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Estudo Intra e Interpopulacional de *Pachycoris torridus* (Scopoli, 1772) (Heteroptera: Scutelleridae)

São José do Rio Preto
2015

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Genética, junto ao Programa de Pós-Graduação em Genética, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientador: Profª. Drª. Mary Massumi Itoyama

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"*Foi o tempo que dedicastes à tua rosa que fez tua rosa tão importante*"

(Antoine de Saint-Exupéry)

SUMÁRIO

RESUMO

O percevejo *Pachycoris torridus* (Scopoli 1772) é considerado praga de grande importância agrícola. Por ser uma espécie fitófaga e polífaga seus registros já foram confirmados em diferentes cultivos, entretanto seus ataques à cultura do pinhão manso (*Jatropha curcas* L), planta que possui em suas sementes uma importante fonte de matéria prima para a produção do biodiesel, são os responsáveis pelo seu valor agrícola. Para complementar as informações referentes a este percevejo, inicialmente realizamos a caracterização da sua distribuição geográfica no Brasil e relatamos a sua ocorrência no Noroeste do Estado de São Paulo, ampliamos assim as informações referentes à sua distribuição. Por haver vários trabalhos de descrição dos padrões desta espécie, realizamos um checklist de todos os padrões descritos e descrevemos três novos, completando uma lista com 30 padrões cromáticos, auxiliando na taxonomia desta espécie, a qual já foi descrita oito vezes como nova. Descrevemos também que o desenvolvimento dos padrões cromáticos na espécie é gradual e que o alto polimorfismo de *P. torridus* possivelmente é um comportamento aposemático. A partir da técnica de Banda C, realizamos a descrição da homogeneidade cromossomal nos diferentes padrões de cores do percevejo. Com a utilização dos marcadores moleculares COI, 28S e 16S concatenados, realizamos a caracterização genética de *P. torridus*, verificamos que devido à sua alta variabilidade genética existem vários padrões genéticos resultando no mesmo fenótipo. A partir da análise populacional com o marcador mitocondrial COI foi realizada a caracterização dos haplótipos, onde verificamos a estruturação da espécie, o seu alto grau de variabilidade genética e sua recente expansão populacional associada à ampliação do plantio de pinhão manso, assim fornecemos informações inéditas sobre este percevejo, que se destaca entre os insetos mais polífagos do mundo.

Palavras-chave: Praga agrícola. Polimorfismo. Haplótipos. Marcadores moleculares. Variabilidade genética.

ABSTRACT

The stink bug *Pachycoris torridus* (Scopoli 1772) is considered a pest of great agricultural importance. For being polyphagous and phytophagous your records have already been confirmed in different cultures, however their attacks on culture of physic nut (*Jatropha curcas* L), plant which has in its seeds an important source of raw material for the production of biodiesel, are responsible for their agricultural value. For additional information regarding this bug, initially we performed the characterization of its geographical distribution in Brazil and report its occurrence in the Northwest of the state of São Paulo, thus expanding the information related to their distribution. By having several studies of description of patterns of this species, we performed a checklist of all patterns described and we describe three new, completing the list with 30 chromatic patterns, assisting in the taxonomy of this species, which was already described eight times as a new species. We also described that the development of color patterns in this species is gradual and that the high polymorphism of *P. torridus* is possibly one aposematic behavior. From the C-band technique, we perform the description of chromosomal homogeneity in different color patterns of the stink bug. With the use of molecular markers COI, 28S and 16S concatenated, we performed the genetic characterization of de *P. torridus*, and we verified that due to its high genetic variability there are several genetic patterns resulting in the same phenotype. From the population analysis with the mitochondrial marker COI was performed the characterization of haplotype, verifying the structure of the species, its high degree of genetic variability and its recent populational expansion associated with expanding the planting of physic nut, thus we provide new information about this bug, that stands out among the most polyphagous insects in the world.

Keywords: Agricultural pest. Polymorphism. Haplotypes. Molecular markers. Genetic variability.

I. INTRODUÇÃO

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Na ordem Hemiptera, a subordem Heteroptera representa o mais diversificado grupo de insetos endopterigotos e não holometábolos, possuindo mais de 40.000 espécies (WERAUCH; SCHUH, 2011) e aproximadamente 80 famílias (SCHUH; SLATER, 1995). Há registro da ocorrência de heterópteros em todos os continentes (exceto Antártica) e algumas ilhas. Sua antiga existência e aparente adaptabilidade tem resultado, sob o ponto de vista evolutivo, em extrema diversidade estrutural e biológica. Nenhum outro grupo de insetos possui tamanha diversidade com relação ao habitat, como os Heteroptera. Eles vivem como parasitas de pássaros e mamíferos (morcegos), em teias de aranha, na água ou na sua superfície, com algumas espécies ocupando os oceanos (SCHUH; SLATER, 1995).

Os Heteroptera possuem alimentação muito variável sendo que a maioria depende, exclusivamente, da seiva de plantas vivas (do xilema ou floema e, às vezes, do parênquima); mas existem espécies não fitófagas que compreendem muitos predadores de fungos e outros artrópodes, algumas hematófagas e necrófagas (GULLAN; CRANSTON, 2008).

Uma das principais características dos Heteroptera, da qual deriva o nome do grupo, é a estrutura das asas anteriores que, em geral, são espessadas na base e membranosa no ápice, para formar hemiélitros. A maioria tem asas muito desenvolvidas, mas existem espécies que são ápteras e algumas com asas muito curtas (SCHUH; SLATER, 1995). Suas ninfas se parecem com os adultos, exceto pela ausência do desenvolvimento das asas e da genitália. Possuem glândulas odoríferas, que usualmente se abrem nos lados do tórax e que emitem um odor desagradável, principalmente quando são perturbados (GULLAN; CRANSTON, 2008).

Pelo fato da maioria dos Heteroptera serem fitófagos, eles recebem destaque como praga agrícola, pois podem causar grandes danos às produções de grãos, frutos, ou transmitir doenças às plantas. A importância econômica de vários heterópteros, também, envolve muitas espécies que são benéficas, utilizadas, por exemplo, no controle de pragas. Algumas espécies são ectoparasitas de humanos e animais domésticos e outros são vetores de doenças (Chagas) (SCHUH; SLATER, 1995).

Suas espécies possuem cromossomos holocêntricos, os quais apresentam atividade cinética restrita à parte final dos autossomos e cromossomos sexuais, sendo denominados telocinéticos. A atividade cinética pode afetar ambos os finais dos cromossomos, sendo que a atividade na segunda divisão é a extremidade oposta da primeira divisão (SCHRADER, 1935, 1940; BUCK, 1967; COMINGS; OKADA, 1972; MOTZKO; RUTHMAN, 1984; RUFAS; GIMÉNEZ-MARTÍN, 1986; WOLF, 1996; GONZÁLEZ-GARCIA et al., 1996).

Os Heteroptera são classificados em sete infraordens, que são: Enicocephalomorpha, Dipsocoromorpha, Gerromorpha, Nepomorpha, Leptopodomorpha, Cimicomorpha e Pentatomomorpha (LI et al., 2012). Na infraordem Pentatomomorpha, a família Scutelleridae é composta por percevejos fitófagos, possuindo 80 gêneros, 450 espécies e ampla distribuição geográfica. As espécies desta família são difíceis de serem identificadas, por exemplo, em relação à coloração dentro de uma mesma espécie, há muita variação (GRAZIA et al., 2010).

A característica mais notável dos scutelerideos é o escutelo, que sobrepõe todo o abdômen, sendo conhecidos popularmente como percevejo-de-escudo. Seu comprimento varia de 12 a 14 mm e sua largura de 8 a 9 mm, podendo ter uma variação maior dependendo da quantidade e do tipo de alimento disponível durante seu desenvolvimento (BONDAR, 1913). Esses insetos alimentam-se de gramíneas, ervas, frutos e flores, produzem um odor desagradável emitido pelos ductos das glândulas produtoras de cheiro, que se abrem na região da metapleura. Como todos os hemípteros, sofrem metamorfose incompleta e não possuem as etapas de pupa e larva; os adultos desenvolvem-se a partir de vários instares de ninfas, geralmente cinco, por meio de sucessivas ecdises (SILVA et al., 1968).

Na família Scutelleridae, encontramos o percevejo *Pachycoris torridus* presente desde os Estados Unidos até a Argentina (FROESCHNER, 1988). Entretanto, seus relatos são mais frequentes na América do Sul (PEREDO, 2002), sendo o único percevejo da família Scutelleridae com importância agrícola no Brasil (GALLO et al., 2002). Adaptado a diferentes condições climáticas, já foram registrados em todas as regiões brasileiras (MICHELOTTO et al., 2006; SILVA et al., 2007; RODRIGUES et al., 2011; ZANARDI et al., 2010; MARQUES et al., 2012;).

São percevejos globosos, com o escutelo bem desenvolvido apresentando diferentes padrões de manchas e cores, sendo o padrão cromático básico de oito manchas no pronoto e 14 no escutelo, as colorações das manchas variam (MONTE, 1937), característica que o fez ser classificado oito vezes como espécie nova (COSTA LIMA, 1940).

Esta espécie é polífaga, sendo descritos infestações às culturas do arroz (Poaceae), laranja (Rutaceae), caju e manga (Anacardiaceae), araçá, eucalipto e goiaba (Myrtaceae), mandioca e tungue (Euphorbiaceae) (SILVA et al., 1968), acerola (Malpighiaceae) (GALLO et al., 2002), aroeira-vermelha (Anacardiaceae) (SANCHEZ-SOTO et al., 2004), cansanção (Euphorbeaceae) (SANTOS et al., 2005), café (Rubiaceae) (PIKART et al., 2011) e, principalmente, à cultura do pinhão manso (*Jatropha curcas*) (Euphorbiaceae). Podem se alimentar de vários hospedeiros, e assim estar presente durante todo o ano. A época de maior intensidade das infestações é durante o verão, devido às condições ambientais, as quais

favorecem o desenvolvimento e a multiplicação do percevejo e das espécies hospedeiras. Com a redução da temperatura ocorre o desaparecimento visual da sua presença na área (AVELAR et al., 2007). As bromélias provavelmente são um sitio de hibernação desta espécie (SCHMIDT; BARCELLOS, 2007).

Em campo, observou-se que as fêmeas de *P. torridus* protegem suas posturas localizadas nas folhas, permanecendo sobre as mesmas constantemente, protegendo-as principalmente de inimigos naturais, como parasitoides e predadores. As ninfas são de coloração verde metálica e permanecem durante o primeiro instar agregadas e sob proteção materna e nos demais instares em grupos menores ou isoladas. Na fase adulta ficam sobre folhas e frutos verdes ou maduros, localizando-se em diferentes estratos das plantas. (BROGLIO-MICHELETTI et al., 2010). No pinhão manso, tanto as ninfas quanto os adultos de *P. torridus* sugam os frutos, afetando a formação do endosperma, podendo ocorrer o aborto prematuro da semente, afetando a qualidade e quantidade do óleo (AVELAR et al., 2007).

O pinhão manso (*Jatropha curcas* L.) caracteriza-se como uma espécie perene, resistente à seca e destaca-se entre as plantas oleaginosas por possuir alto potencial na produção de óleo, utilizado na produção do biodiesel (SATURINO et al., 2005). As plantas levam de 3 a 4 anos para atingirem a idade reprodutiva e podem ter uma longevidade de até 40 anos, com produção mínima de duas toneladas de óleo por hectare. Além da produção de óleo para biodiesel, o pinhão manso pode ser utilizado para outros fins, como no tratamento de doenças, como a disenteria, hemorroidas, gonorreia, infertilidade, infecções de pele, entre outras (AKINTAYO, 2004).

Embora o pinhão manso apresente poucos registros de pragas, devido à exsudação de um látex tóxico e repelente à maioria das espécies, os danos impostos pelo percevejo *P. torridus* são irreversíveis a partir de determinados níveis populacionais, interferindo na produtividade do fruto e do óleo (PEIXOTO, 1973). Ainda não existem medidas de controle deste percevejo, porém já se sabe da ação de um óleo vegetal (azadiractina) que provem da planta *Azadiractina indica* (Meliaceae) sendo mais conhecido como óleo de Nim, que se mostrou eficiente ao controle de *P. torridus* em condições de laboratório (RAMOS et al., 2009). Além desse bioinseticida, já foram relatados o uso de parasitóides como *Telenomes pachycoris*, que se mostraram eficientes ao ataque dos ovos de *P. torridus*, também em condições de laboratório (COSTA LIMA, 1928). Entretanto, pouco se sabe sobre *P. torridus*, havendo necessidade de pesquisas, para que sejam disponibilizados métodos de controle desta praga na cultura do pinhão manso (AVELAR et al., 2007).

Uma importante ferramenta para auxiliar e melhorar as práticas de gerenciamento e controle de pragas é a caracterização de diferenças genéticas intra e interpopulacional. A crescente disponibilidade das sequências de DNA em bancos de dados públicos e os avanços da bioinformática vêm permitindo o desenvolvimento de novas abordagens e estratégias no controle de diversas pragas (PRIMROSE; TWYMAN, 2003).

No final da década de 60, o advento das técnicas moleculares possibilitou o desenvolvimento de hipóteses testadas experimentalmente a partir de dados empíricos sobre os fatores e padrões da variação genética, permitindo abordagens mais precisas e grandes progressos nos estudos populacionais e evolutivos em nível inter e intraespecíficos. Até o ano de 1966 os estudos de polimorfismos nas populações eram realizados com base nos fenótipos dos organismos, o que não permitia uma boa detecção da variabilidade genética populacional, pois tais caracteres apresentavam diferentes tipos de heranças (EIZIRIK, 1996).

A partir da década de 80, diferentes tipos de DNAs começaram a ser utilizados em estudos genéticos e evolutivos. Diferenças nas sequências de nucleotídeos de várias regiões mitocondriais foram utilizadas para gerar informações sobre a estrutura de várias espécies de insetos (GASPARICH et al., 1995; LEHMANN et al., 2000). Uma característica que torna a molécula de DNA mitocondrial atrativa para estudos populacionais é o fato de ela ser citoplasmática, de modo que não segue os padrões mendelianos de segregação e não sofre recombinação. Outra característica fundamental dessa molécula é a sua alta taxa de evolução: 10 vezes superior a um gene de cópia única nuclear (ARIAS; INFANTE-MALACHIAS, 2001).

O genoma mitocondrial de Heteroptera já foi completamente sequenciado, pelo grupo de Dotson e Beard (2001), utilizando *Triatoma dimidiata* (Heteroptera, Reduviidae), vetor da doença de Chagas. O genoma mitocondrial dessa espécie apresentou 17.019 pb, sendo 13 sequências que codificam proteínas, 22 tRNAs, subunidades ribossomais maior e menor e uma região controle. A ordem e a orientação dos genes foram idênticas aos de *Drosophila yakuba*, porém a composição de nucleotídeos foi maior com relação às bases adenina e timina (69,5%). Evidenciando assim as similaridades e diferenças genéticas entre estas espécies.

Dados das sequências de nucleotídeos e aminoácidos do gene mitocondrial citocromo oxidase subunidade I (*COI*), foram analisados em espécies de triatomíneos do complexo Phyllosoma, *Triatoma rubida* e *T. recurva* (Heteroptera: Reduviidae: Triatominae), sendo utilizados para estudos populacionais e filogenéticos. Segundo Pfeiler et al. (2006), nenhum haplótipo foi compartilhado entre estas espécies nas principais regiões geográficas (Baja Califórnia Sur, Sonora e Arizona), confirmando a identidade taxonômica preexistente. Nas

análises das sequências de aminoácidos, todos os membros do complexo Phyllosoma compartilharam cinco substituições no segmento do gene *COI*, demonstrando a importância dos dados moleculares na confirmação dos táxons. Grisales et al. (2010), a partir das análises do gene mitocondrial *ND4*, realizou a caracterização genética de três populações de *Triatoma dimidiata* (Heteroptera), destacando a variabilidade genética existente e suas implicações no controle destes percevejos.

