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Assessing matting patterns, chromosome composition, host suitability, and diapause expression of *Euschistus* strains and species

Frederico Hickmann

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

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DEDICATION

To my beloved dad, Roque (in memoriam), and my beloved mother Crescência

To my brother Sérgio and my sister Rejane

To my bellowed wife Karine

In recognition of their worth

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SUMMARY

RESUMO

Identificação de padrões de acasalamento, composição dos cromossomos, adequação de hospedeiro e expressão de diapausa de linhagens e espécies de *Euschistus*

O Brasil emergiu como um proeminente produtor agrícola, contribuindo significativamente para o suprimento global de alimentos. Percevejos da família Pentatomidae são um grupo de insetos comuns no Brasil que se alimentam de culturas agrícolas, causando uma diminuição na produção e na qualidade. Fatores demográficos e adaptativos históricos e contemporâneos associados com as práticas agrícolas são importantes para determinar quais artrópodes, nativos ou invasores, terão impacto positivo ou negativo sobre os agroecossistemas do ponto de vista humano. Por exemplo, o percevejo-marrom-da-soja, *Euschistus heros* (Fabricius), uma espécie raramente encontrada em campos de soja até os anos 70, evoluiu rapidamente para uma praga importante da soja em poucas décadas. A descoberta de duas linhagens alopátricas de *E. heros* na América do Sul, com uma área de contato secundária no Brasil central, levantou questões sobre seu impacto na dinâmica de pragas no Brasil. Além disso, recentemente, *E. heros* tornou-se uma ameaça significativa para as culturas de algodão. Adicionalmente, na região Neotropical, várias espécies de *Euschistus* foram registradas na soja, mas apenas *E. heros* se tornou altamente predominante nos campos de soja. Os objetivos deste estudo foi investigar as causas de um possível processo de isolamento pré-zigotico no fluxo gênico assimétrico entre as duas linhagens de *E. heros*, bem como caracterizar o comportamento meiótico e a dinâmica do DNA satélite das duas linhagens de *E. heros* e sua progênie híbrida e comparar com as espécies irmãs *E. crenator* (Fabricius) e *E. taurulus* Berg. Também foi caracterizado a preferência e a adequação do hospedeiro das duas linhagens de *E. heros* e seus híbridos recíprocos. Além disso, testamos a adequação da soja e do algodão e a suscetibilidade a inseticidas em três espécies de *Euschistu*s (população híbrida natural de *E. heros*, *E. taurulus* e *E. crenator*) relatadas em soja no Brasil e caracterizamos a expressão/finalização da diapausa de *E. heros* (ambas as linhagens), *E. taurulus* e *E. crenator*. Por fim, empregamos uma abordagem funcional e molecular para caracterizar o perfil de transcritos de *E. heros* e *E. taurulus* submetidos a diferentes condições de fotoperíodo, validando funcionalmente os genes candidatos relacionados a diapausa em *E. servus* (Say), uma espécie Neárctica. Identificamos que a linhagem SS é maior e tem uma coloração mais escura, enquanto a linhagem NS é menor e tem uma cor marrom mais clara. Além disso, descobrimos que o *E. heros* acasala de forma sortida com insetos maiores, favorecendo a SS, e os híbridos recíprocos (fêmeas NS acasaladas com machos SS) apresentaram uma frequência reduzida de quiasmas. Além disso, os satDNAs foram acumulados de forma diferente, principalmente no cromossomo Y, nas duas linhagens de *E. heros*, no entanto, não promovem isolamento reprodutivo entre as linhagens. Nossos bioensaios revelaram que a linhagem SS prefere a soja em vez do algodão, enquanto a linhagem NS escolheu a soja e o algodão de maneira aleatória. As linhagens híbridas recíprocas (HSN e HNS) se comportaram de forma semelhante à NS, escolhendo aleatoriamente entre algodão e soja. A sobrevivência das ninfas das linhagens puras e de seus híbridos recíprocos foi semelhante quando alimentadas com soja, enquanto apenas os híbridos recíprocos atingiram a idade adulta quando alimentados com algodão, embora com viabilidade muito baixa (<1%). Identificamos que uma dieta à base de soja melhorou significativamente os parâmetros biológicos de *E. heros* em comparação com as espécies irmãs. Além disso, a soja é moderadamente adequada para a *E. crenator*, enquanto a *E. taurulus* apresentou baixa adequação para ela, enquanto os ramos de algodão não são adequados para as três espécies. A suscetibilidade a inseticidas de *E. heros*, *E. crenator* e *E. taurulus* a organofosforados e piretroides apresentou baixa variação. A expressão da diapausa varia significativamente entre as linhagens e espécies de *Euschistus* (intensa em *E. taurulus*, moderada em *E. heros* SS, fraca em *E. heros* NS e ausente em *E. crenator*). Além disso, a combinação de comprimento curto do dia e baixa temperatura aumentou a expressão da diapausa na SS e atrasou o término da diapausa em *E. heros* (ambas as linhagens). Por meio dos transcritos de *E. heros* e *E. taurulus* em diapausa e sem diapausa, identificamos e validamos os genes *ftz-f1*, *fpps* e *jheh*, que contribuem significativamente para o desenvolvimento do ovário, e o gene *ftz-f1* reduz o armazenamento de lipídios da espécie neártica *E. servus*. Nossas descobertas mostraram que a dinâmica da praga *E. heros* nas últimas décadas é significativamente associada a expansão dos cultivos de soja na América do Sul que possibilitou uma rápida adaptação dessa espécie para utilizar um hospedeiro exótico como recurso quando comparada as demais espécies de *Euschistus*. Embora tenhamos identificado que as duas linhagens apresentam apenas pequenas diferenças, a introgressão assimétrica que favorece a SS pode estar relacionada ao acasalamento e à meiose. A reunião desses dois pools genéticos sem nenhuma

barreira reprodutiva aumenta a diversidade genética e pode selecionar fenótipos de pragas mais aptos a sobreviver na paisagem agrícola. Além disso, a menor expressão de diapausa das duas linhagens sugere que esse é um ponto de atenção para o *E. heros* explorar novos hospedeiros durante a entre safra da soja e pode estar contribuindo para os surtos de pragas na cultura do algodão. Entre as espécies de *Euschistus* testadas, identificamos uma estrutura de genoma conservada e uma suscetibilidade semelhante a inseticidas, adequação variável à soja, e a expressão da diapausa é distinta. Por fim, nossa abordagem molecular revelou que a diapausa de *Euschistus* está relacionada à sinalização de JH, e a parada da oogênese parece ser regulada pelo gene *ftz-f1*. Com essas respostas, avançamos mais algumas etapas para entender a dinâmica evolutiva e adaptativa das linhagens de *Euschistus* (tanto as linhagens de *E. heros* quanto as espécies *E. taurulus* e *E. crenator*) nas áreas agrícolas, fornecendo informações evolutivas e biológicas inéditas e apoiando o desenvolvimento de estratégias e táticas para o manejo dessa espécie de praga nos agroecossistemas brasileiros.

Palavras-chave: Cultura da soja; Cultura do algodão; Dormência; Adaptação; Agricultura tropical

ABSTRACT

Assessing matting patterns, chromosome composition, host suitability, and diapause expression of *Euschistus* strains and species

Brazil has become a prominent agricultural producer, contributing significantly to the global food supply. Stink bugs of the Pentatomidae family are a group of insects common in Brazil that feed on crops, causing decreased production and quality. Historical and contemporary demographic and adaptive factors associated with agricultural practices are important in determining which arthropods, native or invasive, will positively or negatively impact agroecosystems from a human point of view. For example, the Neotropical brown stink bug, *Euschistus heros* (Fabricius), a species rarely found in soybean fields until the 1970s, quickly evolved into a major soybean pest in just a few decades. The discovery of two allopatric strains of *E. heros* in South America, with a secondary contact area in central Brazil, has raised questions about its impact on pest dynamics in Brazil. Furthermore, *E. heros* has recently become a significant threat to cotton crops. Additionally, several species of *Euschistus* have been recorded in the Neotropical region on soybeans, but only *E. heros* has become highly prevalent in soybean fields. This study aimed to investigate the causes of a possible process of pre-zygotic isolation in the asymmetric gene flow between the two strains of *E. heros*, as well as to characterize the meiotic behavior and satellite DNA dynamics of the two strains of *E. heros* and their hybrid progeny and compare them with the sister species *E. crenator* (Fabricius) and *E. taurulus* Berg. We also characterized the host preference and suitability of the two *E. heros* strains and their reciprocal hybrids. In addition, we tested soybean and cotton suitability and insecticide susceptibility in three *Euschistus* species (natural hybrid population of *E. heros*, *E. taurulus,* and *E. crenator*) reported on soybean in Brazil and characterized diapause expression/termination of *E. heros* (both strains), *E. taurulus* and *E. crenator*. Finally, we employed a functional and molecular approach to characterize the transcript profile of *E. heros* and *E. taurulus* submitted to different photoperiod conditions, functionally validating the candidate genes related to diapause in *E. servus* (Say), a Nearctic species. We found that the SS strain is larger and darker, while the NS strain is smaller and has a lighter brown color. In addition, we found that *E. heros* mates assortatively with larger insects, favoring the SS, and the reciprocal hybrids (NS females mated with SS males) showed a reduced frequency of chiasmas. In addition, satDNAs were accumulated differently, mainly on the Y chromosome, in the two strains of *E. heros*; however, they did not promote reproductive isolation between the strains. Our bioassays revealed that the SS strain prefers soybeans over cotton, while the NS strain chose soybeans and cotton randomly. The reciprocal hybrid strains (HSN and HNS) behaved similarly to NS, choosing randomly between cotton and soybean. The survival of the nymphs of the pure strains and their reciprocal hybrids was similar when fed soybean, while only the reciprocal hybrids reached adulthood when fed cotton, albeit with very low viability (<1%). We identified that a soybean-based diet significantly improved the biological parameters of *E. heros* compared to the sister species. In addition, soybean is moderately suitable for *E. crenator*, while *E. taurulus* showed low suitability, while cotton branches are unsuitable for the three species. The insecticide susceptibility of *E. heros*, *E. crenator,* and *E. taurulus* to organophosphates and pyrethroids showed little variation. Diapause expression varies significantly between *Euschistus* strains and species (intense in *E. taurulus*, moderate in *E. heros* SS, weak in *E. heros* NS, and absent in *E. crenator*). In addition, the short-day length and low-temperature combination increased diapause expression in SS and delayed diapause termination in *E. heros* (both strains). Through the transcriptomes of *E. heros* and *E. taurulus* in diapause and non-diapause, we identified and validated the *ftz-f1*, *fpps,* and *jheh* genes, which contribute significantly to ovarian development, and the *ftz-f1* gene reduces lipid storage in the Nearctic species *E. servus*. Our findings show that the dynamics of the *E. heros* pest in recent decades are significantly associated with the expansion of soybean crops in South America, enabling this species to adapt rapidly to use an exotic host as a resource compared to other *Euschistus* species. Although we have identified that the two strains show only minor differences, the asymmetrical introgression that favors SS may be related to mating and meiosis. Bringing these two gene pools together without any reproductive barriers increases genetic diversity and can select pest phenotypes better suited to survive in the agricultural landscape. In addition, the lower diapause expression of the two strains suggests that this is a point of attention for *E. heros* to explore new hosts during the soybean off-season and may contribute to pest outbreaks in the cotton crop. Among the *Euschistus* species tested, we identified a conserved genome structure, similar insecticide susceptibility,

variable suitability for soybeans, and distinct diapause expression. Finally, our molecular approach revealed that *Euschistus* diapause is related to JH signaling, and the arrest of oogenesis appears regulated by the *ftz-f1* gene. With these answers, we have taken a few more steps towards understanding the evolutionary and adaptive dynamics of *Euschistus* strains (both *E. heros* and the species *E. taurulus* and *E. crenator*) in agricultural areas, providing unprecedented evolutionary and biological information and supporting the development of strategies and tactics for the management of this pest species in Brazilian agroecosystems.

Keywords: Soybean crop; Cotton crop; Overwintering; Adaptation; Tropical agriculture

1. GENERAL INTRODUCTION

The family Pentatomidae (stink bugs) is found worldwide. Most species are phytophagous, obtaining nutrients by forcefully puncturing the plant tissues using their stylets and extracting the required fluids. Their feeding habit on plant structures can significantly impact the quantity and quality of crop harvest (Panizzi et al., 2000; McPherson, 2018; Colk & Borges, 2017). The stink bug genus *Euschistus* (Hemiptera: Pentatomidae) is endemic to the Americas, with representatives noxious to crops, particularly those belonging to the Fabaceae family (McPherson, 2018; Čokl & Borges, 2017). Among *Euschistus*, we can highlight *Euschistus servus* (Say) and *E. variolarius* (Palisot) in the Nearctics and *E. heros* (Fabricius) in the Neotropics as main pests in soybean cultivation (Sosa-Gomes et al*.,* 2020; Panizzi & Lucini, 2022, Pezzini et al., 2019). Other *Euschistus* species have also been reported as secondary soybean pests, such as *Euschistus taurulus* Berg and *E. crenator* (Fabricius) (Hickmann et al., 2019; 2021).

Furthermore, Soares et al. (2018) identified two strains of *E. heros* in Brazil. These two strains showed significant genetic (mitochondrial genes and single nucleotide polymorphism) divergences (Soares et al., 2018; Zucchi et al., 2019; Singh et al., 2023). The strains are found in distinct niches, one mostly related to the Atlantic Forest and Pampa Biomes further south of Brazil, Argentina, and Paraguay (hereafter SS), and another in North Cerrado, Caatinga, and Amazon Forest Biomes further north (hereafter NS). These strains have a secondary contact in the Cerrado biome, forming a hybridization zone with an asymmetric gene flow (Soares et al., 2018; Zucchi et al., 2019; Singh et al., 2023). Meeting two different gene pools, which do not have reproductive isolation, may generate populations with high adaptive capacity even in disturbed environments such as agroecosystems (Aitken & Whitlock 2013, Mallet 2018, Corrêa et al. 2019).

Herbivory is intrinsic to *Euschistus,* which demands detoxification mechanisms capable of metabolizing and excreting toxins or secondary metabolites produced by their hosts (Ali & Agrawal, 2012; Jermy, 1984; Petschenka & Agrawal, 2016). Although *Euschistus* is primarily reported on plants of the Fabaceae family, there have been increasing reports of *E. heros* occurring on alternative hosts such as cotton and sunflower (Frota & Santos, 2007; Soria et al., 2009, 2010, 2011, 2017; Azambuja; et al., 2013; Smanioto & Panizzi 2015). The apparent host range expansion can be attributed to the search for alternative hosts after soybean harvesting, particularly in simplified areas like superabundant monocultures.

The development and establishment of a species are significantly influenced by abiotic factors such as temperature, photoperiod, and humidity, which are correlated with seasonal changes. In arthropods, one of the mechanisms to cope with seasonal challenging periods is diapause (Tauber et al., 1986; Danks, 1987; Denlinger, 2022). Diapause is a neurohormonal mechanism of developmental suppression to survive unfavorable conditions and synchronize insect development. Most *Euschistus* stink bugs express a facultative adult diapause induced by short-day conditions in the immature stages (Saulish & Musolin, 2012; Musolin & Saulich, 2018), allowing synchronization of their development and reproduction to resource availability (often crops). Overall, *Euschistus* stink bugs' diapause induction, expression, and termination leads to a change in their behavior, accumulation of fat bodies, reduction or increase of metabolic rate, and suppression/resumption of reproduction (Mourão & Panizzzi, 2002; Panizzi & Vivan 1997; Musolin & Saulich, 2018). Insects that overwinter play a vital role in the next crop season, and understanding the duration and intensity of their diapause is critical for devising effective management strategies.

Soybean, *Glycine max* (L.) Merrill and cotton, *Gossypium hirsutum* L., are two of the most important crops worldwide, and Brazil is one of the top producers of both (CONAB, 2022; USDA, 2023). *Euschistus* stink bugs cause severe damage to crops such as soybean and cotton, which can lead to reduced yields and quality of the crops. It is worth mentioning that *E. heros* was a secondary pest of soybeans in the 1970s and is now a key pest of soybean cultivation in Brazil (Panizzi & Lucini 2022; Sosa-Gomes et al. 2020). Thus, understanding the adaptive processes of key pest species (e.g., *E. heros*) and of genetically close species (e.g., *E. taurulus* and *E. crenator*) that occur in sympatry in the same agricultural landscape is helpful in its management in crop areas and can help in predicting new population outbreaks of the different species.

Here, we aimed to investigate the reproductive isolation and asymmetrical gene flow between the two allopatric *E. heros* strains through mating patterns bioassays (chapter 2) and a detailed characterization of the meiotic behavior and developed chromosome markers of the two strains of *E. heros* and its hybrid progeny, comparing with *E. taurulus* and *E. crenator* karyotypes (chapter 3); characterize the host preference and host suitability of the two *E. heros* strains and their reciprocal hybrids (chapter 4) and additionally, we tested the suitability of soybean and cotton and susceptibility to selected insecticides of three *Euschistus* species (*E. heros* natural hybrid population, *E. taurulus,* and *E. crenator*) reported on soybeans in Brazil (chapter 5). Moreover, we characterize the diapause expression/termination of *E. heros* (two

strains), *E. taurulus*, and *E. crenator* (chapter 6) and employ a functional (RNA-seq) and molecular approach to characterize the transcriptome profile of *E. heros* and *E. taurulus* subjected to different photoperiod conditions, validating the candidate genes in *E. servus*, a Nearctic species with strong diapause phenotype expression (chapter 7). Lastly, The results obtained from this study had the following goals:

(i) to identify mating patterns and reproductive behavior of the *E. heros* allopatric strains;

(ii) to characterize the meiotic behavior in the two strains of *E. heros* and the hybrid progeny from reciprocal crossings of the strains. In addition, develop chromosome markers for the three *Euschistus* species, *E. heros* (both strains), *E. taurulus,* and *E. crenator*;

(iii) to identify differences in host preference and biological parameters of the two *E. heros* strains and their reciprocal offspring fed with soybean or cotton;

(iv) to test the suitability of soybean and cotton and susceptibility to selected insecticides of three *Euschistus* species (*E. heros*, *E. taurulus*, and E. *crenator*);

(v) to characterize the diapause expression/termination of the two strains of *E. heros*, *E. taurulus*, and *E. crenator*, under different photoperiod and temperature conditions;

(vi) to identify the genetic and molecular mechanisms of diapause of *E. heros* and *E. taurulus* and validate three differential expressed genes using RNA interference in the Nearctic species *E. servus*;

With these answers, we take a step further to understand the implication of the *Euschistus* strains (both *E. heros* strains and the species *E. taurulus* and *E. crenator)*, providing unedited evolutionary and biological information and supporting the development of strategies and tactics for the management of this pest species in the Brazilian agroecosystems.

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2. MATTING PATTERNS DRIVE THE GENE FLOW AND SPATIAL DISPERSAL OF *Euschistus heros* (HEMIPTERA: PENTATOMIDAE)

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Abstract

Euschistus heros (Hemiptera: Pentatomidae) has two allopatric strains with a hybrid zone in central Brazil. Asymmetric dispersal and gene flow between these strains of *E. heros* have been observed, where the South strain (SS) moves more quickly to the northern regions of the country than the North strain (NS) to the southern areas. In addition, SS generally has a bigger body size and presents dark brown coloration, and NS is usually smaller in size and presents light brown coloration. Here, we studied the mating behavior and tested for assortative mating between the two allopatric strains of *E. heros*. Non-random mating was observed in the SS strain based on mating choice trials and the reproductive isolation indexes. SS females and males prefer to mate with their co-specific (same strain) partner, while NS insects showed no mating preference. The insect's pronotum width was positively associated with the mating choice, suggesting size-assortative mating in *E. heros*. The asymmetric gene flow in the hybridization zone that favors SS is associated with the mating behavior of the species, which favors the typical phenotype found in the SS populations.

Keywords: Neotropical brown stink bug; Assortative mating; Hybridization; Reproductive isolation; Population genetics

2.1 Introduction

The Neotropical brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), is one of the main pests of soybean *Glycine max* (L.) Merrill in South America. The primary injuries include senescence of young pods and lower weight and quality of grains (Corrêa-Ferreira and Azevedo 2002, Depieri and Panizzi 2011, Sosa-Gómez et al. 2020). With the expansion of soybean fields from the southern to the central regions of the country (i.e., Cerrado) during the 1980s and more recently to northern regions, population outbreaks of *E. heros* have become more frequent (Corrêa-Ferreira et al. 2009, Cattelan and Dall'Agnol 2018, Sosa-Gómez et al. 2020).

Euschistus heros is widely distributed in Brazil, and two allopatric strains have been recently identified (Soares et al. 2018). The strains represent two distinct and largely preserved gene pools: the northern strain (hereafter NS), which occurs predominantly in the northern part of the country (e.g., Amazon and Caatinga ecoregions), and the southern strain (hereafter SS) in southern Brazil (e.g., the Atlantic Rain Forest and Pampa ecoregions) (Soares et al. 2018). In central Brazil (Cerrado ecoregion), the two strains of *E. heros* co-occur, forming a hybridization zone (Soares et al. 2018, Zucchi et al. 2019).

Population outbreaks of this pest in soybean fields in central Brazil are frequently reported, such as insecticide control failures and expansion of the range of plant hosts (Soria et al. 2009, 2017, Santos et al. 2018, Sosa-Gómez et al. 2020). One hypothesis that could explain the increasing importance of *E. heros* as a pest, mainly in the Brazilian Cerrado, in the last two decades is the hybridization between the two strains of *E. heros*. In addition to increasing genetic diversity, hybridization can lead to a higher vigor of individuals and the rapid evolution of adaptive traits in local populations (Edmands 1999, Hahn and Rieseberg 2017). However, in some cases, hybrid outbreeding with hybrid fitness declination may also occur (Edmands 1999).

Despite in more northern and more southern regions, both strains show a high pure degree, an asymmetric dispersal and gene flow between the allopatric strains of *E. heros* have been observed, where the SS strain contributes more to the hybrid gene pool than the NS strain, given that SS introgressions are more common than NS (Soares et al. 2018, Zucchi et al. 2019). This observation raises questions about the reproductive behavior between the two strains of *E. heros*. *Euschistus heros,* like other stink bugs, display polygamous behavior with multiple copulations performed by females and males (Borges et al. 1987, Blassioli-Moraes et al. 2005, 2014, Silva et al. 2012, Laumann et al. 2013). However, information about the reproductive behavior of *E. heros* is still scarce.

Assortative mating is a typical behavior in species with sexual reproduction (Dittrich et al. 2018), where individuals with similar phenotypes mate more frequently than individuals with different phenotypes. Mating preferences can substantially impact population demography and the evolution of adaptations (Crespi 1989). In insect pest species, mating preferences can indirectly contribute to the rapid fixation of important alleles, such as those responsible for insecticide resistance, significantly affecting our ability to control pest populations (McNamara et al. 2004; Cordeiro et al. 2017). Assortative and disassortative mating has been reported in pentatomid stink bugs, but not in *E. heros* (Capone 1995, Follett et al. 2007, Krupke et al. 2008).

