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MARCADORES MOLECULARES E CITOGENÉTICOS PARA INFERÊNCIA DE RELAÇÕES FILOGENÉTICAS EM QUATRO ESPÉCIES NEOTROPICAIS DE GRILOS *Oecanthus* SERVILLE, 1831 (ORTHOPTERA, GRYLLIDAE)

PORTO ALEGRE
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Orientador: Dr.ª Maríndia Deprá

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SUMÁRIO

RESUMO

A ordem Orthoptera apresenta distribuição mundial e foi utilizada como organismo modelo em diversos estudos, principalmente nas áreas de filogenética e citogenética. Este grupo se divide nas subordens Caelifera e Ensifera, sendo o último considerado o mais antigo. As primeiras filogenias de Ensifera se basearam em taxonomia e bioacústica, e depois a utilização de genes permitiram maior suporte às pesquisas. Em Ensifera, o táxon Grylloidea é considerado o mais basal, sendo composto por 13 subfamílias, dentre elas Oecanthinae. Os grilos desta subfamília são conhecidos como grilos arbóreos, e o gênero *Oecanthus*, é o principal representante, com 72 espécies descritas e ampla distribuição mundial. O gênero é considerado recente na história evolutiva dos grilos, tendo suas relações filogenéticas estudadas apenas para algumas espécies com ocorrência na China. Além disso, os estudos citogenéticos são escassos, uma vez que apenas seis espécies tiveram seus cariótipos descritos, sendo estas: *O. indicus, O. nigricornis* e *O. quadripunctatus* com 2n=19, X0, *O. longicauda* e *O. pellucens* com 2n=20, XY, e *O. valensis* com 2n=18, XY. Todos os cariótipos compartilham a mesma assimetria, incluindo cromossomos grandes e pequenos. Assim, o objetivo deste trabalho foi descrever os cariótipos das espécies Neotropicais dos grilos *O. valensis*, *O. pictus*, *O. pallidus* e *O. lineolatus*, e analisar as relações filogenéticas destas espécies empregando marcadores moleculares e os dados disponíveis nos bancos de dados, de espécies do gênero *Oecanthus*. As filogenias foram reconstruídas por Inferência Bayesiana utilizando os genes COI, 12S, 16S e 18S rDNA. Para as análises citogenéticas foram realizadas técnicas de coloração convencional e Bandeamento C; a técnica de FISH foi empregada em *O. pictus*, utilizando o marcador 18S rDNA. As relações filogenéticas das espécies foram bem suportadas, mostrando que as espécies Neotropicais são as menos derivadas do gênero. Os cariótipos apresentaram variações quanto ao número, morfologia e mecanismo sexual; e as marcações de bandeamento mostraram diferenças principalmente entre os cromossomos grandes. Em *O. pictus* dois pares autossômicos foram marcados pela sonda 18S rDNA. As relações filogenéticas e a descrição dos cariótipos das espécies indicam que estes caracteres estão derivando de forma independente. Os dados mostram um padrão filogenético de separação clara entre grupos Neártico, Paleártico e Neotropical, enquanto os padrões cromossômicos se misturam em relação a morfologia e mecanismo de determinação do sexo.

Palavras chave: Cromossomos, cariótipos, filogenia, Inferência Bayesiana, insetos.

ABSTRACT

The order Orthoptera has a worldwide distribution and has been used as a model organism in several studies, such as the areas of phylogenetics and cytogenetics. This group is divided into the sub-orders Caelifera and Ensifera, the latter being considered the most basal. The first phylogenies of Ensifera were based on taxonomy and bioacoustics, and then the use of molecular genes allowed greater support for research. In Ensifera, the Grylloidea taxon is considered the most basal, being composed of 13 subfamilies, including Oecanthinae. The crickets of this subfamily are known as tree crickets, and the genus *Oecanthus*, is the main representative, with 72 described species and worldwide distribution. The genus is considered recent in the evolutionary history of crickets, having its phylogenetic relationships studied only for some species occurring in China. In addition, cytogenetic studies are scarce, since only six species had their karyotypes described, which are *O. indicus, O. nigricornis*, and *O. quadripunctatus* with 2n=19, X0, *O. longicauda* and *O. pellucens* with 2n=20, XY, and *O. valensis* 2n=18, XY. All karyotypes share the same asymmetry, including large and small chromosomes. The objective of this work was to describe the karyotypes of Neotropical species of the crickets *O. valensis*, *O. pictus*, *O. pallidus* and *O. lineolatus*, and to analyze the phylogenetic relationships of these species using molecular markers and the available data of the genus *Oecanthus*. Phylogenies were reconstructed by Bayesian Inference using the COI, 12S, 16S and 18S rDNA genes. For cytogenetic analyzes, conventional staining and C-Banding techniques were performed; the FISH technique was used in *O. pictus*, using the 18S rDNA marker. The phylogenetic relationships of the species were well supported, showing that Neotropical species are the least derived from the genus. Karyotypes showed variations in number, morphology and sexual mechanism; and the banding markings showed differences mainly between the large chromosomes. In *O. pictus* two autosome pairs were marked by the probe 18S rDNA. The Phylogenetic relationships and description of karyotypes indicate that these characters are deriving independently. The data show a phylogenetic pattern of clear separation between Neartic, Paleartic and Neotropical groups, while chromosomal patterns are mixed in relation to morphology and sex determination mechanism.