Neste contexto, a utilização de sequências de DNA, principalmente o DNAmt representa um instrumento promissor na identificação de espécies. No gene mitocondrial *COI*, um fragmento de 650 pares de bases da extremidade 5' foi proposto como padrão global, a chamada "região do código de barras" de animais (HEBERT et al., 2003 a,b). Esta abordagem de código de barras tem sido aplicada com sucesso em vários vertebrados e invertebrados para a delimitação e identificação de espécies (SMITH et al., 2005; ZHOU et al., 2009).

As espécies são estruturadas em grupos de indivíduos geneticamente mais semelhantes, estando o grau de isolamento diretamente relacionado à dispersão de indivíduos entre estes grupos, este padrão de distribuição da variação genética dentro e entre populações é referido como "estrutura genética populacional" (LAIKRE et al., 2005). Técnicas de genética molecular têm sido amplamente aplicadas em estudos da distribuição da variação genética dentro e entre populações, os quais tem revelado a existência de populações significativamente diferenciadas. As populações naturais apresentam grande diversidade fenotípica e a genética de populações trabalha com essa diversidade que pode ser causada pelas diferenças genotípicas entre os indivíduos (HARTL; CLARK, 2007).

A variabilidade genética é uma condição necessária para a ocorrência das mudanças evolutivas. Novas variações são introduzidas nas populações pela ocorrência de mutações, ou como resultado do fluxo gênico determinado pelo cruzamento entre indivíduos nativos ou imigrantes geneticamente distintos. A presença de variabilidade genética herdável em características adaptativas fornece flexibilidade fenotípica a novas mudanças ambientais, tais como introdução de um predador ou repentina contaminação com patógeno ou poluente. A adaptação de um indivíduo em um ambiente pode ser um prognóstico da adaptação em outro ambiente. Então, torna-se importante a conservação dos componentes herdáveis da variabilidade, maximizando deste modo as oportunidades de sobrevivência (CARVALHO, 1993).

Uma população pode apresentar diferenças na variação genética entre os agrupamentos que a compõem por várias razões evolutivas diferentes (acasalamento não randômico, deriva genética, entre outras). Wright (1965) desenvolveu uma maneira de estimar

a quantidade de diferenciação nas subdivisões de uma população. Estas estimativas são feitas a partir de três diferentes coeficientes F, usados para determinar a variabilidade genética na população total (T), nas subpopulações (S) e nos indivíduos (I). Nei (1977) demonstrou que estes valores podem ser expressos em termos das frequências alélicas e das frequências genotípicas observadas e esperadas. Além disso, estendeu as análises para múltiplos locos, onde os diferentes coeficientes-F podem ser calculados.

Os trabalhos citados confirmam a importância do uso de dados moleculares para o estabelecimento de relações intra e interpopulacionais em Insetos. Portanto, realizamos a caracterização da espécie *P. torridus*, e assim ampliamos as informações referentes a esta espécie, considerada praga de grande valor agrícola no Brasil.

II. OBJETIVOS

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O objetivo geral foi realizar a caracterização da distribuição espacial, da variabilidade fenotípica, da diversidade genética e dos processos de colonização e expansão de *Pachycoris torridus.*

Os objetivos específicos foram:

a) Caracterizar a distribuição geográfica de *P. torridus* no Brasil*;*
b) Descrever a variabilidade fenotípica existente na espécie*;*
c) Registrar os aspectos do desenvolvimento das cores de *P. torridus;*
d) Caracterizar a distribuição da heterocromatina entre diferentes padrões de cores;
e) Identificar e descrever os padrões de haplótipos de *P. torridus;*
f) Caracterizar a variabilidade genética intra e interpopulacional relacionando-a com as diferentes localidades;
g) A partir da caracterização da estrutura populacional inferir a dinâmica das populações nas diferentes áreas amostradas.

III. CAPÍTULOS

ARTIGO 1

Artigo 1 - Occurrence of *Pachycoris torridus* (Scopoli, 1772) (Hemiptera: Scutelleridae) on physic nut (*Jatropha curcas*) in northwest of Sao Paulo, Brazil

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Abstract

We notified for the first time the occurrence of *Pachycoris torridus* (Scopoli, 1772) (Hemiptera: Scutelleridae) in different localities of Northwest region of the State of Sao Paulo, attacking the culture of physic nut (*Jatropha curcas*). The stink bug *P. torridus* shows longevity, are phytophagous and polyphagous, characteristics that emphasize the importance of their records for a better understanding of their infestations in the culture of physic nut, a plant whose seeds are an importance source of raw material for the production of biodiesel.

Keywords: *Jatropha curcas*, *Pachycoris torridus*, stink bug, entomological lifting

Scientific Note

The physic nut (*Jatropha curcas*) is a plant of the family Euphorbiaceae, with an increasing highlight agricultural in Brazil by the high oil content in the seeds, low cost of production cost and their capacity to produce in sandy soils with low fertility, besides of the ease of cultivation and harvesting [1]. With the possibility of using of physic nut to produce biodiesel, several planting areas and researches have been installed in different regions of Brazil, being a plant of easy propagation and with longevity of 30-50 years and can live for over a century [2].

The geographical distribution of physic nut is wide, being adaptable to the adversities of the soil and the climate. Is found in a wide range climatic and pluviometric, can survive in adverse conditions such as 200 mm of annual rains or three consecutive years of drought, because, as adaptation, paralyze their growth in those periods, losing their leaves and surviving from the water stored in the stems [3, 4].

For being a perennial crop, can be used in soil conservation, once covers it with a layer of dry matter, reducing erosion and loss of water by evaporation and enriching the soil with organic matter decomposed [2]. The fruit is capsular, ovoid shape, with a diameter of 1.5 cm to 3.0 cm. It is trilocular with a seed in each cavity, consisting of a ligneous pericarp, indehiscent, initially green, becoming yellow, brown and finally black, when it reaches the maturity stage. It comprises 53% to 62% of seeds and from 38% to 47% of bark, each weighing 1.53g to 2.85g. In seed are found 7.2% of water, 37.5% of oil and 55.3% of sugar, starch, albuminoid and mineral materials [1].

In domestic medicine, the latex of this plant is used as a healing agent, the roots are considered diuretic and effective against rheumatism. The seeds and the oil extracted from these are often used as purgative, in the treatment of skin diseases, gout, paralysis, hemorrhoids and rheumatism. However, the ingestion of a single fresh seed can cause vomiting and diarrhea and, when eaten in excess, can be fatal due to toxic properties of compounds (globulin and acid) presents in the seed [2].

The phisic nut has as defense the caustic latex exudation which acts as a repellent and is toxic to most species [1]. However, the species *Pachycoris torridus* (Scopoli, 1772) is able to colonize the pinion, which gives the insect highlighted as agricultural pest of this culture vegetal. The fruits attacked by *P. torridus* become unviable, presenting initially dark and deformed appearance, which results in subsequent fall and loss of product [5]. Thus, the

damage caused by these insects to the fruits of physic nut has been reported to decrease the productivity of the oil used as a raw material for biodiesel [5, 6].

P. torridus belongs to the Hemiptera order and Heteroptera suborder, insects popularly called stink bug. This Heteroptera presents longevity, can live for up to 600 days [7], are phytophagous and polyphagous, with a record of their attacks in 16 vegetable crops [8], with wide distribution in the Neotropical region [9]. In Brazil, the occurrence of *P. torridus* was reported in at least 15 states (Table 1). In Sao Paulo, the species was observed only in the cities of Jaboticabal, Araras, Campinas, Itapira, Piracicaba and Tatui [7, 9, 10, 11, 12]. In this context, this work notifies, for the first time, the presence of *P. torridus* in northwestern of Sao Paulo, Brazil.

Specimens of *P. torridus* were collected in Sao Jose do Rio Preto (20º46’48.2’’S, 49º 21’18.3’’W), Americo de Campos (20°17'43.1"S, 49°44'12.2"W), Pontes Gestal (20°10'18.1"S, 49°42'21.2"W) and Monte Aprazivel (district of Engenheiro Balduino (20°40'50.6"S, 49°42'09.6"W) (Figure 1). The insects were analysed at the Laboratory of Cytogenetics and Molecular of Insects, localized in Institute of Biosciences, Humanities and the Exact Sciences, Sao Paulo State University – Julio de Mesquita Filho (UNESP/IBILCE), Sao Jose do Rio Preto, Sao Paulo, Brazil. The identification of insects was based on work of the Monte (1937) [13] and Costa Lima (1940) [14].

These insects were collected in plantations of physic nut, which were familiar plantations, used mainly as a living fence. It was possible to observe the presence of all developmental stages of *P. torridus*, being five nymphal stages (N1, N2, N3, N4 and N5) and adult males and females. During the first and second instar, the insects remained aggregates and under maternal protection, mainly in the lower surface of the leaves, once parental care is a characteristic well known for this species [15]. In the other nymphal instars (N3, N4 and N5), these heteropteran were found in smaller groups or isolated. We emphasize that all stages occurred simultaneously in physic nut, and from the third instar, the stink bug remained on leaves and unripe and ripe fruits located in different strata of the plants. All adult insects presented the same chromatic patterns with eight spots on pronotum and 14 on scutellum.

The presence of *P. torridus* in northwest region of the State of Sao Paulo, is of great economic importance for the nature of the damage described for culture of physic nut. Furthermore, we highlight that the characteristic of polyphagous of *P. torridus*, which allows this insect can colonize and mainly become an agricultural pest to other crops of economic importance.

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Table 1 – States with record of *Pachycoris torridus*

States	References
Alagoas	[5]
Amapa	[16]
Amazonas	[10]
Bahia	[8]
Goias	[17]
Maranhao	[10, 18]
Mato Grosso do Sul	[6]
Minas Gerais	[10, 19]
Para	[20]
Parana	[21]
Piaui	[22]
Rio de Janeiro	[10, 13]
Rio Grande do Sul	[23]
Rondonia	[24]
Sao Paulo	[7, 9, 10, 11, 12]

Figure 1. Map of Brazil highlighting the state of Sao Paulo. The points represent the locations of the collects of *P. torridus* in the northwest of the state of Sao Paulo.

ARTIGO 2

Artigo 2. Checklist and description of three new chromatic patterns of *Pachycoris torridus* (Scopoli, 1772) (Hemiptera: Scutelleridae)

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Abstract

In the present paper, 27 chromatic patterns of the species *Pachycoris torridus* (Scopoli, 1772) were grouped and three new patterns are described. Because of this high phenotypic polymorphism, *P. torridus* already been registered eight times as a new species, highlighting the importance of the application of different tools to assist in taxonomy of this hemipterous of economic importance.

Key words: *i*nsecta, Heteroptera, polymorphism, agricultural pest

Introduction

The insects of the Scutelleridae family are popularly known as shield-backed bug, due to its scutellum which covers the whole body (Grazia & Schwertner 2011). A striking feature of Scutelleridae is intraspecific phenotypic variability, as can be observed in the species *Poecilocoris lewisi* (Distant, 1883), *Pachycoris klugii* (Burmeister, 1835) and *Pachycoris torridus* (Scopoli, 1772) (Miyamoto & Kosaku 2002, Peredo 2002).

The stink bug *P. torridus* is a phytophagous and polyphagous that has great prominence as an agricultural pest, with emphasis on the cultures of physic nut (*Jatropha*

curcas Linnaeus), a raw material for the production of biodiesel (Borges Filho et al. 2013). This hemipterous has wide distribution in the Neotropical region and shows a wide variation in chromatic patterns, characteristic that made *P. torridus* be registered several times as a new species (Costa Lima 1940), getting names like: *Tetyra schousboei, Pentatoma fabricii, Scutellera decorata, Pachycoris Klungii, Pachycoris linaei, Pachycoris aquila, Pachycoris stallii* and *Poecilocoris aeneiventris* (Maes 1994).

Monte (1937) initiated the descriptions of stains patterns of this stink bug and registered 13 different patterns, terming standard 1 to 13. The author highlights the pattern 13 (Figure 1) as the basic for *P. torridus*. Currently, there are 27 chromatic patterns described for this hemipterous in different scientific articles (Monte 1937, Sanchez-Soto 2004, Santos et al. 2005, Pikart 2011, Souza et al. 2012). Thus, in this study we performed a checklist of all the patterns of stains described in the literature for *P. torridus* and we describe three new (Figure 2).

Material and Methods

The checklist of twenty-seven chromatic patterns described for *P. torridus* was compiled from the literature (Monte 1937, Sanchez-Soto 2004, Santos et al 2005, Pikart 2011, Souza et al. 2012). For description of three new patterns, specimens of *P. torridus* were collected in Brotas, Sao Paulo, Brazil (22°14'17.6" S, 48°07'56.0" W) in *Jatropha curcas*. The description was based on basic pattern of spots described by Monte (1937). Vouchers were deposited in the collection of the Laboratory of Cytogenetics and Molecular of Insects, of Institute of Biosciences, letters and the Exact Sciences (UNESP/IBILCE), Sao Jose do Rio Preto, Sao Paulo, Brazil.

Results

Checklist of the 30 chromatic patterns identified for the species *P. torridus* (Figure 3).

Pattern 1. Features 15 spots. Pronotum with six, being two near the head, two on the sides and two in the posterior region, and scutellum with nine, arranged in four rows, respectively 2, 4, 2 and 1 spots starting from the base to the apex (Monte 1937).

Pattern 2. Features 4 spots. Pronotum and scutellum with two spots, both of paired (Monte 1937).

Pattern 3. Features 8 spots. Pronotum with four, being two in the middle region disposed in parallel and two on the sides, and scutellum with four, arranged in three rows, being the last with two spots (Monte 1937).

Pattern 4. Features 16 spots. Pronotum with six, arranged in two rows with 2 and 4 stains, respectively, and scutellum with ten spots, arranged in four rows of 2, 2, 4 and 2 spots each, starting from the base to the apex (Monte 1937).

Pattern 5. Features 14 spots. Pronotum with four, being two rows with two spots each, and scutellum with ten, arranged in four rows of 2, 2, 4 and 2 spots, respectively, from base to apex (Monte 1937).

Pattern 6. Features 12 spots. Pronotum with six, being two near the head, two on the sides and two in the posterior region, and scutellum with six, arranged in three rows of 1, 4, 1 stains, being one big spot next to the base region (Monte 1937).

Pattern 7. Features 15 spots. Pronotum with six, being two near the head, two on the sides and two in the posterior region, and scutellum with nine, arranged in four rows with 2, 4, 2 and 1 spots, respectively, from base to apex (Monte 1937).

Pattern 8. Features 7 spots. Pronotum with two big spots, and scutellum with five, being one big in the region near the base, two on the sides and two near the apex (Monte 1937).

Pattern 9. Features 2 spots. Pronotum and scutellum with one big spot on each, featuring the spaces that would be the spots on basic pattern (Figure 1) (Monte 1937).

Pattern 10. Features 12 spots. Pronotum with four, being two in the middle, and two on the sides, and scutellum with eight, arranged in three rows with 2, 4 and 2 spots each (Monte 1937).

Pattern 11. Features 13 spots. Pronotum with six, being two near the base, two near the apex and two on the sides, and scutellum with seven, arranged in four rows with 2, 2, 2 and 1 spots respectively from base to apex (Monte 1937).

Pattern 12. Features 16 spots. Pronotum with six, being two near the base, two near the apex and 2 on the sides, and scutellum with ten, arranged in three rows of 4, 4 and 2 spots, respectively, from the base to the apex (Monte 1937).

Pattern 13. Features 22 spots. Pronotum with eight spots, and scutellum with fourteen, neatly arranged in four rows, respectively of 5, 4, 3 and 2 spots from base to apex (Monte 1937).

Pattern 14. Features 22 spots. Pronotum with eight spots, and scutellum with fourteen, with the spots 10, 11 and 12 higher than the basic pattern (Figure 1) and very close together (Sanchez-Soto et al. 2004).

Pattern 15. Features 6 spots. Pronotum with a single spot, the eight spots of the basic pattern (Figure 1) joined from lateral and anterior margins, and scutellum with five spots, the spots 1, 6, 7, 8, 9 and 5, joined and formed a continuous strip, and spots 10, 11, 12, 13 and 14 joined leaving a small space near the apex (Sanchez-Soto et al. 2004).