Long periods of reproductive isolation, through sympatric or allopatric processes, may lead to the development of reproductive barriers between genetically distinct groups (Payseur and Rieseberg 2016). These phenomena are continuous, and it is not possible to know precisely at what point in evolutionary history the reproductive barriers arise (Dopman et al. 2009). Reproductive barriers can result from either pre-zygotic or post-zygotic mechanisms. Prezygotic mechanisms reduce encounters and copulations between males and females, reducing the transfer of sperm or ovule fertilization (Dobzhansky 1937).

Our goal was to investigate whether there was assortative mating between the two different strains of *E. heros* that are geographically separated. We used size, specifically the width across the pronotum, as the phenotypic trait that is positively linked to fitness components, as suggested by McLain (1991). We conducted morphological characterization and mate-choice bioassays on both strains. Additionally, we analyzed the effects of mate choice and assortative mating on the gene flow and dispersal of *E. heros* strains.

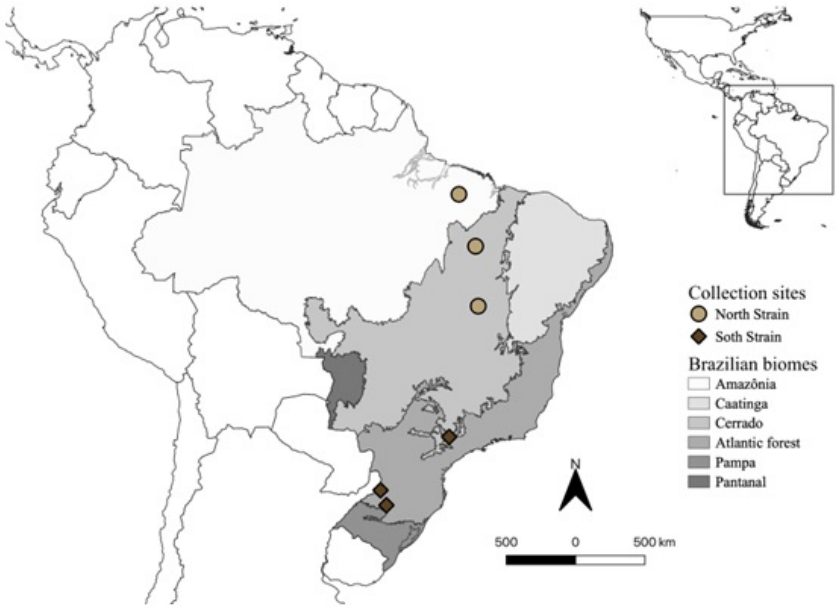
2.2 Material and Methods

2.2.1 Collection and maintenance of *E. heros* experimental colonies

Strain collection: individuals from localities representing the two strains of *E. heros*, i.e., Balsas, state of Maranhão (NS – 07°13'44.50" S, 45°58'35.32" W) and Itapiranga, state of Santa Catarina (SS – 27°08'41.59" S, 53°43'52.85" W), were sampled to establish laboratory stock populations (Figure 1). Approximately 150 adult insects of each locality were sent to the Laboratory of Molecular Ecology of Arthropods at the University of São Paulo, USP/ESALQ (Piracicaba, São Paulo, Brazil).

Strain maintenance: the insects were kept in cages 36.5 × 25.5 × 14.4 cm (length, width, and height, respectively) and covered with organza fabric. Egg masses were maintained in Petri dishes 1.0 cm high with a radius of 4.5 cm, containing moistened cotton. Nymphs were kept in

the dishes until reaching the 2nd instar (1st instars do not feed) and then transferred to cages with dimensions 15.5 × 15.5 × 8.0 cm (length, width, and height, respectively) and covered with voile until they reached adulthood. As described above, adults were kept in 35.5 × 25.5 × 14 cm cages. To the adults and immature forms, we offered a natural diet [dry soybean seeds (*G. max*), dry peanut seeds (*Arachis hypogaea* L.), dry sunflower seeds (*Helianthus annuus* L.), and green bean pods (*Phaseolus vulgaris* L.)], with water *ad libitum* with moistened cotton. Laboratory conditions were 25 ± 1°C, relative humidity of 60% ± 10, photophase of 14 h, and scotophase of 10 h.



Sampling site	Strain	N (female / male)	Latitude S / Longitude (W)
Balsas*	**NS**	**10 / 10**	**07°13'44.50'' / 45°58'35.32''**
Luiz Eduardo Magalhães#	NS	10 / 10	12°05'25.63'' / 45°46'49.95''
Paragominas#	NS	10 / 03	03°00'09.95'' / 47°21'11.19''
Itapiranga*	**SS**	**10 /10**	**27°08'41.59'' / 53°43'52.85''**
Santa Bárbara do Sul#	SS	10 / 10	28°22'01.95'' / 53°15'06.23''
Anhembi#	SS	10 / 10	22°47'17.09'' / 48°07'52.29''

*Collection in this study; #Collections of Soares et al. (2018); N = the number of individuals analyzed. NS = North Strain = NS; South Strain = SS.

Figura 1. Map showing collection sites (including localities and coordinates and the number of insects analyzed) of the *Eushistus heros* strains. The scale of gray indicates Brazilian biomes.

2.2.2 Identification and characterization of *E. heros* strains

Molecular strain characterization: twelve *E. heros* F_0 adults of each population (Itapiranga, Santa Catarina State, and Balsas, Maranhão State) were used to confirm the strain. The total genomic DNA was extracted using a leg with thorax muscle tissues according to the protocol proposed by Clark et al. (2001).

Two specific primer sets developed by Soares et al. (2018) were used to amplify the 607 bp fragment of the *cytochrome c oxidase subunit I* (COI) gene. The polymerase chain reaction (PCR) was performed in a total of 25 µl containing 3-µl gDNA, 10.7-µl Milli-Q water, 2.5-µl buffer (10X), 2.5-µl MgCl2 (25 mM), 2-µl dNTP (2.5 mM), 2 µl of each primer (5 pmol), and 0.3-µl Taq (5 U/µl). The PCR cycle conditions were 95°C for 3 min, then 30 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 2 min, and a final extension of 72°C for 10 min.

The PCR products were evaluated by electrophoresis on 1.5% (wt/vol) agarose gels stained with SYBR Safe (Life Technologies) and were purified using 0.33 µL EXO I, 0.33 µL FastAp and 0.34 µL of ultra-pure water together with 10 µL of each PCR product, held at 37 °C for 30 min, then at 80 °C for 15 min. The bidirectional Sanger sequencing was performed using the DNA sequencing services of the Agricultural Biotechnology Center (CEBTEC) from the University of São Paulo. The sequences were checked and edited manually in the software Sequencher 4.8 (Gene Codes Corp., Ann Harbor, MI). The sequences generated here were aligned with COI sequences by Soares et al. (2018) using the ClustalW algorithm in MEGA X (Kumar et al. 2018). The number of haplotypes was calculated using the software DNAsp (Librado and Rozas 2009). A Bayesian phylogeny tree was generated to identify the relationships among the haplotypes. The best substitution model of evolution used was GTR+I+G using MRMODELTEST v2.3 (Nylander 2004). The Bayesian phylogenetic tree was carried out in MRBAYES v3.1.2 (Ronquist and Huelsenbeck 2003), using two simultaneous runs of 5 million generations each, with one cold and three heated chains in each run. At the end of the runs, the first 25% of the trees were discarded as burn-in samples. The consensus tree was obtained with posterior probabilities >0.50.

Morphological strain characterization: morphological differences between the two strains were evaluated using qualitative and quantitative morphological characters. Qualitative morphological characters were based on the description provided by Rolston (1974), including pronotum, scutellum, hemelytra, and connexiva, highlighting the body coloration. The body size of the adults of both sexes (ten females and ten males) from each collection site (Balsa, MA and Itapiranga, SC) was indexed by measuring the distance between the humeral angles,

hereafter the pronotum width (McLain et al. 1990, McLain 1991), using a caliper. In addition to the populations used in behavioral bioassays (see below), we also measured the pronotum width of individuals from four other populations (two NS and two SS populations) to avoid population bias (Figure 1). The means of pronotum width were compared with a *t-test* (P = 0.05) in SAS Studio (SAS Studio 2021). The visual representation of this data was plotted in R (R Core Team 2021) with the package 'ggplo2' (Wickham 2016).

2.2.3 Mating-choice trials

One-day-old, emerged adults of both strains (NS and SS) were sexed, their pronotum width was measured and then were placed in individual plastic containers [530 mL volume (7.5 cm high, 11 cm diameter)] covered with voile and containing natural diet and water *ad libitum* until sexual maturation (≈ two weeks after emergence).

Female- and male-choice mating trials: after sexual maturation, mate-choice triads composed of randomly selected individuals were placed in transparent plastic arenas (9.0 cm high, radius 14.0 cm) containing diet and water and filmed for a period of 13 h in a climate-controlled room (25 ± 2°C, relative humidity 60 ± 10%) under artificial light. The experiment included female- and male-choice trials, as follows: (a) ♀ NS × (♂NS and ♂ SS); (b) ♀SS × (♂NS and ♂ SS); (c) ♂ NS × (♀SS + ♀ NS); and (d) ♂ SS × (♀NS + ♀ SS), with each mating trial repeated 16 times. To identify the chosen insect in the video, we marked the insects of the same sex on the scutellum with blue or red gouache ink (the strain marked was reversed each repetition to avoid color bias). We evaluated the mating-choice preference in the first and second copulations if more than one copulation occurred within 13 h. The results of the mating-choice trials were analyzed with a binomial generalized linear model (GLM) to determine the significant factors that influence the mating choice of the two strains of *E. heros*. These analyses were performed in R (R Core Team 2021) 'with the package 'arm' (Gelman et al. 2020)'. To compare the first and second mate choice of females and males of both strains were analyzed the binomial data with a Fisher's Exact Test ($p < 0.05$) followed by an exact binomial test ($p < 0.5$, confidence interval = 0.95) in R (R Core Team 2021) 'with the package 'dplyr' (Wickham et al. 2021)'.

Pair total index (PTI): to identify and quantify the degree of reproductive isolation that could result from female- and male-first choice preferences, we analyzed each chooser's mating choice to estimate the pair total index (PTI) (Rolán-Alvarez and Caballero 2000). We analyzed female- and male-chooser trials separately (mating behavior may differ between sexes). PTI statistics estimate and assess the deviations from random mating, by comparing the observed and expected mating pairs (assuming random mating between individuals) among the four

possible combinations of mating pairs [♀ NS × (♂NS and ♂ SS); ♀SS × (♂NS and ♂ SS); ♂ SS × (♀NS + ♀ SS); ♂ NS × (♀SS + ♀ NS)]. We estimated the sexual-isolation effects for each mating-pair combination separately and the two-tailed probability of rejecting the null hypothesis being true (coefficient = 1), obtained by bootstrapping using JMATING v. 1.0.8 (Carvajal-Rodriguez and Rolan-Alvarez 2006).

Reproductive isolation: to estimate the prezygotic barriers and probability of gene flow, we calculated the reproductive isolation (RI) metrics for males and females of both strains (first choice data) using the linear equation proposed by Sobel and Chen (2014):

$$RI = 1 - 2\left(\frac{H}{H + C}\right)$$

where H represents the frequency of heterospecific success, and C represents the frequency of co-specific success. The probability of gene flow and the degree of reproductive isolation ranges from 1 at complete isolation (all matings occur within a strain), 0 at random mating (indicates equal pairing between strains), and −1 when gene flow probably is facilitated (all matings occur between strains).

Assortative mating: subsequently, the first mating choice data from female- and male-choice mating trials were analyzed with a pairwise correlation test of the variable's "pronotum width of the chosen" and "pronotum width of the chooser" in R (R Core Team 2021) with the package 'ggpubr' (Kassambara 2020).

2.3 Results

2.3.1 Identification and morphological characterization of *E. heros* strains

The Bayesian phylogenetic analysis confirmed that the COI haplotypes originated from individuals collected in Itapiranga from SS, and the insects collected in Balsas are from NS (Figure 2). The 134 COI sequences generated 91 haplotypes. Specifically, the COI sequences of Balsas generated five haplotypes (Genbank accession number: MZ895188-MZ895198), and the Itapiranga population generated eight haplotypes (Genbank accession number: MZ895176-MZ895187). The Bayesian analysis of COI haplotypes confirmed that the Balsas haplotypes (in orange) are from the NS, and the Itapiranga haplotypes (in red) are from SS (Figure 2).

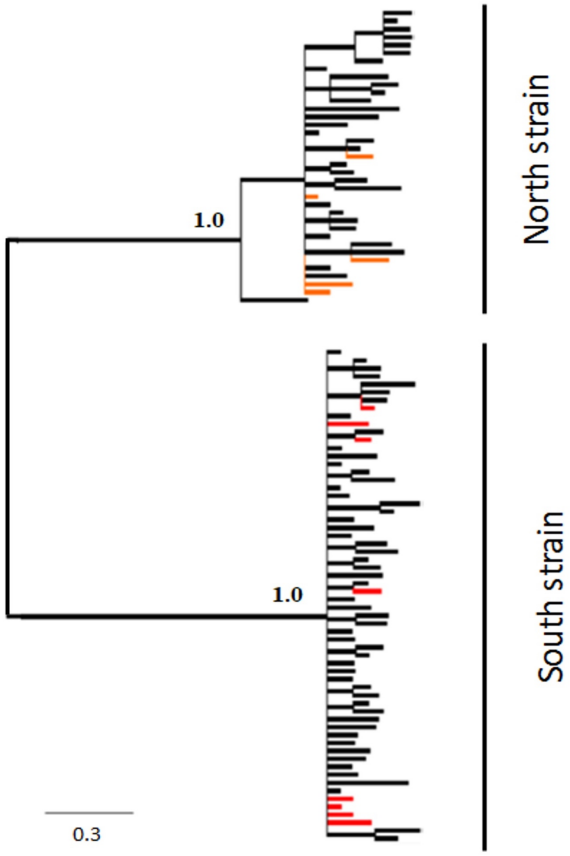


Figura 2. Bayesian phylogeny tree of 91 COI haplotypes of *Euschistus heros*. In Orange, *cytochrome c oxidase subunit I* (COI) haplotypes from Balsas (North strain), and in red, COI haplotypes from Itapiranga (South strain). The tree did not record node support values below 0.50 (posteriori probability).

We observed differences in size and general body coloration between the two strains (Figure 3. A–F). NS individuals have a light-colored body, with the dorsum, antennae, and legs chestnut to yellowish-brown; the connexiva and vittae on the abdominal venter mostly light brown; and the dark humeri contrasting strongly with the body coloration. SS individuals have a dark-colored body, with the dorsum, antennae, and legs chestnut to dark brown; the connexiva and vittae on the abdominal venter darker; and the humeri contrasting less strongly with the body coloration, and the cream-colored maculae on hemelytra and the ivory apex of the scutellum contrasting more strongly. Somatic and genital structures used for the definition of the species (i.e., distribution of the punctuation, presence of a black fovea at the basal angles of the scutellum, and the humeri developed into a spine; Rolston (1974)) are similar in both strains.

NS individuals had narrower pronotum widths in both males [mean ± standard error] (NS: 7.77 mm ± 0.12 vs. SS: 8.38 mm ± 0.10; $t_{51} = 3.95$; $p < 0.0001$) and females (NS: 8.05 mm ± 0.19 vs. SS: 8.63 mm ± 0.12; $t_{58} = 2.51$; $p = 0.015$) (Figure 3G).

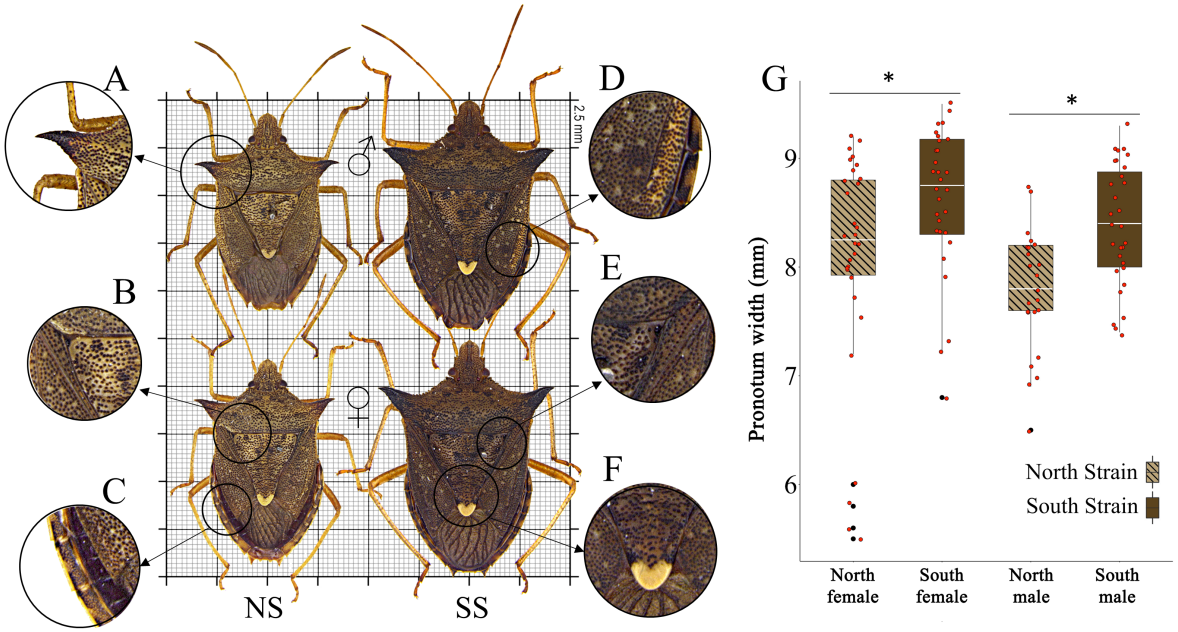


Figura 3. (A–F) Differences between individuals of the North (A–C) and South strains (D–F): C, dark humeral angles contrasting with the black ground coloration of the body; D and E, details of the pronotum, scutellum, hemelytra, and connexiva, highlighting the light body coloration; E–F, details of the pronotum, scutellum, hemelytra, and connexiva, highlighting the dark body coloration. (G) Pronotum width of females and males of *Euschistus heros* North (light brown) and South strains (dark brown). Means for pronotum width were submitted to a t-test (P = 0.05); the asterisk indicates a significant difference between strains.

2.3.2 Mating-choice trials

The GLM binomial regression analysis identified that the significant contributors of the mating choices where the factors strain (estimated = 1.63064 ± 0.66616, z = 2.448, p = 0.0144) and pronotum width (estimated = 0.16148 ± 0.04999, z = 3.230, p = 0.0012). Additionally, it also showed a significant difference between first and second mating choices (estimated = 2.0794 (± 0.9465), z = 2.197, p = 0.028) (Table 1). The selected model showed a lower AIC value (AIC = 49.967, estimate = –12.986 ± 4.198, z = –3.094, p = 0.001) when compared to the null model (AIC = 68.508, estimate = 0.7503 ± 0.2943, z = 2.55, p = 0.0108) (Table 1).

Tabela 1. Results of the general linear model (GLM) binomial regression analysis exploring the significant contributors in the mating choice trials of the two strains of *Euschistus heros*.

Source of variation (1st mate choice)	Estimated (± SE)	z value	P
Strain	1.63064 (± 0.66616)	2.448	**0.0144 ***
Sex	-0.3567 (± 0.5950)	-0.60	0.549
Pronotum width	0.16148 (± 0.04999)	3.230	**0.00124 ****
First x second mate choice	2.0794 (± 0.9465)	2.197	**0.028 ***
Best model	-12.986 (± 4.198)	-3.094	**0.001****
Null model	0.7503 (± 0.2946)	2.55	**0.0108***

Bolded significant values and asterisks denoting the level of significance of the general linear model (GLM) binomial regression (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).

Comparison of the first choice showed a deviating choice preference between strains (Fisher's Exact Test, p = 0.01769). The choice tests showed that females (Exact binomial test, p = 0.02246, CI (95%) = 0.01920667–0.45447106, n = 13, choice proportion = SS: 0.85 and NS: 0.15) and males (Exact binomial test, p = 0.02246, CI (95%) = 0.01920667–0.45447106, n = 13, choice proportion = SS: 0.85 and NS: 0.15) from SS preferred their co-specific partner for the first copulation (Figure 4). NS individuals showed no strain mating preference in the first copulation for males (Exact binomial test, p =1.00, CI (95%) = 0.2658613–0.7873333, n =15, choice proportion = SS: 0.58 and NS: 0.42) and females (Exact binomial test, p = 0.5811, CI (95%) = 0.3157776–0.8614207, n = 12, choice proportion = SS: 0.47 and NS: 0.53) (Figure 4).

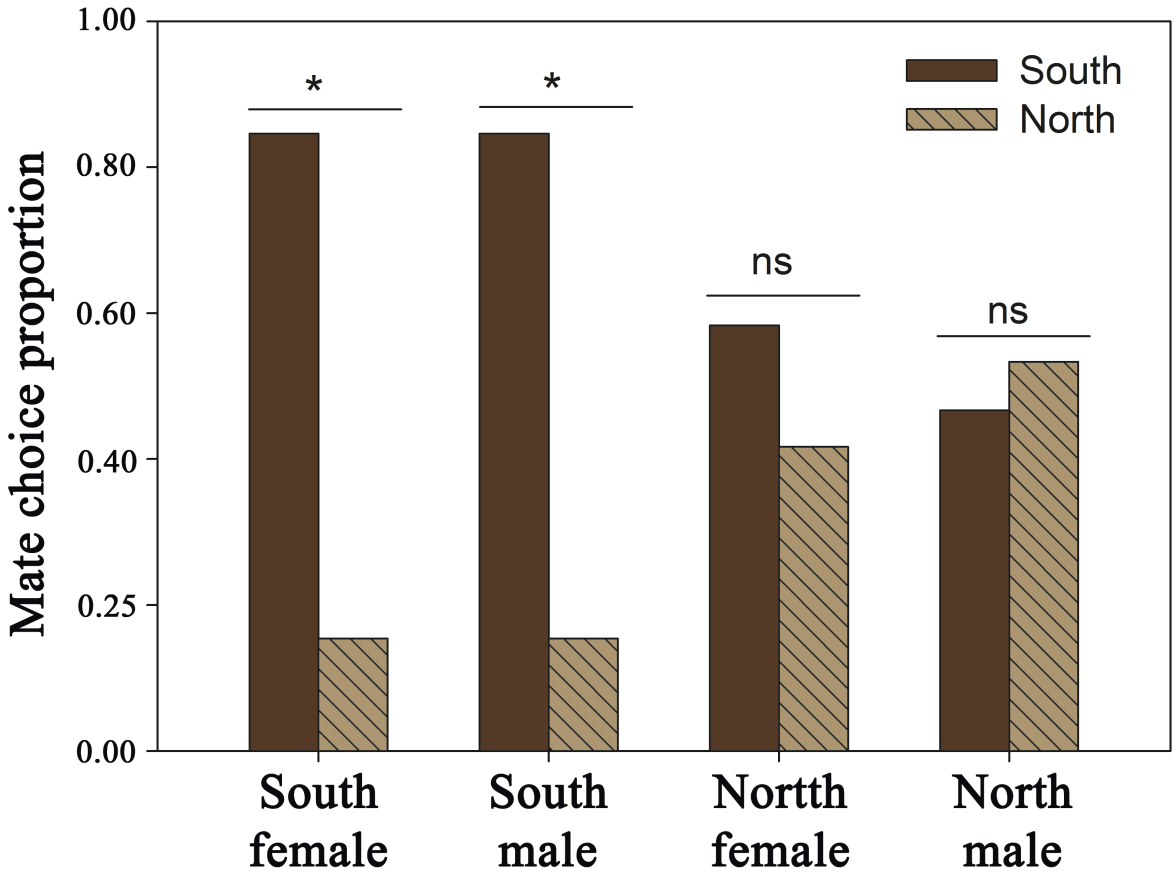


Figura 4. Mate choice of females and males of two strains of *Euschistus heros* in the first mate choice. The binomial data of the first mate choice of females and males of both strains were submitted to the exact binomial test (P = 0.5, confidence interval = 0.95). Asterisk indicates a significant difference (P ≤ 0.05), and ns indicates no significant difference (P > 0.05).