Keywords: Chromosomes, karyotypes, phylogeny, Bayesian Inference, insect.

CAPÍTULO I
INTRODUÇÃO GERAL

1.1. Filogenética

1.1.1. Ordem Orthoptera

A Ordem Orthoptera é considerada uma das mais diversas e antigas em Insecta, constituída por mais de 28.000 espécies válidas (FLOOK E ROWELL, 1998; CIGLIANO et al., 2019). Estima-se que essa ordem tenha origem no Carbonífero e suas duas subordens, Caelifera (gafanhotos) e Ensifera (grilos e esperanças), tenham divergido no Permiano (LEGENDRE et al., 2010; SONG et al., 2015; CIGLIANO et al., 2019), sendo Ensifera o grupo mais antigo em Orthoptera, com mais de 16.000 espécies válidas (GWYNNE, 1995, ZHOU et al., 2017, CIGLIANO et al., 2019).

As primeiras filogenias realizadas para a ordem Orthoptera foram baseadas em diferentes caracteres morfológicos, como nervuras das asas, escleritos fálicos, aparelho estridulatório, entre outros (SHAROV, 1971; DESUTTER-GRANDCOLAS, 2003). Estas foram muito utilizadas para inferir as primeiras espécies com capacidade de produzir som e como essa característica se manteve ou foi perdida em diferentes gêneros (ALEXANDER, 1962; DESUTTER-GRANDCOLAS, 2003; ROBILLARD et al., 2007). Já para Ensifera, as primeiras filogenias foram propostas por Gwynne (1995) e Desutter-Grandcolas (2003), ambas utilizando caracteres morfológicos, e a partir do trabalho de Jost e Shaw (2006) marcadores moleculares passaram a ser inseridos nos estudos. Foi observado que existe um grande conflito entre dados morfológicos e moleculares disponíveis para Ensifera (SONG et al., 2015).

As infraordens que compõem Ensifera são Tettigoniidea e Gryllidea, sendo esta dividida nas superfamílias Grylloidea e Gryllotalpoidae (DESUTTER-GRANDCOLAS, 2003; GÄDE et al., 2003; ZHOU et al., 2017; CIGLIANO et al., 2019). Grylloidea pode ser considerado um grupo basal na linhagem dos ensíferas, sendo o primeiro a se diversificar no início do Triássico, continuando a diversificação no decorrer do Mesozoico (FLOOK et al., 1999; SONG et al., 2015). Esta superfamília é composta por seis famílias válidas, sendo uma delas Gryllidae dividida em treze subfamílias, onde se encontra Oecanthinae. Dentro desta, se dispõem os gêneros *Oecanthodes*, *Viphyus*, *Oecanthus*, *Xabea*, *Neoxabea* e *Paraphasius* (CIGLIANO et al., 2019).

O DNA mitocondrial é comumente utilizado como marcador molecular em estudos de filogenia e genética de populações (ZHANG E HEWITT, 1997). Seus genes, em geral, evoluem em taxas mais rápidas do que os nucleares (LIN E DANFORTH, 2004). O gene Citocromo c Oxidase (COI) é considerado um dos mais conservados no genoma animal, decorrente disso, é amplamente utilizado em estudos de diferenciação de espécies (FOLMER et al., 1994). Por outro lado, os genes das subunidades 12S e 16S do DNA ribossomal são considerados informativos em níveis subapical e apical das filogenias. Em estudos na subfamília Eneopterinae o 12S apresenta menos variação, sendo mais informativo em nível basal, e o 16S é informativo para nós mais intermediários (ROBILLARD E DESUTTER-GRANDCOLAS, 2006).

