures [the infolded portion of the ventral rim] appear to have limited functional significance, because they are usually immovable and not provided with muscles; they may provide tactile clues to the female and/or provide support to the various movable structures during copulation.'	Schaefer (1977)	Partially rejected
Pygophore	Pentatomidae	H6. 'The aesthetic aspect of the arrangement [of the genital chamber] in many of the higher species, [...], is very remarkable, but I do not think there is at present evidence that would justify us in attaching any special biological importance to it.'	Sharp (1890)	Partially rejected

Column 'structure' refers to the terminology used here, while the original terminology is indicated in bold within the hypothesis quote. Column 'support' denotes whether the hypothesis was supported herein.

terminology with a few additions of other recent studies (Genevcius *et al.*, 2012).

The male genitalia is roughly a tube-like sclerotized capsule (=pygophore) with associated structures (e.g. a pair of claspers) and an internal phallus. Although some authors refrain to use the terms 'external' and 'internal' genitalia, we designate internal genitalia as the movable intromittent parts that penetrate the female internal tract, whereas the capsule itself, the parameres and the tenth segment are considered as external genitalia. The pygophore can be divided into a dorsal and a ventral wall. Since it remains twisted in 180° inside the male's body while in rest position, the ventral and the dorsal sides are opposite to the body's plans (Schaefer, 1977). All structures can be seen in dorsal view, including the posterior extremity of the ventral wall, denominated ventral rim (Fig. 1).

We follow Grazia *et al.* (2008) to the female parts, which compiled the nomenclature and reviewed homology statements. The morphology of the female genitalia is relatively simpler, comprising a series of soft tubes and chambers (the internal genitalia) covered by various sclerotized plates (the external genitalia). The opening of the female internal tract gets covered by the larger genital plates, the gonocoxites 8 (Fig. 1E, F). The terminology of all genital parts and respective abbreviations used in this work are described in Figure 1 and Table 2.

SPECIES CHOICE, COLLECTION AND REARING

We investigated the functional morphology of male and female external genitalia in five species of Pentatomidae in a hypothesis-driven approach