Comparison of the first choice with the second choice showed a deviating choice preference (Fisher's Exact Test, p = 0.03618), meaning that the second choice was not random and did not follow the same pattern as the first choice. In the second mate choice, NS males (NS: Exact binomial test, p = 0.01172, CI (95%) = 0.5872201–0.9977010, n = 11, choice proportion = same: 0.10 and different: 0.90) chose a different mate partner when compared with the first mate choice (Figure 5). The SS males did not show a difference in the second mate choice (SS: Exact binomial test, p = 0.125, CI (95%) = 0.4212768–0.9963897, n = 7, choice proportion = same: 0.15 and different: 0.85). Females from the two strains (SS: Exact binomial test, p = 1.00, CI (95%) = 0.1570128–0.8429872, n = 8, choice proportion = same: 0.50 and different: 0.50; NS: Exact binomial test, p = 0.625, CI (95%) = 0.006309463–0.805879550, n = 4, choice proportion = same: 0.75 and different: 0.25) did not choose the second mating partner based on the partner in the first mate choice (Figure 5).

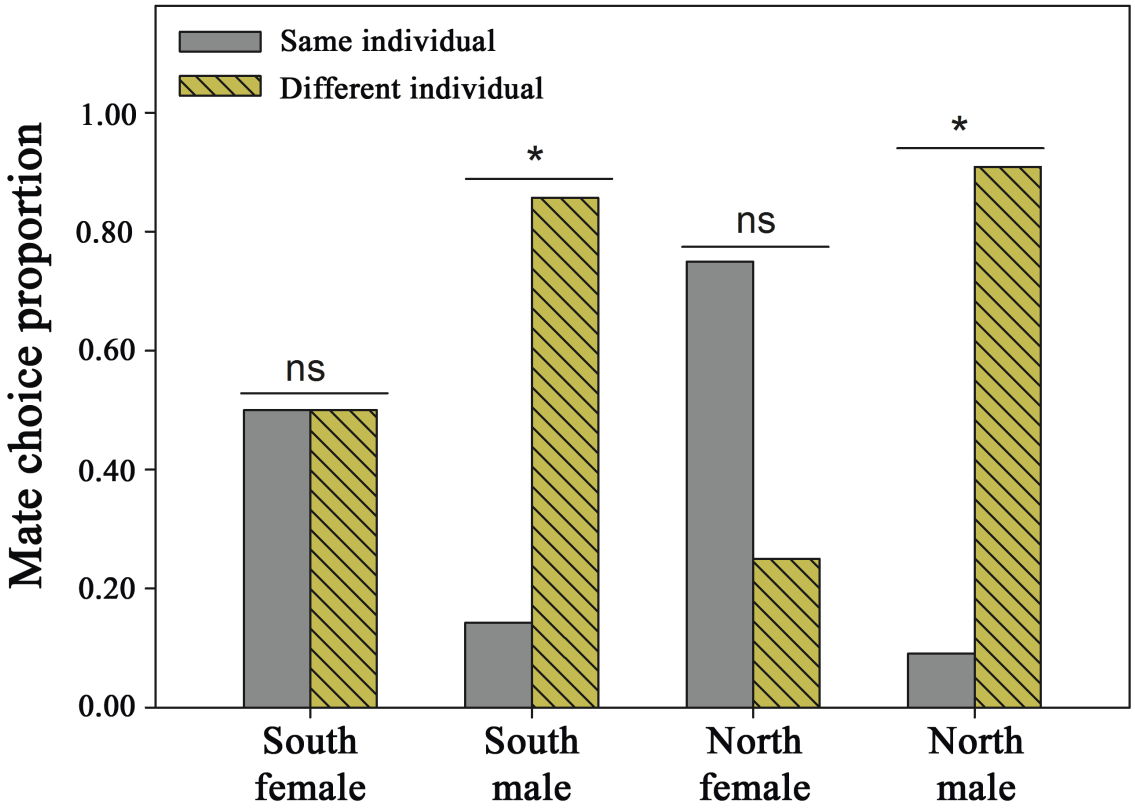


Figura 5. Mate preference of females and males of two strains of *Euschistus heros* in the second mate choice. The binomial data of the second mate choice of females and males of both strains were submitted to the exact binomial test (P = 0.5, confidence interval = 0.95). Asterisk indicates a significant difference (P ≤ 0.05), and ns indicates no significant difference (P > 0.05).

The results of the first-choice trials indicated that SS females and males showed a non-random mating pattern. When females were the choosing sex, the PTI value above 1, SS PTI: 1.8625, p =0.026, showed a pronounced assortment of SS which preferentially chose SS males as mating partners (Table 2). When males were the choosing sex, SS males showed a PTI higher than 1 (SS PTI: 1.7949, p = 0.035), revealing an assortative preference for SS females (Table 2). The female- and male-choice trials for SS showed an RI of 70% of SS females and males (Table 2). The RI values found for NS females was 34% and NS 6% meaning random mating (Table 2).

Tabela 2. Estimates of the pair total index (PTI) coefficient and isolation indices (RI) in the female- and male-choice trials of the two *Euschistus heros* strains (SS: south strain and NS: north strain).

Sex, strain of choosing partner	Chosen partner				Isolation Index
	SS (PTI) ± SD	P	NS (PTI) ± SD	P	RI
Female:					
SS	1.8625 ± .79	**0.026**	0.3402 ± .27	0.074	0.70
NS	1.0864 ± .48	0.407	0.5441 ± .31	0.192	0.34
Male:					
SS	1.7949 ± .74	**0. 035**	0.3550 ± .29	0.090	0.70
NS	0.8676 ± .25	0.457	0.9927 ± .26	0.797	-0.06

PTI values ± SD and the isolation index (RI) are given for each choice scenario. Deviations of PTI values from 1 were tested by resampling, and the RI values were calculated following Sobel & Chen (2014) linear equation. Significant p-values of PTI are in bold.

Disregarding the effect of strain, the pronotum width was positively correlated with the individuals' mating preference (r = 0.42; P = 0.001), which supports the hypothesis of size-assortative mating in *E. heros* (Figure 6).

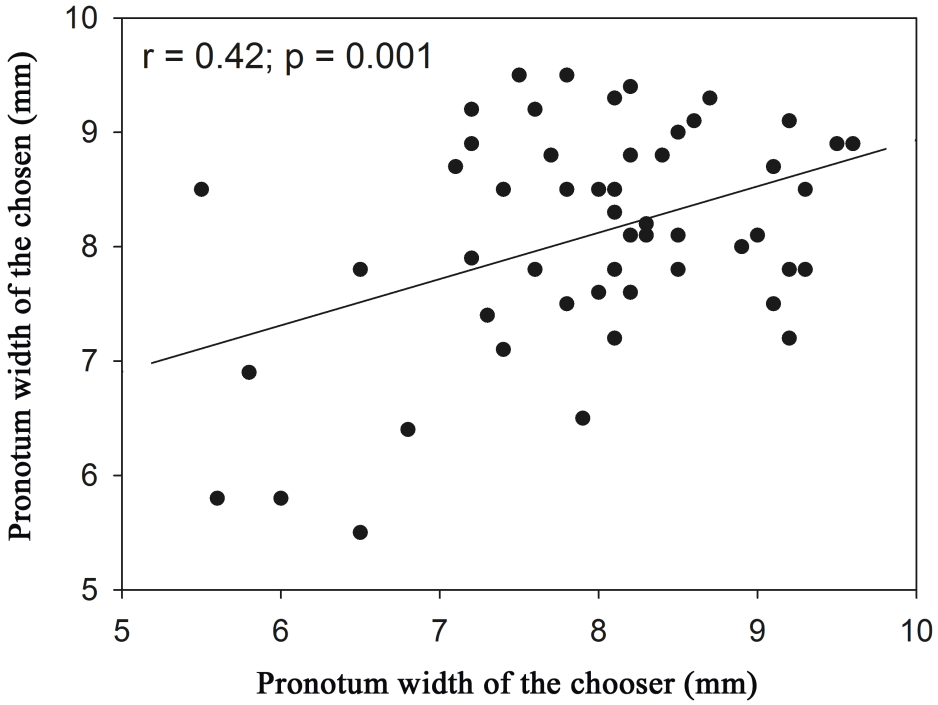


Figura 6. Correlation between pronotum width resulting from first mate-choice tests of females and males of *Euschistus heros*.

2.4 Discussion

The two *E. heros* strains, although genetically and morphologically distinct, do not have a significant reproductive barrier that prevents gene flow between these two genetic pools. The viability of the hybrid offspring supports this conclusion, based on the reciprocal mating between the two strains of *E. heros* when compared to the offspring from the mating of co-strains. However, we found that SS females and males preferred to mate with co-strain individuals, whereas NS insects did not show strain preference. The asymmetric mating choice between the two *E. heros* strains reinforces the hypothesis that the evolution of reproductive traits between two groups of organisms is not predictable because it depends on how selection and genetic drift have shaped the genomes of the different groups' (Seehausen et al. 2014, Martin et al. 2019, Poikela et al. 2019).

Polygamy of males and females is ubiquitous in all species of stink bugs, and the female often chooses the mate (Grazia and Schwertner 2017). Our results revealed mate-choice behavior in males of *E. heros* since males from NS chose a different female from that selected in the first mating (virgin female). In addition, it is worth mentioning that SS males showed a similar trend where 6 of 7 insects chose a different partner (see Figure 5). The absence of

significance is probably due to the low number of second mattings during the experimental period. Although this behavior has been recognized within the Pentatomidae (Krupke et al. 2008), it is reported here for the first time in *E. heros*. Sexual selection can reduce gene flow [e.g., shown in *Drosophila* flies and crickets (Oh et al. 2012, Debelle et al. 2016)] or increase gene flow and phenotypic divergence among allopatric populations that experience secondary contact (e.g., neutral genes are under selection or traits that reflect local adaptation (Servedio and Boughman 2017)). Specifically, for *E. heros*, the choice behavior of females and males may be favoring the dispersal of SS to northern areas of Brazil. Furthermore, the behavior of males increases the number of mating partners and may positively contribute to the gene flow among populations and the admixture between strains.

Here, according to the PTI coefficient and RI indexes, we show a non-random mate-choice pattern of females and males of SS. We also found that NS males and females showed no deviations from random mating. Our results indicate an incomplete premating isolation and that premating RI likely could play a role in the population and/or strain diversification. Indexes of reproductive isolation are useful metrics to determine which factors could be involved in the speciation process. Furthermore, non-random mating has been related to several important evolutionary patterns and processes, e.g., the origin of exaggerated traits (Andersson 1994), maintenance of the genetic diversity (M'Gonigle et al. 2012), local adaptation (Thibert-Plante and Hendry 2009) and speciation (Dieckmann and Doebeli 1999, Servedio 2000, Boughman 2001, Kirkpatrick and Ravigné 2002, Seehausen et al. 2008).

When we analyzed the mate-choice pattern of the first copulation, when strains and sex were not considered, we found a significant correlation between mating choice and the pronotum width, suggesting size-assortative mating. The size-assortative mating found in *E. heros* is described as the most common mating pattern in animals and was reported in Gastropods, Chelicerates, Crustaceans, Insect, Amphibians, Fish, Birds, and Mammals (Crespi 1989, Jiang et al. 2013). In addition, it has been reported in other Pentatomidae stink bugs as *Edessa contermina* Walker (Moura and Gonzaga 2019), *Thyanta pallidovirens* Stål (Wang and Millar 1997), and *Chinavia hilaris* (Say) (Capone 1995). There is strong evidence that size-assortative mating improves beneficial characteristics in the gene pool of the offspring (Jiang et al. 2013, Cordeiro et al. 2017, Kopp et al. 2018, Janicke et al. 2019).

Assortative mating may be key in speciation and generating reproductive barriers between genetic groups. Assortative mating can act similarly to disruptive selection when only one allele is under the selection (Kirkpatrick and Ravigné 2002). When more genes are under

selection, a balance of evolutionary forces can occur, maintaining differences between populations but not necessarily leading to speciation (Kirkpatrick and Ravigné 2002, Irwin 2020). In *E. heros*, we can suggest more than one gene is under selection due to high genetic structuration among populations and the absence of reproductive isolation between strains (Soares et al. 2018, Zucchi et al. 2019).

Here, we showed that the greater dispersal of SS individuals northward compared to the dispersal of NS individuals southward, as well as the asymmetric gene flow in the hybridization zone, seems to be associated with the reproductive pattern of the species, which favors the common phenotype in the SS populations. However, all populations in the hybridization zone have genetic mixtures between SS and NS (Soares et al. 2018, Zucchi et al. 2019). There is no doubt that the northward expansion of soybean cultivation in South America has accelerated encounters between the two strains of *E. heros*. Thus, we must consider that SS individuals have been associated with soybean cultivation longer than NS individuals and consequently have been subjected to pressure from different crop varieties, cultivation strategies, and insecticides for a longer time (Tuelher et al. 2018, Somavilla et al. 2020, Sosa-Gómez et al. 2020). These facts may also favor the SS phenotype in the hybrid zone.

The reunion of two genetic pools, without reproductive isolation, adapted to different biotic and abiotic conditions can maximize the evolution of adaptive characteristics due to the increase in the genetic diversity that is the basis for evolution (Aitken and Whitlock 2013, Mallet 2018, Corrêa et al. 2019). We still need additional information about the consequences of the hybridization of this agricultural pest to understand important evolutionary processes such as adaptation to new hosts, climatic conditions, and evolution of insecticide resistance. This first description of the reproductive patterns of *E. heros* will help understand the evolution of the adaptive characteristics of this pest in an agricultural scenario where large and diversified crops and non-crops shape a complex landscape in South America.

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3. CYTOGENOMIC CHARACTERIZATION OF *Euschistus* (HETEROPTERA: PENTATOMIDAE) SPECIES AND STRAINS OF REVEALS LOW CHROMOSOMAL AND REPETITIVE DNAS DIVERGENCES

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Running title: Low chromosomal divergence among *Euschistus*.

Abstract

Euschistus stink bugs are important pests on soybean crops, including the Neotropical representatives *E. heros*, *E. crenator*, and *E. taurulus*. Despite their importance, little genomic and chromosomal information is available. Genomic and chromosomal differences can play an important role in establishing reproductive barriers between species and populations. Here we investigate the chromosomes, some repetitive DNAs, and genome sizes of three Euschistus, including two *E. heros* strains (North strain-NS and South strain-SS), to address chromosomal evolution and genomic differentiation associated with the soybean crop in South America. Our data revealed conservative karyotypes and only one possible inversion among *Euschistus* species. Moreover, we observed the Y chromosome reorganization by differential microsatellite accumulation. The nuclear genome sizes were slightly variable among species. We noticed a differential accumulation of satellite DNAs, mainly on Y chromosome, in the two strains of *E. heros*. Although typical meiotic behavior, demonstrating full compatibility, was noticed on hybrids despite interference on chiasmata frequency. This data shows that chromosomal and repetitive DNAs do not prevent secondary contact between *E. heros* strains, contributing to genetic variability in hybrids. Therefore, we offer data about *Euschistus* pests and their chromosomal characteristics, being the first step in understanding their genomic organization and evolution.

Keywords: Chiasmata frequency; Genome size; Holocentric chromosome; Inversion; Microsatellite repeats; Multigenic families mapping; SatDNA; Speciation; Stink bugs

3.1. Introduction

Speciation is a dynamic process resulting from several factors. The origin of a new species can be linked to niche occupation, which can restrict the mating possibilities and, consequently, gene flow, resulting in an accumulation of variations (e.g., of the genome, morphology, physiology, behavior, reproduction etc.) and rise pre- or post-zygotic reproductive barriers (Dobzhansky, 1937; Mayr, 1942; Feder et al., 1994; Coyne & Orr, 2004; Egan & Funk, 2009). The reproductive isolation between populations is promoted by genomic changes, such as differential accumulation of DNA mutation, gene duplication, and chromosomal changes (White, 1973; King, 1995; Kraaijeveld, 2010; Nosil & Feder, 2012). Among insects, genomic differences and chromosomal rearrangements have been investigated and are prone to play an essential role in establishing reproductive barriers between species or populations (Kobayashi et al., 2000; Noor et al., 2001; Coluzzi, 2002; Kawakami et al., 2011; Mills & Cook, 2014).

The genus *Euschistus* Dallas, 1851 comprises 67 species, including species frequently found on crops, e.g., soybeans, *E. heros* (Fabricius, 1798), *E. crenator* (Fabricius, 1974), and *E. taurulus* Berg, 1878 (Hickmann et al., 2019; 2021b; Sosa-Gómez et al., 2020). These species are considered sister species, and morphologically similar with a distribution overlap in South America (Rolston, 1974; Bianchi et al., 2017). *Euschistus heros* is an important pest on soybean, initially considered as a secondary pest in the seventies, it became a key pest of soybeans a few years later (Panizzi et al., 2000; Sosa-Gómez et al., 2020). Furthermore, Soares et al. (2018) identified two strains of *E. heros* in Brazil that diverged from Pliocene, about 4.5 Myr. These two strains showed significant genetic (*Cytochrome c Oxidase Subunit I*, *Internal Transcribed Spacer* markers, and single nucleotide polymorphism), phenotypic (body size and coloration), and ecological (host preference) divergences (Soares et al., 2018; Zucchi et al., 2019; Hickmann et al., 2021a; 2022; Singh et al., 2023). The strains are found in distinct niches, one mostly restricted to the Atlantic Forest and Pampa Biomes further south of Brazil (hereafter SS), and another occurs in Caatinga and Amazon Forest Biomes further north (hereafter NS). These strains have a secondary contact in the Cerrado biome, forming a hybridization zone with an asymmetric gene flow (Soares et al., 2018; Zucchi et al., 2019; Hickmann et al., 2021a; 2022; Singh et al., 2023).

Mating in hybrid zones could be affected by ecological aspects, mating preferences, chromosome rearrangements, chromosome races within populations, reinforcement etc. (Gompert et al., 2012; Abbott et al., 2016; Kost et al., 2016; Lavrenchenko & Bulatova 2016; Corrêa et al., 2019). Hybridization may cause interactions involving a wide range of types and

levels of genetic divergence between the parental forms. In addition, unbalanced introgression can also play an important role, affecting recombination between two genetic pools (Noor et al., 2001; Emelianov et al., 2004). Therefore, the secondary contact of the two *E. heros* strains may either represent beneficial allele exchanges between the strains and generate highly adapted phenotypes, or they may also show irregular meiotic behavior decreasing their fitness and contributing to the speciation process (Shaw, 1981; Fitzpatrick & Shaffer, 2007; Gompert et al., 2017).

The chromosomes encompass the genetic elements of the entire nuclear genome, regardless of gene expression, age, or stage of development, which makes them an excellent source of information for evolutionary studies (Stebbins, 1966; Stace, 2000; Dobigny et al., 2004). Differences in karyotypes may indicate differentiation between evolutionary strains. These divergences can be caused by chromosomal rearrangements and/or accumulation/elimination and differentiation of repetitive sequences (such as mobile elements and satellite DNAs, satDNAs) that promote nuclear genome size variations (Coyne & Orr, 2004; Kirkpatrick & Barton, 2006; Faria & Navarro, 2010).

Here, we characterized the karyotype, applying classical and molecular cytogenetics, and measured the nuclear genome size of three species of *Euschistus* (*E. taurulus*, *E. crenator*, *E. heros*), including two strains of *E. heros* (SS and NS). Moreover, we sequenced the genomes of *E. heros* SS and NS to study the satDNAs. We aimed to describe in detail the chromosome composition and organization of these species to assess possible genomic divergence at inter- and intra-species levels and contribute to a better understanding of the chromosomal evolution and genomic differentiation processes of *Euschistus* genus and *E. heros* strains associated with the soybean crop in South America that has its genome recently assembled (Singh et al., 2023), but the repetitive fraction was not explored.

3.2. Material and methods

3.2.1 Samples and laboratory stock populations maintenance

We collected three *Euschistus* species in soybean fields, including males and females, as follows: *E. heros* SS was collected in Itapiranga, Santa Catarina state (27º 14' 00" S / 53º 73' 13" W); *E. heros* NS in Balsas, Maranhão state (7º 22' 90" S / 45º 97' 64" W); *E. taurulus* specimens were collected in Piracicaba, São Paulo state (22° 50' 49" S / 48° 01’ 08" W); and *E. crenator* was collected in Boa Vista, Roraima state (2° 39' 41.3" N / 60° 46' 58.9" W). A stock population of *E. heros* strains, *E. taurulus,* and *E. crenator* was established in the laboratory (Piracicaba, São Paulo, Brazil). Stock colonies and insect maintenance followed procedures

described in Hickmann et al. (2021a). In brief, they were kept in cages in a controlled environment (25 ± 1 ºC, 65 ±10 RH, and 14:10 (light: dark), and a natural diet and water were offered *ad libitum*.

3.2.2. Chromosome preparations and banding

To study mitotic and meiotic chromosomes, we dissected the testes and fixed them in Carnoy's solution (3:1, 100% ethanol: glacial acetic acid). The slides were obtained by squash technique for chiasmata analysis or by spreading under a hot plate at about 45ºC for the other analysis, including conventional staining, fluorochrome staining, and Fluorescence *in situ* Hybridization (FISH). Slides for conventional analysis were stained using Giemsa 5%. We also obtained mitotic chromosomes from embryos following the protocol of Webb et al. (1978). These slides were stained with Giemsa 5% to observe the occurrence of heteromorphic sex chromosomes in males and confirm which chromosome is the X and the Y. The fluorochrome banding with Chromomycin A_3 (CMA_3) and 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was performed according to Schweizer et al. (1983).

3.2.3. Nuclear genome size measurement

Adult males and females of *E. heros* strains, *E. taurulus* and *E. crenator* (samples), and female *Partamona helleri* (Friese, 1900) (standard) were used to measure the 2C nuclear DNA content. Due to the number of individuals needed, we used *P. helleri* as the standard. Thus, its 2C DNA value was previously measured from female *Scaptotrigona xanthotricha* Moure, 1950 [standard 2C = 0.88 pg (Lopes et al., 2009)]. From ten female *P. helleri*, the mean 2C value was equivalent to 1.20 pg (1C = 0.60 pg, Fig. S1A). This mean 2C value was used as a reference to measure the 2C value of the male and female *Euschistus* species and *E. heros* strains.