Pattern 16. Features 3 spots. Pronotum with one spot, formed by the union of the eight spots of the basic pattern (Figure 1), and scutellum with two spots, one is the spot 2 of the basic pattern (Figure 1), and the other formed by the union of spots 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 (Sanchez-Soto et al. 2004).

Pattern 17. Features 3 spots. Pronotum with one spot, formed by the union of the 8 spots of the basic pattern (Figure 1), and scutellum with two spots formed by the union of spots 1, 2, 3, 4, 5, 6, 7, 8 and 9, near the base, and spots 10, 11, 12, 13 and 14 near the apex (Sanchez-Soto et al. 2004).

Pattern 18. Features 8 spots. Pronotum with two near the head, and scutellum with six, being two near the base, two on median region, and two on the sides (Santos et al. 2005).

Pattern 19. Features 4 spots. Pronotum with two near the head, and scutellum with two on the medium region (Santos et al., 2005).

Pattern 20. Without spots (Santos et al., 2005).

Pattern 21. Features 21 spots. Pronotum with eight spots, and scutellum thirteen, with the spots 7 and 8 of the basic pattern joined (Figure 1) and the spots 10 and 12 larger than the basic pattern (Pikart et al. 2011).

Pattern 22. Features 17 spots. Pronotum with six, being spots 1 and 8 of the basic pattern (Figure 1) joined, respectively, with the spots 3 and 7, and scutellum with eleven, presented the spots 7 and 8 united, and spots 10, 11 and 12 joined forming a continuous strip (Souza et al. 2012).

Pattern 23. Features 20 spots. Pronotum with eight, and scutellum with twelve, the spots 10, 11 and 12 of the basic pattern (Figure 1) joined forming a continuous strip (Souza et al. 2012).

Pattern 24. Features 19 spots. Pronotum with eight spots, and scutellum with eleven, the spots 7 and 8 of the basic pattern joined (Figure 1) and spots 10, 11 and 12 grouped forming a continuous strip (Souza et al. 2012).

Pattern 25. Features 20 spots. Pronotum with eight spots, being the spots 4 and 5 very close together, and scutellum with 12, being the spots 6 and 9 joined, respectively, with spots 7 and 8 (Souza et al. 2012).

Pattern 26. Features 20 spots. Pronotum with eight spots, and scutellum with twelve, the spots 6 and 7 joined, as well as the spots 8 and 9, and the spots 10, 11 and 12 are larger and closer together (Souza et al. 2012).

Pattern 27. Features 17 spots. Pronotum with seven, the spots 4 and 5of basic pattern joined (Figure 1), and scutellum with ten, the spots 2, 3 and 4 grouped near the base forming a continuous strip and spots 6 and 9 joined, respectively, with spots 7 and 8 (Souza et al. 2012).

Pattern 28. Features 23 spots. Pronotum with eight spots, and scutellum fifteen, with one additional spot between the spots 7 and 8 of the basic pattern (Figure 1). (This paper) (Figure 2a).

Pattern 29. Features 21 spots. Pronotum with eight spots, and scutellum with thirteen, the spots 7 and 8 of the basic pattern joined (Figure 1) (This paper) (Figure 2b).

Pattern 30. Features 22 spots. Pronotum with eight spots, and scutellum with fourteen, the spots 7 and 8 are larger and closer together, that the basic pattern (Figure 1) (This paper) (Figure 2c).

Discussion

The stink bug *P. torridus* shows different phenotypes with several variations in the pattern of spots and colors of your body. Monte (1937) described the basic chromatic patterns as being of eight spots on pronotum and 14 on scutellum and recorded 13 different patterns in 13 of 16 specimens collected on an unidentified plant. Years later, Sanchez-Soto et al. (2004) recorded in *Schinus terebinthifolius* Raddi more four patterns, Santos et al. (2005) more three in *Cnidoscolus pubescens* Pohl, Pikart et al. (2011) described a new pattern in *Coffea arabica* Linnaeus and Souza et al. (2012) described six patterns with specimens collected in *Jatropha curcas.* In this article we performed a checklist of the 27 color patterns described and we describe three new patterns, completing a list of 30 color patterns described for *P. torridus.*

The high polymorphism of *P. torridus* already led to great taxonomic mistakes. Costa Lima (1940) described that the specie has been already registered eight times as new. According to Monte (1937), the color variations of *P. torridus* are not hereditary and the factors that may contribute to the differentiation of a color are diverse and complex, however the factors involved in the polychromatism of *P. torridus* have not yet been identified.

Grazia & Schwertner (2011) in a checklist about biodiversity of the State of Sao Paulo, Brazil, emphasized that the Scutelleridae family needs of taxonomic revision and highlighted the importance of the developing of tools to identification and dissemination of the knowledge of the group. Souza et al. (2012) also emphasize that the high chromatic variation of *P. torridus* makes necessary to describe new phenotypes to assure its correct taxonomic identification. Thus the checklist and description of three new chromatic patterns of *P. Torridus*, is a new tool for taxonomy of this stink bug, synthesizing all the chromatic patterns described for the specie.

Conclusion

Due the high chromatic polymorphism observed in present checklist, we highlight the importance of applying different tools to aid in the taxonomy of this insect of economic importance.

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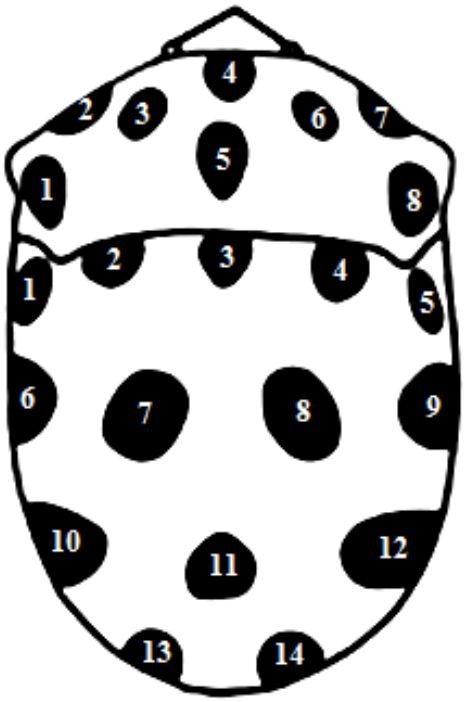

Figure 1. Basic pattern of *P. torridus* described by Monte (1937), with 22 spots, being eight on pronotum and 14 on scutellum.

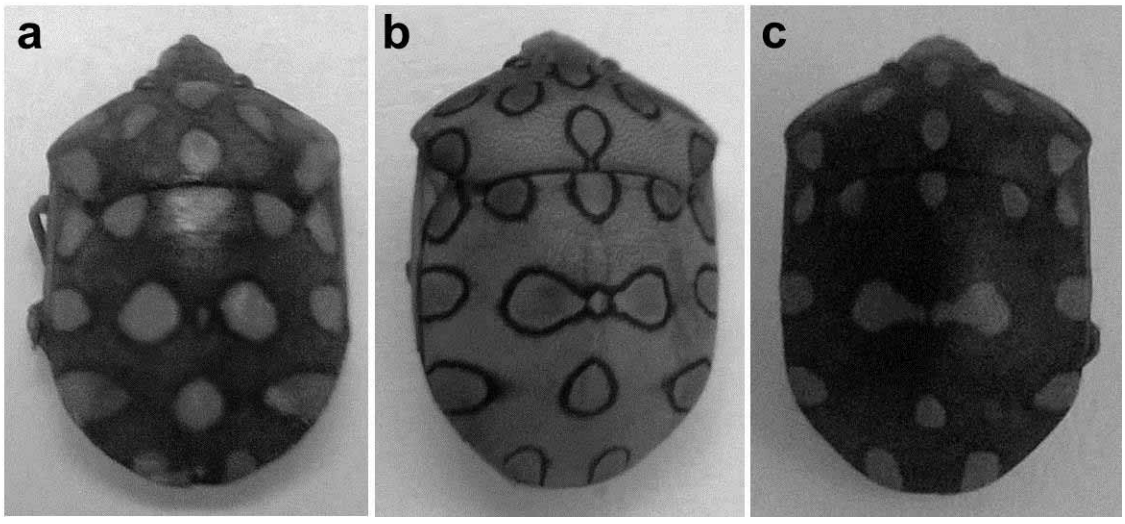

Figure 2. New chromatic patterns observed in the present work. a) Pattern 28; b) Pattern 29; c) Pattern 30.

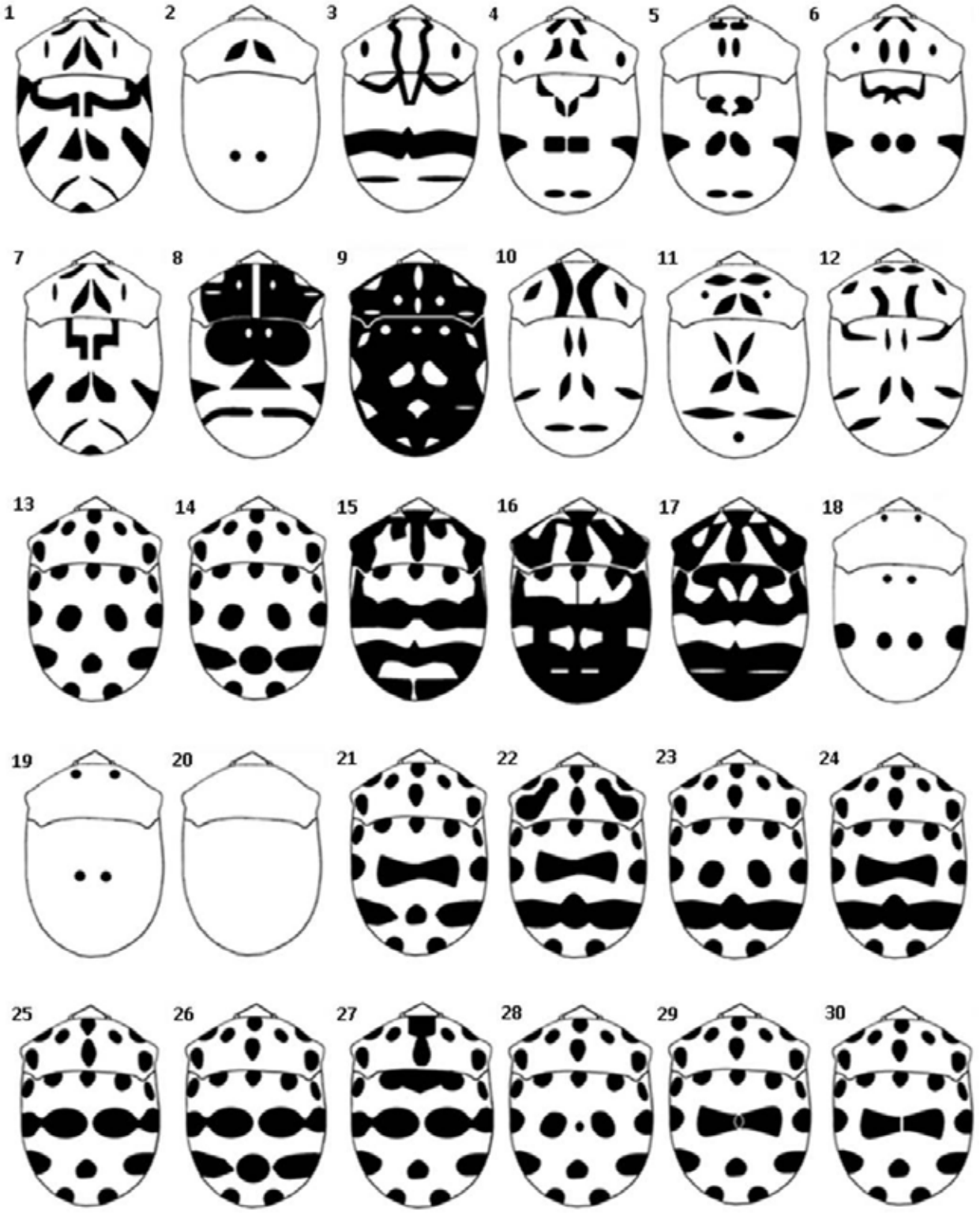

Figure 3. Schemes of 30 chromatic patterns of *P. torridus*. In black the spots.

ARTIGO 3

Artigo 3. Aspects of the color development in *Pachycoris torridus* (Scopoli, 1772) (Heteroptera: Scutelleridae)

Running Title: Aspects of the color development in *Pachycoris torridus*

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Resumo

We notify the aspects of the coloration development of *Pachycoris torridus* (Scopoli, 1772), species that presents high polymorphism, characteristic that made be described eight times as new. Thus, we recorded that the development of color patterns in the species is gradual and that the high polymorphism of *P. torridus* possibly is an aposematic behavior. However, new analyzes should be conducted to test the hypothesis.

Keywords: stink bug, polymorphism, agricultural pest

Scientific Note

The species *Pachycoris torridus* (Scopoli 1772) is popularly known as “stink bug of physic nut”, due to their attacks to culture of physic nut (*Jatropha curcas*) (Silva et al., 1968). Is widely distributed in America, being registered from the United States to Argentina (Froeschner, 1988). For present variations in patterns of colors and stains from their body, has already been described eight times as a new species (Costa Lima, 1940). The colors of the spots vary from yellow to red (Monte, 1937). In this context, this work was conducted with the objective to contribute with details about the development of coloration of *P. torridus.*

Adults, nymphs and eggs of *P. torridus* were colected in physic nut in the city of São José do Rio Preto-SP (20º46’48.2’’S, 49º 21’18.3’’W) and transported to the Laboratory of Cytogenetics and Molecular of Insects of UNESP/IBILCE. The nymphs were separated from adults in glass box (35x25x25 cm), containing leaves and fruits of physic nut and acerola, which were covered with hammock to avoid insects output (Figure 1a).

The insects were analyzed daily. The changes of instars were detected to be noted the exuviae, and by himself growth of nymphs. To analyse the development of the coloring of *P. torridus*, after the last ecdysis (after the 5th instar) the specimens were individualized in plastic pots of 80 ml, containing leaves and fruits of physic nut and acerola (Figure 1b). Colour development was monitored and registered, which can be observed in Figure 2.

Gabriel and Franco (2012) in a study about the morphological aspects of *P. torridus* observed that the color of the spots of the descendants may differ or not of the color of the female that they were born, and in the same oviposition can be born descendants of different colors. And all adults, to emerge, showed yellow color and, in the course of time, have become orange, red or remained with the source color.

In our analysis we confirmed that all adults, after the last ecdysis, present the yellow color to emerge, which is certainly the color of chitin in this species; however we verified that the color development is gradual. The ecdysis process occurs through hormonal stimulation, the Ecdysone, called molting hormone, causing the insect leaves the exoskeleton (exuvia), providing a short period of development with subsequent production of a new exoskeleton.

When emerge as adult, *P. torridus* presents the yellow color (head, chest, abdomen and legs) (Figure 2a). First develops the pattern of spots, in a gradual onset, of the pronotum to the scutellum. After 12 hours begins to develop the coloration, both of the spots as the carapace, also in a gradual manner, of the pronotum to the scutellum. After 48 hours of ecdysis, the bug already presents its permanent adult form (Figure 3).

The chromatic variations can interfere in evolutionary ecology of these different phenotypes, and may differ in their fitness. Moreover, the polymorphism may influence the communication between the sexes and, therefore, on the probability of mating within different phenotypes. However, some insects are unpalatable to predators and often use warning colors like orange, red and yellow to advertise them (Ruxton et al., 2004). Many predators know to associate the presence of chemical defense with visual signals, and polymorphism may perform this role to avoid predators (Joron et al., 1999). The presence of chemical defenses in *P. torridus* has not been studied, but is possible that it get toxic compounds during feeding in physic nut, thus as occurs with other species of the same genus (Williams et al., 2001).

Therefore, we recorded that the develop of the color in the species *P. torridus* is gradual and the possible mechanism responsible for high polymorphism in the species would be an aposematic behavior. But further analysis should be conducted to test the hypothesis.

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Figure 1. a) glass box for accommodation of insects; b) plastic pot for the verification of the color development after the last ecdysis.