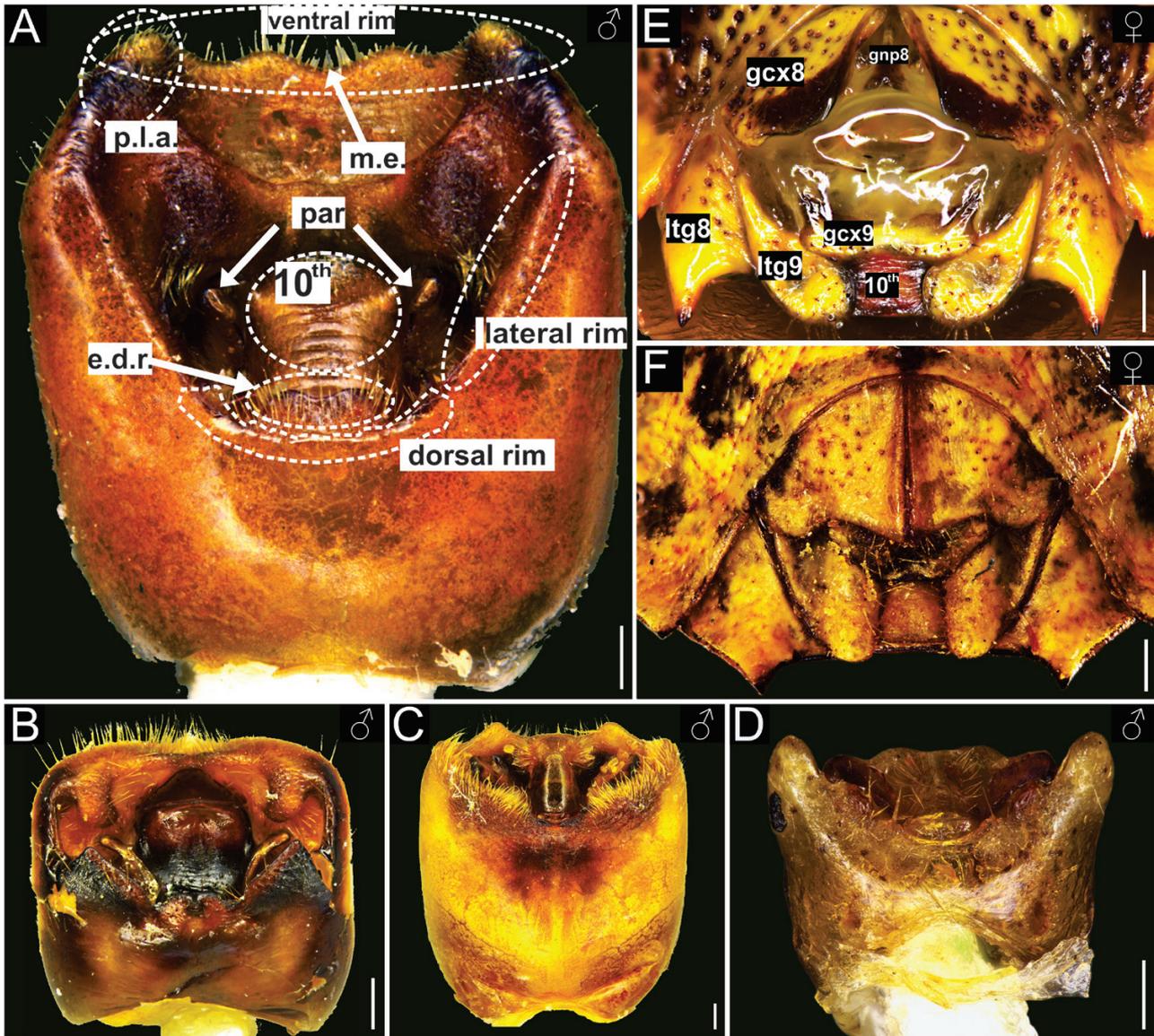


Figure 1. Male (A–D) and female (E, F) external genitalia of the studied genera, with terminology and abbreviations indicated. Female genitalia are represented with the internal tract exposed (E) and unexposed (F). A and F = *Euschistus heros*; B and E = *Mormidea v-luteum*; C = *Edessa meditabunda*; D = *Podisus nigrispinus*. Scale bar is 0.25 mm.

(Table 1). To examine whether the general system of attachment between the genitalia vary within the family, we chose species with different levels of relatedness. Even though a complete phylogeny of the family does not exist, different phylogenetic studies support the recognition of different groups within Pentatomidae (Gapud, 1991; Bistolos *et al.*, 2014; Banho, 2016; Wu *et al.*, 2016) with certain congruence with the current taxonomic classification in tribes and subfamilies (Rider *et al.*, 2017). The five species studied herein represent three of the four major and most diverse lineages of Neotropical pentatomids

(i.e. Asopinae, Discocephalinae, Edessinae and Pentatominae).

We selected two species from the same genus, *Mormidea v-luteum* (Lichtenstein) and *Mormidea maculata* (Dallas), and a third species from the same tribe as the *Mormidea*, *Euschistus heros* (Fabricius). The three species belong to the group of the Neotropical Carpocorini (subfamily Pentatominae). The fourth and the fifth species belong to other subfamilies: *Podisus nigrispinus* (Dallas) (Asopinae) and *Edessa meditabunda* (Fabricius) (Edessinae). We manually collected specimens in the municipality of Diadema, São Paulo,

Table 2. Abbreviations of the genital parts used in text and figures

Abbreviation	Structure
Female	
gcx8	Gonocoxite 8
gcx9	Gonocoxite 9
gnp8	Gonapophysis 8
ltg8	Laterotergite 8
ltg9	Laterotergite 9
Male	
e.d.r	Extension of dorsal rim
m.e.	Median excavation
p.l.a.	Posterolateral angle
Par	Paramere
Pyg	Pygophore
s.p.	Superior process

Brazil (−23.7204, −46.6276) and maintained them in laboratory inside plastic cages of 2 L. Males were reared separately from females prior to the experiments using the following conditions to all species: 26 ± 2°C, 70 ± 10% relative humidity and photophase of 14 L:10 D. Individuals of *E. heros* and *Ed. meditabunda* were fed on bean pods (*Phaseolus vulgaris*) and peanut seeds (*Arachis hypogaea*), *M. v-luteum* and *M. maculata* on branches of *Brachiaria* sp. and *P. nigrispinus* on larvae of *Tenebrio molitor*.

EXPERIMENTAL APPROACH

We randomly formed couples which were maintained in separate cages during the mating trials. The number of couples observed per species (*n*) varied from three to 12 (*E. heros* = 12, *Ed. meditabunda* = 3, *M. v-luteum* = 10, *M. maculata* = 8, *P. nigrispinus* = 3). All observations were consistent showing no differences among pairs of the same species. Mating pairs were frozen in copula in a −20°C freezer. Because pentatomids commonly tend to copulate for several hours (McLain, 1980; Rodrigues *et al.*, 2009), we were able to wait several minutes after copula had started to guarantee that genitalia were properly coupled. After 20 min in the freezer, mating pairs were pinned and promptly analysed in a stereomicroscope Leica MZ205C. Photographs were taken firstly of the attached genitalia and secondly after slight manipulations, using a Leica DFC450 and the Leica Application Suite software with Z-stacking acquisition.

RESULTS

The arrangement between male and female genitalia from a dorsal view of the pygophore was similar in all

species. Left and right gcx8 were the only mobile structures of the female genitalia. They touch the dorsal side of the pygophore and are pressed against the lateral rim (Fig. 2) by the parameres internally (Fig. 3). This connection apparently comprises the tightest point of attachment between the two genitalia. In *P. nigrispinus*, the gcx8 is also grasped externally by the superior processes (=genital plates according to some authors). The parameres and the superior processes function as tweezers to keep the gcx8 opened (Fig. 3). The opening angle of the gcx8 differed slightly among species. In *M. v-luteum* and *Ed. meditabunda*, the gcx8 remains virtually parallel to the male's body plan (Fig. 2C, D), whereas the angle is around 45° in the remaining species (Fig. 2A, B). In all five species, the connection between genitalia is probably mediated by several sensory setae mostly concentrated on the e.d.r. and p.l.a. of males and on the internal angles of the gcx8 of females (Fig. 3).