Adult males and females have their cerebral ganglia dissected in commercial saline solution. Nuclei suspensions were separately (only the sample or the standard) or simultaneously (sample and standard) prepared from cerebral ganglia in a 1.5 mL tube containing 100 µL OTTO I nuclear extraction buffer supplemented with 0.1 M citric acid, 0.5% Tween 20, 2.0 mM dithiothreitol and 50 µg mL^{-1} RNAse, pH = 2.3. The nuclei were extracted using a pestle (Lopes et al., 2009), and the resulting suspensions were incubated for 5 min. After 1.0 mL OTTO I was added, the suspensions were filtered through a 30 µm nylon filter into a 2.0 mL tube and centrifuged at 100 *x*g for 5 min. The supernatants were poured out, and the pellets were homogenized in 100 µL OTTO I and kept at 10 min. The nuclei suspensions were

stained for 30 min in the dark with 500 µL OTTO II buffer containing 0.4 µM $Na_2HPO_4.2H_2O$, 75 µM propidium iodide, and 50 µg mL^{-1} RNAse, pH = 7.8, and then filtered through a 20 µm filter into a cytometry tube. The suspensions were analyzed in a BD Accuri™ C6 Flow Cytometer (Accuri, Belgium) equipped with a 488 nm laser source to promote emissions of the propidium iodide at FL2 (615 – 670 nm) and FL3 (> 670 nm). The monoparametric and biparametric histograms were analyzed using the BD Accuri™ C6 software. G_0/G_1 peaks from each sample and the standard with a coefficient of variation below 5% were considered for 2C value measurement. From the mean 2C value in pg, the mean 2C or 1C value in Mbp [1 pg = 978 Mbp, (Dolezel et al., 2003)] was also determined for the male in 2C due to XY chromosomes and for the female in 1C due to XX chromosomes.

After the definition of the flow cytometry parameters (gain and channel of the G_0/G_1 peaks from each sample and the *P. helleri* standard) by external flow cytometry procedure, different internal flow cytometry analyses were performed: (a) cerebral ganglia of each sample and *P. helleri* standard; (b) cerebral ganglia of *E. taurulus* and *E. crenator*; (c) cerebral ganglia of the male and female for each *Euschistus* species and each *E. heros* strains. At least five repetitions (individuals of each *Euschistus* species and *E. heros* strain) were performed for each analysis, and more than 10,000 nuclei were analyzed for each nuclei suspension.

3.2.4. Meiotic behavior and chiasmata frequency of *E. heros* strains

The meiotic behavior and the chiasmata frequency from pure *E. heros* strains (SS and NS) offspring and hybrid offspring from reciprocal crosses between strains (♀NS x ♂SS and ♀SS x ♂NS) were accessed. For this, we dissected and fixed in Carnoy's solution (3:1, 100% ethanol: glacial acetic acid) the testes from one-week-old adult males randomly chosen from pure strains and hybrids stock populations, and the slides for chiasmata analysis were stained with lactic/acetic Orcein 2%. For each strain and reciprocal crosses offspring, five slides were prepared, and the whole slide was analyzed to evaluate the number of the rod (one chiasma) and ring chromosomes (two chiasmata) at diplotene–diakinesis (metaphase I) (Fig. S2A–D). Slide observations were made under a Nikon microscope Eclipse E2000 in 40- and 100-times magnification. The chiasmata frequency was estimated according to the number of rod and circular chromosomes found in each cell analyzed. These data were submitted to an ANOVA analysis and Tukey's test ($p \geq 0.05$).

3.2.5. Satellite DNA identification on *E. heros* strains

To advance in detail about the genome composition between the two strains of *E. heros,* we sequenced their genomes from male individuals in low coverage to prospect satDNAs. The genomic DNA from males of NS and SS was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's protocol. Genomic libraries were prepared using the NEBNext Ultra DNA Library Prep Kit (Biolabs, Massachusetts, EUA). The genomes of both strains were sequenced by paired-end sequencing (2 x 150 bp) using the Illumina HiSeq 4000 system, performed by Novogene (HK) Co., Ltd. (Hong Kong, China). The Illumina reads are available on GenBank SRR22807635 (North stain) and SRR22807636 (South strain). The reads obtained by sequencing both *E. heros* individuals belonging to NS and SS were processed, filtered, and interlaced using the preprocessing of reads tools available on the Galaxy/RepeatExplorer platform (Novak et al., 2013). The clustering pipeline by TAREAN (Novak et al., 2017) was performed using 500,000 reads from each strain genome, totaling 1 million paired reads. Then, the consensus sequence of each identified cluster in high and low confidence categories was annotated and checked for similarities between more clusters (Superclusters grouping two or more clusters).

For the calculation of the abundance and divergence of each satDNA family, we used the RepeatMasker software (Smit et al., 2017), comparing the NS and SS genomes. Sequence divergences were estimated by the average counts of Kimura 2-parameter distances (K2P) using the calcDivergenceFromAlign.pl script from the RepeatMasker utility tool (Smit et al., 2017). The reads of each genome were filtered by quality, concatenated, and transformed to fasta format with the "rexp_prepare_normaltag.py" script (https://github.com/ fjruizruano/ngs-protocols) using all sequenced libraries resulting in 5,086,288 paired reads for north genome and 5,546,672 paired reads for south genome. Following the same method of (Ruiz-Ruano et al., 2017), the abundance of each satDNA family was estimated as genome proportion for normalization, calculated by the number of mapped nucleotides concerning the library size. Likewise, the satDNAs families were named by decreasing abundance considering the mean value of quantity between both strain genomes. Through satDNA composition comparison, landscapes gathering all satDNA families identified were designed for each strain showing relative abundance on one axis and the K2P distance levels on the other. All sequences were analyzed to determine possible similarities with previously identified sequences by comparison with sequences deposited on GenBank/NCBI (Altschul et al., 1990) and Repbase v20.10 (Bao

et al., 2015). Monomers for each satDNA family were deposited on GenBank under the accession numbers OP499992-OP500039.

3.2.6 Probes obtaining and fluorescence in situ hybridization (FISH)

We extracted the genomic DNA from male individuals from *E. heros* belonging to NS using the Qiagen DNeasy kit (Qiagen Inc., CA, USA), following the manufacturer's protocol. The genomic DNA was used for Polymerase Chain Reaction (PCR) using the primers described by (Cabral-de-Mello et al., 2010) to obtain the 18S rDNA and 5S rDNA and by (Pineau et al., 2005) for the H4 histone sequence. Some satDNAs differentially enriched in one of the strains of *E. heros* (more than 1.5 times) were selected and amplified by PCR using specific primers for each satDNA family (Table S1). The probes were labeled with biotin-14-dATP or digoxigenin-11-dUTP by the second round of PCR using the product from the first PCR as a template. The three synthetic microsatellites oligonucleotide probes, i.e., $(A)_{30}$, $(CAC)_{10}$, and $(GACA)_4$, were labeled with biotin-14-dATP during their synthesis at the 5′ end (Sigma, St Louis, MO, USA). In the cross-hybridization experiments with genomic DNAs between two strains of *E. heros,* we labeled 500 ng of male genomic DNA by nick translation with digoxigenin-11-dUTP for 90 min.

FISH was performed according to (Cabral-de-Mello et al., 2021). To map the three multigene families in the same chromosome spreading to check if they are or are not in the same chromosome, we first mapped the 18S and 5S rDNAs. After the documentation of the results, the slides were washed three times in 2 × SSC, and the second round of FISH was performed using the probe H4 histone gene. Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin rhodamine (Roche), and the biotin-14-dATP labeled probes were detected using streptavidin, Alexa Fluor 488 conjugated (Invitrogen). The chromosomes were counterstained using DAPI, and the slides were mounted with VECTASHIELD (Vector, Burlingame, CA, USA). The chromosomes and hybridization signals were observed using an Olympus microscope BX61. Fluorescence images were recorded using a DP71-cooled digital camera in grayscale. The images were pseudocolored, merged, and optimized for brightness and contrast using Adobe Photoshop version 20.0.

3.3. Results

3.3.1 Karyotypes and fluorochrome banding

In males of the three species, including the two strains of *E. heros*, we observed identical karyotypes composed of 2n=14 (12+XY) and chromosomes slightly decreasing in size (Fig. 1A–H). Although the chromosomes decreased slightly in size, by repeated and combined analysis of metaphase I and mitotic metaphases from embryos, we recognized the six autosomal pairs, plus the X and Y chromosomes, based on differences in chromosome total length. In all species, the X chromosome was bigger than the Y chromosome, the smallest karyotype element, with a similar size to the sixth autosomal pair (Fig. 1A–H). We evidenced in mitotic metaphases a constriction near the middle of chromosome pair 3 (Fig. 1B, D, F, H) [holocentric chromosomes do not have primary constriction]. Chromosome banding with CMA_3 and DAPI revealed uniform staining for DAPI and one CMA_3 positive block interstitially in the pair three (Fig. 1I–L), corresponding to the constriction noticed by conventional staining.

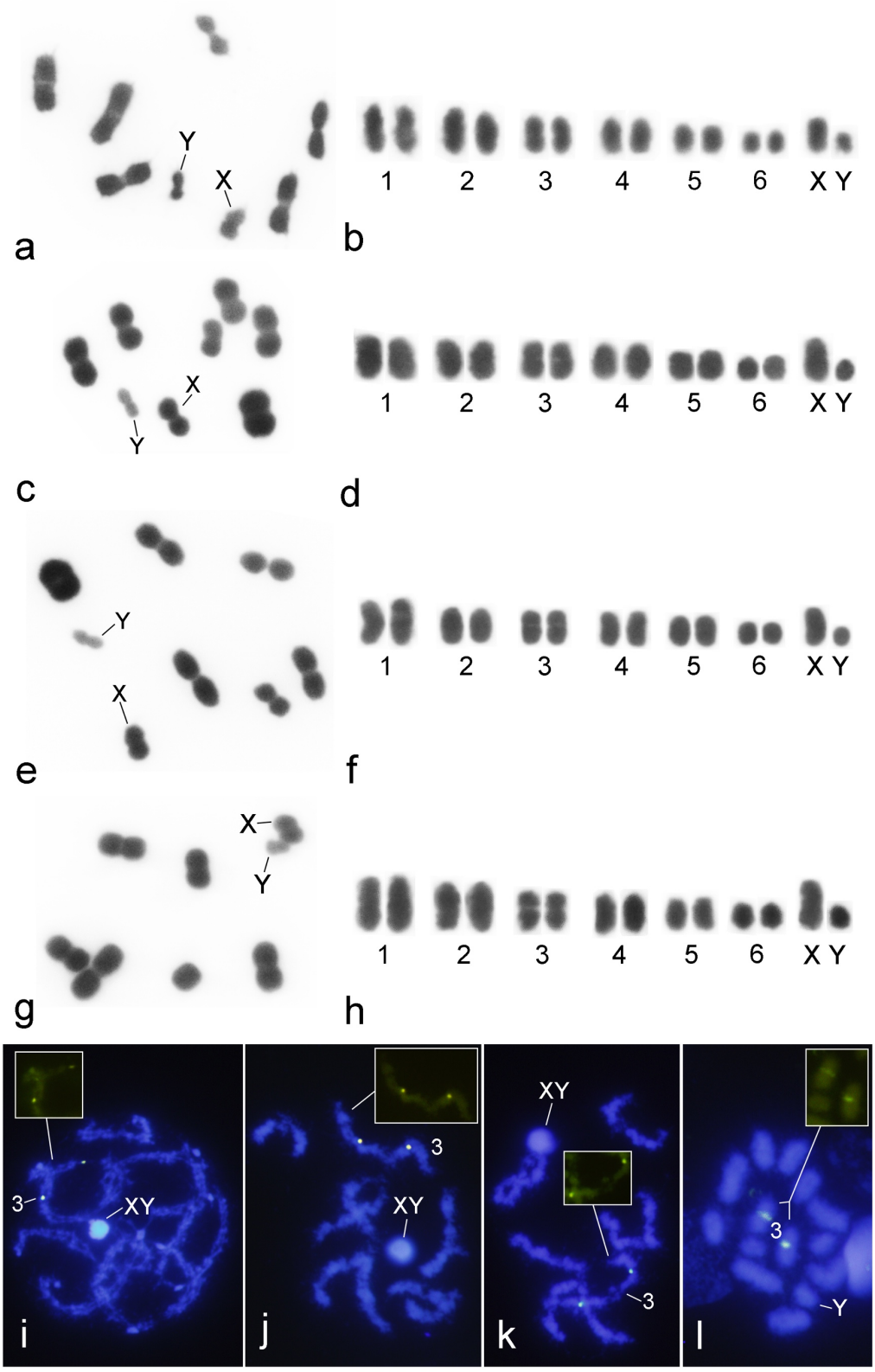

Figura 7. Conventional analysis (A-H) and banding with CMA_3/DAPI fluorochromes (I-L) of chromosomes of three *Euchistus* species, (A,B,I) *E. taurulus*, (C,D,J) *E. crenator*, (E,F,K) *E. heros* North strain and (G,H,L) *E. heros* South strain. (A,C,E,F) metaphase I, (B,D,F,H) upper: male karyotype from mitotic spermatogonial metaphase, bellow: female karyotype from mitotic embryo chromosomes (I) pachytene, (J,K) diplotene, (L) mitotic spermatogonial metaphase. The sex chromosomes are indicated in all cells. In (I-L) the insets show the chromosome

harboring positive CMA_3 signal. Note the occurrence of CMA_3 positive blocks on the middle of pair three (I-L), corresponding to the constriction observed in mitotic metaphase (B,D,F,H). Bar = 10 µm.

3.3.2. Nuclear genome size in *Euschistus* species

Based on the channel of the G_0/G_1 peaks (coefficient of variation below 4.41%) determined by external and internal flow cytometry procedures, we measured the mean 2C value of *Euschistus* species. The mean 2C values were: *E. taurulus* male 2C = 2.70 pg, *E. taurulus* female 2C = 2.74 pg, *E. heros* male 2C = 2.82 pg, *E. heros* female 2C = 2.92 pg, *E. crenator* male 2C = 2.92 pg, and *E. crenator* female 2C = 3.01 pg (Table 1 and Fig. S3A–C').

Table 1. Genome size measurements for *Euschistus* species.

Species	Sex	Genome Size		
		2C [pg]*	SD**	Mbp***
E. taurulus	Male	2.70	0.148	2,637.93 (2C)
	Female	2,74	0.105	1,337.64 (1C)
E. heros NS	Male	2,79	0.029	2,727.79 (2C)
	Female	2.94	0.060	1,437.66 (1C)
E. heros SS	Male	2,84	0.025	2,773.96 (2C)
	Female	2.90	0.035	1,414.99 (1C)
E. crenator	Male	2.92	0.010	2,853.98 (2C)
	Female	3.01	0.013	1,473.67 (1C)

A 2C = 0.05 pg difference was found between the mean 2C values of the North and South male *E. heros* strains, equivalent to ~1.76% of the nuclear genome size. The difference between the females was 2C = 0.04 pg, equivalent to ~1.37%. The G_0/G_1 peaks of these two strains (NS *E. heros* x SS *E. heros*) occurred in the same channel, confirming the previous results obtained from internal standard *P. helleri*. Therefore, both strains have the same nuclear genome size.

The mean 2C value and comparative flow cytometry histograms from male *E. taurulus* x male *E. crenator* and female *E. taurulus* x female *E. crenator* showed that *E. taurulus* possesses the lowest nuclear genome size (Fig. S1B). Considering the mean value measured by males and females, *E. taurulus* has 2C ~0.15 pg lesser than *E. heros*, and 2C ~0.25 pg lesser than *E. crenator*. *Euschistus heros* has 2C ~0.098 lesser than E. crenator. Therefore, we noted an interspecific mean 2C value variation (Fig. S1C).

From the internal standard *P. helleri* measurements, the male *E. crenator* and *E. heros* showed a smaller mean 2C value than the respective females. This result was confirmed by nuclear suspensions containing both males and females of each species. From this, we noted that the G_0/G_1 peaks of the male positioned in the different channels about the female. Differently, male *E. taurulus* (2C = 2.70 ± 0.148 pg) and female *E. taurulus* (2C = 2.74 ± 0.105 pg) G_0/G_1 peaks were identified in the same channel (coefficient of variation 3.00%) in the FL2 and FL3 filters to detect propidium iodide fluorescence (Fig. S1B and S1C). Therefore, we appointed that if there is a difference between them, the value is lesser than ~2C = 0.04 pg, equivalent to ~1.46% of the nuclear genome size.

3.3.3. Chromosomal mapping of multigene families and microsatellites of *Euschistus* species

Through FISH mapping, we invariably observed one pair of clusters (one autosomal bivalent) for each of the three multigene families mapped, i.e., 18S rDNA, 5S rDNA, and the H4 histone gene (Fig. 2A–D). The 18S rDNA cluster was located interstitially on pair 3 near the middle of this chromosome, corresponding to the constriction and CMA_3 positive block (Fig.1). The 5S rDNA was placed interstitially on pair one. The H4 histone cluster was on pair 2, mapped interstitially for *E. taurulus* and terminally positioned for *E. crenator* and *E. heros* (both strains) (Fig. 2A–D).

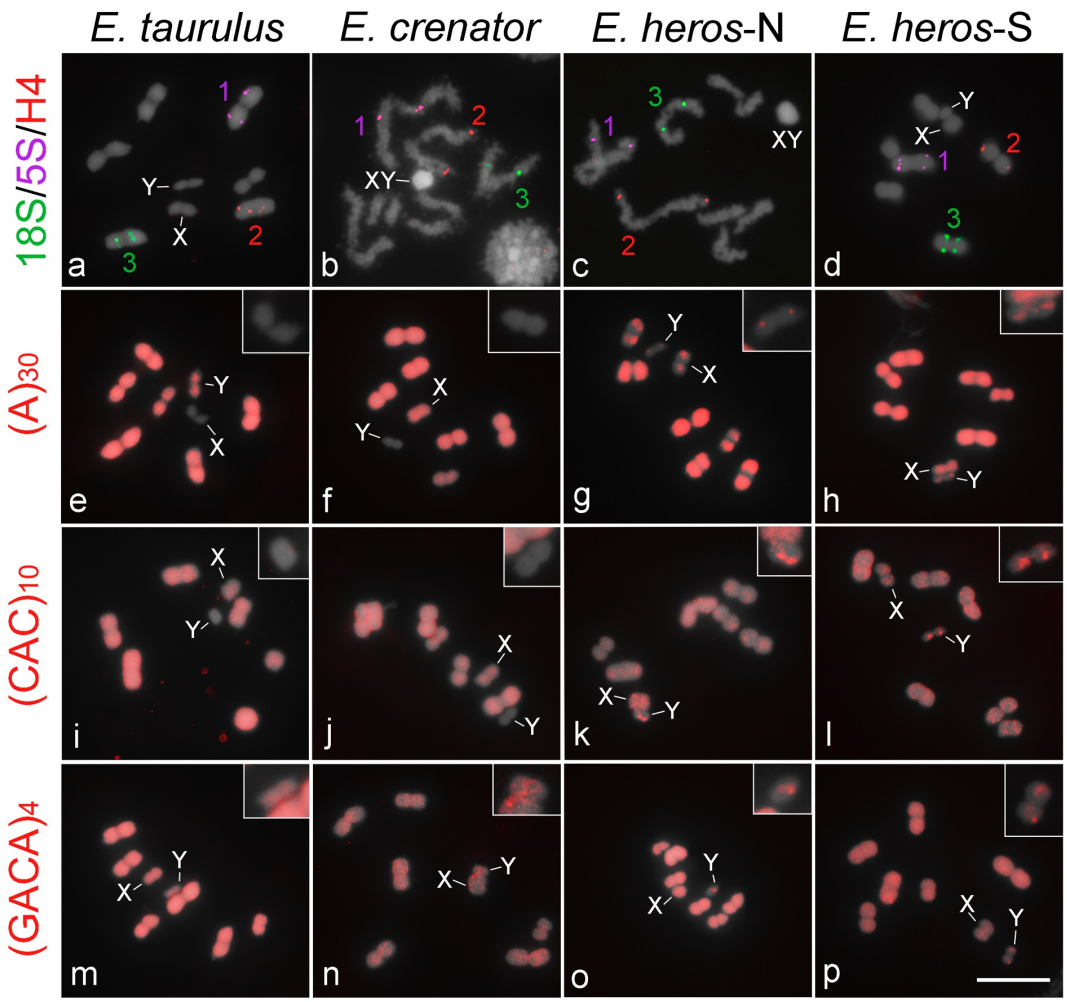

Figura 8. Chromosomal mapping of repetitive DNAs on meiotic chromosomes of *Euchistus* species. (A,D,E-N,P) metaphase I, (B,C) diplotene and (O) metaphase II. In (A-D) the chromosomes harboring signals are identified. In all cells the sex chromosomes are indicated. The insets in (E-P) shows enlarged Y chromosome, showing in detail the signal distribution. The name of species and specific probes are directly indicated in the images. Bar = 10 µm.

The mapping of microsatellites $(A)_{30}$ (Fig. 2E–H), $(CAC)_{10}$ (Fig. 2I–L), and $(GACA)_4$ (Fig. 2M–P) revealed enrichment on all autosome chromosomes. The labeling of $(A)_{30}$ was more intense than the other two microsatellite motifs (Fig. 2E–P). The X chromosome was strongly labeled, as the autosomes, for the three microsatellites (Fig. 2E–P). The Y chromosome presented variability between species. For $(A)_{30}$ and $(CAC)_{10}$, the Y chromosomes of *E. taurulus* and *E. crenator*, no signals were observed (Fig. 2E, F, I, J), while in *E. heros*, the Y chromosome revealed one small signal near to the middle region (Fig. 2G, H, K, L). The $(GACA)_4$ showed spread signals on the Y chromosome of all species (Fig. 2M–P), but for *E. heros* strains, besides spread signals, a stronger signal was noticed near the middle of this chromosome (Fig. 2O–P).

3.3.4. Cross-mapping of genomic DNA, chiasmata frequency, and satDNAs of two strains of *Euschistus heros*

Aiming to go into deeper details on possible differences between the strains of *E. heros*, we cross-mapped the genomic DNA of NS and SS to check if there is any part of the chromosomes differentiated. Moreover, we verified if there any type of meiotic incompatibility between the strains by reciprocal crosses and offspring analysis of chromosome pairing and chiasmata formation. Finally, we studied the satDNA populating the genomes of NS and SS of *E. heros*.

Our cross-strain mapping of genomic DNA revealed spread signals along all chromosomes, with no virtual variability of signal intensity between chromosomes. Independent of the FISH direction, no signal intensity or distribution differences, i.e., gDNA from NS on SS chromosomes or vice-versa, were observed (Fig. 3).