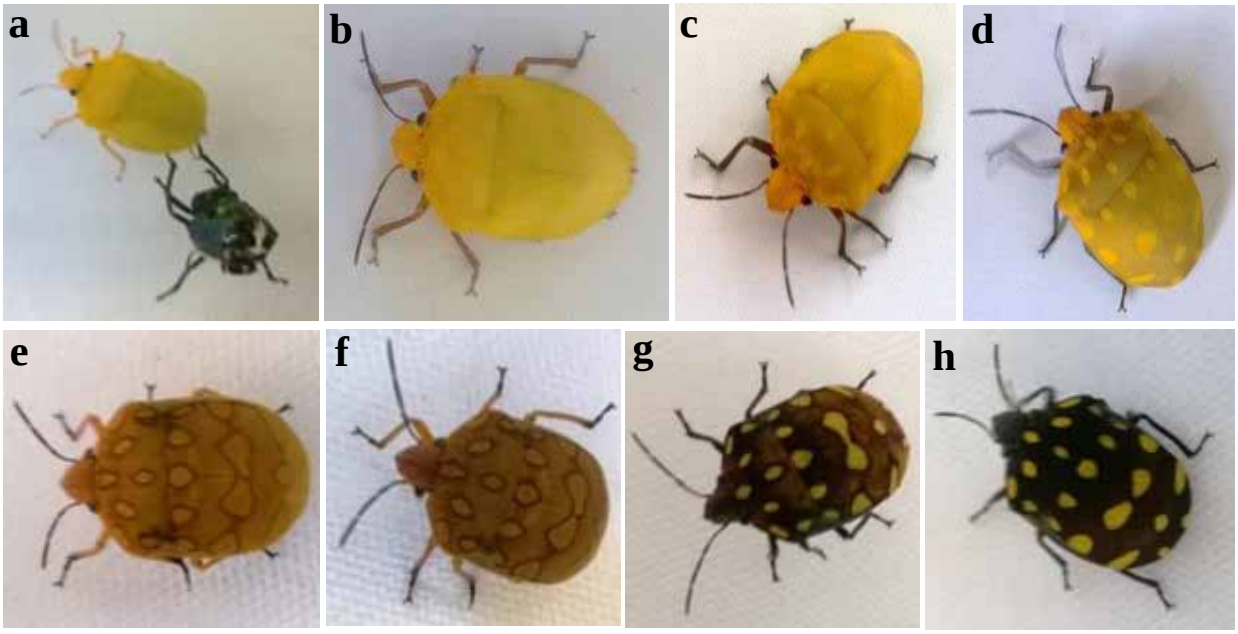

Figure 2. Development of coloration in *P. torridus*. a) ecdysis; b) 1 hour after ecdysis; c) 3 hours after ecdysis; d) 5 hours after ecdysis; e) 7 hours after ecdysis; f) 9 hours after ecdysis; g) 12 hours after ecdysis; h) 24 hours after ecdysis.

Figure 3. Color patterns observed 48 hours after ecdysis. a) red; b) orange; c) yellow; d) brown.

ARTIGO 4

Artigo 4. Distribution of constitutive heterochromatin in *Pachycoris torridus* (Hemiptera, Scutelleridae) of different chromatic patterns

Running Title: Constitutive heterochromatin in *Pachycoris torridus*

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ABSTRACT

The stink bug *Pachycoris torridus* is a pest of great featured agricultural, due their records in the culture of physic nut (*Jatropha curcas*), raw material for biodiesel production. An interesting feature of this insect is its high phenotypic variability, characteristic that made eight times be classified as a new species. In the Heteroptera suborder, the pattern of heterochromatin is specific and allows, in many cases, the differentiation of the species. Aiming to check if there differentiation between different color patterns (yellow, orange, brown and red), specimens of *P. torridus* were analyzed cytogenetically, using the technique band C. During meiotic prophase, the four color patterns analyzed showed a big chromocenter heterochormatic, consisting of the combination of both sex chromosomes (XY). Thus, the present study reports chromosomal homogeneity in different color patterns of *P. torridus* and highlights the importance of this tool in the description of new species.

Keywords: Heteroptera; Spermatogenesis; Meiosis; Chromosomal homogeneity

INTRODUCTION

The Hemiptera Order is composed of three suborders: Auchenorrhyncha, Heteroptera and Sternorrhyncha (Cryan and Urban 2012). The Heteroptera suborder presents great economic importance, because many species can interfere with agricultural crops as agricultural pests (phytophagous) or the biological control of insect pests (predators), as well as also may interfere with public health (hematophagous), as vectors of protozoan *Trypanosoma cruzi*, etiologic agent of Chagas disease.

Between the phytophagous, the species *Pachycoris torridus* (Heteroptera: Scutelleridae) is the only in the family Scutelleridae with agricultural importance in Brazil. This insect is popularly known as "stink bug of physic nut", due to their attacks to culture of physic nut (*Jatropha curcas*), raw material for biodiesel production (Silva el al., 1968). However, this is not the only plant where these stink bug were found, for be polyphagous and consequently pest of various agricultural crops, such as rice, guava, orange, cassava, mango, among others (Silva et al., 1968). Are widely distributed in America, being found of the United States to Argentina (Froeschner, 1988).

The *P. torridus* shows variations in the pattern of spots and the color of the spots is diversified, varying from red to yellow (Monte, 1937). Have been reported 27 different staining patterns (Souza et al., 2012), characteristic it took the species to be described eight times as new (Costa Lima, 1940).

Due its importance economic, several papers were recently publicated about this species. Gomes et al. (2013) described some characteristics morphologic, as testicles oval, peritoneal sheath transparent and seven lobes elongated. Gabriel and Franco (2012) in a biologic study, verified that this Scutelleridae is longevity and can live out to 600 days, already Rodrigues et al. (2011) related a short period of development, with average of 85.6 days between egg and adult stage. Totaling these features Souza-Firmino et al. (2015) highlighted the presence of *P. torridus* in 15 brazilian states and emphasized their infestations in the culture of physic nut.

However, genetic analysis of these insects are scarce, restricting only to the karyotype of the species, namely, 2n= 12 (10A + XY) (Souza et al., 2014). Therefore, this paper describes the heterochromatic pattern in four different color patterns of *P. torridus* (yellow, orange, brown and red), in order to analyze whether there intraspecific chromosomal variations.

MATERIAL AND METHODS

Were used three adult males of *P. torridus* of each color of spots (yellow, orange, brown and red) (Figure 1), collected in Municipal reservoir in city of São José do Rio Preto, Sao Paulo, Brazil and transported in pots, to the Laboratory of Cytogenetics and Molecular Genetics of Insects (LACIMI) of UNESP / IBILCE. The species were fixed in methanol: acetic acid (3:1) and then dissected being the testicles extracted, torn apart and stained with the cytogenetic technique of C-banding (Sumner, 1972). The best images were captured in the microscope ZEISS Axio Scope A1, using the program for the analysis of images AXIO VISION LE version 4.8.

RESULTS

In the initial prophase the heterochromatic blocks are easily visible, because the chromatin is decondensed. By the analysis of prophases, we observed that all the chromatic

variations analyzed of *P. torridus* showed heterochromatic blocks only in chromocenter, formed by the sex chromosomes (X and Y) (Figure 2a-d).

DISCUSSION

The disposition of constitutive heterochromatin in chromatin and chromosomes is an important tool in studies taxonomic, evolutionary and population genetics of heteropteran (Panzera et al., 1992, 1997, 2004; Pérez-Crossa et al., 2002; Gómez-Palacio et al., 2008; Alevi et al., 2013, 2014a, b; 2015a, b; Chirino et al., 2013).

Intraspecific chromosomal variations in heterochromatic block have been reported in *Triatoma infestans* (Panzera et al., 1992, 2004), *T. sordida* (Panzera et al., 1997), *Panstrongylus geniculatus* (Pérez-Crossa et al., 2002) and *Rhodnius pallescens* (Gomez-Palacio et al., 2008). This feature was extremely important for understanding the evolutionary history of *T. infestans*, since it possible differentiate populations in the Andean and non-Andean and mainly to associate the loss of heterochromatin with the occupation of different environments (Panzera et al., 1992). Already the species *P. torridus* had no variation in the distribution of heterochromatin blocks between the different color patterns. Absence of intraspecific variation was also observed for different populations of *T. brasiliensis* (Panzera et al., 2000), *R. neglectus* (Alevi et al., 2015a) and *P. megistus* (Alevi et al., 2015b).

P. torridus has been described eight times as a new species (Costa Lima, 1940). Currently, the morphological data should be combined with other tools to validate the specific status of a taxon. Cytogenetic analysis are important taxonomic tools, they often aid in the differentiation of species morphologically and evolutionarily related, for example, species of the genus *Rhodnius* that are considered cryptic species (morphologically identical) (Pita et al., 2014). In the case of *P. torridus*, according to our results, if classical cytogenetic analysis, as karyotype and C-banding pattern had been used in the initial analysis of the eight morphotypes, this error could have been avoided because all would present the same number of chromosomes and the same pattern of bands.

Thus, the present study describes the heterochromatic pattern of *P. torridus*, reports chromosomal homogeneity in different color patterns and highlights the importance of this tool in the description of new species. We emphasize that further studies as crosses experimental of hybrid and molecular analyzes must be conducted in order to clarify how occurs the segregation of color patterns of these insects with economic importance.

ACKNOWLEDGMENTS

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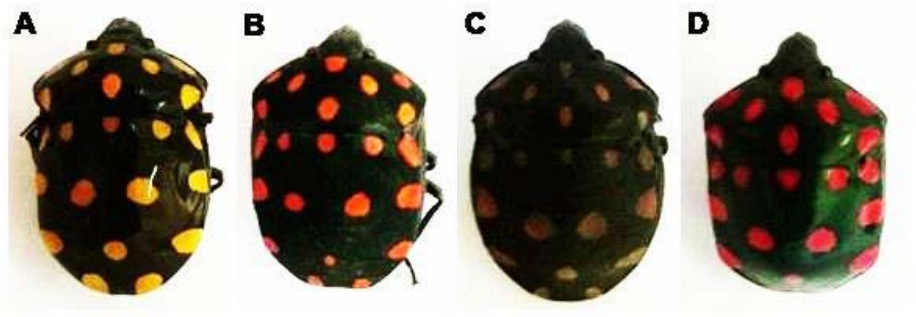

Figure 1. Specimens of *P. torridus* representing the different color patterns. A) yellow, B) orange, C) brown and D) red.

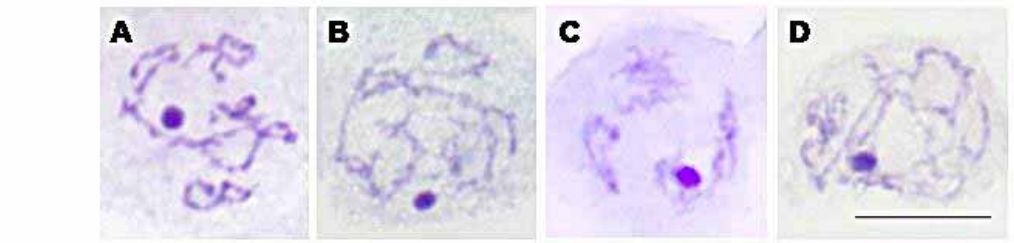

Figure 2. Heterochromatic pattern of different color patterns of the *P. torridus*. A) yellow, B) orange, C) brown and D) red. Note that only the sex chromosomes were shown to be heterochromatic. Bar: 10 µm.

ARTIGO 5

Artigo 5. High genetic variability and polychromatism of *Pachycoris torridus* (Scopoli, 1772) (Heteroptera: Scutelleridae)

Running Title: High genetic variability of *Pachycoris torridus*

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ABSTRACT

The stink bug *Pachycoris torridus* (Scopoli, 1772) is listed among the insects most polyphagous in the world, being pointed as a major pest of diverse cultures, in special the physic nut (*Jatropha curcas L.*), raw material for biodiesel production. A peculiar characteristic of this species is its high phenotypic variability, characteristic that difficult its identification and it took the stink bug to be described eight times as a new species. Thus, the genetic characterization of this insect was performed. We verified that, due to the high genetic variability of *P. torridus*, exist several genetic patterns resulting in the same phenotype.

Keywords: genetic variability, stink bug, molecular analysis

INTRODUCTION

The Scutelleridae family gathers Heteropteran of the most varied colors (red, yellow, orange, among other), frequently iridescent. Among Scutelleridae is common occurrence of species with polychromatism (Sanches-Soto et al., 2004), as was observed in the species of the genus *Pachycoris.* This polychromatism have created confusion for the identification of species as *P. klungii*, *P. stallii* and *P. torridus* (Peredo, 2002).

The Stink bug *Pachycoris torridus* (Scopoli, 1772) is the only species in the family Scutelleridae with agricultural importance in Brazil, popularly known as "stink bug of physic nut", due to their attacks to culture of physic nut (*Jatropha curcas L.*), raw material for biodiesel production (Silva el al., 1968). However, this is not the only plant where these insects were found. There are reports in Anacardiaceae (*Anacardium occidentale*, *Mangifera indica*, *Schinus terebinthifolius*), Borraginaceae (*Cordia* sp.), Euphorbiaceae (*Aleurites fordii*, *Cnidoscolus pubescens*, *Jatropha curcas*, *Jatropha* sp., *Manihot esculenta*, *Sapium haematospermum*), Malpighiaceae (*Malpighia glabra*), Myrtaceae (*Eucalyptus* sp., *Psidium araca*, *Psidum guajava*), Poaceae (*Oryza sativa*), Rubiaceae (*Coffea* sp.) and Rutaceae (*Citrus sinensis*) (Marques et al., 2012), is considered, therefore, one of the most insects polyphagous of the world. These stink bugs are widely distributed in America, being found of the United States to Argentina (Froeschner, 1988).

Possess several variations in the pattern of spots and colors of your body (Monte, 1937), characteristic it took the species to be described eight times as new (Costa Lima, 1940), getting names like: *Tetyra schousboei, Pentatoma fabricii, Scutellera decorata, P. klungii, P. linaei*, *P. aquila*, *P. stallii* and *Poecilocoris aeneiventris* (Maes, 1994). The most common form of this stink bug is the one that presents 22 spots, being eight in the pronotum and 14 in the scutellum, neatly arranged in the scutellum where present in four rows respectively of 5, 4, 3 and 2 spots, starting from the base to the apex. The color of the spots is diversified, varying of red to yellow (Monte, 1937).

The variety of colors and patterns are governed by a complex set of enzymes, pathways, control elements and genetics. Some studies have been done about the developmental genetics of cuticular pigmentation in organisms, such as *Drosophila melanogaster*, that is providing valuable information about the fundamentals of this important source of polymorphism in all the class Insecta (Sugumaran, 1998; Sugumaran, 2002; Sugumaran, 2009; Wittkopp and Beldade, 2009).

The identification of haplotypes from the simultaneous use of multiple markers has contributed with successfully to the determination of the genetic variability of insects (Dotson and Beard, 2001). Therefore, using the markers *COI*, *28S* and *16S* concatenated, we perform the genetic characterization of *P. torridus* and thus, we expanded the information regarding this species that is considered pest of great economic value in Brazil.

MATERIAL AND METHODS

The specimens of *P. torridus* were collected in the cities of Americo de Campos-SP (20° 17' 43.1" S, 49° 44' 12.2" W) and Sao Jose do Rio Preto-SP (20º 46' 48.2" S, 49º 21' 18.3" W). The specimens were conducted to the Laboratory of Cytogenetics and Molecular of Insects, of Universidade Estadual Paulista "Julio de Mesquita Filho", Campus of Sao Jose do Rio Preto, Sao Paulo, Brazil. The identification of the insects was based on the work of Monte (1937) and Costa Lima (1940).

For DNA extraction, was used, the muscles of the thorax of specimens fixed in absolute ethanol, which was removed with tweezers. Were performed extractions in 40 specimens, being 20 of each population for a total of 10 with yellow spots, 15 oranges and 15 red (Figure 1).