The ventral rim of the pygophore makes direct contact with the female plates in all species except *P. nigrispinus*. However, we found three different modes of accommodation between these two traits, each mode corresponding to one genus. In *E. heros*, the ventral rim of the pygophore is pronouncedly differentiated to engage with the female plates (Fig. 4A); the posterolateral angles fit between the ltg8 and ltg9 while the sinuosity of the ventral rim matches the ltg9 and tenth segment (Fig. 4A). In the *Mormidea*, the ventral rim of the pygophore is less modified showing only a simple v-shaped median excavation (Fig. 1B); the m.e. fits the gcx9, whereas the ltg8, ltg9 and tenth segment remain untouched by the pygophore (Fig. 4B, C). In *Ed. meditabunda*, the p.l.a. of the pygophore makes contact with the outer side of the ltg8 (Fig. 4D). In such species, both the ltg9 and tenth segment lie in the median excavation of the pygophore (Fig. 4D), and the tenth segment is untouched by the ventral rim. We could not visualize whether the gcx9 engages with a specific portion of the male genitalia in *E. heros* and *Ed. meditabunda* because it was covered by the pygophore ventrally and by the gcx8 dorsally. In *P. nigrispinus*, the ventral rim of the pygophore is not well developed and does not engage with any of the female plates. In this species, the attachment between the genitalia is mediated exclusively by the parameres, lateral rim and superior processes (Fig. 3A).

In the Carporini (i.e. *E. heros*, *M. v-luteum* and *M. maculata*), the e.d.r. of the pygophore is well developed and bifurcated (Fig. 1A, B). This structure is used to accommodate the gnp8 (Fig. 5A), which is covered by the gcx8 while in rest position (Fig. 1E, F). In these three species, the bifurcation of the e.d.r. fits thoroughly the median longitudinal elevation of the gnp8 (Fig. 5A). In *P. nigrispinus* and *Ed. meditabunda*, the