Os genes nucleares tendem a evoluir de forma mais lenta do que os mitocondriais (LIN E DANFORTH, 2003). O marcador 18S rDNA é uma das sequências mais bem estudadas em insetos, e geralmente, é utilizado para reconstrução de níveis mais basais das filogenias (FLOOK E ROWELL, 1998). Em Orthoptera, o táxon Grylloidea apresenta sequências do gene 18S rDNA disponíveis em banco de dados, e que são extremamente divergentes das encontradas em outras espécies da ordem (FLOOK et al., 1999).

1.1.2. Subfamília Oecanthinae

Os indivíduos da subfamília Oecanthinae, foco deste trabalho, são comumente conhecidos como grilos arbóreos, contudo, muitas espécies são encontradas em gramíneas e arbustos. Este táxon se distingue de outros Gryllidae por apresentar corpo delgado, cabeça quase prognata e pernas posteriores muito delgadas (WALKER, 1962). Dentro deste grupo, o gênero *Oecanthus*, apresenta distribuição mundial com 72 espécies válidas, sendo sua origem considerada recente na história evolutiva dos grilos (SHAROV, 1971; CIGLIANO et al., 2019).

Os grilos desta subfamília apresentam hábito onívoro e podem ser considerados benéficos por atacarem insetos que danificam plantas, e prejudiciais por causarem danos em flores e frutos em desenvolvimento (WALKER, 1962). Foi observado que espécies Neárticas, causam danos por ovipositar nas hastes de árvores frutíferas e arbustos, podendo transmitir fungos que atacam macieiras (WALKER, 1962; EISEMAN et al., 2010).

As espécies de *Oecanthus* em geral são facilmente identificadas por caracteres morfológicos, porém algumas espécies do grupo *nigricornis* geram dúvidas quanto ao seu reconhecimento (WALKER, 1963). Este grupo ocorre na Região Neártica e é considerado um dos mais difíceis de analisar suas relações taxonômicas (COLLINS et al., 2014; WALKER,

1963), sendo necessária a utilização de marcadores moleculares em alguns casos, como na confirmação da divergência entre as espécies *O. symesi* e *O. pini* (COLLINS et al., 2014).

No estudo de Liu et al. (2018) foram inferidas as relações filogenéticas das espécies de *Oecanthus* com ocorrência na China. Na filogenia, a primeira separação ocorreu entre o grupo *Oecanthus* spp. e *Xabea levíssima*, pertencentes a mesma subfamília (LIU et al., 2018; CIGLIANO et al., 2019), sendo *O. antennalis* a primeira espécie a divergir dentro do gênero. Foi visto que *O. euryelytra* é um grupo monofilético, e as espécies *O. longicauda* e *O. similator* mostraram ser filogeneticamente próximas, sendo provável que *O. similator* tenha derivado a partir do grupo de *O. longicauda* (Figura 01) (LIU et al., 2018).