The extraction methodology was based second protocol of Bargues and Mas-Coma (1997), using the markers *COI*, *28S* and *16S* concatenated (Table 1).

The sequences obtained were aligned and adjusted manually, using the program BioEdit version 7.1.3.0. (Hall, 1999). To carry the genetic characterization of *P. torridus*, was calculated the nucleotide diversity (π), the number of haplotypes (h), the haplotype diversity (Hd) and the average number of nucleotide differences (k), using the program DnaSP 5.10

(Rozas and Librato, 2009). The program Mega 6.06 (Kumar et al., 2013) was employed to infer the genetic relationships among the specimens, using the method of maximum likelihood with the model of nucleotide substitution Kimura 2 Parameters (Kimura, 1980). The network of connections among haplotypes was obtained to facilitate the visualization of the distribution of chromatic patterns among the haplotypes, with the program Networt 4.6.1.2. (Bandelt et al., 1999).

The sequences used in this work are found available in GenBank. The access codes are presented in Table 2.

RESULTS

Analyzing the population of Sao Jose do Rio Preto and Americo de Campos, we verified that no had visible phenotypical difference between the two populations, and presented the same proportion of insects with yellow spots, orange and red, there was no phenotype with more frequently between them. After alignment of the sequences concatenated of the genes *COI*, *28S* and *16S*, we could observe that the sequences presented when aligned, a total of 1634 sites, of which 1597 were conserved, 37 varied, 32 parsimonious and 5 single. A total of 40 sequences were analyzed, which presented a nucleotide proportion of T= 26.8%, A= 32.9%, C= 21.3% and G= 19.0%, reaffirming the richness of bases A and T described for the mitochondrial genome of insects (Hoy, 2003). The indices of genetic diversity of the species *P. torridus* are presented in Table 3.

In topology obtained, we can observe the distribution of the phenotypes analyzed and their evolutionary relationships (Figure 2). We observed the separation of the phenotypes yellow and red, being the red more basal in the topology and yellow derived. The orange phenotype appeared widely distributed, this being related to the other two phenotypes studied.

Considering the total number of informative sites, from the analysis of Minimum Evolution, by the program Network, was possible to observe the connections between haplotypes and the phenotypes studied. To facilitate the visualization of the phenotypic distribution, each haplotype was represented with the color of the stains of specimens (Figure 3). The distribution of color patterns was similar to that obtained in the topology, confirming the relationship between phenotypes.

DISCUSSION

The index of haplotype diversity obtained was elevated (Hd= 0.872), evidencing the high genetic diversity present in the species *P. torridus*. Grisales et al. (2010) in a study with 40 specimens of *Triatoma dimidiata* (Heteroptera), using the mitochondrial gene *ND4*, also observed high values of diversity with Hd= 0.863, revealed the large variations of Heteroptera studied.

We observed the separation of the phenotypes yellow and red, and the orange phenotype appeared widely distributed, this being related to the other two phenotypes studied. Almeida et al. (2002) analyzing four chromatic patterns of *Triatoma rubrovaria* (Heteroptera), verified the existence of distinct genetic patterns related to the different phenotypes. However, in our analysis there was no relationship between genetic and phenotypic pattern, demonstrated that due to the high genetic variability of *P. torridus*, there are several genetic patterns resulting in the same phenotype.

The species *P. torridus* is highly polymorphic, characteristic it difficult to identify and have already led to great taxonomic mistakes. According to Monte (1937), color variations of *P. torridus* are not hereditary and the factors that may contribute to the differentiation of a

color are diverse and complex, such as: temperature, digestion, existence of oxidases, sensitivity of the cells, humidity, age and the sex.

Gabriel and Franco (2012) in a study about the morphological aspects of *P. torridus* observed that the color of the spots of the descendants may differ or not of the color of the female that they were born, and in the same oviposition can born descendants with different colors. And all adults, to emerge, showed yellow color and, in the course of time, have become orange, red or remained with the source color. They also noted that under laboratory conditions, the adults red color are longer-lived in relation to adults oranges and yellows, and that the age and sex did not influence the color of the spots. Consequently, were eliminated the hypotheses raised by Monte (1937), that the age and sex could contribute to color variability in *P. torridus*.

Hollocher et al. (2000) demonstrated that the divergent melanization between light and dark color of the abdomen of *Drosophila* involves a complex genetic architecture, including factors related to the X chromosome and to the autosomes, well as paternal and maternal effects, revealing that there are genetic factors involved in the chromatic variations of insects. Although the genetics factors involved in the polychromatism of *P. torridus* have not yet been identified. Therefore we conclude that the variations found are singles, due to the high genetic variability of *P. torridus*, exist several genetic patterns resulting in the same phenotype.

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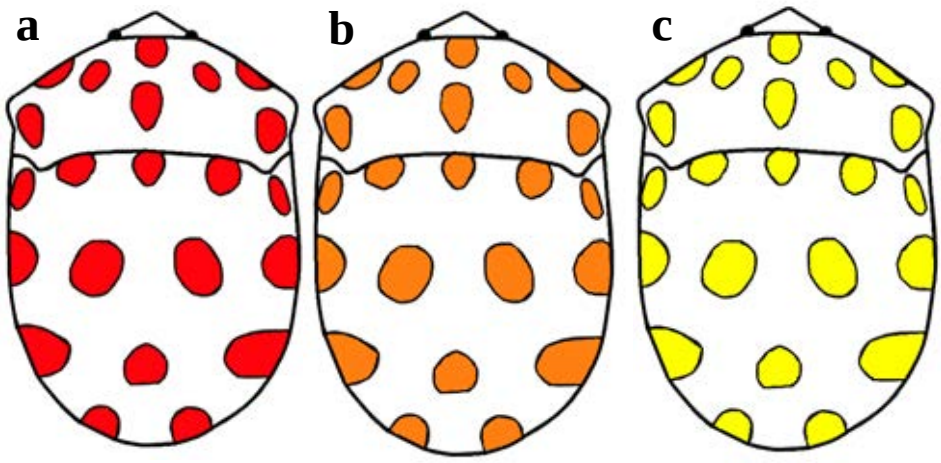

Figure 1. Specimens of *Pachycoris torridus,* representative of colors in study. a) red; b) orange; c) yellow.

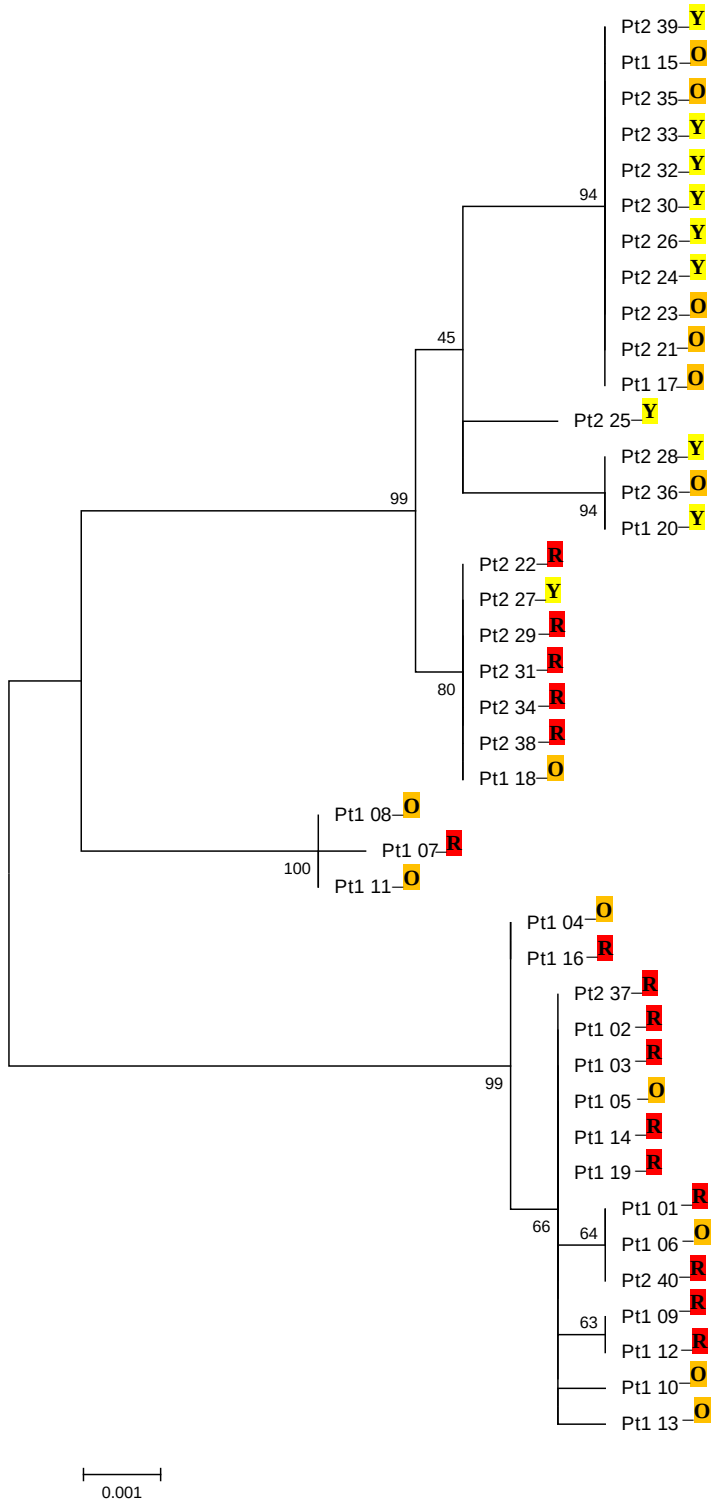

Figure 2. Topology generated for specimens of *Pachycoris torridus*, obtained from alignment of 40 sequence with 1634 sites each, using the markers COI, 28S and 16S concatenated. The evolutionary history was inferred using the Maximum Likelihood method with Kimura 2 parameters and bootstrap of 1000 replicates. Using the MEGA 6.06 program. In featured the color of each specimen analyzed.

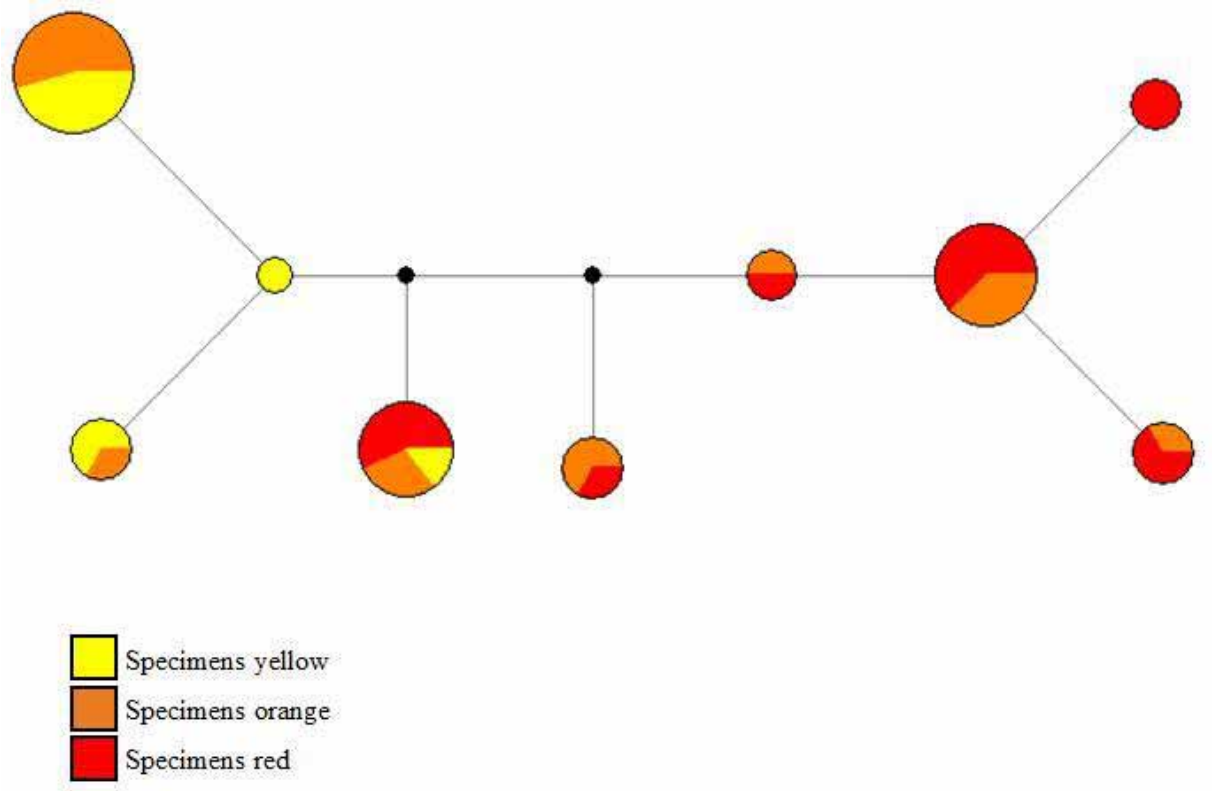

Figure 3. Network of connections between the haplotypes obtained from the analysis of the Minimum Evolution by Network program. The diameters of the circles are proportional to the frequency of each haplotype. The black spots represent a haplotype not sampled or extinct. Each color in legend represents the coloration of the specimens analyzed.

Table 1. Primers used for amplification of gene segments, COI, 28S and 16S, discriminated by sequence (Foward and Reverse), expected size and reference.

Primes	Foward	Reverse	Size	Reference
COI	TTTCAACAAATCATAAAGATATTGG	TAAACTTCAGGGTGACCAAAAATCA	700 pb	Folmer et al. (1994)
28S	CCCGTCTTGAAACACGGACCAA	CCACAGCGCCAGTTCTGCTTAC	600 pb	Muraji and Tachikama (2000)
16S	CRCCTGTTTAACAAAAACAT	AAAAAAATTACGCTGTTATCCCTAAAGTAA	600 pb	Lyman et al. (1999)

Table 2. Access codes of the sequences utilized. Discriminated by Population of Sao Jose do Rio Preto (Pt1_01 to Pt1_20) and Population of Americo de Campos-SP (Pt2_21 to Pt2_40).