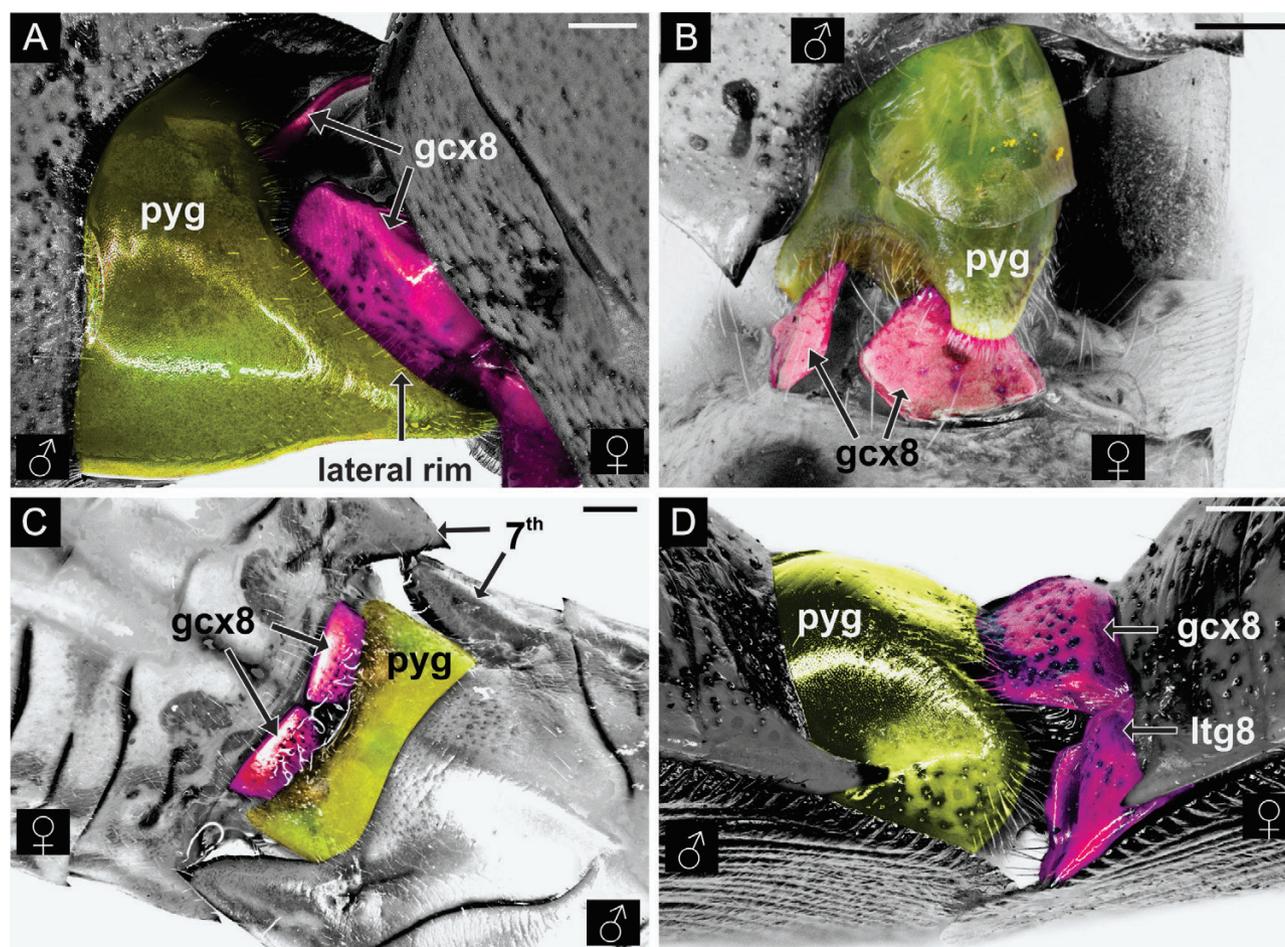


Figure 2. Attached genitalia after 20 min in copula, dorsolateral perspective of the pygophore. Male traits are highlighted in green and female traits in pink. A = *Euschistus heros*; B = *Podisus nigrispinus*; C = *Edessa meditabunda*; D = *Mormidea v-luteum*. Scale bar is 0.4 mm.

e.d.r. is vestigial and do not participate in the connection with the gnp8 (Fig. 3A). We could also visualize the interaction between some anatomical parts that were not focus of our study but can be relevant to interpret mechanisms of evolution (see ‘Discussion’ section). In *Ed. meditabunda*, the last pre-genital abdominal segment (i.e. the seventh segment) is strongly extended and thickened. The male projections of the seventh segment anchor on the inner side of the female projections (Fig. 2C). Such anchoring may be important to avoid the rotation of the individuals in copula. After slight manipulation to decouple the mating pairs, we could visualize the intromittent male genitalia (=phallus) inflated inside the female tract (Fig. 5B). While the external parts could be easily untied, this internal connection was much tighter.

In summary, both the dorsal rim of the pygophore and the parameres work jointly to support and keep

the gcx8 opened in all species (Figs 2, 3). In addition, the similarity among all species (except *P. nigrispinus*) was the perpendicular connection between the pygophore and the female genitalia in which the female plates accommodate the ventral rim of the pygophore. However, each genus exhibited a different pattern with respect to which plates engage with the curvatures of the ventral rim and in which portion of the ventral rim the plates get supported (Fig. 4). In *E. heros*, the ventral rim touches all the unmovable plates; in the *Mormidea*, only the gcx9 interacts with the ventral rim; in *Ed. meditabunda*, the Itg9 and the tenth segment lie in the median excavation and the p.l.a. touches the outer side of the Itg8; in *P. nigrispinus*, the ventral rim does not touch the female genitalia at all. Furthermore, the Carpocorini (i.e. *Euschistus* and *Mormidea*) showed an additional point of stabilization, between the e.d.r. and the gnp8.

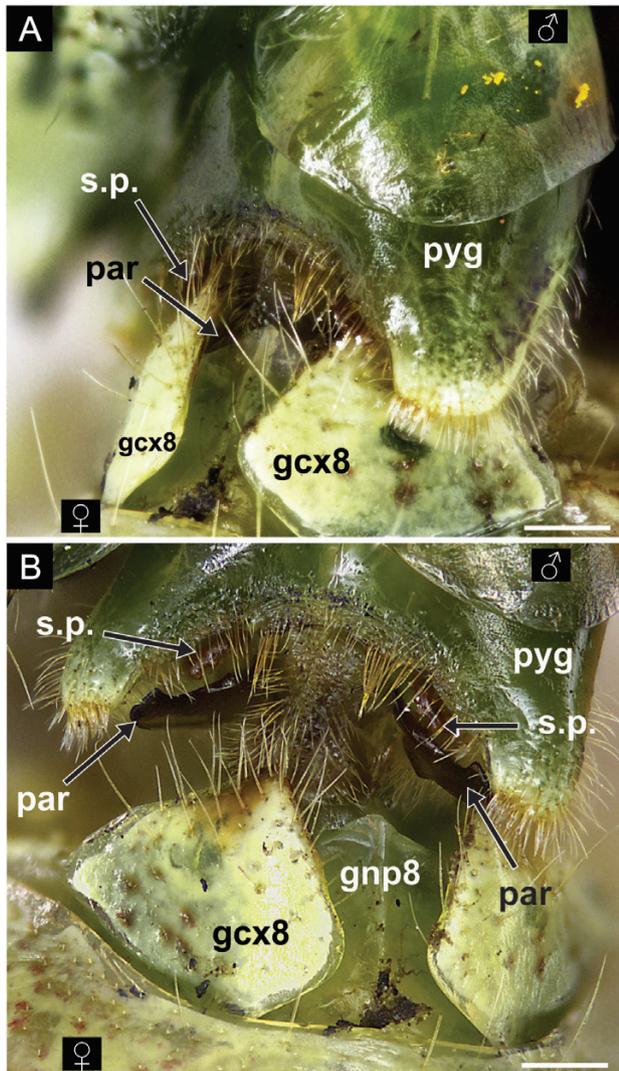


Figure 3. Genitalia of *Podisus nigrispinus* during copula (A) and after a slight manipulation, with the structures hidden by the gcx8 indicated (B). Scale bar is 0.2 mm.