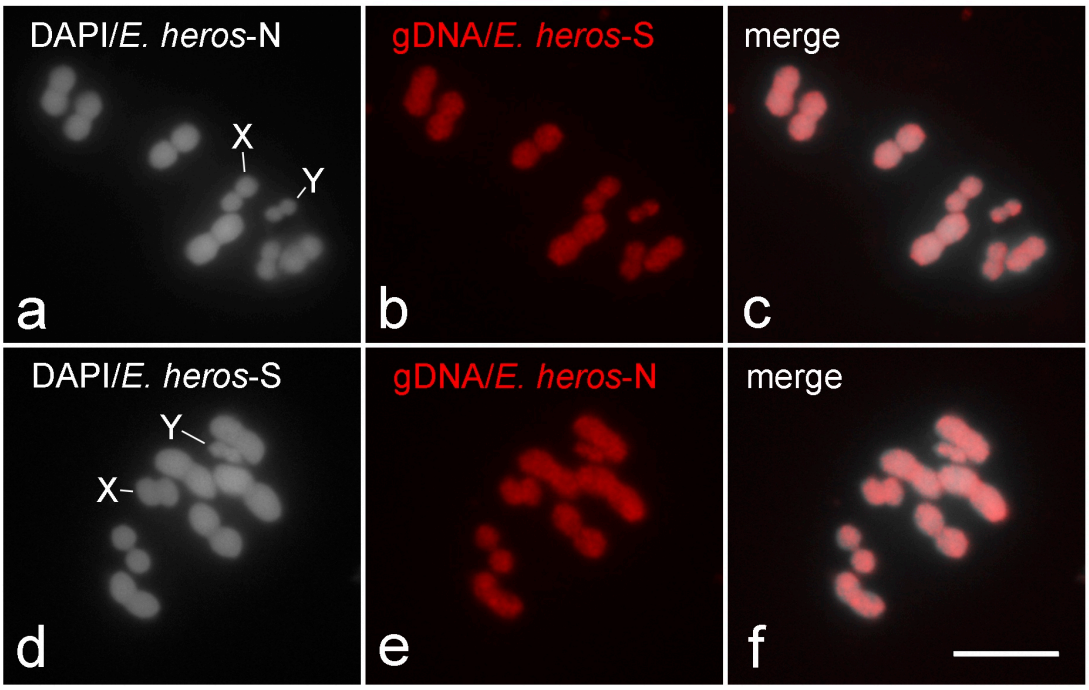

Figura 9. Cross-mapping of genomic DNAs from males in metaphase I of males belonging to the two *E. heros* strains. (A) chromosomes stained with DAPI from one individual belonging to NS, (B) signal obtained with genomic DNA probe from one individual belonging to SS, (D) chromosomes stained with DAPI from one individual belonging to SS, (E) signal obtained with genomic DNA probe from one individual belonging to NS. In (C,F) both channels are merged. Note the uniform distribution of signals in all chromosomes. The sex chromosomes are indicated. Bar = 10 µm.

The cytological observations of reciprocally crossed offspring did not show meiotic incompatibility, as the meiotic phases showed the same pattern as the parental strains, with standard chromosome pairing (Fig. S2A–D). The NS showed the highest chiasmata frequency and significantly differed compared to the offspring from the reciprocal cross ♀NS x ♂SS [F = 4.07, df = 3 and p = 0.0251] (Table 2). The SS and the reciprocal cross between ♀SS x ♂NS did not show a difference in chiasmata frequency. On the other hand, the cross ♀NS x ♂SS reduced the chiasmata frequency of its offspring. Although we did not observe meiotic incompatibility, we found that the crosses between *E. heros* strains can potentially affect the chiasmata frequency of the offspring.

Table 2. Number of chiasmata (q) and mean chiasma frequency at diplotene–diakinesis (metaphase I) in specimens of *Euschistus heros* (south strain - SS and north strain - NS), reciprocal crosses between the two *E. heros* strains (♀SS x ♂NS and ♀NS x ♂SS).

Chiasmata frequency (q)							
Euschistus heros - SS							
	Number of cells with:						
ID	6q	7q	8q	9q	10q	Total	Mean chiasma frequency
1	30	49	10	2	-	91	6.82
2	37	33	8	-	-	78	6.63
3	55	27	5	3	-	90	6.51
4	101	45	6	-	-	152	6.38
5	61	55	5	-	-	121	6.54
							Mean ± SE: 6.58^{ab} ± 0.06
Euschistus heros - NS							
	Number of cells with:						
ID	6q	7q	8q	9q	10q	Total	Mean chiasma frequency
1	34	50	6	-	1	91	6.73
2	81	29	2	-	-	112	6.29
3	81	76	8	-	-	165	6.56
4	49	76	12	-	-	137	6.73
5	36	53	9	-	-	97	6.79
							Mean ± SE: 6.63^{a} ± 0.08
Euschistus heros - ♀SS x ♂NS							
	Number of cells with:						
ID	6q	7q	8q	9q	10q	Total	Mean chiasma frequency
1	33	42	16	2	1	94	6.89
2	43	48	24	3	1	119	6.92
3	53	17	5	-	-	75	6.36
4	33	21	2	-	-	56	6.45
5	71	30	6	-	-	107	6.39
							Mean ± SE: 6.60^{ab} ± 0.11
Euschistus heros - ♀NS x ♂SS							
	Number of cells with:						
ID	6q	7q	8q	9q	10q	Total	Mean chiasma frequency

1	79	19	2	-	-	100	6.23
2	53	15	-	-	-	68	6.22
3	46	11	1	-	-	58	6.22
4	85	40	4	-	-	129	6.36
5	86	37	5	-	-	128	6.31
							Mean ± SE: 6.27^{b} ± 0.03

The analysis of satDNAs by RepeatExplorer allowed the identification of 48 satDNA families on the genomes of both strains of *E. heros*. These satDNA families were mostly A+T rich (mean 68,64%) and ranged in monomer length from 43 bp to 6.512 bp. None of these satDNAs presented similarities with previously described sequences. The abundance for each satDNA family ranged from 0.00753% to 0.46180% on NS and from 0.00610% to 0.57622% on SS. The total amount of satDNA on the NS genome was 4.08157%. At the same time, in the SS, the satDNAs represented slightly more of the genome proportion, corresponding to a total abundance of 4.74768%, which is about 14% morem abundant for satDNAs on this strain than NS. Some satDNAs were more enriched on SS strain than NS and vice-versa, but in general, more satDNA families were enriched on SS, causing an increase in the total abundance of satDNAs. However, the most striking example of the difference in abundance was for the EheSat30-547, which is about six times more enriched on the NS strain. The EheSat38-539 is the only other satDNA with more than 1.5 times enriched on the NS. On the SS, we identified eight satDNAs with more abundance compared to NS, at least 1.5 times more abundant, EheSat05-165, EheSat12-560, EheSat24-130, EheSat32-489, EheSat33-702, EheSat36-126, EheSat39-129, EheSat43-1089 (Table 3). The patterns of amplification and homogenization could also be noticed by analysis of landscape (abundance versus divergence), in which more abundance of repeats with lower divergence in SS in comparison to NS (Fig. 4).

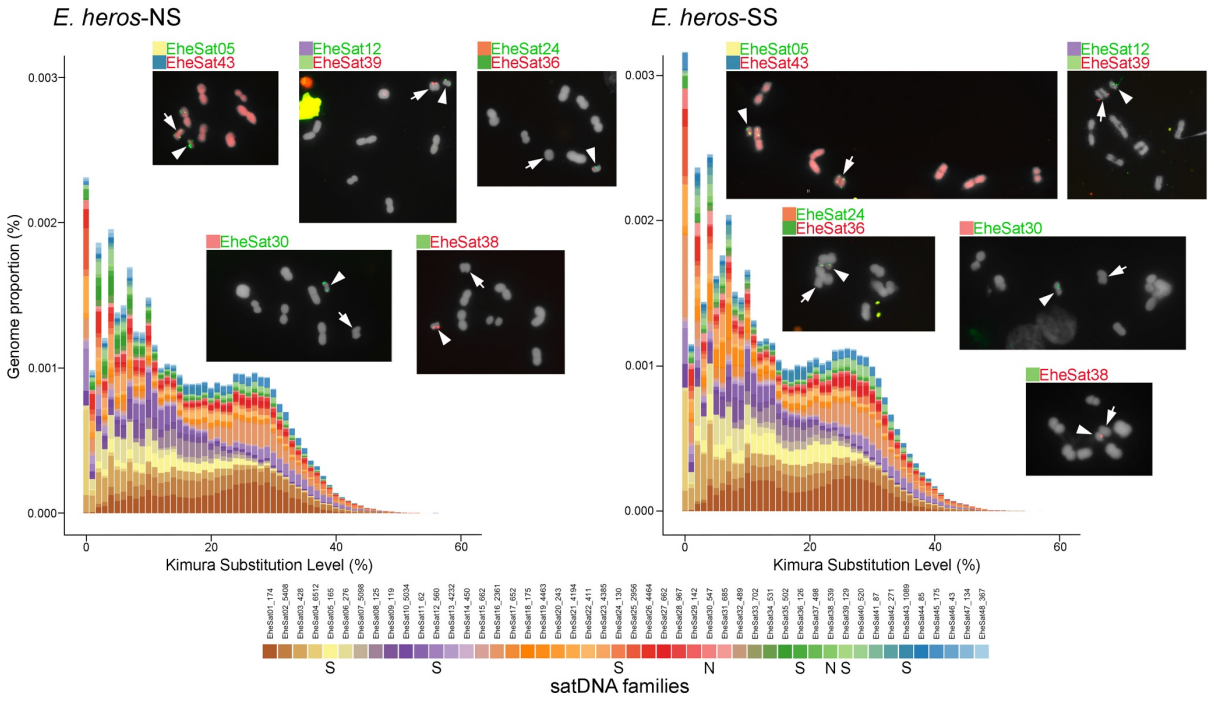

Figure 10. satDNA landscapes (genome proportion versus sequence divergence based on Kimura substitution level) and FISH mapping of some repeats in the two strains of *E. heros*. Note the slightly higher, more general abundance for some repeats with lower Kimura substitution levels on SS. The arrow points to the X chromosome on FISH images, and the arrowheads to the Y chromosome. Note the recurrent occurrence of signals on the sex chromosomes, mostly on the Y chromosome.

Among the ten satDNA families more enriched in one of the genomes, we successfully amplified through PCR eight of them and mapped them on the chromosomes. The satDNA EheSat43-1089 revealed spread signals along all chromosomes of the karyotype, while the other satDNAs presented discrete bands on specific chromosomes. EheSat05-165 was located in the terminal region of X and Y chromosomes and on chromosome 3; EheSat39-129 was placed exclusively in the terminal region of chromosomes X and EheSat12-560, EheSat24-130, EheSat30-547, EheSat36-126, and EheSat38-539 were mapped solely on the terminal part of the Y chromosome. Although there is a difference in abundance observed by genomic analysis, from the chromosomal point of view, it is not evidenced, with clusters presenting similar sizes (Fig. 4).

Table 3. Main features of the 48 satDNA families found on the genomes of *Euchistus heros* strains (north strain – NS and south strain - SS). ML (monomer length) and K2P (Kimura 2-parameter divergence). Genomic-enriched satDNA families are bolded.

			North Strain (NS)		South Stain (SS)			
satDNA families	ML	AT %	K2P %	Abundance	K2P %	Abundance	Coefficient NS/SS	Coefficient SS/NS
EheSat01-174	174		21.33	0.46180%	19.98	0.57622%	0.80	1.25
EheSat02-5408	5408		21.41	0.33953%	21.84	0.32812%	1.03	0.97
EheSat03-428	428		9.86	0.24742%	9.58	0.33821%	0.73	1.37
EheSat04-6512	6512		21.05	0.27349%	20.47	0.30209%	0.91	1.10
EheSat05-165*	**165**		**14.36**	**0.16800 %**	**15.19**	**0.29061 %**	**0.58**	**1.73**
EheSat06-276	276		6.79	0.17146%	6.75	0.20269%	0.85	1.18
EheSat07-5098	5098		23.43	0.16612%	24.32	0.16987%	0.98	1.02
EheSat08-125	125		16.29	0.13784%	16.7	0.15046%	0.92	1.09
EheSat09-119	119		10.98	0.12873%	10.37	0.15727%	0.82	1.22
EheSat10-5034	5034		23.46	0.12319%	23.09	0.13498%	0.91	1.10
EheSat11-62	62		11.94	0.13425%	11.77	0.11190%	1.20	0.83
EheSat12-560*	**560**		**15.89**	**0.06606 %**	**10.91**	**0.17014 %**	**0.39**	**2.58**
EheSat13-4232	4232		6.7	0.09652%	6.22	0.10511%	0.92	1.09
EheSat14-450	450		19.45	0.09701%	18.3	0.10368%	0.94	1.07
EheSat15-662	662		16.32	0.08813%	20.39	0.11162%	0.79	1.27
EheSat16-2361	2361		26.94	0.09036%	24.11	0.09813%	0.92	1.09
EheSat17-652	652		8.07	0.09754%	7.22	0.08921%	1.09	0.91
EheSat18-175	175		16.63	0.07176%	13.9	0.10053%	0.71	1.40
EheSat19-4463	4463		10.59	0.08311%	9.82	0.08324%	1.00	1.00
EheSat20-243	243		20.87	0.07556%	24.44	0.08876%	0.85	1.17
EheSat21-4194	4194		9.03	0.07672%	7.93	0.08302%	0.92	1.08
EheSat22-489	489		16.29	0.07943%	19.85	0.05922%	1.34	0.75
EheSat23-4385	4385		13.6	0.06325%	11.71	0.07379%	0.86	1.17
EheSat24-130*	**130**		**20.8**	**0.05011 %**	**20**	**0.07916 %**	**0.63**	**1.58**
EheSat25-2956	2956		8.31	0.06155%	8.41	0.05679%	1.08	0.92
EheSat26-4464	4464		6.08	0.05686%	6.04	0.06041%	0.94	1.06
EheSat27-662	662		12.16	0.04654%	13.38	0.06561%	0.71	1.41
EheSat28-967	967		13.28	0.05899%	13.64	0.04434%	1.33	0.75
EheSat29-142	142		17.43	0.03944%	15.57	0.05877%	0.67	1.49

EheSat30-547**	**547**	**8.01**	**0.07926 %**	**17.93**	**0.01292 %**	**6.14**	**0.16**
EheSat31-685	685	8.55	0.03663%	9.41	0.03288%	1.11	0.90
EheSat32-489*	**489**	**10.47**	**0.02054 %**	**8.03**	**0.04728 %**	**0.43**	**2.30**
EheSat33-702*	**702**	**15.16**	**0.02117 %**	**11.93**	**0.04661 %**	**0.45**	**2.20**
EheSat34-531	531	8.63	0.03747%	9.46	0.02853%	1.31	0.76
EheSat35-502	502	9.46	0.03041%	8.33	0.03012%	1.01	0.99
EheSat36-126*	**126**	**9.94**	**0.01821 %**	**8.19**	**0.03722 %**	**0.49**	**2.04**
EheSat37-498	498	13.78	0.02244%	13.71	0.03217%	0.70	1.43
EheSat38-539**	**539**	**11.77**	**0.03215 %**	**21.61**	**0.01714 %**	**1.88**	**0.53**
EheSat39-129*	**129**	**20.15**	**0.01483 %**	**18.43**	**0.03299 %**	**0.45**	**2.22**
EheSat40-520	520	11.53	0.01968%	9.97	0.02625%	0.75	1.33
EheSat41-87	87	22.29	0.01683%	20.14	0.01977%	0.85	1.17
EheSat42-271	271	11.72	0.01542%	11.53	0.01626%	0.95	1.05
EheSat43-1089*	**108 9**	**3.68**	**0.01172 %**	**2.07**	**0.01858 %**	**0.63**	**1.59**
EheSat44-85	85	10.33	0.01246%	10.31	0.01758%	0.71	1.41
EheSat45-175	175	16.1	0.01327%	16.12	0.01037%	1.28	0.78
EheSat46-43	43	6.79	0.01194%	7.18	0.00907%	1.32	0.76
EheSat47-134	134	13	0.00885%	12.83	0.01193%	0.74	1.35
EheSat48-367	367	14.37	0.00753%	14.7	0.00610%	1.23	0.81
Total			**4.08157 %**		**4.74768 %**		

3.4. Discussion

3.4.1. Interspecies chromosomal evolution of *Euschistus* species

Here by a detailed analysis of nuclear DNA content and karyotype features, we advance in understanding genome organization and differentiation in three soybean pests of the *Euschistus* genus. The three species presented similarities concerning the karyotypes, nuclear genome sizes, and repetitive DNA distribution. In addition, only slight differences were noticed, contrasting with the previous differences reported in genetic and morphological features (Bianchi et al., 2017; Hickmann et al., 2021a; 2021b; 2019).

The karyotype described for the *Euschistus* species is in accordance with the previous description for *E. heros* (Aguiar et al., 2017) and with most other Pentatomidae bugs, 2n=14, XY (Ueshima, 1979; Rebagliati et al., 2005; Bardella et al., 2013a; 2013b; Bardella et al., 2016). Based on our karyotype and nuclear genome size data of both sexes, we define that the Y chromosome is smaller than the X. This pattern has been described in multiple species of Pentatomidae but based only on an analysis of males (Rebagliati et al., 2005; Papeschi &

Bressa, 2006). The smaller size of the Y chromosome has been related to degeneration during evolution (Charlesworth & Charlesworth, 2000), suggesting that this genome decrease is common for Pentatomidae.

The chromosomal markers studied here revealed stasis in the karyotypes of *Euschistus,* and the minor differences helped understand chromosomal evolution in the genus. All species presented the same number of CMA_3+ bands, 18S and 5S rDNAs, and H4 histone located on homoeologous chromosomes. One single cluster for these multigene families is quite common on Heteroptera, only with few divergent and putatively derived patterns (Bardella et al., 2016a; Bardella et al., 2013b). Although, the interspecies difference was detected for the position of H4 histone that occurred terminally in *E. heros* and *E. crenator*, but interstitially in *E. taurulus*. Moreover, the terminal 18S blocks in *E. cornutus* Dallas, 1851 found by (Bardella et al., 2013b) contrast with the species studied here. Interstitial sites of 18S rDNA, as observed here for *Euschistus*, are uncommon in Heteroptera and Pentatomidae, they are usually terminally located, evidencing a chromosomal inversion for these *Euschistus* species (Bardella et al., 2013b). Furthermore, our data suggest an inversion in the common ancestor of these closely related species (Bianchi et al., 2017), indicating a synapomorphy. Occurrence of inversions and reshuffling of the position of multigene families were also postulated on other holocentric chromosome insects (Šíchová et al., 2015; Anjos et al., 2016; Provazníková et al., 2021).

The microsatellite repeats were similarly spread along most chromosomes of *Euschistus*, likewise in other insects (Cuadrado & Jouve, 2010; Milani & Cabral-de-Mello; 2014, Palacios-Gimenez & Cabral-de-Mello, 2015; Elizeu et al., 2021). The extent distribution of microsatellites found here can be related to mobile elements that may contain microsatellite sequences (Messier et al., 1996; Nadir et al., 1996; Wilder & Hollocher, 2001; Li et al., 2004; Grandi & An, 2013; Elizeu et al., 2021). Particularly on the X and Y, the microsatellite repeats distribution and enrichment were divergent, revealing molecular differentiation between them. While the X chromosome has a similar distribution to autosomes, the Y chromosome was impoverished on microsatellites, suggesting differentiation of this chromosome to the rest of the genome. Moreover, for the $(A)_{30}$ and $(CAC)_{10}$ microsatellite repeats, the distribution was divergent among the three *Euschistus* species, evidencing plasticity for the Y chromosome among these close relatives. These findings align with other species with heteromorphic sex chromosomes that also showed differential accumulation of microsatellites (Pokorná et al., 2011; Matsubara et al., 2016; Yano et al., 2017).

The mean nuclear 2C value for the three *Euschistus* species is similar to other Hemiptera (Hanrahan & Johnston, 2011; Alfsnes et al., 2017) and other *Euschistus* (Hughes-Schrader & Schrader, 1956), including *E. heros* (Singh et al., 2023). Comparative nuclear genome size analysis revealed slight interspecific variations, supporting that their genomes experienced genome increasing or reduction events. Nuclear C-value differences between closely related species have been reported in insects, e.g., flies, grasshoppers, and bed bugs (Gregory & Johnston, 2008; Sadílek et al., 2019; Mao et al., 2020). In addition, we noticed nuclear 2C value differences between sexes, with males presenting smaller nuclear genome sizes. This orthodox intraspecific variation is generally associated with the sex determination chromosome (XX and XY) in other species (Gregory & Johnston, 2008; Mao et al., 2020), proposing the same pattern for *Euschistus* species.

3.4.2. *Euschistus heros* lineages are not isolated by chromosomal characteristics

Our detailed analysis of the two allopatric stains of *Euschistus* revealed that they are fully reproductive compatible from the chromosomal point of view. We evidenced that the two *E. heros* strains are similar in general genomic composition with positive cross-hybridization in both directions, suggesting no incompatibility between NS and SS (Fig. 3). Moreover, the two allopatric strains of *E. heros* (SS and NS) did not show extensive differences in nuclear genome size (Table 1 and Fig. S3A–C'), suggesting that the mechanisms that cause genomic size variations (Blommaert, 2020) were not able to differentiate in a high extension the genomes of the two strains.

Although no extensive difference was observed for genome size between *E. heros* strains, the satellitome study revealed slight enrichment of satDNAs on the SS (Table 3), suggesting recent amplification of this type of repeat on this strain. By FISH analysis, we observed that most of these repeats are located on the Y chromosome (Fig. 4), and it is in accordance with the difference in genome size between males from NS and SS, in which males from SS presented a slightly bigger genome. The amplification of repeats on sex chromosomes, mainly on a heteromorphic sex chromosome (Y or W), is quite common and accounts for their evolutionary differentiation (Ezaz & Deakin, 2014; Hobza et al., 2015). Between populations or even in the same population, differential accumulation of repeats or heterochromatin on the Y chromosome was seen in some insects (Giardini et al., 2015; Hall et al., 2016; Chirino et al., 2020; Ferretti et al., 2020). These differences in the sex chromosome abundance of satDNAs

have apparently no relevant impact on the reproduction of the two stains, as no incompatibility was observed (Hickmann et al., 2021a).

The reproductive compatibility between the two *E. heros* strains was also corroborated by the usual meiotic behavior we observed in hybrid offspring, with all chromosomes with regular pairing and segregation (Fig. S2A–D). The absence of any meiotic incompatibility supports the lack of reproductive barriers found between the two *E. heros* strains (Hickmann et al., 2021a). These results support the hypothesis that gene flow and allelic exchange between the two strains can occur in the secondary contact regions (Soares et al., 2018; Zucchi et al., 2019; Hickmann et al., 2021a; Singh et al., 2013). Given that the two strains of *E. heros* diverged around 4.5 Myr, their satDNA pool still showed high similarity. The only few satDNAs differentially amplified between the two *E. heros* strains occurred primarily on the sex chromosomes, mainly the Y chromosome, and did not prevent the secondary contact. The secondary contact can increase allelic variability, which, summed with the extra variability found on the Y chromosome and on lesser extent on the X chromosome, may contribute to adaptability on the contact zone, providing additional evidence of the sudden increase of economic importance of this pest in the recent decades. Furthermore, the two strains co-occurrence region (Cerrado biome) is one of the most important Brazilian agriculture frontiers with extensive soybean and cotton fields (Silva et al., 2020). The reunion of these two gene pools (prone to allelic exchange) represents a massive potential for selecting (by natural selection and/or genetic drift) highly adapted phenotypes to these agroecosystems (Corrêa et al., 2019).

Besides the stable meiotic behavior in hybrid offspring, the chiasmata frequency was significantly reduced in reciprocal cross ♀NS × ♂SS compared with pure NS (Table 2). This finding is in line with the asymmetric gene flow previously reported, where SS shows higher genome introgression compared to NS in the co-occurrence region (Zucchi et al., 2019; Singh et al., 2013). This asymmetric gene flow has been related to the mating behavior of the *E. heros* strains, where insects with wider pronotum have more chance to mate, favoring the SS phenotype (SS is bigger in size than NS) (Hickmann et al., 2021a). Thus, the mating choice behavior and the reduction of chiasmata frequency provide additional evidence **of** why NS introgression is reduced in the hybrid zones. This scenario of apparently extensive gene flow between the two allopatric strains may favor the complete admixture between the two gene pools (Abbott et al., 2013; Cordeiro et al., 2020).