Specimen	Primer	number of access	Primer	number of access	Primer	number of access
Pt1_01	*COI*	KM257865	*28S*	KM821050	*16S*	KM676295
Pt1_02	*COI*	KM257866	*28S*	KM821051	*16S*	KM676296
Pt1_03	*COI*	KM257867	*28S*	KM821052	*16S*	KM676297
Pt1_04	*COI*	KM257868	*28S*	KM821053	*16S*	KM676298
Pt1_05	*COI*	KM257869	*28S*	KM821054	*16S*	KM676299
Pt1_06	*COI*	KM257870	*28S*	KM821055	*16S*	KM676300
Pt1_07	*COI*	KM257871	*28S*	KM821056	*16S*	KM676301
Pt1_08	*COI*	KM257872	*28S*	KM821057	*16S*	KM676302
Pt1_09	*COI*	KM257873	*28S*	KM821058	*16S*	KM676303
Pt1_10	*COI*	KM257874	*28S*	KM821059	*16S*	KM676304
Pt1_11	*COI*	KM658553	*28S*	KM821060	*16S*	KM676305
Pt1_12	*COI*	KM658554	*28S*	KM821061	*16S*	KM676306
Pt1_13	*COI*	KM658555	*28S*	KM821062	*16S*	KM676307
Pt1_14	*COI*	KM658556	*28S*	KM821063	*16S*	KM676308
Pt1_15	*COI*	KM658557	*28S*	KM821064	*16S*	KM676309
Pt1_16	*COI*	KM658558	*28S*	KM821065	*16S*	KM676310
Pt1_17	*COI*	KM658559	*28S*	KM821066	*16S*	KM676311
Pt1_18	*COI*	KM658560	*28S*	KM821067	*16S*	KM676312
Pt1_19	*COI*	KM658561	*28S*	KM821068	*16S*	KM676313
Pt1_20	*COI*	KM658562	*28S*	KM821069	*16S*	KM676314
Pt2_21	*COI*	KM257875	*28S*	KM821070	*16S*	KM676315
Pt2_22	*COI*	KM257876	*28S*	KM821071	*16S*	KM676316
Pt2_23	*COI*	KM257877	*28S*	KM821072	*16S*	KM676317
Pt2_24	*COI*	KM257878	*28S*	KM821073	*16S*	KM676318
Pt2_25	*COI*	KM257879	*28S*	KM821074	*16S*	KM676319
Pt2_26	*COI*	KM257880	*28S*	KM821075	*16S*	KM676320
Pt2_27	*COI*	KM257881	*28S*	KM821076	*16S*	KM676321
Pt2_28	*COI*	KM257882	*28S*	KM821077	*16S*	KM676322
Pt2_29	*COI*	KM257883	*28S*	KM821078	*16S*	KM676323
Pt2_30	*COI*	KM257884	*28S*	KM821079	*16S*	KM676324
Pt2_31	*COI*	KM658563	*28S*	KM821080	*16S*	KM676325
Pt2_32	*COI*	KM658564	*28S*	KM821081	*16S*	KM676326
Pt2_33	*COI*	KM658565	*28S*	KM821082	*16S*	KM676327
Pt2_34	*COI*	KM658566	*28S*	KM821083	*16S*	KM676328
Pt2_35	*COI*	KM658567	*28S*	KM821084	*16S*	KM676329
Pt2_36	*COI*	KM658568	*28S*	KM821085	*16S*	KM676330
Pt2_37	*COI*	KM658569	*28S*	KM821086	*16S*	KM676331
Pt2_38	*COI*	KM658570	*28S*	KM821087	*16S*	KM676332
Pt2_39	*COI*	KM658571	*28S*	KM821088	*16S*	KM676333
Pt2_40	*COI*	KM658572	*28S*	KM821089	*16S*	KM676334

Table 3. Index of genetic diversity of *Pachycoris torridus.*

Índex	**Genetic diversity**
Informative sites (S)	32
Nº of haplotype (H)	12
Haplotype diversity (Hd)	0.872
Nucleotide diversity (π)	0.00737
Average number of nucleotide differences (k)	12.049
Total number of mutations found (η)	37

ARTIGO 6

Artigo 6. Populational expansion of *Pachycoris torridus* (Scopoli, 1772) associated with expanding of the planting of physic nut (*Jatropha curcas* L.) demonstrated by mitochondrial marker COI

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Abstract

The stink bug *Pachycoris torridus* (Scopoli, 1772) is considered pest of great agricultural importance. To be phytophagous, their records have been reported in several countries, being considered the species with the highest number of host plants and also the most widely distributed in Brazil. However, their attacks to culture of physic nut (*Jatropha curcas* L), raw material for biodiesel production, are responsible for their agricultural value. From the sequencing and analysis of the mitochondrial marker COI was performed the characterization of haplotypes. We verified the structuration of the species, its high degree of genetic variability and its recent populational expansion, associated with expanding of the plantation of physic nut. Thereby, we confirmed that the identification of the genetic structure of a population is possible through the use of mitochondrial marker COI.

Keywords: populational genetics, stink bug, haplotypes, cytochrome oxidase I

Introduction

The Heteroptera are the largest and most diverse group of insects with incomplete metamorphosis. This suborder has seven infra-orders and approximately 75 families (Souza et al., 2009). Due to the fact of the most of Heteroptera be phytophagous, they are highlighted as agricultural pest, causing extensive damage to production of grains and fruits (Schuh & Slater, 1995).

The Scutelleridae are popularly known as shield-backed bugs, due to the enlargement of the last section of their thorax into a continuous shield over the abdomen and wings. Are all

phytophagous and in phylogenetic studies showed monophyletic. The identification of specimens in this family is difficult, because they vary greatly in coloration within a species (Grazia et al., 2008), characteristic that led *Pachycoris torridus* to be described eight times as a new species (Costa Lima, 1940).

The stink bug *Pachycoris torridus* is a native species of Mexico (Henry & Froeschner, 1988). The most common form this stink bug, is that has 22 spots, with eight on pronotum and 14 on scutellum (Monte, 1937). Records of this insect were carried out in Guatemala, Nicaragua, Costa Rica, United States, Mexico, Argentina, Colombia, Panama and Brazil (Maes & Téllez, 1988; Froeschner, 1988; Maes, 1994; Sanchez-Souto et al., 2004).

This Scutelleridae is longevous and can live out to 600 days (Gabriel & Franco, 2012), they are also phytophagous and polyphagous, with records of their attacks in 16 vegetable crops (Marques et al., 2012), characteristics that highlights as an agricultural pest, with emphasis on the culture of physic nut, a raw material for biofuel production (Borges Filho et al., 2013).

The physic nut (*Jatropha curcas*) is a plant of the Euphorbiaceae family, and is believed to be originally from tropical America, having probably been dispersed around 1783 by Portuguese ships, from the Cape Verde islands to other countries like India and to other continents as the American. It is adapted to various climatic conditions, whose seeds contain a high amount of oil, with high potential for use as raw material for biodiesel and biofuels for aviation (Alves et al., 2008).

Agencies of International development have researched and divulged this culture in countries as South Africa, Asia and Central America, as a producer of oil for the production of biofuels, which aroused the interest of brazilian businessmen, who since 2004 has planted physic nut in Brazil (Saturnino et al., 2005). The Embrapa Agroenergia coordinates a network with 22 institutions of research that are studying this culture. Damage caused by *P. torridus* has been reported to decrease the productivity of oil (Rodrigues et al., 2011).

An important tool to support and improve management practices and pest control is the characterization of genetic differences intra and interpopulational. The mtDNA is an important data source, whose knowledge through research is a powerful fusion between molecular and evolutionary biology, between empirical and theoretical approaches (Moritz et al., 1987). This is a very peculiar molecule that presents maternal inheritance, lack of recombination, the absence of introns, repetitive DNA, pseudogenes, transposable elements, and a high rate of evolution (Moritz et al., 1987). Such features allow that accumulated

differences between matrilineal lineages are especially useful in population studies, allowing evolutionary analyzes of organisms (Matioli & Passos-Bueno, 2001).

Mitochondrial genes are generally considered excellent markers for genealogical inferences the intra-specific level, and particularly the gene of cytochrome oxidase I (*COI*), which is one of the most widely used genes in population and systematic studies (Avise, 2004), showing the genetic diversity, population structure, phylogeography and phylogeny of insects from different orders (Clark et al., 2001; Smith-Caldas et al.,2001; Finn et al., 2006; Vandergast et al., 2007; Boehme et al., 2010; Morales & Freitas, 2010; Wilson et al., 2010; Barbosa et al., 2014).

The description of molecular markers based on PCR (polymerase chain reaction) has increased the efficiency of detection of polymorphisms in the DNA level and is effective at several polymorphic sites from small sampling amounts of DNA (Matioli & Passos-Bueno, 2001). It is common that studies of mtDNA reveal variations between populations from different geographic areas (Avise, 1987). Therefore, we decided to use this technique of sequencing to be the most sensitive for this study, with analyzes of sequences of the mitochondrial gene *COI*.

Material and Methods

The specimens of *P. torridus* were collected in the cities of Americo de Campos-SP, Pontes Gestal-SP, Sao Jose do Rio Preto-SP, Maringa-PR, Sao Luis-MA and Sao Joao do Araguaia-PA (Table 1, Figure 1). The samples were conducted at the Laboratory of Cytogenetics and Molecular of Insects of the Paulista State University "Julio de Mesquita Filho", Campus of Sao Jose do Rio Preto, Sao Paulo, Brazil. The identification of the insects was based on the work of Monte (1937) and Costa Lima (1940).

For DNA extraction, the muscles thorax of the specimens fixed in absolute ethanol was removed with use tweezers. Extractions of 60 specimens were performed, being 10 of each population. The extraction methodology was based on the protocol of Bargues and Mas-Coma (1997), using the oligonucleotide initiator *COI* (Table 2). The *COI* sequences used in this study are available in GenBank access codes KM257865 - KM257924.

The sequences obtained were aligned and adjusted manually using BioEdit software version 7.1.3.0. (Hall, 1999). The fragment of 643 bp of the mitochondrial gene *COI* amplified, had 85% of identity when blastado with the sequence *COI* of the specie

Solenostethium rubropunctatum (Heteroptera: Scutelleridae) (access code, GenBank Nº. HQ236465.1), validating thus the sequences used.

To describe the genetic variation in the population under study, we calculated the nucleotide diversity (π), the number of haplotypes (h), the haplotype diversity (HD) and the average number of nucleotide differences (k) using the program DnaSP 5.10 (Rozas & Librato, 2009). The Harlequin software version 3.5 (Excoffier et al., 2010) was used to estimate the population structure of *P. torridus*, through the analysis of molecular variance (AMOVA). Mega 6.06 program (Kumar et al., 2013) was used to infer genetic relationships between populations and their haplotypes. The estimated of genetic distance (d) was performed using the template nucleotide substitution Kimura 2 parameter (Kimura, 1980), which considers that the transitions are more frequent than transversions, a fact that is observed in insect mtDNA. The network of connections among haplotypes was obtained to facilitate visualization of the relationship between haplotypes and their distribution in different populations, with program Networt 4.6.1.2. (Bandelt et al., 1999).

Using DNASP 5.10, was constructed a histogram (Mismatch distribution) that represents the distribution of genetics differences within populations. This distribution has graphic patterns featuring different types of demographic history (Rogers & Harpending, 1992).

For the analysis of correlation between geographic and genetic distances, the Mantel test (Mantel, 1967) was applied, using the UPGMA program version 1.3 (Miller, 2005). The average number of haplotypes migrants per generation (Nm) between populations was calculated using the Fst equation (Nm = {(1 / Fst) -1} / 2) by program GenA1Ex 6.5 (Peakall & Smouse, 2012).

Results

After alignment of the sequences of the *COI* gene was observed that the populations presented, when aligned, a total of 634 sites, of which 607 were conserved 27 variables, 26 parsimonious and one single site. Table 3 shows the values obtained for each population. The population of Maringa-PR was the only that showed no genetic variability, without sites variables, parsimonious and unique. Already the population of Pontes Gestal-SP stood out with the highest variability, followed by the population of Sao Jose do Rio Preto-SP.

A total of 60 sequences from the initial portion of the *COI* gene were analyzed, presenting a nucleotide ratio of T= 29.6%, A= 33.4%, C= 20.4% and G= 16.6%, reaffirming

the richness of bases A and T described for the mitochondrial genome of insects (Hoy, 2003). The distance between populations was calculated and the interrelationships between them can be seen in the phenogram obtained by Neighbor-joining method (Figure 2) using the values of distance (d) of each population. In highlighted the lower and higher values of genetic distance found (Table 4).The obtained indices of genetic diversity can be seen in Table 5.

Table 6 shows the 26 polymorphic sites determinants of the 14 haplotypes identified in our analyzes with *COI* gene. Considering the total number of informative sites of the six populations analyzed, based on the analysis of Minimum Evolution at Network program, it was observed that the network of connections among haplotypes was consistent with the results of alignments, demonstrating the great genetic variation exists in populations of *P. torridus*, supporting the distribution and relationship of haplotypes. A total of 14 networks were formed based on the dataset of COI marker, six of these networks were unique haplotypes (H01, H02, H03, H08, H12 and H13) and five haplotypes were shared by more than one population (H04, H05, H09, H10 and H14). The H05, H10 and H14 haplotypes were the most frequent, with 12 representatives in each (Figure 3).

The demographic history of populations was inferred by analyzing mismatch distribution (Figure 4), which is based on the number of differences observed between all pairs of haplotypes and is represented by the frequency distribution of the differences. The population of Maringa showed no genetic variation, so it was not possible to generate a graph. Populations of Sao Jose do Rio Preto-SP and Pontes Gestal showed bimodal curve, however the populations of Americo de Campos-SP, Sao Joao do Araguaia-PA and Sao Luis-MA showed unimodal pattern.

Results of the Analysis of Molecular Variance (AMOVA) to assess the distribution of genetic diversity within and among populations, revealed a lower percentage of intra-population variation (27.75%) (Table 7). The Fst value obtained (0.72251), with P = 0.00000 being P <0.05, showed a low gene flow, because the highest rate of variability was inter-population (72.25%) (Table 7). The number of haplotypes migrants (Nm) was low (0.192), corroborating the inference of low gene flow between populations.

The Mantel test evaluates the correlation of two matrices, one with estimates of genetic distance and the other with the geographical distance between populations (Table 4). The test indicated that there is no correlation between genetic and geographic distances (r = -0.1875; p = 67%) (Figure 5).

Discussion

The stink bug *P. torridus* is one of leading agricultural pest, being among the most polyphagous insects, oldest old and widely distributed in the world, however the genetic structure of this insect was previously unknown. During this study, was possible verify that the genetic distance between populations is high, averaging 0.015 and values that reach 2.5%. Barbosa et al. (2014), also with the *COI* gene in a population study of *Chrysoperla externa* (Insecta: Neuroptera), found values close to zero, revealing the high similarity between populations collected. Already Smith-Caldas et al. (2001) using the *COI* gene in phylogenetic study verify the value of 0.033 genetic distance among 15 species of insects (Diptera), evidencing that values above 3% are found between different taxonomic groups.

In the present work, of the 14 haplotypes obtained from the alignment of 60 sequences, 6 were exclusives, which evidences a recent expansion and restriction in gene flow, inference confirmed with Nm estimate (0.192), which showed restriction in the flow of migrants haplotypes. Grisales et al. (2010) in a population study with *Triatoma dimidiata* (Heteroptera), using the mitochondrial gene *ND4* also found restriction in gene flow (Nm= 0.157) of 3 Colombian populations. The insect *P. torridus* despite having wings, is not a good flyer, when threatened, their defense system is to fall and blend on burlap. However with the expansion of planting physic nut, also increases the distribution of this stink bug (Alves et al., 2008).

The populations of Sao Jose do Rio Preto-SP and Pontes Gestal-SP were the presented the greatest variability, with five haplotypes each. In contrast the population of Maringa-PR did not showed variation, being all representatives included in a single haplotype. The population of Sao Luis-MA (Northeast of Brazil) shares their haplotypes with population of Sao Joao do Araguaia-PA (North). There are 823 km of distance between them. The population of Americo Campos-SP has not shared any haplotype with populations of Pontes Gestal-SP e Sao Jose do Rio Preto-SP, to which are distant 18,7 Km and 86,7 Km, respectively. The only haplotype of population of Maringa is one of haplotypes present in population of Sao Jose do Rio Preto (440 km between they).

Intraspecific variations and low gene flow were also observed for specimens of the genus *Triatoma* (Heteroptera: Reduviidae) (Giordano et al, 2005; Piccinali et al, 2009) and *Halobates* (Gerridae) (Andersen et al., 2000) when collected in different locations and relatively isolated. In our studies a high genetic variability was found even in nearby populations as Pontes Gestal and Americo de Campos (18 km). The maintenance of the

genetic diversity is of extremely important so that the populations can respond to continuous environmental changes (Frankham et al., 2004).

The index of haplotype diversity (Hd= 0.871) observed was high, confirming the great genetic variability on the specie *P. torridus*. Pfeiler et al. (2006) using the mitochondrial marker *Cyt b* in *T. rubida* (Heteroptera: Reduviidae), also found elevated values of diversity haplotítica (Hd= 0.913).

The results for the analysis of the genetic structure of populations obtained by AMOVA, showed that most of the haplotype diversity is interpopulational. The value of genetic differentiation between populations was high (72.25%), thereby statistically there is structure in populations of *P. torridus*. Low nucleotide diversity was found (0.01335), This suggests that most haplotypes have recently appeared (Ferreri et al., 2011). According to Grant and Bowen (1998), Low values of nucleotide diversity is evidence of recent events founders. The Mantel test indicated no correlation between genetic and geographical distances (r= -0.1875; p= 67%), confirming the result of low gene flow among populations.