DISCUSSION

Our study revealed a unique pattern of strong functional integration among multiple parts of male and female external genitalia. Several male parts, mostly located in the dorsal face, accommodate one or more parts of the female external genitalia. Some of such male structures are apparently modified and specialized to this function. Interestingly, the mechanism of attachment between the genitalia varied among the lineages once certain homologous parts of the male genitalia in different species engage with different parts of the female genitalia. Below we discuss how our data fit the functional hypotheses derived from literature, the evolutionary trends of the genitalia and the probable underlying mechanisms.

HYPOTHESES OF FUNCTIONAL MORPHOLOGY

Sharp (1890) suggested that the pygophore does not participate directly in the copulatory process and it should instead function to protect the internal parts (H6; Table 1). Although it is not possible to discard this 'protective hypothesis' with our data, we uncovered an important role of accommodation of the female parts by the pygophore, rejecting his hypothesis at least partially. The most explicit fastening structure of the male genitalia was the ventral rim of the pygophore, which fits either the ltg8, ltg9 and the tenth segment or the gcx9. The ventral rim has apparently evolved to retract in its parts that touch the female plates. Since the female genitalia is being pushed towards the outside by the parameres, such fit between the ventral rim and the female plates probably helps to avoid the male capsule do slide laterally. Particularly in *P. nigrispinus*, where the ventral rim does not participate in the genital attachment, the superior processes appear to perform this function. These results are to a certain extent in disagreement with Schaefer's (1977) hypotheses that the ventral rim has limited functionalities and should mainly support the internal parts of the male genitalia (H4 and H5). Although the ventral rim *per se* is clearly engaged with the female genitalia externally, it is possible that certain structures derived from the ventral rim (e.g. the cup-like sclerite) interact with the internal parts during and after intromission. Unfortunately, we were not able to visualize the operation of the internal parts because they were completely covered by the male capsule and the female plates.

We showed that the parameres operate in holding the female gcx8 opened to provide access of the phallus to the internal female genitalia. This result is in line with Baker's (1931) hypothesis (H1) and with the operation mode observed in true bugs of other families (e.g. Moreno-García & Cordero, 2008). However, the parameres keep the gcx8 separate by pressing their inner surface, contrary to Leston's (1955) hypothesis which suggest contact with the outer surface of the gcx8 (H3). Our results also refute Singh-Pruthi's (1925) hypothesis (H2) by showing that the female gonapophyses 8 are supported by the e.d.r. of the pygophore and not by the parameres. In summary, we fully rejected H2 and H3, partially rejected H4, H5 and H6 and corroborated H1.

FUNCTIONAL INTEGRATION AND GENITAL EVOLUTION

Anatomically diverse genitalia are usually thought to be also diverse in function (Huber, 2004; Song & Wenzel, 2008), implying that distinct selective pressures should operate within a single genitalia (Rowe & Arnqvist, 2012). This has been shown true even to structures that are physically connected (Song & Wenzel,