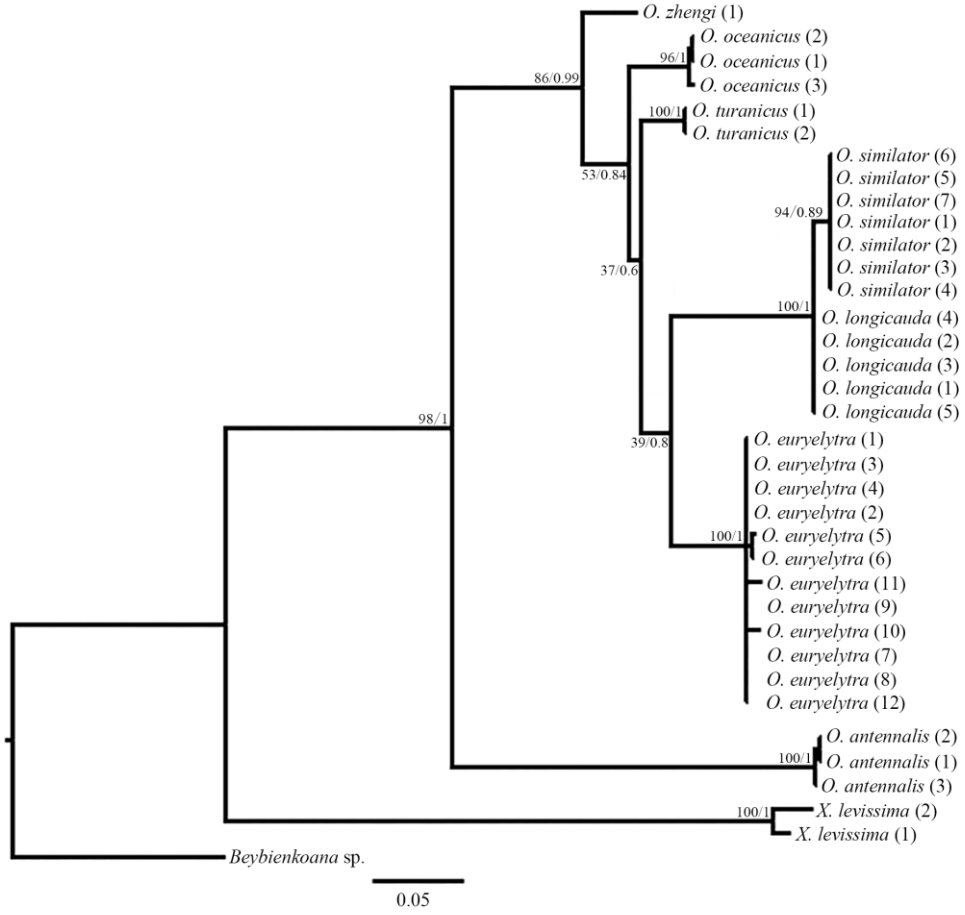

Figura 01 – Árvore de Maximum-likelihood de grilos do gênero *Oecanthus* com ocorrência na China baseada no gene COI. Indicado em cada nó o suporte de Bootstrap (esquerda) e a probabilidade posterior Bayesiana (direita) (Liu et al., 2018).

A utilização de marcadores moleculares nucleares e mitocondriais pode permitir melhor entendimento sobre evolução e relações filogenéticas entre as espécies de *Oecanthus*. Permitindo assim um avanço no conhecimento deste grupo que apresenta ampla distribuição

global. Além disso, este trabalho servirá de base para futuros estudos sobre o gênero e grupos relacionados.

1.2. Citogenética

A ordem Orthoptera se destacada pela grande contribuição para o entendimento da biologia cromossômica e citogenética, em geral por apresentar cromossomos grandes e número diploide baixo (HEWITT, 1979; BLACKMON et al., 2017; CIGLIANO et al., 2019). Nesta ordem, a subfamília Oecanthinae apresenta 172 espécies válidas, e dentro desta ordem, encontra-se o gênero *Oecanthus* sp. (Serville, 1831) com 72 espécies descritas e uma distribuição mundial (CIGLIANO et al., 2019). Neste gênero, apenas seis espécies tiveram seus cromossomos estudados, focando em descrições do número cromossômico e sistema de determinação do sexo (OHMACHI, 1927, 1935; JOHNSON, 1931; MAKINO, 1932; KITADA, 1949; HEWITT, 1979; MILACH et al., 2016).

Dentre as espécies estudadas citologicamente, *O. indicus, O. nigricornis* e *O. quadripunctatus* apresentam 2n=19, X0 (Figura 02) (JOHNSON, 1931; OHMACHI, 1935; KITADA, 1949; BEAUDRY, 1973), *O. longicauda* e *O. pellucens* (Figura 02) 2n=20, XY (OHMACHI, 1927, 1935; MAKINO, 1932, HEWITT, 1979), e *O. valensis* 2n=18, XY (Figura 02) (MILACH et al., 2016). Essas espécies compartilham a mesma assimetria cariotípica, incluindo autossomos grandes, junto com o cromossomo sexual X, e autossomos pequenos, incluindo Y, quando presente (JOHNSON, 1931; MAKINO, 1932; OHMACHI, 1927, 1935; KITADA, 1949; MILACH et al., 2016).

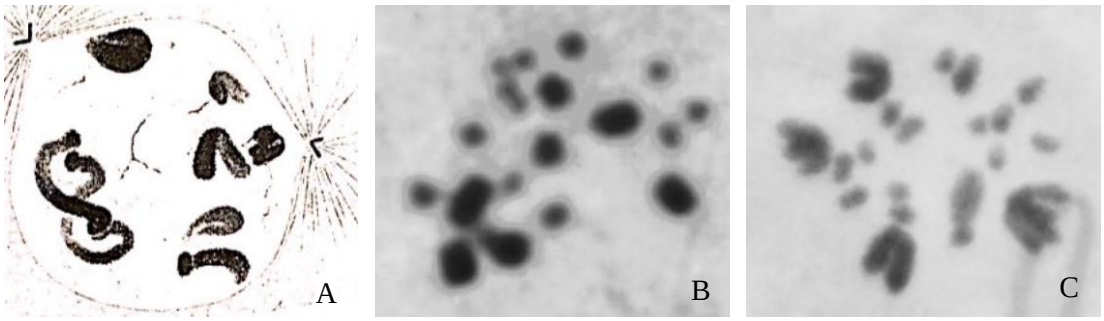

Figura 02 – Cromossomos de *Oecanthus nigricornis* com 2n= 19, X0 (A) (JOHNSON, 1931), *O. longicauda* com 2n= 20, XY (B) (YOU et al, 2007) e *O. valensis* com 2n= 18, XY (C) (MILACH et al, 2016).