The patterns of distribution proposed by the analysis of Mismatch distribution corroborate with the inference of the occurrence of an event of population expansion, presenting bimodal curves for populations of Sao Jose do Rio Preto and Pontes Gestal and unimodal for populations of Americo de Campos, Sao Joao do Araguaia and Sao Luis. The population of Maringa showed no genetic variation, which suggests that the low variability of this population is a result of the occurrence of a recent colonization, probably allied with the recent expansion of the distribution of the culture of physic nut. According to Rogers and Harpending (1992) patterns uni and bi modal indicate processes of population expansion.

The marker COI showed a high genetic diversity for *P. torridus*, which explains its high capacity to adapt and resistance to natural enemies, because the genetic drift effects tend to be minimized by high variability. A description of the population structure of *P. torridus* is a consequence of the choice of a marker that presents sensitivity sufficient for the detection of events of recent fragmentation (Nei et al., 1978).

Conclusions

This study enabled us to understand the processes of colonization and expansion of *P. torridus*, revealing the existence of populations significantly differentiated, with high genetic variability. Thus, highlight the recent populational expansion of *P. torridus*, coupled with the expansion of the plantation of physic nut.

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Table 1. Data of the populations of *Pachycoris torridus*.

Populations	Geographic coordinates	Collectors
Sao Jose do Rio Preto-SP	20º 46' 48.2" S; 49º 21' 18.3" W	Tatiani Seni de Souza Firmino
Americo de Campos-SP	20° 17' 43.1" S; 49° 44' 12.2" W	Mary Massumi Itoyama
Pontes Gestal-SP	20° 10' 18.1" S; 49° 42' 21.2" W	Mary Massumi Itoyama
Maringa-PR	23° 25' 38.3" S; 51° 56' 15.0" W	Satiko Nanya
Sao Joao do Araguaia-PA	05° 25' 06.9" S; 48° 41' 26.3" W	Diego de Macedo Rodrigues
Sao Luis-MA	02° 35' 17.8" S; 44° 12' 32.0" W	Keneson Klay Gonçalves Machado

Table 2. Oligonucleotide initiator (COI) used in the amplification of gene segments, broken down by sequence (Foward and Reverse), expected size and reference.

Primer	Foward	Reverse	Size	Reference
COI	TTTCAACAAATCATAAAGATATTGG	TAAACTTCAGGGTGACCAAAAATCA	700 pb	Folmer et al. (1994)

Table 3. Results of analyzes of the sequences of each population.

Sites (Total 634)	Sao Jose do Rio Preto	Americo de Campos	Pontes Gestal	Maringa	Sao Luis	Sao Joao do Araguaia
Conserved	617	627	615	634	632	632
Variables	17	7	19	0	2	2
Parsimonious	17	3	17	0	2	2
Single	0	4	2	0	0	0

Table 4. Distances genetic and geographic observed in the populations of *P. torridus.*

Populations	S. J. Rio Preto	Am. Campos	Pontes Gestal	Maringa	S. Joao Araguaia	Sao Luís
Sao Jose do Rio Preto		86.7	95.5	440.0	1898.0	2501.0
Americo de Campos	**0.025**		18.7	471.0	1921.0	2524.0
Pontes Gestal	0.021	0.008		487.0	1918.0	2521.0
Maringa	0.021	0.019	0.020		2336.0	2939.0
Sao Joao do Araguaia	0.023	0.006	0.008	0.019		823.0
Sao Luis	0.024	0.005	0.007	0.019	**0.002**	

Table 5. Indices of genetic diversity of *P. torridus.*

Índex	**Genetic diversity**
Informational sites (S)	26
Number of haplotypes (H)	14
Haplotype diversity (Hd)	0.871
Nucleotide diversity (π)	0.01335
Average number of nucleotide differences (k)	8.495
Total number of mutations found (η)	27

Table 6. Polymorphic sites determinants of the 14 haplotypes of *P. torridus*. Referring the fragment of 634 bp of the *COI* gene.

	Polymorphic sites																									
Haplotypes				**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**3**	**4**	**4**	**4**	**5**	**5**	**5**	**5**	**5**	**6**
	4	**8**	**8**	**2**	**2**	**8**	**9**	**0**	**0**	**1**	**2**	**4**	**5**	**5**	**8**	**1**	**4**	**3**	**4**	**8**	**0**	**3**	**3**	**8**	**8**	**1**
	2	**3**	**6**	**2**	**5**	**2**	**7**	**6**	**9**	**8**	**1**	**8**	**4**	**8**	**4**	**1**	**1**	**7**	**1**	**5**	**9**	**0**	**3**	**1**	**4**	**7**
H01	T	T	T	T	C	C	G	A	T	C	A	A	G	C	G	G	A	G	A	A	A	T	A	C	T	A
H02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.
H03	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	.
H04	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.
H05	C	C	C	C	T	T	.	.	C	T	.	.	.	.	A	A	G	A	G	.	G	.	.	T	C	G
H06	.	.	C	C	T	.	A	G	.	T	G	.	A	T	A	.	G	A	G	.	G	C	G	T	C	.
H07	.	.	C	C	T	.	A	G	.	T	.	.	A	T	A	.	G	A	G	.	G	.	.	T	C	.
H08	.	.	C	C	T	.	A	G	.	T	.	.	A	T	A	.	G	A	G	G	G	.	G	T	C	.
H09	.	.	C	C	T	.	A	G	.	T	.	G	A	T	A	.	G	A	G	.	G	.	G	T	C	.
H10	.	.	C	C	T	.	A	G	.	T	.	G	A	T	A	.	G	A	G	.	G	.	G	T	C	G
H11	.	.	C	C	T	.	A	G	.	T	.	G	A	T	A	.	G	A	G	.	G	.	G	T	C	.
H12	.	.	C	C	T	.	A	G	.	T	.	.	A	T	A	.	G	A	G	G	G	.	G	T	C	.
H13	.	.	C	C	T	.	A	G	.	T	G	.	A	T	A	.	G	A	G	.	G	.	G	T	C	.
H14	.	.	C	C	T	.	A	.	.	T	.	G	A	T	A	.	G	A	G	.	G	.	G	.	C	.

Table 7. Results of the Hierarchical analysis of molecular variance (AMOVA) of *P. torridus.* P: probability of obtaining the value of Fst larger than expected. *Nm:* average number of haplotypes migrants per generation.

Components of Variance	**% of Total**	**P**	**Fst**	**Nm**
interpopulational	72.25	0.00000	0.72251	0.192
intrapopulational	27.75			

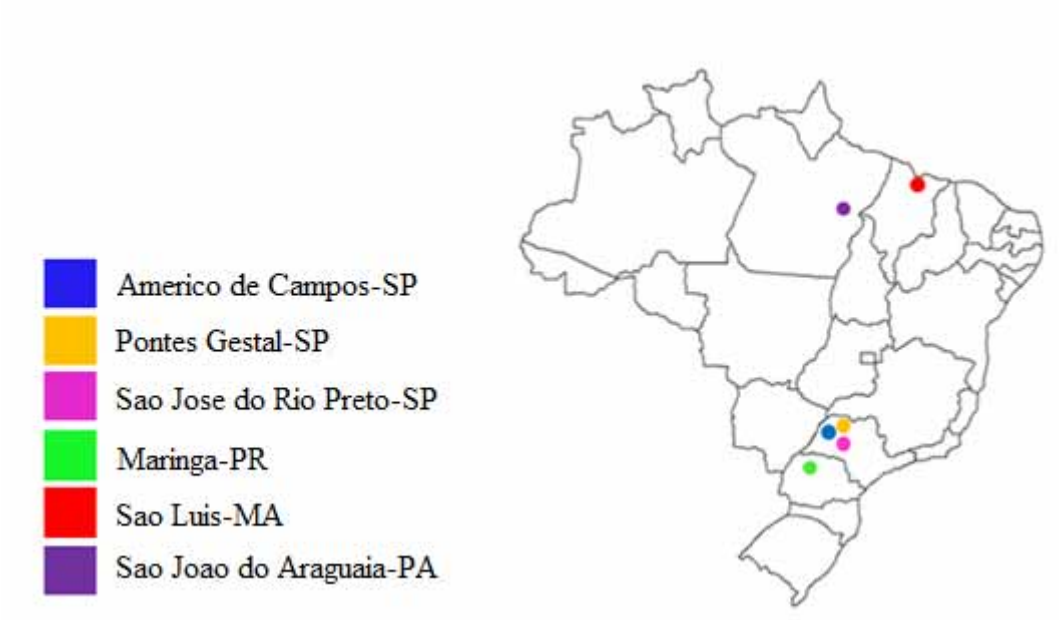

Figure 1. Map of Brazil, indicating the regions of the collects of *P. torridus.*

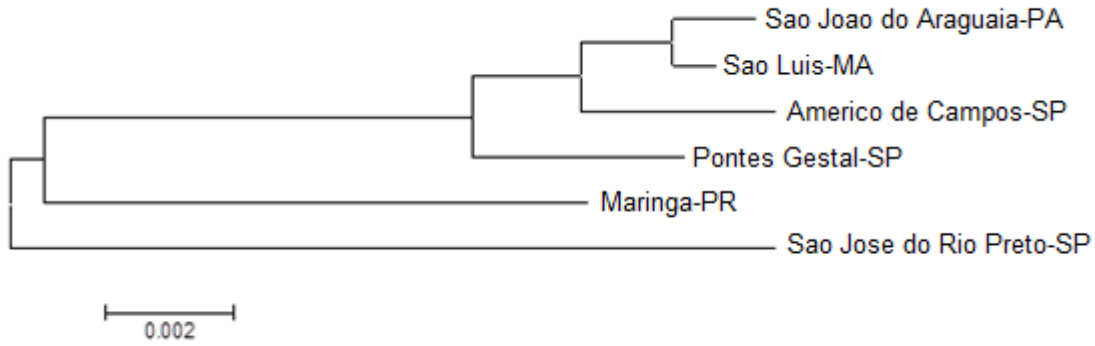

Figure 2. Phenogram of interrelationships among populations, obtained by the Neighbor-joining method from the distance values of each population, with the initiator oligonucleotide COI. Bootstrap of 1000 replicates.

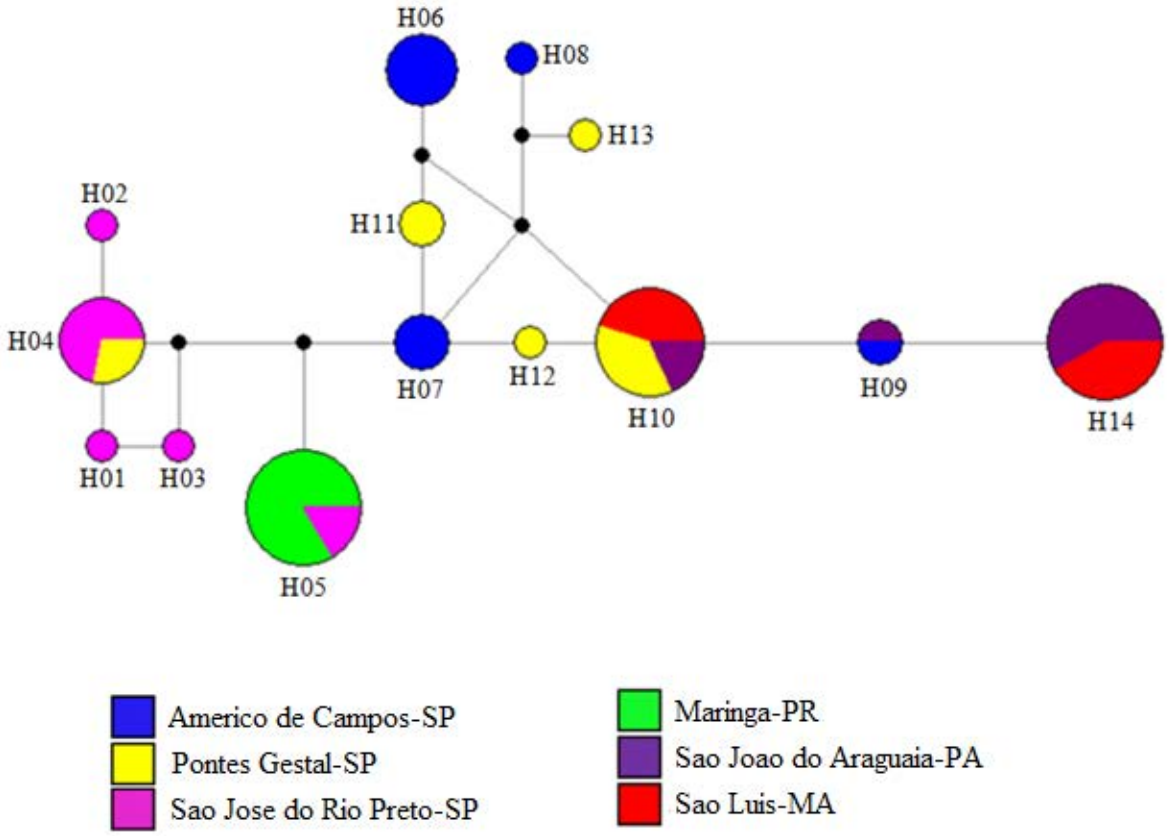

Figure 3. Network of connections between the haplotypes obtained from the analysis of the Minimum Evolution by Network program. The diameters of the circles are proportional to the frequency of each haplotype. The black dots represent a haplotype not sampled or extinct. Each color represents a population according to legend.

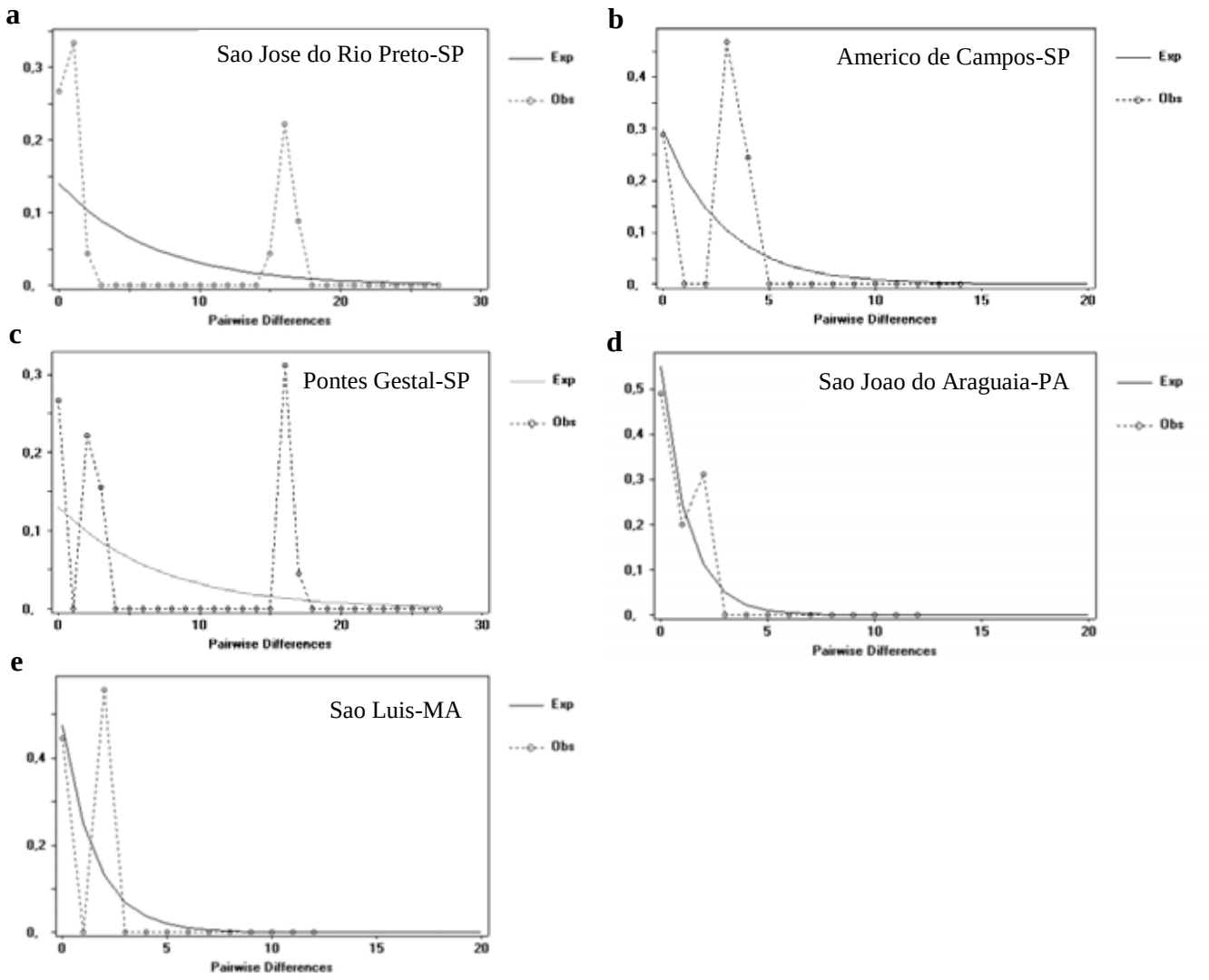

Figure 4. Histograms showing the differences pair-to-pair observed (dotted lines) and expected (solid lines) between the sequences of the *COI* gene of each population. a) Sao Jose do Rio Preto; b) Americo de Campos; c) Pontes Gestal; d) Sao Joao do Araguaia; e) Sao Luis.