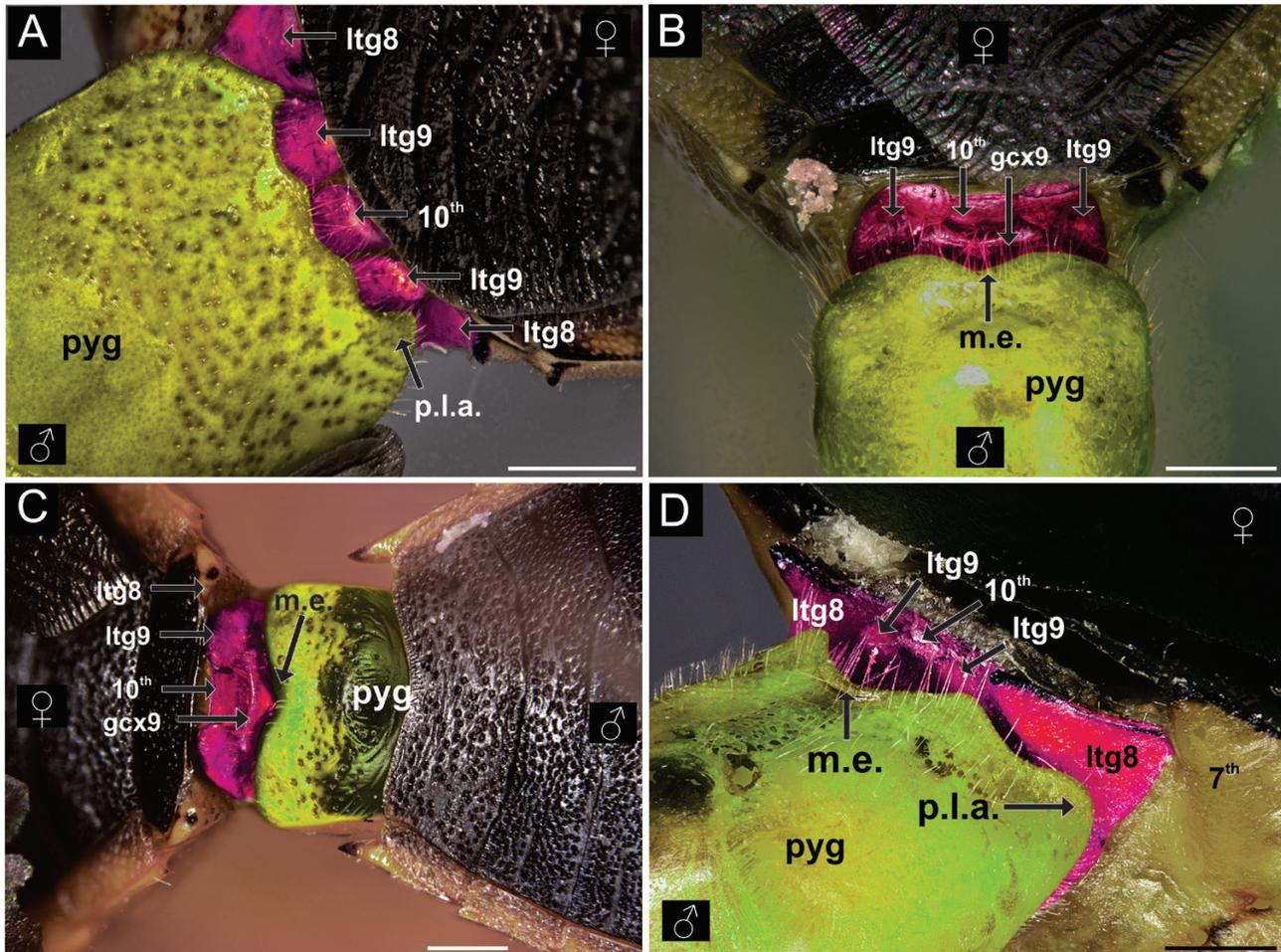


Figure 4. Attachment between the ventral rim of the pygophore (green) and the female plates (pink) from ventral (A–C) and ventrolateral (D) perspective of the pygophore. A = *Euschistus heros*; B = *Mormidea maculata*; C = *Mormidea v-luteum*; D = *Edessa meditabunda*. Scale bar is 0.5 mm.

2008). In contrast with this general view, we show that the multiple components of the Pentatomidae external genitalia are integrated to function exclusively as anchoring structures. The ventral rim of the pygophore is the most obvious example since it interacts with at least three of the five female external parts in most species (i.e. Itg8, Itg9 and tenth segment). The female gcx8 is analogous and shows a similar level of integration, interacting simultaneously with the lateral rim, e.d.r and the parameres. These results indicate that the external genitalia of the Pentatomidae comprise a system of strong level of functional integration, which means that their parts are prone to vary in a combined and coordinated manner. Accordingly, we suggest that virtually all external genital parts studied here should be directly or indirectly integrated to each other to some degree, a process similar to the one shown in a dung beetle (House & Simmons, 2005; Werner & Simmons, 2008).

In systems as such, it is intuitive to predict that changes in one component would entail changes in another to maintain the coordination integrity among the parts (Klingenberg, 2014). For instance, as the gcx8 is supported on one side by the parameres and by the lateral rim on the other, some level of evolutionary correlation among these three traits would be expected. Nevertheless, our data are limited in supporting an intersexual coevolutionary process between the genitalia. While various anchoring parts of the male genitalia are morphologically peculiar and species specific, the female plates were relatively more constant among the species we studied. For example, the e.d.r. of *E. heros*, *M. v-luteum* and *M. maculata* is differentiated to grasp the female gnp8, but the gnp8 is mostly invariable among all species we analyzed. Several other structures of the male genitalia seem much more diverse among species than the female plates such as the parameres, the tenth segment and the ventral rim,