3.5. Conclusions

Our data reveal stability in the karyotypes of related *Euschistus* species, with only minor nuclear 2C value differences, intrachromosomal rearrangements, like inversions, and accumulation of microsatellites on the Y chromosome. Although some slight differences were accumulated between the two strains of *E. heros* (NS and SS) for satDNAs, we found no apparent chromosomal incompatibility between them. Therefore, we offer data about these agriculture pests and their genome characteristics, being the first step in understanding their genomic organization and differentiation, as well as that of other *Euschistus* species.

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Disclosure

The authors declare they have no conflicts of interest.

Authors contributions

FH, ASC, MM, WRC, and DCC conceived the study and designed the experiments; FH, VBB, DM, DCC, MM, WRC, ASC, FF, and RFC, performed the experiments and analyzed data; FH, ASC, MM, DCCM wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary files

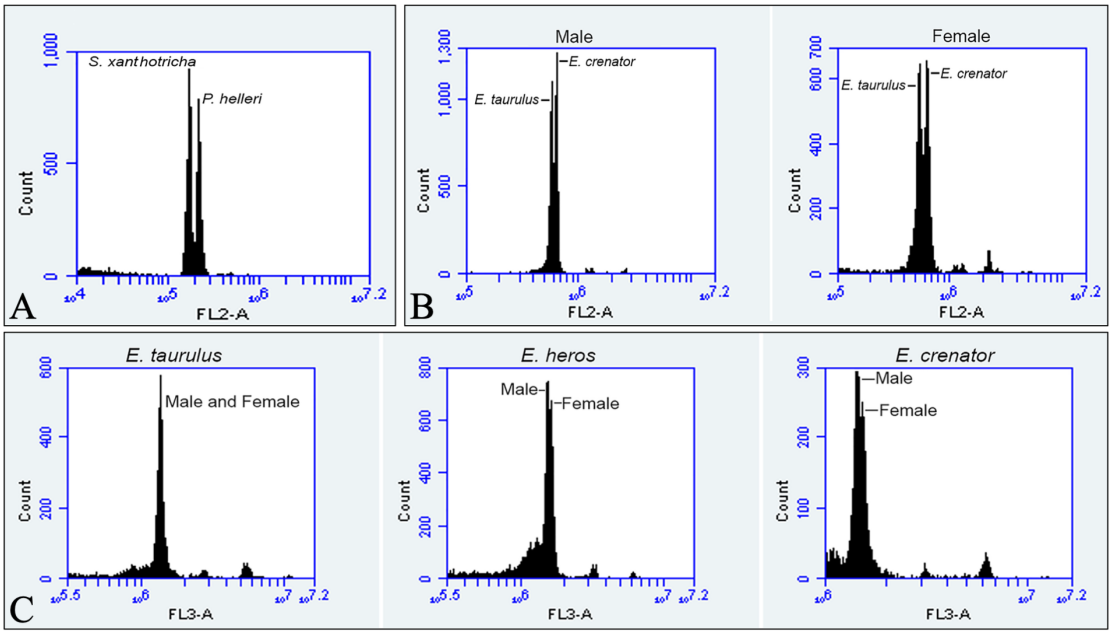

Figure S1. (A) Representative flow cytometry histogram about the 2C value of the female *P. helleri* from the internal standard *S. xanthotricha*. Considering the G_0/G_1 nuclei peaks and 2C value of the female *S. xanthotricha* (2C = 0.88 pg), the 2C value of the female *P. helleri* is equivalent to 1.20 pg. (B) Nuclear genome size differences between *Euschistus* species. Based on the G_0/G_1 nuclei peaks of *E. taurulus* and *E. crenator*, we confirmed the interspecific 2C value variation between the males and the females. (C) Representative flow cytometry histograms evidencing the same 2C value between the male and female *E. taurulus*, and the 2C value difference between male and female *E. heros* and *E. crenator*, confirming our data (Figure S3).

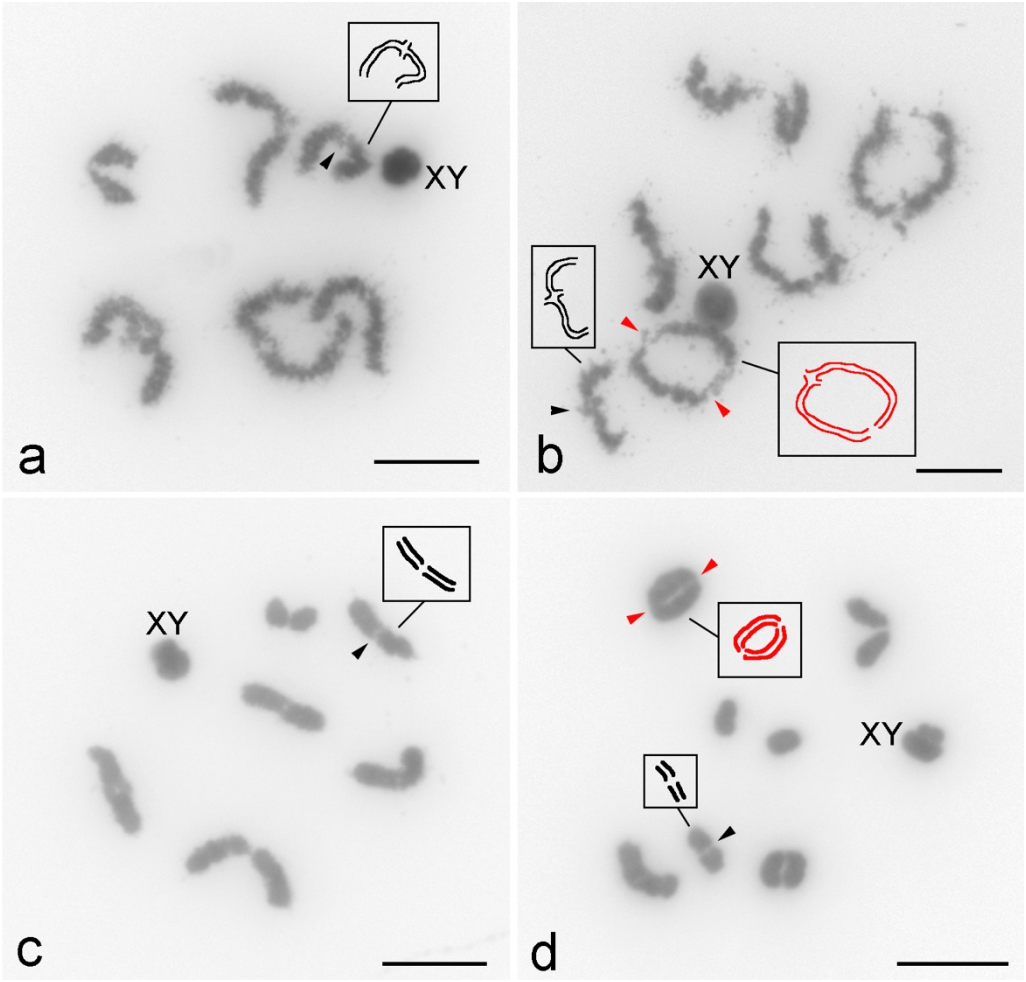

Figure S2. Conventional analysis of meiosis I of *Euschistus heros* showing different chiasmata configurations. (A) late diplotene, (B) early diplotene, (C) early diakinesis and, (D) late diakinesis. The sex chromosomes are indicated in all cells. Black arrows show bivalents with one chiasma and red arrows show bivalents with two chiasmata. Schematic representations of chromosomes with one chiasma (black) or two chiasmata (red) are also presented. Bar = 10 µm.

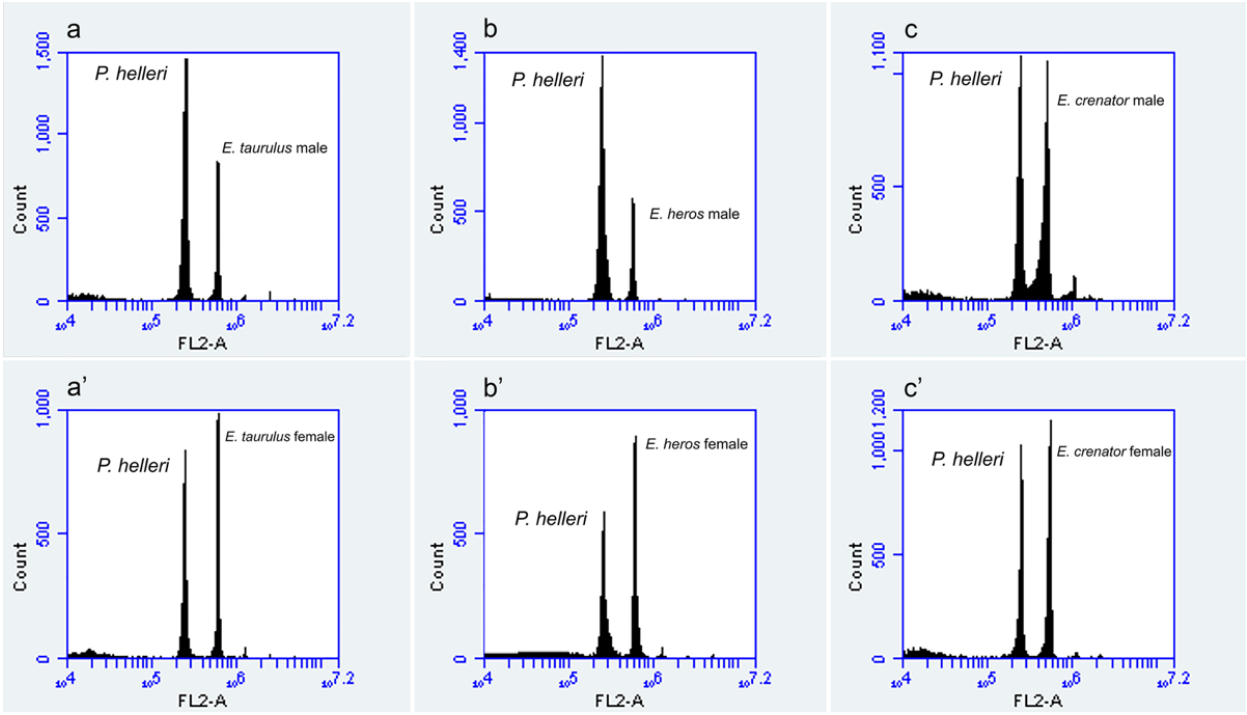

Figure S3. Nuclear genome size of the *Euschistus* species. Based on the G_0/G_1 nuclei peaks and 2C value of the internal standard female *Partamona helleri* (2C = 1.20 pg), the mean 2C values were: A) *E. taurulus* male 2C = 2.70 pg, A') *E. taurulus* female 2C = 2.74 pg, B) *E. heros* male 2C = 2.82 pg, B') *E. heros* female 2C = 2.92 pg, C) *E. crenator* male 2C = 2.92 pg, and C') *E. crenator* female 2C = 3.01 pg.

4. HOST PREFERENCE AND SURVIVORSHIP OF *Euschistus heros* STRAINS ON COTTON AND SOYBEAN

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Abstract

The Neotropical brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), is a key pest of soybeans, *Glycine max* (L.) Merrill (Fabaceae) recently became an economically important pest of cotton, *Gossypium hirsutum* L. (Malvaceae), in Brazil. This stink bug has two allopatric strains, one prevalent in southern Brazil (SS), and another in the north (NS). The two strains hybridize in central Brazil. Knowledge of host preferences and host suitability of these strains can clarify the contribution of the various gene pools to contemporary adaptive features such as the ability to harm cotton crops. We tested the attraction of the *E. heros* strains and reciprocal hybrids – ♀N × ♂S (HNS) and ♀S × ♂N (HSN) – to soybean and cotton leaves and reproductive structures and evaluated the nymph development and survivorship of the two strains and reciprocal hybrids fed on soybean or cotton. We conducted host-choice experiments with fourth instars and adult females and evaluated the survival of immatures on soybean and cotton plants under laboratory conditions. The SS strain preferred soybean over cotton. Hybrid and NS strains chose randomly between soybean and cotton plants. All strains developed on soybean, with similar survival rates. On cotton, pure strains did not reach adulthood; however, hybrids developed on cotton but with a survival rate of <1%. Our results showed that the SS strain of *E. heros* was more attracted to soybeans, and NS and hybrid strains had a polyphagous choice behavior, suggesting that current host selection has been mediated by historical and, mainly, contemporary relationships of *E. heros* strains with these hosts.

Running title: Differential host choice among *Euschistus heros* strains

Keywords: Neotropical stink bug; Heteroptera; Genetic strains; Host choice; Insect adaptation; Tropical agriculture; Bionomy; Fabaceae, Soybean crop; Cotton crop; Emergent pest; Hemiptera; Pentatomidae

4.1. Introduction

Herbivorous insects have a close relationship to their host plants and are subject to continuous variation over time (Simon et al., 2015). Expansion of agricultural frontiers dramatically changes the landscape by replacing complex natural areas with simplified agroecosystems (Silva et al., 2020). Insect populations exploiting crops encounter large patches of single hosts, resulting in diet specialization or evolution of novel plant-insect interactions (Jaenike, 1990; Wetzel et al., 2016; Jiao et al., 2018). Therefore, agroecosystems are important areas for evolutionary change (especially in superabundant monocultures) and may lead to rapid selection for host use in populations of crop pests, such as corn leafhopper, *Dalbulus maidis* (DeLong & Wolcott), western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter) (Gray et al., 2009; Barman et al., 2012; Medina et al., 2012; Dávila-Flores et al., 2013; Bernal & Medina, 2018).

The Neotropical brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), is a polyphagous pest recorded on 21 plant species and can complete its life cycle on six, primarily Fabaceae (Smaniotto & Panizzi, 2015). Considered in the 1970s as a secondary pest on soybeans, *Glycine max* (L.) Merrill (Fabaceae), *E. heros* is now a serious pest that feeds on soybean pods, causing them to abort and reducing grain weight and quality (Panizzi & Slansky, 1985; Panizzi et al., 2000; Sosa-Gómez et al., 2020). Furthermore, in the last decade, reports of *E. heros* moving to cotton, *Gossypium hirsutum* L. (Malvaceae), after soybean harvest has become frequent, mainly in the central region of Brazil (Soria et al., 2009, 2010, 2011, 2017). This behavior has been hypothesized to be a host range expansion because an increasing number of field reports indicate that *E. heros* increases annually in cotton crops in Brazil.

Two allopatric strains of *E. heros* occur in Brazil, one prevalent in the south (hereafter SS) and another common in the north/northeast (hereafter NS) (Soares et al., 2018). Despite the long period of geographic isolation between the northern and southern strains, these allopatric strains have formed a hybridization zone in central Brazil without a significant reproductive isolation (Soares et al., 2018; Zucchi et al., 2019; Hickmann et al., 2021). The reunion of two gene pools, without complete reproductive isolation, can mediate evolutionary events through recombination, hybrid vigor, and selection of adaptive alleles in local populations (Gasperi et al., 1991; Wilding et al., 2001; Corrêa et al., 2019).

In Brazil, cotton is grown mainly in the central part of the country, which coincides with the E. heroshybridization zone. However, soybean, the preferred host of *E. heros*, is also

commonly grown in these areas during the first crop season. Thus, inferring the contribution of *E. heros* strains (pure and their hybrids) to dispersal to cotton-growing areas is crucial to understand this phenomenon and the evolutionary aspects of exploiting a new host. Therefore, our objectives were: (1) to test whether there is a preference for a host (soybean or cotton) among the *E. heros* pure strains and their reciprocal hybrids ♀SS × ♂NS (hereafter HSN) and ♀NS × ♂SS (hereafter HNS), and (2) to evaluate the development and survivorship of nymphs of the two *E. heros* pure strains and their reciprocal hybrids on soybean and cotton. The evolutionary implications of the *E. heros* strains and the impacts on the management of this pest are discussed.

4.2. Material and methods

4.2.1. Collection and maintenance of *Euschistus heros* stock colony strains

Individuals from the NS were collected in the municipality of Balsas, state of Maranhão (07°13’44.50” S, 45°58’35.32” W), and individuals from the SS were collected in Santa Maria, state of Rio Grande do Sul (29°42'17.9" S, 53°35'47.9" W). Approximately 200 adults from each locality were collected and taken to the Laboratory of Arthropod Molecular Ecology at the University of São Paulo, USP/ESALQ (Piracicaba, São Paulo, Brazil). The insects were reared and maintained as described by Hickmann et al. (2021). In brief, the insects were kept in cages under controlled laboratory conditions (25 ºC, 65% r.h., and L14:D10 light regime), and a natural diet and water were offered *ad libitum*.

Both *E. heros* strains were collected from soybean fields at least 180 km from the nearest cotton field, as cotton is grown mainly in central Brazil (AMAPA, 2021/2022; CONAB, 2022). Both strains were maintained for approximately five generations without contact with soybean or cotton plants or the introduction of field-collected insects before being used in the trials.

4.2.2. Cultivated plants

To obtain the reproductive structures and branches in the reproductive phase, seeds of soybean cv. Brasmax Desafio RR (an undetermined cultivar) and cotton cv. DP 1742 RF were sown at approximately 30 and 10 plants per m^2, respectively, in the experimental area of the Department of Entomology and Acarology (Piracicaba, SP, Brazil) in October (cotton) and November (soybean) of 2020. The experimental area has soil composed primarily of red latosol, and we incorporated about 8 g of fertilizer (4:20:20 N:P:K) per m^2 before sowing.

4.2.3. Host-choice bioassays

We used individuals from populations of the two *E. heros* strains (SS and NS) established in the laboratory to test for host preference. Reciprocal hybrids were obtained by crossing a ♀NS with a ♂SS (HNS) and a ♀SS with a ♂NS (HSN; 15 pairs for each cross). Host-choice tests were carried out with fourth instars (2 days after instar change) and adult females of reproductive age (ca. 2 weeks old) from both strains of *E. heros* and the reciprocal hybrids HNS and HSN. The insects were starved for 24 hours before testing, and water was offered ad libitum. The experiments comprised 30 replications for each strain, reciprocal hybrid, and life stage.

The host-choice tests were performed in a dark room with controlled temperature and humidity (25 ± 1 °C and 65 ± 10% r.h.). The choice tests were carried out in transparent acrylic cages measuring 40 × 20 × 20 cm. The soybean host used in the experiments was at reproductive stages R_3–R_4 (Fehr et al., 1971); in each choice test, six pods in the grain-filling phase plus a leaf of the upper trefoil were inserted into one end of the cage. For the cotton host, plants in principal growth stage 7 (71–79; Munger et al., 1998) were used; a developed cotton boll (2–4 weeks old – stink bugs showed a preference for this cotton boll stage; Siebert et al., 2005) and a healthy cotton leaf were placed at the opposite end of the cage. For each replication, the cage was cleaned with 70% ethanol, and the plant structures were replaced and inserted in alternate ends of the cage to prevent bias. A fasting nymph or adult female was released into the center of the cage and could choose between the soybean or cotton host plant structures. Visual evaluations were made after 10, 20, and 30 min for nymphs and after 20, 40, and 60 min for females, noting which host the insects chose. If after the third inspection a choice had not occurred, the insect/replicate was discarded.

4.2.4. Nymph development and survivorship of *Euschistus heros* SS, NS, HSN, and HNS on soybean or cotton

To identify developmental differences between strains and reciprocal hybrids fed exclusively on soybean or cotton, 70-second instar nymphs (the first instar does not feed and is gregarious) of SS, NS, HSN, and HNS were transferred to cylindrical PVC cages (29.5 cm high, 24.5 cm diameter); one side of each cage was attached to a polystyrene plate (30 × 30 cm), and the other side was covered with voile fabric. The cages contained soybean branches (at least three major or secondary soybean branches, containing leaves and at least ten pods in the filling grains stage per branch) or cotton branches (at least three mainstem, monopodial, or sympodial

cotton branches, containing leaves and at least three cotton bolls – one of which at least 2–4 weeks old – per branch), arranged in 100-mL flasks with distilled water. The experiments consisted of three repetitions for each strain and reciprocal hybrid and host. The branches were replaced every two days. The development time (days), weight (mg), and survivorship of each stage were evaluated daily for the nymphs on both hosts. The sex ratio was determined within 24 hours of adult emergence.

4.2.5. Statistical analysis

Data from the host-choice assays were processed with Fisher's exact and χ^2 tests in R (R Core Team, 2022). The biological data were tested for normality with the Shapiro-Wilk test in R. Data that did not fit a normal distribution were log-transformed. Data were then submitted to a variance analysis (ANOVA) and means were separated by Tukey test ($\alpha = 0.05$). Treatments that have no insects survived or only one individual survived were excluded from the statistical analysis. Survival over time of the immature phases was submitted to a log-rank test in the R package *nph* (Ristl, 2020).

4.3. Results

4.3.1. Host Choice

Preference differed between soybean and cotton (Fisher's exact test: $P = 0.039$). Nymphs and females of SS showed a preference for soybean (females: $\chi^2 = 6.533$, $P = 0.011$; nymphs: $\chi^2 = 8.533$, $P = 0.0035$, both d.f. = 1; Figure 1). On the other hand, nymphs and females of NS (females: $\chi^2 = 0.533$, $P = 0.47$; nymphs: $\chi^2 = 0$, $P = 1$), HSN (females: $\chi^2 = 1.2$, $P = 0.27$; nymphs: $\chi^2 = 1.2$, $P = 0.27$), and HNS (females: $\chi^2 = 0.533$, $P = 0.47$; nymphs: $\chi^2 = 0.533$, $P = 0.47$, all d.f. = 1) did not show a preference, choosing randomly between soybean and cotton (Figure 1).

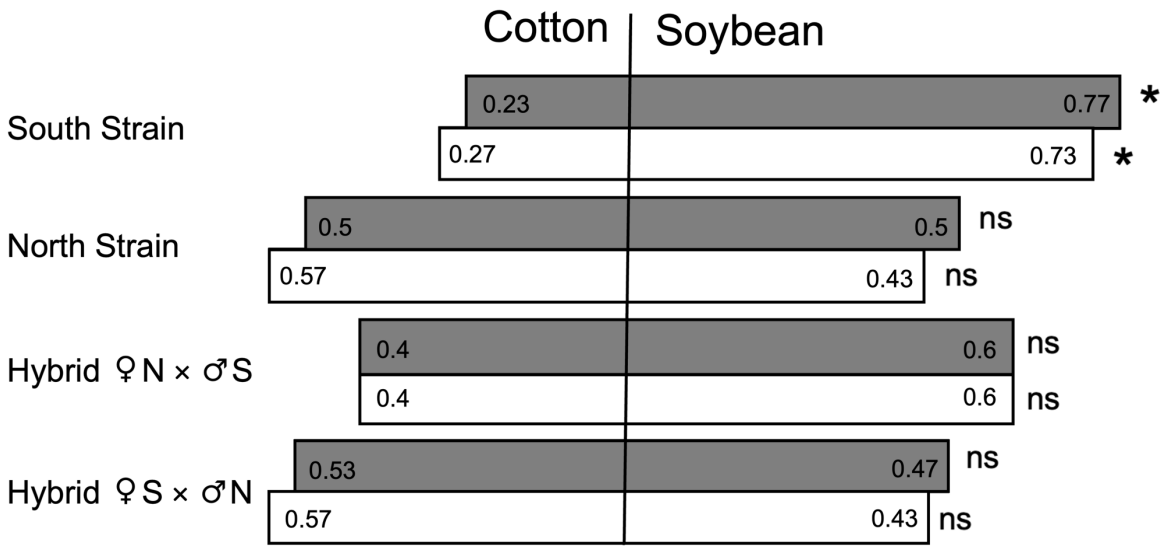

Figure 1. The choice proportion of fourth instar nymphs (grey bars) and adult females (white bars) of *Euschistus heros* strains South (S), North (S), and two F_1 hybrids, ♀N × ♂S and ♀S × ♂N, between leaves and reproductive tissue (pods and bolls) of soybean and cotton plants. Asterisks indicate a significant deviation from a 50:50 distribution (= preference) (χ^2 test: $P < 0.05$; ns, $P > 0.05$).