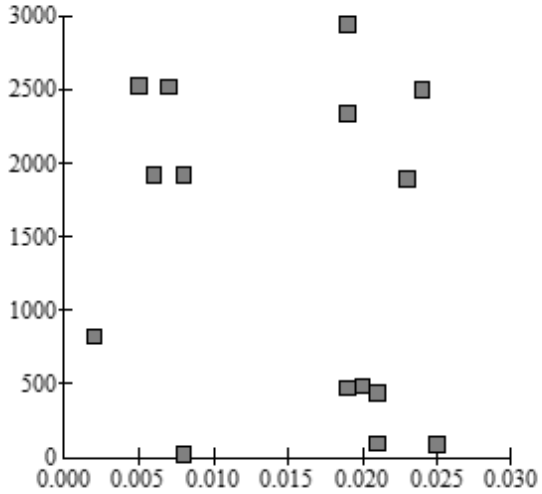

Figure 5. Test Mantel. Indicating no correlation between geographic distance and genetic.

IV. DISCUSSÃO GERAL

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O percevejo *Pachycoris torridus* está elencado entre os insetos mais polífagos do mundo, sendo apontado como a principal praga de diversas culturas, em especial o pinhão manso, matéria prima para a produção do biodiesel. Uma característica peculiar desta espécie é a sua alta variabilidade fenotípica, característica que dificulta a sua identificação, e que levou o percevejo a ser descrito oito vezes como espécie nova (COSTA LIMA, 1940). Os aspectos da variabilidade genética deste inseto ainda não eram conhecidos, e os estudos biológicos são carentes de informações. Atualmente é necessária a utilização de múltiplas abordagens metodológicas que visem o esclarecimento da biologia desta tão importante praga agrícola.

Com o intuito de contribuir com informações sobre *P. torridus*, o presente trabalho propôs, inicialmente, a caracterização da distribuição geográfica de *P. torridus* no Brasil, relatando adicionalmente a sua ocorrência no Noroeste do Estado de São Paulo, ressaltando a grande infestação deste inseto no Brasil. Seguida pelo checklist e descrição de três novos padrões cromáticos de *P. torridus*, a fim de evitar novos registros errôneos e facilitar na identificação e analise biológica deste percevejo polimórfico. Descrevemos também os aspectos do desenvolvimento dos padrões cromáticos na espécie. E a partir da técnica de Banda C registramos a homogeneidade cromossomal, nos diferentes padrões de cores do percevejo. Posteriormente realizamos a caracterização da variabilidade genética de *P. torridus*, a partir dos marcadores *COI, 28S* e *16S* concatenados. Por fim, populações de *P. torridus* foram analisadas a partir do marcador mitocondrial *COI*, permitindo a descrição da expansão populacional da espécie aliada ao aumento do plantio de pinhão manso no Brasil.

Segundo Monte (1937), as variações de cores de *P. torridus* não são hereditárias e os fatores que podem levar para a diferenciação de uma cor são diversos e complexos, tais como: temperatura, digestão, existência de oxidases, sensibilidade das células, umidade, idade e sexo. Entretanto, Hollocher et al. (2000) demonstraram que a melanização divergente entre a cor clara e escura do abdôme de *Drosophila* envolve uma arquitetura genética complexa, incluindo fatores ligados ao cromossomo X e aos autossomos, bem como efeitos paternos e maternos, revelando assim, que há fatores genéticos envolvidos nas variações cromáticas dos insetos. Todavia os fatores genéticos envolvidos no policromatismo de *P. torridus* ainda não foram identificados. Contudo, a partir de nossas análises verificamos que não há correlação entre haplótipo e fenótipo.

Gabriel e Franco (2012) em estudo sobre os aspectos morfológicos de *P. torridus* observaram que a cor das manchas dos descendentes pode diferir, ou não, da cor da fêmea que lhes deu origem, e que de uma mesma oviposição nascem descendentes de diferentes cores. E que todos os adultos ao emergirem, apresentaram a cor amarela e com o passar do tempo tornaram-se laranja, vermelho ou permaneceram com a cor de origem. Foi observado, também, que em condições de laboratório, os adultos de cor vermelha são mais longevos com relação aos adultos laranjas e amarelos. E que a idade e o sexo não influenciam na cor das manchas. Portanto, eliminam as hipóteses levantadas por Monte (1937), de que a idade e o sexo poderiam contribuir com a variabilidade de cor em *P. torridus*. Contudo, em nosso estudo, foi verificado que as variações encontradas são individuais e não há relação entre padrão genético e fenotípico na espécie.

Em nossas análises confirmamos também que todos adultos, após a última ecdise, apresentam a cor amarela ao emergirem, a qual certamente é a cor da quitina neste percevejo, contudo verificamos que o desenvolvimento da coloração é gradual. As variações cromáticas podem interferir na ecologia evolutiva destes diferentes fenótipos, podendo diferir no seu valor adaptativo. Além disso, o polimorfismo pode influenciar na comunicação entre os sexos e, portanto, na probabilidade de acasalamento dentro dos diferentes fenótipos. Entretanto, alguns insetos são intragáveis aos predadores e muitas vezes usam cores de advertência como laranja, vermelho e amarelo para anunciá-los (RUXTON et al., 2004).

Muitos predadores sabem associar a presença de defesa química com sinais visuais, e o polimorfismo pode desempenhar este papel para evitar predadores (JORON et al., 1999). A presença de defesas químicas em *P. torridus* ainda não foi estudada, mas é possivel que ele obtenha compostos tóxicos durante a alimentação em pinhão manso, assim como acontece com espécies do mesmo gênero (WILLIAMS et al., 2001).

A análise citogenética é uma ferramenta taxonômica importante, que muitas vezes ajuda a verificar a diferenciação de espécies morfologicamente e evolutivamente relacionadas (PITA et al., 2014). A disposição de heterocromatina constitutiva na cromatina e nos cromossomos é uma importante ferramenta em estudos taxonômicos e de genética populacional em heteropteros (GÓMEZ-PALACIO et al., 2008, CHIRINO et al., 2013). Variações cromossômicas intraespecíficas em blocos heterocromáticos foram relatados em *Triatoma infestans* (PANZERA et al., 1992), *T. sordida* (PANZERA et al.), *Panstrongylus geniculatus* (PEREZ et al., 2002) e *Rhodnius pallescens* (GOMEZ-PALACIO et al ., 2008). Já a espécie *P. torridus* não apresentou nenhuma variação na distribuição dos blocos heterocromáticos entre os diferentes padrões de cores analisados. Ausência de variação

intraespecífica foi também observada em diferentes populações de *T. brasiliensis* (PANZERA et al., 2000), *R. neglectus* (ALEVI et al., 2014a) e *P. megistus* (ALEVI et al., 2014b).

Após o alinhamento das sequências concatenadas dos genes *COI*, *28S* e *16S*, foi possível observar que o índice de diversidade haplotípica obtido foi alto (*Hd*= 0,872), evidenciando a alta diversidade genética presente na espécie *P. torridus.* Grisales et al. (2010) em estudo com 40 espécimes de *Triatoma dimidiata* (Heteroptera) com o gene mitocondrial *ND4* também observou altos valores de diversidade, com *Hd*= 0,863, revelando a grande variabilidade dos Heteroptera estudados.

Analisando o relacionamento evolutivo dos fenótipos amarelo, laranja e vermelho, observamos a separação dos fenótipos amarelo e vermelho, estando o vermelho basal na topologia e o amarelo derivado. O fenótipo laranja apresentou-se amplamente distribuído, estando este relacionado com os outros dois padrões fenotípicos estudados. Almeida et al. (2002) analisando quatro padrões cromáticos de *T. rubrovaria* (Heteroptera), verificou a existência de padrões genéticos distintos relacionados aos diferentes padrões fenotípicos. Todavia em nossas análises não houve o relacionamento entre padrão genético e fenotípico, demonstrando que devido à alta variabilidade genética de *P. torridus*, existem vários padrões genéticos resultando em um mesmo fenótipo.

A partir dos resultados obtidos com o marcador mitocondrial *COI*, verificamos que a composição nucleotídica para esse gene é rica em bases AT. Hua et al. (2008) analisando o genoma mitocondrial de Pentatomomorpha (Heteroptera) observaram que a composição nucleotídica estava entre 68,86 a 77,8% do conteúdo de AT. Estes resultados são semelhantes aos valores observados em nosso estudo.

Foi possível verificar que a distância genética entre as populações de *P. torridus* é alta, com média de 0,015 e valores chegam a 2,5%. Barbosa et al. (2014), também com o gene *COI*, em estudo populacional de *Chrysoperla externa* (Insecta: Neuroptera), encontrou valores próximos a zero, revelando a alta similaridade entre as populações coletadas. Já Smith-Caldas et al. (2001) utilizando o gene *COI* em estudo filogenético, verificou o valor de 0,033 de distância genética entre 15 espécies de insetos (Diptera), evidenciando que valores acima de 3% são encontrados entre grupos taxonômicos diferentes.

No presente trabalho, dos 14 haplótipos obtidos a partir do alinhamento de 60 sequências com o gene *COI*, seis eram exclusivos, o que evidencia uma expansão recente e restrição no fluxo gênico, inferência confirmada com a estimativa *Nm* (0,192), a qual revelou restrição no fluxo de haplótipos migrantes. Grisales et al. (2010) em estudo populacional com *T. dimidiata* (Heteroptera), utilizando o gene mitocondrial *ND4* também encontrou restrição

no fluxo gênico (*Nm*= 0,157) de 3 populações Colombianas. O inseto *P. torridus* apesar de ter asas, não é um bom voador, quando ameaçado, o seu sistema de defesa é cair e se misturar na serapilheira. Todavia com a expansão do plantio do pinhão manso, aumenta-se também a distribuição deste percevejo (ALVES et al., 2008).

As populações de São José do Rio Preto-SP e Pontes Gestal-SP foram as que apresentaram maior variabilidade, com cinco haplótipos cada. Em contraste a população de Maringá-PR não apresentou variação, estando todos representantes incluídos em um único haplótipo. A população de São Luís-MA (Nordeste do Brasil) compartilha os seus haplótipos com a população de São João do Araguaia-PA (Norte), há 823 Km de distância entre elas. A população de Américo de Campos-SP não compartilhou nenhum haplótipo com as populações de Pontes Gestal-SP e São José do Rio Preto-SP, as quais distam 18,7 e 86,7 Km, respectivamente. O único haplótipo da população de Maringá é um dos haplótipo presente na população de São José do Rio Preto (distância de 440 Km entre elas).

Variações intraespecíficas e baixo fluxo gênico foram observados também para os espécimes dos gêneros *Triatoma* (Heteroptera: Reduviidae) (GIORDANO et al, 2005; PICCINALI et al, 2009) e *Halobates* (Gerridae) (ANDERSEN et al., 2000) quando coletados em locais diferentes e relativamente isolados. Em nossos estudos uma alta variabilidade genética foi encontrada mesmo nas populações mais próximas como Pontes Gestal e Américo de Campos (18 km de distância). A manutenção da diversidade genética é extremamente importante para que as populações possam responder às continuas mudanças ambientais (FRANKHAM et al., 2004).

O índice de diversidade haplotípica obtido a partir do marcador *COI* (*Hd*= 0,871) foi alto, confirmando a grande variabilidade genética existente na espécie *P. torridus*. Pfeiler et al. (2006) utilizando o marcador mitocondrial *Cyt b* em *Triatoma rubida* (Heteroptera: Reduviidae), também encontrou valores elevados de diversidade haplotítica (*Hd*= 0,913).

Os resultados para as análises de estrutura genética das populações, obtidos por meio da AMOVA, mostraram que grande parte da diversidade haplotípica é interpopulacional. O valor de diferenciação genética entre as populações foi alto (72,25%), assim estatisticamente há estruturação nas populações de *P. torridus*. Uma baixa diversidade nucleotídica foi encontrada (0,01335), o que sugere que a maior parte dos haplótipos têm surgido recentemente (FERRERI et al., 2011). Segundo Grant e Bowen (1998), valores de baixa diversidade nucleotídica são evidencia de eventos fundadores recentes. O Teste de Mantel indicou que não há correlação entre distâncias geográfica e genética (*r*= - 0,1875; *p*= 67%), confirmando o resultado de baixo fluxo gênico entre as populações.

Os padrões de distribuição propostos pela análise de *Mismatch distribution* corroboram com a inferência da ocorrência de um evento de expansão populacional, apresentando curvas bimodais para as populações de São José do Rio Preto e Pontes Gestal e unimodal para as populações de Américo de Campos, São João do Araguaia e São Luis. A população de Maringá não apresentou variação genética, o que sugere que a baixa variabilidade desta população seja resultado da ocorrência de uma colonização recente, provavelmente aliada à recente ampliação da distribuição da cultura do pinhão manso. Segundo Rogers e Harpending (1992) padrões uni e bi modais indicam processos de expansão populacional.

O marcador mitocondrial *COI* mostrou uma alta diversidade genética para *P. torridus*, o que explica a alta capacidade de adaptação deste percevejo e sua resistência a inimigos naturais, pois os efeitos da deriva genética tendem a ser minimizada pela sua alta variabilidade genética. A descrição da estrutura populacional de *P. torridus* é consequência da escolha de um marcador que apresenta sensibilidade suficiente para a detecção de eventos de fragmentação recente (NEI et al., 1978). Desta maneira, contribuímos com informações inéditas e relevantes, principalmente pelo valor agrícola agregado aos danos causados por este percevejo e pela escassez de informações literárias sobre *P. torridus.*

V. CONCLUSÕES

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O presente trabalho teve como objetivo geral realizar a caracterização da distribuição espacial, da variabilidade fenotípica, da diversidade genética e dos processos de colonização e expansão de *Pachycoris torridus,* podendo-se concluir que:

- O percevejo *P. torridus* está amplamente distribuído pelo Brasil, com registros em 15 Estados brasileiros;
- A presença de *P. torridus* na região Noroeste do Estado de São Paulo, é de grande relevância econômica pela natureza dos danos descritos para a cultura do pinhão manso. Além disso, destacamos a característica de polífagia deste percevejo, o que permite que ele possa colonizar e, principalmente, tornar-se praga agrícola para outras culturas vegetais de importância econômica;
- O desenvolvimento da cor na espécie *P. torridus* é gradual e o possível mecanismo responsável pelo alto polimorfismo na espécie seria um comportamento aposemático;
- Não há variação na disposição da heterocromatina constitutiva entre os diferentes padrões de cores observados na espécie;
- Devido à alta variabilidade genética de *P. torridus*, existem vários padrões genéticos resultando em um mesmo fenótipo;
- Estatisticamente há estruturação nas populações de *P. torridus*;
- Houve uma recente expansão populacional de *P. torridus*, aliada a ampliação do plantio de pinhão manso;
- Há baixo fluxo gênico entre as populações de *P. torridus;*
- A identificação da estrutura genética de uma população é possível mediante a utilização do marcador mitocondrial *CO*I.

VI. REFERÊNCIAS BIBLIOGRÁFICAS

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