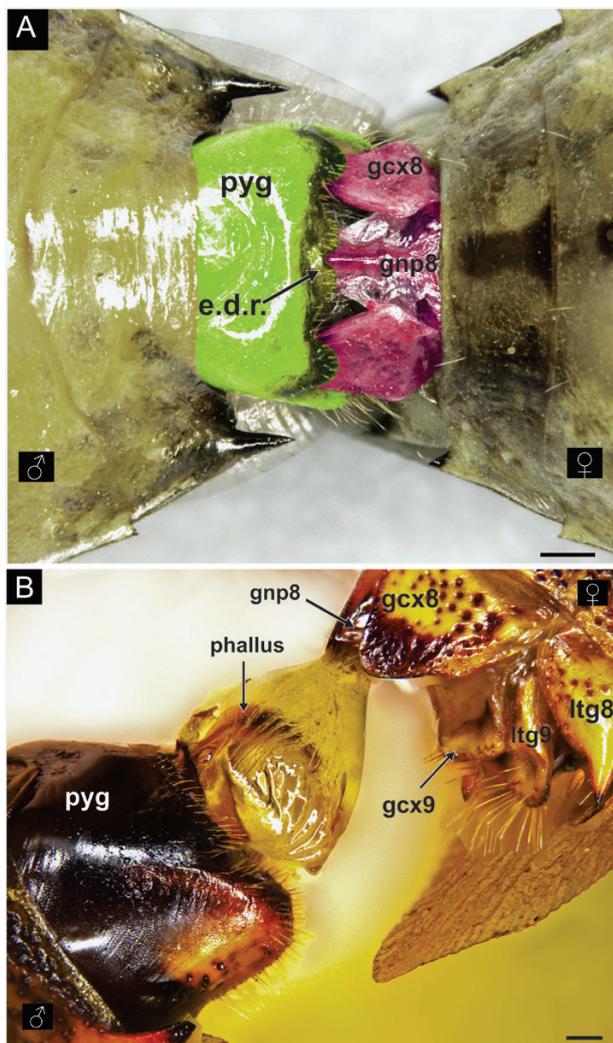


Figure 5. Genitalia of *Mormidea maculata* in copula (A) illustrating the connection between the e.d.r. of the pygophore (green) and the female gnp8 and gcx8 (pink); connection of the internal genitalia of *Mormidea v-luteum* exposed after manipulation (B). Scale bar is 0.3 mm.

what is consistently observed across the taxonomic literature (e.g. Ferrari *et al.*, 2010; Genevcius *et al.*, 2012). These observations suggest that a probable process of selection enhancing the mechanical fitness of the genitalia should be acting essentially or predominantly over male genitalia, while female genitalia should be subjected to a weaker selective pressure (Genevcius *et al.*, 2017). Alternatively, female genitalia may be constrained due to other processes such as intersexual differences in gene expression and regulation during the developmental process (Aspiras, Smith & Angelini, 2011).

Although we found no explicit evidence of coevolution, it should be noted that our approach only allows for examination of qualitative variation exhibited by

the genitalia. Thus, we cannot rule out the hypothesis that female genitalia may evolve in response to male genitalia in a small scale, detectable only by approaches that take continuous variation into account. This hypothesis is somewhat in line with a recent study with stink bugs which shows lesser changes in female genitalia compared to fast-evolving male genitalia in a coevolutionary scenario (Genevcius *et al.*, 2017). Because rates of genitalia change have rarely been quantified to males and females simultaneously, similar scenarios with other groups are unknown and we are not able to speculate about its prevalence across animals. We believe that various structures of the Pentatomidae genitalia are candidate to be tested for coevolution using continuous data: the margins of the gcx8 and the curvatures of the dorsal rim of the pygophore, the shape of the parameres and the concavity of the gcx8, the length of the spines of the seventh segment, among others. Future fine-scale studies will allow one to test whether different levels of functional integration exhibited by different lineages are good predictors of evolutionary correlation.

CONVERGENCE AND EVOLUTIONARY TRENDS OF THE PENTATOMIDAE GENITALIA

The overall taxonomic literature of stink bugs documents high levels of pygophore species specificity. However, if pygophore conformation has fitness consequences and female plates are more evolutionarily conserved, one would expect the repeated evolution of certain male shapes across different lineages. Within *Euschistus*, the biconvex ventral rims of the pygophore in several species are similar to the observed to *E. heros*, for instance in *E. atrox*, *E. acutus*, *E. cornutus*, *E. emoorei*, *E. irroratus*, *E. nicaraguensis*, *E. schaffneri* and *E. stali* (Rolston, 1974; Bunde, Grazia & Mendonça-Junior, 2006). As at least four of these species belong to well-separated lineages (Weiler, Ferrari & Grazia, 2016; Bianchi *et al.*, 2017), we may presume at least four episodes of convergent evolution within this genus. By briefly analyzing the taxonomic literature, we found five other species belonging to other tribes and subfamilies that exhibit similar ventral rims: *Acledra* spp. (Faúndez, Rider & Carvajal, 2014), *Cahara incisura* (Fan & Liu, 2013), *Braunus sciocorinus* (Barão *et al.*, 2016), *Edessa puravida* (Fernandes *et al.*, 2015), *Mecocephala bonariensis* (Schwertner, Grazia & Fernandes, 2002). This series of potential convergences reinforce that shape changes of the pygophore in the parts that touch the female plates (and vice versa) are advantageous strategies to perform an effective genital coupling in Pentatomidae.