4.3.2. Nymph development and survivorship

4.3.2.1. Nymph development time

The development time of second instars of the various strains differed between the hosts ($F_{1,7} = 16.83$, $P<0.001$; 4.0–5.7 days on soybean vs. 7.3–11.7 days on cotton) (Table 1). Second instars of the NS fed on cotton developed the slowest of all strain/host combinations. The development time of third instars did not differ among strains or hosts ($F_{1,7} = 1.11$, $P = 0.40$). The development time of fourth instars on soybean differed among strains ($F_{1,3} = 7.75$, $P = 0.009$) – fourth instars of SS developed the slowest (Table 1). Also, the development time of fifth instars on soybean differed among strains ($F_{1,3} = 12.21$, $P = 0.002$) – here the fifth instars of NS developed slowest. Overall, the time needed to reach adulthood (second – fifth instar development) on soybean did not differ among the pure/hybrid strains ($F_{1,3} = 5.02$, $P = 0.085$; Table 1).

Table 3 Mean (± SE) development time (days), weight (mg), and sex ratio (♀/(♀+♂)) of – various developmental phases of – Euschistus heros strains South (SS), North (NS), and reciprocal hybrids ♀SS × ♂NS (HSN) and ♀NS × ♂SS (HNS) fed only on soybean or cotton under laboratory conditions

Variable	Phase	Soybean						Cotton					
		SS	NS	HSN	HNS	$F_{1,3}$ [4]	P [4]	SS	NS	HSN	HNS	$F_{1,3}$ [4]	P [4]
Development time (days)	Second	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab	5.7 ± 0.5ab
	Third	10.3 ± 0.9a	11.7 ± 1.2b	6.0 ± 1.2a	0.55 ± 0.1a	74.3 ± 2.2a	29.6 ± 3.1a	16.3 ± 1.9a	6.2 ± 0.6a	34.0 ± 2.4a	10.3 ± 0.9a	11.7 ± 1.2b	6.0 ± 1.2a
	Fourth	14.7 ± 0.5b	7.3 ± 1.4ab	8.3 ± 1.9a	0.58 ± 0.2a	57.7 ± 2.7b	33.7 ± 3.2a	17.0 ± 1.7a	5.4 ± 0.6a	36.0 ± 4.0a	14.7 ± 0.5b	7.3 ± 1.4ab	8.3 ± 1.9a
	Fifth	8.7 ± 0.7a	5.3 ± 0.3a	5.0 ± 0.5a	0.66 ± 0.1a	66.3 ± 3.0ab	38.1 ± 3.6a	16.3 ± 1.8a	8.9 ± 2.0a	23.0 ± 1.2a	8.7 ± 0.7a	5.3 ± 0.3a	5.0 ± 0.5a
	Second-adult	8.3 ± 0.3a	4.3 ± 0.3a	4.3 ± 0.3a	0.50 ± 0.1a	67.3 ± 1.1ab	32.1 ± 2.2a	14.3 ± 0.3a	6.3 ± 0.2a	23.0 ± 0.5a	8.3 ± 0.3a	4.3 ± 0.3a	4.3 ± 0.3a
Weight (mg)	Third	12.21	7.75	–	0.39	5.61	0.90	0.36	–	5.02	12.21	7.75	–
	Fourth	0.002	0.009	–	0.76	0.023	0.48	0.78	–	0.085	0.002	0.009	–
	Fifth												
	Adult	–	–[1]	7.0 ± 0.9a	–	–	–	10.1[2]	6.6 ± 0.9a	–	–	–[1]	7.0 ± 0.9a
Sex ratio	Adult	–	–	7.7 ± 1.4a	–	–	–	17.3 ± 0.3[2]	7.2 ± 1.1a	–	–	–	7.7 ± 1.4a

Means within a row followed by the same letter do not differ significantly (Tukey's test: P>0.05). [1]No survival data available for this instar. [2]Data were excluded from statistical analysis because only one individual survived or too many repetitions were lost. [3]Statistical analyses were applied for 8 treatments. [4]Statistical analyses were applied for 4 treatments.

4.3.2.2. Wheigth and sex ratio

The weight of the third instars, ranging from 4.2 to 8.9 mg, did not differ among pure/hybrid strains when fed on cotton or soybean hosts ($F_{1,7} = 1.66$, P = 0.19; Table 1). Also, the weight of fourth and fifth instars on soybean did not differ among the strains ($F_{1,3} = 0.36$, P = 0.78 and $F_{1,3} = 0.90$, P = 0.48, respectively). However, the weight of adults on soybean differed among the strains ($F_{1,3} = 5.61$, P = 0.023; Table 1) – the SS adults were heaviest, and the NS adults were lightest. No difference was found in adult sex ratio, ranging from 0.55 to 0.66, among the pure and hybrid strains fed on soybean ($F_{1,3} = 0.39$, P = 0.76; Table 1).

4.3.2.3. Nymph survivorship

The average survivorship of pure/hybrid strains when fed exclusively with soybean or cotton was different among strains and hosts (log-rank test: P = 0.0001; Figure 2). Pure and hybrid strains of *E. heros* completed their life cycle on soybean, however, no difference was found between them (proportional values (0.0–1.0), SS: 0.07 ± 0.01, NS: 0.14 ± 0.06, HSN: 0.13 ± 0.00, and HNS: 0.24 ± 0.11; $F_{3,11} = 1.55$, P = 0.28; Figure 3). On cotton, only the hybrid strains developed, although with very low survivorship (<1% (<0.01); Figures 2 and 3).

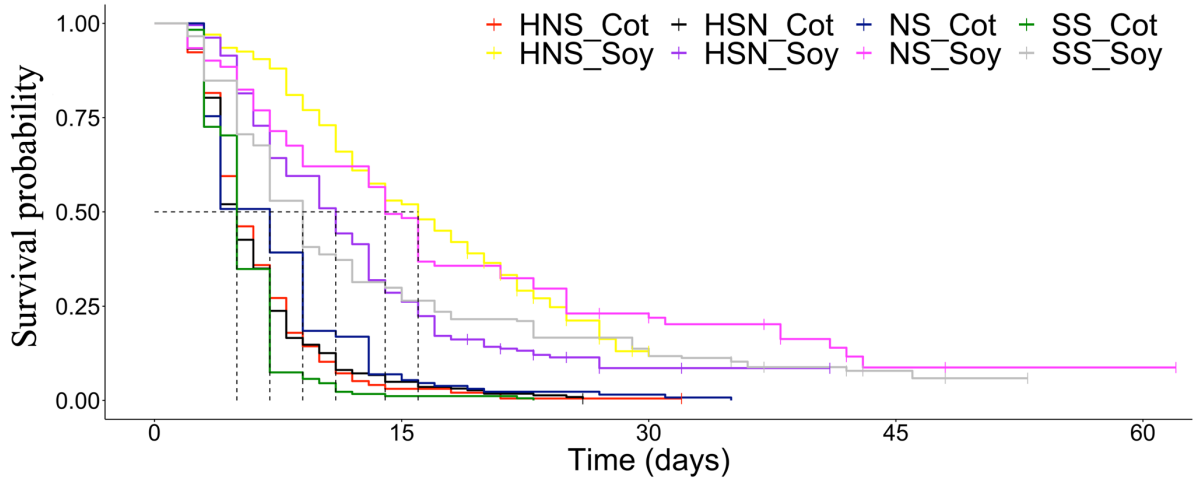

Figure 2. Survival probability of South strain (SS), North strain (NS), and reciprocal hybrids ♀NS × ♂SS (HNS) and ♀SS × ♂NS (HSN) of *Euschistus heros* when fed on soybean (Soy) or cotton (Cot) branches. The hatched lines indicate median survival period (time at which 50% of the population survived). Median survival differed between strains/hybrids and hosts (log-rank test: P < 0.0001).

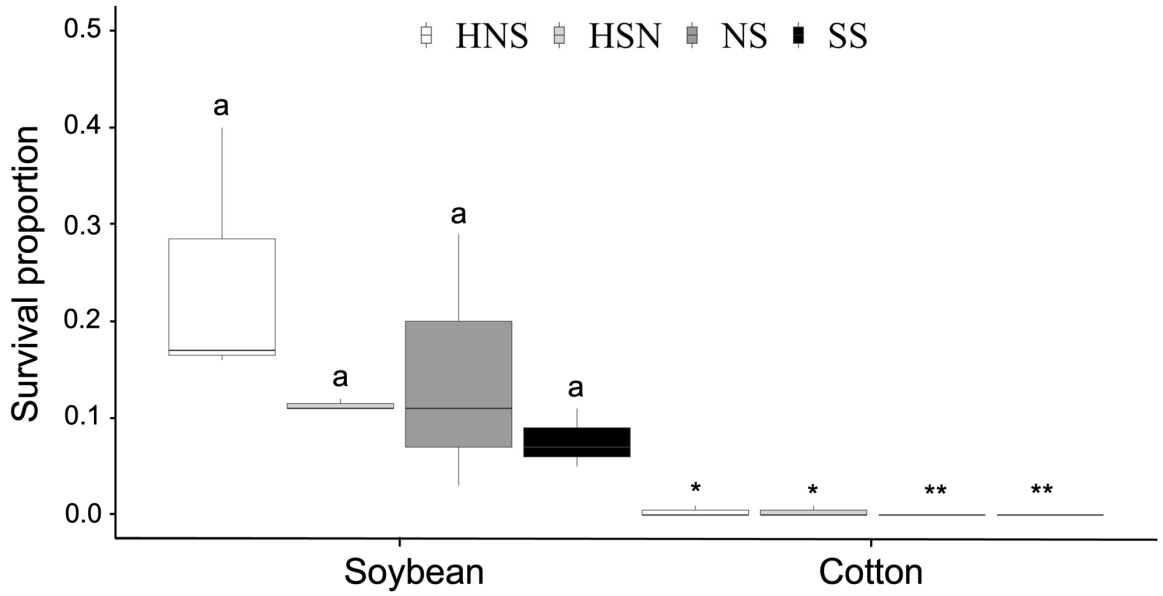

Figure 3. Survival proportion of *Euschistus heros* nymphs (second instar–adult) of South strain (SS), North strain (NS), and F1 hybrids (HSN, ♀SS × ♂NS and HNS, ♀NS × ♂SS) fed on cotton or soybean branches. The top and bottom of each boxplot represent the upper and lower quartile, respectively; the horizontal line within the box represents the mean; the whiskers extend to the minimum and maximum values within 1.5× the interquartile range. Means capped with the same letter are not significantly different (Tukey test: P>0.05). Asterisks indicate that only one individual (*) or no individuals (**) survived – these data were excluded from statistical analysis.

4.4. Discussion

The strains of *E. heros* showed preferences between leaves and reproductive tissue (pods and bolls) of soybean and cotton plants, depending on the strain. The SS strain chose soybean over cotton, suggesting a higher attraction to soybean, whereas the NS strain chose soybean and cotton evenly, suggesting a more polyphagous behavior. The reciprocal hybrid strains (HSN and HNS) behaved similarly to NS, choosing randomly between cotton and soybean. Nymph survivorship of both the pure strains and their reciprocal hybrids was similar when fed on soybean, whereas only reciprocal hybrids reached adulthood when fed on cotton, although with very low viability. The suitability of soybean and the unsuitability of cotton as a host for nymph development for *E. heros* strains has been related to the nutritional quality and allomone substances of hosts (Panizzi, 2000; Azambuja et al., 2013).

The soybean attraction displayed by SS indicates that this strain may be adapted to this non-native host. These results agree with a previous study that reported the preference of *E. heros* for soybean vs. wheat, *Triticum aestivum* L., or canola, *Brassica napus* L. var. *oleifera*

(Possebom et al., 2020). Molina & Trumper (2013) reported the absence of specific attraction to soybean by *Piezodorus guildinii* (Westwood) and *Nezara viridula* (L.), two other stink bug pests of soybean in South America.

The tightened relationship of SS *E. heros* to soybean and the lack of preference of NS may be attributed to the historical and contemporary distribution of soybean and cotton crops in Brazil. After soybean was introduced into Brazil in 1901, it was grown only in the southern region until 1980, when soybean cultivation began to expand to the central and northern/northeastern regions (Cattelan & Dall'Agnol, 2018). Specifically, in northern/northeastern Brazil, where *E. heros* NS is predominant, soybean began to be cultivated in the 2000s. Thus, SS has been associated with soybean in Brazil for 12 decades, whereas NS has been associated with soybean for only 2 decades. Cotton-growing areas have a contrasting history of geographic expansion. Cotton has been cultivated for centuries in northeastern Brazil, and many perennial cotton cultivars are found in the northern/northeastern regions. In the last century, cotton cultivation expanded to the southeastern and, mainly, central parts of the country (Barros et al., 2022). The historical expansion of soybean and cotton crops matches the occurrence areas of the allopatric strains of *E. heros* in Brazil and may account for the preferences of NS and SS.

In central Brazil, cotton fields are interspersed with soybean fields, which favors dispersal of insects from soybean to cotton (Soria et al., 2010; Silva et al., 2020). *Euschistus heros* colonizes soybean and after soybean senescence the insects disperse to cotton crops (Soria et al., 2009, 2010, 2011, 2017). In this scenario, *E. heros* may expand its host range because cotton becomes a resource for *E. heros* (adults and/or nymphs). *Euschistus heros* is a multivoltine species with rare long-dispersal behavior (Panizzi, 1997; Soares et al., 2018). Thus, *E. heros* must shift between host plants when seeking new resources in a limited spatial area (Todd & Herzog, 1980; Tillman et al., 2009; Venugopal et al., 2014; Carrasco et al., 2015; Blaauw et al., 2019).

The landscape changes experienced by *E. heros* in recent decades imply that the populations are under host-selection pressure, which may lead to host specialization or even ecological speciation (Funk, 2010; An et al., 2016; Zucchi et al., 2019). Host shift/expansion usually starts with recognizing the host. The absence of preference between cotton and soybean by NS and hybrids suggests that *E. heros* may evolve to expand to cotton (Knolhoff & Heckel, 2014). Similarly, the preference of *E. heros* SS for soybean suggests that this strain is more adapted to this exotic host.

Both *E. heros* strains and their reciprocal hybrids completed their life cycle on soybean with similar biological parameters, in agreement with previous reports that soybean is a highly suitable host for *E. heros* (Azambuja et al., 2013; Possebom et al., 2020). The *E. heros* strains displayed no or very low viability on cotton branches, denoting low or no suitability of cotton. The faster adaptation of *E. heros* strains to the non-native species soybean than to cotton matches the prevalence of Fabaceae species as suitable native hosts for *E. heros* (Link & Grazia, 1987; Smaniotto & Panizzi, 2015; Panizzi & Lucini, 2017). In contrast, native Malvaceae species have been reported to be unsuitable for *E. heros* (Azambuja et al., 2013; Panizzi & Lucini, 2017).

Although the reciprocal hybrids performed poorly on cotton, a few nymphs did reach the adult phase. These observations are highly concerning, especially because a very large number of insects move annually from soybean to cotton fields. The recurrent attacks and colonization of cotton fields may be selecting insects with a higher capacity to colonize cotton plants (Joshi & Thompson, 1995). The suitability of a host plant is mediated by an insect's capacity to metabolize the primary nutrients and secondary metabolites (allelochemicals) contained in the host (Schoonhoven et al., 1998a; War et al., 2012). Thus, our results suggest that *E. heros* has mostly overcome the defenses of soybean and is still in the early steps of adapting to cotton (a gossypol-rich plant).

Adults of *E. heros* may enter a facultative diapause triggered by short-day (<12 h per day) conditions (Mourão & Panizzi, 2000a, b, 2002). Most Brazilian soybean and cotton fields are in latitudes above 8° South (CONAB, 2022; IBGE, 2022) and experience at least 3 months of short-day conditions per year. Thus, after soybean harvest, adults may disperse to cotton fields, seeking shelter and food to survive the coming mild winter and not necessarily to develop on the host. Therefore, it is imperative to determine whether or not adults of *E. heros* that arrive in cotton fields are in diapause, and how they contribute to the dispersal of insects from cotton to soybean fields in the next crop season. Furthermore, insecticides are applied to cotton more frequently than to soybean, with reports of 20–25 applications a year, with two applications for *E. heros* control in some areas (Miranda, 2010; Lamas & Chitarra, 2014; Pitta et al., 2018). The movement of *E. heros* individuals between cotton and soybean crops could accelerate the selection of insecticide-resistant populations of *E. heros*, raising serious concerns for management of this species (Tuelher et al., 2018).

Intra- and interpopulation variation in host use has been attributed to different plants in different regions or to genetic differences among populations and strains (Jaenike, 1990;

Carrière & Roitberg, 1995; Schoonhoven et al., 1998b). The agricultural expansion in Brazil has changed the landscape dramatically and has directly affected native insect communities, introducing novel selection pressures (Corrêa et al., 2019). For instance, rapid selection of crop pests for host use has been reported in other insect groups (Gray et al., 2009; Bernal & Medina, 2018).

In conclusion, we demonstrated differences among *E. heros strains* in host-choice responses and survivorship on soybean and cotton. *Euschistus heros* SS showed a preference for soybean, unlike NS and laboratory reciprocal hybrids, suggesting that the host selection process may have been mediated by the historical and contemporary relationship of *E. heros* strains with these hosts. These different *E. heros* phenotypes characterized in the laboratory need to be tested in field conditions to better understand the role of these strains (pure and hybrids) in host-range adaptation. It is imperative to continue genotypic and phenotypic monitoring of *E. heros* from different agricultural landscapes because the changes in host ranges would affect other ecological traits such as insecticide susceptibility, overwintering, and interactions with natural enemies.

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5. HOST SUITABILITY AND INSECTICIDE SUSCEPTIBILITY OF NEOTROPICAL *Euschistus* SPECIES

Abstract

Ecological interactions among native and exotic species have complex adaptive consequences in both directions. Soybean, *Glycine max*, is an exotic crop extensively cultivated, covering almost 60 million hectares in South America. Several *Euschistus* species occur sympatrically and have been reported on soybean but only *E. heros* became a key pest of soybean and, lately, cotton. Conventionally *E. heros* has been managed on soybeans and cotton through chemical insecticides. Recently, several studies have revealed a reduction in insecticide susceptibility. Here, we aim to identify host suitability and selected insecticide susceptibility of three *Euschistus* species (*E. heros*, *E. crenator*, and *E. taurulus*). Our objectives were 1) to test the suitability of soybean and cotton in the immature stages of the three species, 2) to Identify the reproductive performance of adults feeding the whole life cycle on soybean, and 3) to test the insecticide susceptibility of the three species to organophosphate and pyrethroid insecticides. A soybean-based diet significantly improved the biological parameters of E. heros, resulting in higher immature survivorship, adult longevity, and offspring production compared to the *E. crenator* and *E. taurulus*. The cotton branches are unsuitable for all three species. Insecticide susceptibility of *E. heros*, *E. crenator*, and *E. taurulus* to organophosphates and pyrethroids showed low variation. Our results suggest that *E. heros* quickly adapted and specialized to the soybean crop. The increasing cultivating area provides resources to increase this species in number, leading to its prevalence. The similar susceptibility of the three species suggests insecticide susceptibility is conserved among the tested species, impacting similarly on *Euschistus* species. Understanding pest dynamics requires knowledge of host suitability and insecticide susceptibility of major and secondary pests, which is crucial in predicting pest outbreaks.

Keywords: Stink bugs; Soybean crop; Cotton crop; Tropical agriculture; bionomy; Neotropical brown stink bug

5.1. Introduction

Ecological interactions among native and exotic species have complex adaptive consequences for both groups (Britton et al., 2017; Godoy, 2019; D'Antonio et al., 2017). In agricultural areas where anthropic actions intentionally and unintentionally introduce exotic species, these ecological interactions directly affect species diversity, abundance, and sustainable management. Brazilian agriculture areas are complex landscapes where exotic and native plant crops are directly affected by native or exotic herbivorous insects, resulting in frequent changes in insect abundance and pest status (Horikoshi et al., 2021; Panizzi et al., 2022).

Soybean is an exotic crop extensively cultivated, covering almost 60 million hectares in South America (Song et al., 2021). Currently, many native species of lepidopteran and heteropteran insects have adopted soybean as a new food source, thereby reaching the status of pests for this crop (Horikoshi et al., 2021; Saldanha et al., 2024). The Neotropical brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), was only occasionally observed on soybeans before the 1990s. However, this species has become the most prevalent one affecting soybean crops in Brazil (Panizzi et al., 2022; Saldanha et al., 2024). Furthermore, in the last decade, there have been frequent reports of *E. heros* individuals moving to cotton crops after the soybean harvest, causing damage to cotton plants, especially in areas cultivated near soybean crops (Soria et al., 2009; 2010; 2011; 2017). However, despite cotton being a native species, it is not a suitable host for *E. heros*, which can barely complete their life cycle on this crop (Azambuja et al., 2013; Hickmann et al., 2023a).

Due to its widespread occurrence, *E. heros* has conventionally been managed on soybeans and cotton through the application of chemical insecticides (e.g., Castellanos et al., 2019; Tuelher et al., 2017; Somavilla et al., 2020, Tibola et al., 2021; Steinhaus et al., 2022). Until 2004, only organophosphates and organochlorides were used to control stink bugs in Brazil. A mix of neonicotinoids and pyrethroids was introduced in 2004, and pure pyrethroid molecules were added to the market in 2005 (Sosa-Gomes et al., 2020). Over the past few decades, several studies have revealed a reduction in the susceptibility of *E. heros* to insecticides in both field and laboratory settings (Sosa-Gomes et al., 2001; Tuelher et al. 2017; Tibola et al. 2021; Castellanos et al., 2019; Sosa-Gomez et al., 2020; Steinhaus et al., 2022), implying that this factor may drive the population dynamics of soybean pests.

Besides *E. heros*, *E. cornutus* Dallas, *E. picticornis* (Stål), *E. taurulus* Berg, and *E. crenator* (Fabricius); *E. picticornis* (Stål) are other species that have been recorded on soybean

since the establishment of this crop in South America (Quintanilla et al., 1981; Panizzi & Slansky, 1985; Link & Grazia 1987; Panizzi et al.; 2000; 2017; Hickmann et al., 2019; 2021). The pest dynamics of the *Euschistus* species are intriguing because several representatives occur sympatrically in the Neotropics, but only *E. heros* became a key pest of soybean and, lately, cotton. The change in economic importance of the prevalence of *E. heros* in Brazilian agroecosystems has been linked to several hypotheses, such as multiple cropping, no-tillage cultivation, suitability of warmer regions, insecticidal tolerance, host suitability, symbionts, etc. (Panizzi et al., 2022; Rodrigues et al., 2023; Sosa-Gomes et al., 2020; Moro et al., 2022; Smaniotto & Panizzi, 2015). Although many hypotheses have been proposed, only a few have been thoroughly tested.