Interestingly, our analyses revealed that pygophores of different species have evolved in distinct directions

to achieve morphological stability in copula. This idea is supported by the fact that certain male parts in different species engage with different parts of the females. For instance, the ventral rim of the pygophore engages with the female ltg8, ltg9 and tenth segment in *E. heros*, with the gcx9 in the *Mormidea* spp., with the ltg8 in *Ed. meditabunda* and does not engage with the female genitalia at all in *P. nigrispinus*. We observed certain particularities in the modes of interaction among male and female parts to the four genera studied here, despite the fact that female genitalia are relatively similar in these species. This raises the intriguing question of how many other modes of morphological correspondence exist within Pentatomidae. We believe the extraordinary diversity of pygophores and the existence of peculiar female plates across several lineages of Pentatomidae (Schuh & Slater, 1995; Rider *et al.*, 2017) suggest the existence of a high diversity of alternative mechanisms of genital coupling.

Another intriguing implication of our results concerns the use of genital characters in phylogenetic analyses of pentatomids. We hypothesize that several genital structures of the Pentatomidae, if not all, may be more phylogenetically dependent among each other than previously thought due to a mechanism favouring their morphofunctional integrity. This raises the question of whether using disproportional amounts of genital characteristics in phylogenetic reconstructions may result in strongly genitalia-biased phylogenies that rely on few dependent evolutionary processes. We are not arguing that genital characters should be rejected *a priori*, especially considering their proven phylogenetic usefulness in insect systematics (Song & Bucheli, 2010). However, since character independence is basically a presumption of the majority of phylogenetic methods (O'Keefe & Wagner, 2001), this issue should be considered with caution.

WHICH EVOLUTIONARY MECHANISMS ARE MOST LIKELY?

The majority of studies on sexual behaviour of pentatomids report long copulations, sometimes spanning several days. Such mechanism of prolonged copulation seems to be controlled by the male to avoid male–male competition for mates and thereby avoid sperm competition (McLain, 1980; Wang & Millar, 1997). The mechanism employed by males to hold the females is unknown to date, but our results shed some light on this topic. We showed that the external structures of the genitalia are not associated to sperm transfer/storage and thereby should not influence paternity because they interact externally and work as anchoring structures. By manipulating the genitalia to investigate the tightest points of attachment between the

individuals, we observed that the external connection was relatively fragile and could be easily untied. On the other hand, the attachment between the inflated phallus and the female internal tract was much stronger (Fig. 5B), indicating that such internal connection might be the determinant mechanism to avoid female access to other males. It should be considered the possibility that while individuals were alive, the parameres could be boosted by muscles to hold the females and the external connection could be actually stronger than we observed with recently dead specimens. However, a functional study with other terrestrial true bug with relatively similar genitalia suggest a passive mode of operation of the male parts coupled with a cooperative movement of the female plates (Moreno-García & Cordero, 2008). While it remains to be tested whether the inflated phallus has a role in physically displacing rival sperm, our study suggest that they participate at least indirectly in the avoidance of sperm competition by holding females and preventing them from subsequent copulations.

Our results coupled with other experiments with true bugs suggest that both sperm transfer/storage and female holding are performed by interactions of the internal genitalia (Moreno-García & Cordero, 2008; Stacconi & Romani, 2011; Genevicius *et al.*, 2017). Accordingly, the external traits are probably disassociated to any function that may directly influence paternity and intersexual conflict for the control of mating. The apparent absence of male–female coevolution and damaged genitalia in museum collections, as well as the passive mating behaviours exhibited by pentatomids (e.g. Wang & Millar, 1997), provide additional support for this hypothesis. Therefore, we believe our data are more indicative of a scenario of natural selection to the external genitalia, which could happen essentially via pure morphological accommodation or species specificity reinforcement (Brennan & Prum, 2015). Since different studies with pentatomids report viable copulation between species with differentiated external genitalia (Foot & Strobell, 1914; Kiritani, Hokyo & Yukawa, 1963), we believe selection favouring the interlocking effectiveness of genitalia rather than species reinforcement is more plausible (Richmond *et al.*, 2016). However, because we do not know whether and how the external genitalia may interact with the internal parts, an additional aspect should be considered. If the pygophore is used to provide support to the movable internal structures as hypothesized by Schaefer (1977), the morphological diversity exhibited by the external genitalia may have arisen also as a by-product of sexual selection acting on the shape of internal parts. Because most of these mechanisms are not mutually exclusive, discerning among them will be possible through an examination

of the internal and external parts using histological and micro-computed tomography techniques.

CONCLUSIONS

Our study revealed an interesting interlocking genital system in which male and female external structures are functionally integrated to stabilize the genitalia during mating. Furthermore, species from different lineages have evolved to engage with distinct parts of the opposite sex. Because female genitalia are greatly more constant than male genitalia when comparing species, the processes that lead to such morphological stability are certainly more directed to the male parts. Literature data and our observations indicate neither intersexual conflict nor a direct participation of the external parts in sperm transfer and storage. Accordingly, we believe the genital traits we studied here are more prone to a process of natural selection, most probably enhancing the morphological accommodation rather than species reinforcement. To discern among mechanisms of evolution, further studies should attempt to determine why selection should favour a stable and strong coupling, which may be either cooperative or conflicting. Fine-scale analyses using histology and micro-CT scan techniques will make possible to investigate the functioning of the internal parts and to test whether these parts are functionally integrated to the external genitalia.

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