Species genetically close are excellent models for understanding evolutionary adaptive processes because they show conservative genome structure, behavior, habitat, and physiology. Thus, in this study, we performed a comparative study with three genetically close species, *E. heros*, *E. crenator*, and *E. taurulus* (Hickmann et al., 2023b), to estimate their suitability to soybean and cotton and their tolerance to two widely applied insecticides for stink bug control. The impact of host suitability and insecticide susceptibility and their relationship to the abundance and pest status in Brazil's agricultural landscape of these three *Euschistus* species is discussed.

5.2 Conclusion

In conclusion, we showed that related *Euschistus* species express different levels of host adaptation. The species *E. heros*, the key pest on soybeans, is well-suited and specialized in soybean crops. The species *E. crenator* and *E. taurulus* still face nutritional or metabolic challenges to perform on soybeans. Even though the species are genetically close, the evolutionary forces they faced led to differences in exploit hosts. However, unlike speculations, the insecticide susceptibility found was similar, suggesting that detoxification mechanisms are conserved and shared among the three species. Lastly, our findings suggest that introducing soybean crops in South America and the innate and/or acquired capacity of *E. heros* to exploit this resource led it to become a key soybean pest. However, caution should be taken as testing was limited to one soybean cultivar, one population of each species, and a few insecticides, and monitoring is mandatory to broaden our understanding of insecticide susceptibility and host suitability of stink bugs associated with crops.

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6. VARIATION IN DIAPAUSE PHENOTYPE EXPRESSION IN NEOTROPICAL *Euschistus* (HEMIPTERA: HETEROPTERA) STINK BUGS

Abstract

Diapause is an evolutive mechanism to face the challenges imposed by annual cycles. Diapause is a regulated delay in development triggered by environmental and hormonal factors for responding to unfavorable conditions, and the incidence and timing of the diapause are highly correlated to latitudinal clines. For Pentatomidae stink bugs, the reduction in day length in immature stages generally induces diapause expression, causing reproduction arrest. The Neotropical stink bugs species *Euschistus taurulus*, *E. crenator*, and *E. heros* [including two strains, the south strain found under higher latitudes and the northern strain found under lower latitudes] can be found sympatrically and/or allopathically in The Americas associated with native and agricultural areas in high and low latitudes. We aimed to characterize diapause in three species of *Euschistus heros* (two strains, and *E. crenator* and *E. taurulus*) found in different latitudes in South America. Our goals were to identify the diapause induction, expression, and termination under different combinations of temperature and photoperiod. Our experiments revealed that diapause expression varies significantly among these closely related strains and species (strong in *E. taurulus*, moderate in *E. heros* SS, weak *in E. heros* NS, and absent in *E. crenator*), suggesting a latitude-based variation in the evolution of diapause phenotype in *Euschistus* species. The distinct diapause expression and termination expressed by the two *E. heros* strains indicate that their diapause expression allows survival and synchronization of their life cycle to crop hosts. The southern strain must survive under harsher conditions, a shorter development window, and fewer yearly generations. In contrast, the strain found under lower latitudes expresses a less intense diapause and resumes reproduction faster, leading possibly to more generations per crop season. Understanding the phenology and diapause are powerful input data for implementing sustainable management strategies and provide information to understand a species' dynamics better and should be considered for IPM programs.

Keywords: Off-season; Reproductive arrest; Dormancy; Phenology; Photoperiod; Temperature

6.1. Introduction

The organisms face numerous challenges due to seasonal cycles, including low winter temperatures, drought seasons, heat waves, floods, food scarcity, etc. Arthropods have developed behavioral, morphological, and physiological adaptations to manage effectively the challenges presented by seasonal changes (Denlinger, 2022). Diapause is one of the most widely spread forms of insect dormancy. Diapause is a regulated delay in development triggered by environmental and hormonal factors for responding to unfavorable conditions (Denlinger, 2022, Tauber et al., 1986; Danks, 1987). It is physiologically dynamic and is divided into three phases: initiation/induction, maintenance, and termination.

Diapause onset is primarily induced by external signals such as daylength and other factors; in maintenance, the fat body undergoes active growth, tissues experience significant biochemical changes, and oxygen consumption is noticeably reduced (Denlinger, 2022). Diapause termination occurs by unblocking neurosecretory centers because of spontaneous or induced reactivation (Saulich & Musolin, 2012; Tauber et al., 1986; Denlinger, 2022). Besides survival of detrimental conditions, diapause also synchronizes adult emergence and reproduction to the abundance of suitable hosts.

Pentatomidae stink bugs are model organisms of diapause expression because the species expresses diapause induced mainly by the reduction in day length (Musolin & Saulich, 2018). Particularly, the genus *Euschistus* includes 67 species restricted to the Americas, and as most other Heteropterans studied so far, express a facultative adult diapause with reproductive arrest, food intake reduction, and behavioral change in the adult stage (Saulich & Musolin, 2012; Musolin & Saulich, 2018). Both male and female adults exhibit a seasonal phenotype, and the reduction in daylength before winter is the main trigger for diapause in neotropical and Nearctic *Euschistus* species (Saulich & Musolin, 2012; Musolin & Saulich, 2018). The sensitive phase is the immature (nymphs), and adults generally have a differential coloration in the diapausing stage (Saulich and Musolin 2012, and references therein). In addition to the photoperiod, the temperature can be an enhancer of the expression of the diapause phenotype, as reported in some other stink bugs such as *Piezodorus guildinii* (Westwood), *Nezara viridula* Linnaeus, and *Dybowskyia reticulata* (Dallas) (Zerbino et al., 2014; Ali & Ewiess, 1977; Musolin & Numata, 2003; Nakamura and Numata 1998).

The incidence and timing of the diapause are highly correlated to latitudinal clines (Tauber et al., 1986; Danks, 1987). The Neotropical stink bugs species *Euschistus taurulus*, *E. crenator,* and *E. heros* [including the two strains of the species characterized by Soares et al.

(2018)] can be found sympatrically and/or allopathically in the American territory associated with native and agriculture areas in high and small latitudes (Soares et al., 2018, Hickmann et al., 2019; 2021). *Euschistus taurulus* can be found from Venezuela to Uruguay, while *E. crenator* is found from northern Brazil to the southern USA. One strain of *E. heros* is predominant in the Pampa and Atlantic Forest biomes in southern Brazil, Paraguay, and Argentina (hereafter SS) (Soares et al., 2018; Balbi et al., 2023). At the same time, the other is predominant in northern Brazil's North Cerrado, Caatinga, and Amazon Forest (hereafter NS). *Euschistus heros* strains hybridize in the Central region from Brazil (Hickmann et al., 2021b; Singh et al., 2023; Zucchi et al., 2019). Among the Neotropical *Euschistus* species, only *E. heros* has a characterized diapause and, probably, the studies were carried out only with *E. heros* SS based on the latitude where these populations were collected (Niva & Panizzi, 1994, Mourão & Panizzi, 2000a; b; 2002). Thus, additional research regarding diapause induction and expression intensity and the impact of photoperiod and temperature on *Euschistus* strains and species at different latitudes is crucial to understanding these stink bugs' diapause phenology.

As ectothermic organisms, stink bugs' development rate and population dynamic are primarily regulated by temperature, but diapause seems triggered mainly by photoperiod (Musolin & Saulich, 2018). Here, we conducted bioassays to examine how photoperiod and temperature cues influence the expression and termination of diapause in three different *Euschistus* species originating from different latitudes (*E. taurulus*, *E. crenator*, and *E. heros* pure strains). Our goals were to determine the 1) impact of photoperiod and temperature on immature development and survival, 2) determine the diapause phenotype induction of the species under short- and long-day conditions at temperatures of 21 and 25ºC, and 3) characterize diapause termination of the *Euschistus* strains/species. The impacts of photoperiodic and temperature effects on diapause induction and termination and its relevance to pest management of these species are discussed.

6.2 Conclusions

Diapause can greatly impact pest management in agriculture and forest areas. Our experiments revealed that diapause expression varies significantly among these closely related strains and species (strong in *E. taurulus*, moderate in *E. heros* SS, weak *in E. heros* NS, and absent in *E. crenator*), suggesting a latitude-based variation in the evolution of diapause phenotype in *Euschistus* species. The NS and *E. crenator*, only found in lower latitudes, exhibit a less intense diapause or no reproductive arrest, resulting in more life cycles per year and a

greater need for a suitable host, such as crops. This can increase the pressure of crop pests and select populations that thrive on their hosts. Understanding the phenology and diapause are powerful input data for implementing sustainable IPM strategies and provide information to better understand a species' dynamics. Therefore, to gain a deeper understanding of the phenology and diapause dynamics within this vital pest complex, additional trials and tests must be conducted on other populations of the *Euschistus* species, including natural *E. heros* hybrid populations.

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7. GENETIC AND FUNCTIONAL CHARACTERIZATION OF *Euschistus* STINK BUGS DIAPAUSE

Abstract

Climatic conditions vary enormously with latitude and are usually directly linked to a species' establishment. Diapause is a neurohormonal mechanism of developmental suppression used by arthropods to survive unfavorable climate conditions. The genus *Euschistus* is one of the most critical soybean pests in the Americas (e.g., *Euschistus heros* and *E. servus* are key soybean pests) and expresses a facultative adult diapause induced by short-day conditions in the immature stages. We aimed to identify and functionally characterize genes linked to diapause in *Euschistus* stink bugs. We employed the transcriptome approach (RNA-seq) to characterize the transcription profile of the two tropical species, *E. heros* SS (the prevalent strain on soybean in Brazil) and *Euschistus taurulus* (express a strong diapause) under different photoperiodic conditions (non- and diapause phenotype). In addition, we characterized a set of candidate genes differentially expressed in the Nearctic species *E. servus* by the RNA interference technique. Through transcriptomic profiling, we have successfully annotated 17485 genes for *E. heros* and 13647 genes for *E. taurulus*. A set of three genes differentially expressed were selected: the gene *juvenile hormone epoxide hydrolase* (*jheh*), *farnesyl pyrophosphate* (*fpps*), and the nuclear receptor *Fushi-tarazu factor 1* (*ftz-f1*). The silencing of the target genes led to RNAi-induced diapause phenotype in *E. servus,* revealing that *fpps* and *jheh* genes significantly contribute to ovary development, and *ftz-f1* is related to oogenesis and reduces lipid storage in *E. servus*. Furthermore, the diapause mechanisms appear to be conserved among *Euschistus* from Neotropical and Nearctic regions. Here, we provide a step further in the molecular and functional characterization of *Euschistus* pests diapause and offer possibilities for future off-season and biorational management approaches.

Keywords: RNA interference; Gene silencing; Transcriptome, Overwintering, Lipid storage

7.1 Introduction

Insects have developed various mechanisms to adapt to the annual rhythms of climate and survive through seasonal changes. One such mechanism is diapause, a programmed stage-specific arrest or retardation of development commonly used to circumvent an adverse season (Tauber et al., 1986; Danks, 1987; Denlinger, 2022). The onset of diapause is characterized by assessing the environmental conditions, such as daylength, energy regulation, upregulating stress response, balancing hormone levels, and seeking overwintering sites (Meuti & Denlinger, 2013; Saunders 2020; Hahn & Denlinger 2011; Short & Hahn 2023; Denliger et al. 2001; Sim & Denlinger, 2013; Denlinger et al., 2012; Short & Hahn, 2023; Denlinger, 2022). During maintenance, the insect is unresponsive to environmental changes with metabolic, respiratory, and metabolism arrest (Koštál, 2006). Diapause termination represents the resumption of average growth (metabolic, respiratory, and hormonal signaling resumption) (Hodek, 1996; 2002).

Most stink bugs (Hemiptera: Pentatomidae) present an adult facultative diapause induced by short daylength during immature stages (Saulich & Musoli, 2012; Musolin & Saulich, 2018). *Euschistus* stink bugs are of great economic importance in the Americas, including several crop pests (McPherson, 2000). Besides their importance, diapause characterization has only been conducted on a limited number of species, with a mere handful of Nearctic representatives, namely *Euschistus servus*, *E. tristigmus tristigmus*, *E. conspersus* Uhler, and *E. ictericus*, as well as a single Neotropical species, *E. heros* (Fabricius) (Musolin & Saulich, 2018).

Diapause has been extensively investigated in temperate species due to the substantial variations in photoperiod and temperature that they experience; however, diapause is also widespread among tropical species (Denlinger, 1986; 2022). Both the brown stink bug, *E. servus*, and the Neotropical brown stink bug, *E. heros*, exhibit adult facultative diapause in response to the shorter daylight periods they encountered during their early stages of life. They both choose to overwinter on field borders, under-field litter, and wooded habitats to survive the extreme and moderate winter conditions (Borges et al., 2001; Rolston & Kendrick, 1961; McPherson & McPherson, 2000; Mourão & Panizzi, 2000a; b; 2002; Panizzi & Vivan, 1997). Although phenotypically and morphologically well-defined, the facultative diapause of *Euschistus* lacks a characterization on the molecular level, such as the genes involved in diapause expression, induction, and signaling pathways that have yet to be identified or characterized.

Experimental tools such as RNA-seq and RNA interference (RNAi) have proven powerful for determining gene functions. RNA-seq analysis is a powerful tool that allows the investigation of transcripts for various purposes, such as detecting differential expression (Deshpande et al., 2023; Raghavan et al., 2022). RNA interference is another potent functional genomic tool that enables manipulation of the levels of specific transcripts and a better understanding of their functional roles (Hannon, 2002). RNAi is a highly conserved mechanism triggered by introducing sequence-specific double-stranded RNA (dsRNA) molecules, leading to target-specific endogenous gene silencing (Ipsaro & Joshua-Tor, 2015). The complete or partial suppression of target gene expression using RNAi helps reveal the target gene's function and allows the annotation of cellular functions (Voorhoeve & Agami, 2003). Induced RNAi has been successfully exploited for studying physiologically relevant hemipteran insect genes involved in embryogenesis, regeneration, development, reproduction, behavior, virus transmission, and insecticide resistance (Jain et al., 2020). RNAi also showed great potential in pest management, especially in developing transgenic crops expressing dsRNA against key genes of insect pests (Yan et al., 2020; Zhang et al., 2017; Baum et al., 2007; Guo et al., 2018), thus characterizing the genes regulating diapause in stink bugs could lead to the discovery of new targets for pest control.

Therefore, here we aim to identify the genetic and molecular mechanisms associated with the diapause of *Euschistus* stink bugs. For this, we employed the transcriptome approach (RNA-seq), where we characterized the transcription profile of the two tropical species, the *E. heros* SS (the prevalent strain on soybean in Brazil) and *Euschistus taurulus* (express a strong diapause) under different photoperiodic conditions (non- and diapause phenotype). In addition, we characterized a set of candidate genes differentially expressed in the Nearctic species *E. servus* by the RNA interference technique. Insights into the diapause mechanisms of stink bugs and potential targets for modern pest management strategies are discussed.

7.2 Conclusions

Our research offers valuable insights into the genes that play a significant role in diapause expression in *Euschistus*. Diapause is the primary mechanism in *Euschistus* species to survive overwintering, where the abiotic conditions and food availability are unfavorable. The genes here identified and validated (*jheh, fpps and ftz-f1*) are associated with the JH pathway and arrested reproduction in *E. servus*. The nuclear receptor *ftz-f1* is crucial for *Euschistus* oogenesis and lipid storage; its depletion leads to high mortality. Finally, studying the

underlying mechanisms of diapause regulation can provide solid input in developing effective population management approaches since overwintering/off-season survival is critical in the insect population dynamic.

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8. FINAL REMARKS AND PERSPECTIVES

The discovery of two distinct strains of *E. heros* in South America, one in the southern and the other in the northern regions, with a secondary contact area in central Brazil, raised questions about their impact on pest dynamics in the agroecosystems. It was found that the two strains did not express pos-zygotic barriers, but the hybrid population had an asymmetric introgression, with the SS genome being more prevalent. In our studies, we identified significant differences in the morphology of two strains of *E. heros*; the SS strain is larger and has a darker coloration, whereas the NS strain is smaller and has a lighter brownish color. Furthermore, we found that *E. heros* mates assortative with bigger insects and reciprocal hybrids (NS females mated with SS males) had a reduced frequency of chiasmata. This suggests that this asymmetric introgression can be attributed to mating behavior and the meiotic behavior of *E. heros*, where the larger SS strain has a higher chance of mating. In addition, the satDNAs (repetitive and non-coding fractions of the genome) were differentially accumulated, mainly on the Y chromosome, in the two strains of *E. heros*. Although differences were found among strains from a chromosomal perspective, we do not find incompatibilities between the two genetic pools, indicating that they can interbreed and exchange genetic material. The results suggest that these two groups have a high degree of compatibility and can potentially merge into a single population.

The chromosomes encompass the genetic elements of the entire nuclear genome, making them an excellent source of information for evolutionary studies. Genome evolution and chromosome reorganization can shed light on understanding the pest status of a species. Thus, here, we characterized three soybean pests [*E. heros* (both strains), *E. crenator*, and *E. taurulus*] from the cytogenomic perspective. Our data revealed that the three *Euschistus* species present conservative karyotypes and only one possible inversion in the *E. taurulus* species. Moreover, we observe Y chromosome reorganization through differential microsatellite accumulation. The nuclear genome sizes are slightly variable among species. Although these organisms may display varying morphological and biological characteristics, their genome remains highly conserved. This remarkable stability of their genetic material may allow them to become potential pests.

The recurrent pest outbreaks of *E. heros* on cotton crops correspond with the admixture zone of the two strains. Our bioassays and trials revealed that the SS strain chose soybean over cotton, suggesting a higher attraction to soybean, whereas the NS strain chose soybean

and cotton evenly. The reciprocal hybrid strains (HSN and HNS) behaved similarly to NS, choosing randomly between cotton and soybean. Nymph survivorship of the pure strains and their reciprocal hybrids was similar when fed on soybean, whereas only reciprocal hybrids reached adulthood when fed on cotton, although with very low viability. This indicates that SS, which is already longer in contact with the soybean host (due to historical soybean cultivation in southern Brazil), has already evolved a higher specialization to this host than NS and hybrids. The random choices between a host of NS can be linked to the historical cotton production region and natural cotton species in northern Brazil, and the hybrids also express this feature. The suitability of soybean and the unsuitability of cotton as a host for nymph development for *E. heros* strains can be related to the nutritional quality and allomone substances of hosts. Another aspect that our data suggests is that cotton is unsuitable for *E. heros* (all strains), and the stink bugs that move to cotton fields are most likely seeking shelter and food sources before or after moving to overwintering sites.

Besides *E. heros*, several *Euschistus* species have been recorded on soybeans, including *E. taurulus* and *E. crenator*, considered secondary pests; many aspects of their biology and management are still lacking. Due to the fast host specialization experienced by *E. heros*, it is imperative to monitor host suitability and management strategies of sibling species of the genus. We identified that a soybean-based diet significantly improved the biological parameters of *E. heros*, resulting in higher immature survivorship, adult longevity, and offspring production compared to the sibling species. Soybean was found to be moderately suitable for *E. crenator*, while *E. taurulus* showed low suitability for the same. In contrast, cotton branches are unsuitable for all three species. Additionally, the insecticide susceptibility of *E. heros*, *E. crenator*, and *E. taurulus* to organophosphates and pyrethroids showed low variation, suggesting that it impacts *Euschistus* species similarly. The distinct adaptability of *E. heros* to soybean, a prevalent monoculture, in comparison to other *Euschistus* species, provides evidence that the fast adaptation of *E. heros* to use this exotic host as a resource is a critical process in the contemporary population dynamic of *E. heros* in the agriculture landscape. This suggests that the introduction of soybean cultivation in South America has facilitated *E. heros* in broadening its host range, specializing in soybean, and emerging as a primary pest of this crop. Consequently, this expansion has led to an increase in its effective population size and abundance, bearing implications for integrated pest management (IPM) strategies on other crops during the soybean offseason, such as cotton. Furthermore, in northern Brazil, where *E. crenator* naturally occurs, soybean fields

have been colonized by this species. Interestingly, *E. crenator* has displayed similar susceptibility to insecticides and reasonable biological parameters when feeding on soybean plants. This underscores the urgency of monitoring *E. crenator*, as its secondary pest status may transition into a primary pest status, as similar observed in recent decades with *E. heros*.

Another aspect that can greatly influence the pest status of a species is its capacity to overwinter and survive crop off-season. One of these mechanisms to survive harsh conditions and synchronize reproduction is diapause. Our trials identified that diapause expression varies significantly among *Euschistus* strains and species (strong in *E. taurulus*, moderate in *E. heros* SS, weak in *E. heros* NS, and absent in *E. crenator*), suggesting rapid evolution and latitude-based variation. The photoperiod is the main cue for inducing diapause in *E. taurulus* and both strains of *E. heros*. Moreover, the short daylength and lower temperature combination increased diapause expression and delayed diapause termination in SS of *E. heros*. Despite testing different photoperiods and temperatures, diapause was not induced in *E. crenator*, implying that this species does not express diapause or uses other cues for induction. The lower diapause expression and termination identified for *E. heros* NS strain provide insights about this species dynamic during the offseason and could justify the increasing pest population outbreaks in other crops, such as happening on cotton crops. The warmer winter in the Cerrado region leads *E. heros* to resume activity earlier, feeding on available food sources after overwintering. Furthermore, *E. crenator* is reproductive even under short daylength and can potentially colonize soybeans in northern areas in winter under shorter days.

Understanding how insects adapt and survive harsh environments from a molecular perspective is crucial to developing long-term management strategies. Diapause is the primary mechanism in *Euschistus* species overwintering, where the abiotic conditions and food availability are unfavorable. Through transcriptomic profiling, we identified and validated genes associated with the JH pathway and arrested oogenesis in *E. servus*. Our results indicate that gene KO effectively arrests oogenesis and reduces lipid storage, and the depletion of the *ftz-f1* gene led to significant mortality of *E. servus*. Studying the underlying mechanisms of diapause regulation is crucial to developing effective population management approaches since overwintering/off-season survival is critical in the insect population dynamic. Moreover, RNAi is a potential tool for biorational pest management.

Finally, our findings showed that *E. heros* pest dynamics are majorly related to the introduction of soybean crops in South America. Although we identified that the two strains show only minor differences, reuniting these two genetic pools with no apparent reproductive barrier can increase variability and select more suitable phenotypes. Additionally, the lower diapause expression of the two strains suggests that it could contribute to the pest outbreaks in other crops during the soybean offseason, such as in the cotton crops. Among the *Euschistus* species tested, we identified a conserved genome structure and a similar insecticide susceptibility, variable soybean suitability, and diapause expression is distinct. Monitoring the secondary pest is essential to predict future pest outbreaks. Finally, our molecular approach revealed that the diapause of *Euschistus* is related to JH signaling, and oogenesis arrest seems to be regulated by the *ftz-f1* gene. This information provides a broader view of *Euschistus*' diapause, which could lead to modern and biorational management approaches.