Em Orthoptera o mecanismo sexual considerado mais comum e menos derivado é o X0♂-XX♀ e, geralmente, o cromossomo X apresenta heteropicnose positiva em relação aos autossomos (WHITE, 1978, HEWITT, 1979). A diferença de picnose permite a identificação

dos cromossomos sexuais em relação aos autossomos durante o processo meiótico, principalmente nas fases iniciais da Prófase I (SAEZ, 1963). A conversão do mecanismo XX-X0 para XX-XY em Orthoptera provavelmente ocorreu inúmeras vezes no processo evolutivo das espécies, sendo possível observar que, quando o neo-Y tem origem recente, ele pode não ter sido fixado na população apresentando um estado eucromático. A eucromatina consiste em regiões geneticamente ativas no cromossomo, que podem se tornar heterocromatina, ou seja, regiões inertes e estáveis de DNA (SWANSON et al., 1981).

Rearranjos entre o cromossomo X e cromossomos autossomos podem originar diferentes mecanismos de determinação do sexo (WHITE, 1954, 1957; SAEZ, 1963), como é o caso do mecanismo neo-XY formado pela fusão entre o cromossomo sexual X e um cromossomo de um par autossômico (OHMACHI, 1927; SAEZ, 1963; KAISER E BACHTROG, 2010; CASTILLO, 2010); o mecanismo X_1X_2Y, originado a partir de dois rearranjos subsequentes entre o cromossomo X e autossomos (MESA E BRAN, 1964; FERREIRA E CELLA, 2006); e também, o mecanismo X_1X_20, derivado da dissociação do cromossomo X original como primeiro passo, e a ocorrência de inversão pericêntrica ou adição de cromatina como segundo passo (MESA et al., 2002; PALACIOS-GIMENEZ E CABRAL-DE-MELLO, 2014; ZEFA et al., 2014).

As técnicas clássicas de bandeamento cromossômico, como banda C e Regiões organizadoras nucleolares (NOR), são vastamente utilizadas em estudos citogenéticos para descrever translocações e inversões, processos de translocação eucromática e amplificação de sequências de DNA (MANSUETO, 1989; WARCHAŁOWSKA-ŚLIWA E MARYAŃSKA-NADACHOWSKA, 1992; WARCHALOWSKA-ŚLIWA et al., 1994). Com a utilização do bandeamento C é possível realizar uma detalhada caracterização do cariótipo, sendo uma ferramenta importante na detecção de polimorfismos de segmentos pequenos de heterocromatina. Em geral a técnica utiliza cromossomos mitóticos, porém o uso de cromossomos meióticos permite a observação de diferenças morfológicas entre homólogos, como em casos de condições heterozigóticas (VILARDI, 1986).

Além das técnicas de bandeamento, a utilização de hibridização *in situ* fluorescente (FISH) se tornou uma importante ferramenta para análises comparativas de cromossomos, melhor entendimento da organização genômica e identificação de rearranjos cromossômicos em diferentes organismos (CABRAL-DE-MELLO et al., 2011). Marcadores como o 18S rDNA podem gerar informações importantes quanto a organização genômica e variações entre espécies (WARCHAŁOWSKA-ŚLIWA et al., 2013; PALACIOS-GIMENEZ E CABRAL-DE-MELLO, 2015).

Apesar do amplo conhecimento em citogenética de Orthoptera, as espécies do gênero *Oecanthus* apresentam poucos dados gerados até o momento. Portanto este trabalho permitirá incrementar o conhecimento sobre os conjuntos cromossômicos do grupo, abrindo caminho para compreender a evolução cariotípica em Oecanthinae.

OBJETIVOS

2.1. Geral

Caracterizar o cariótipo das espécies neotropicais dos grilos *O. lineolatus*, *O. valensis*, *O. pallidus* e *O. pictus*, com destaque ao número e morfologia cromossômica, bem como o mecanismo sexual. Assim como, estabelecer as relações filogenéticas entre as espécies do gênero *Oecanthus* utilizando marcadores moleculares.

Específicos

- Determinar o número cromossômico diploide de quatro espécies de *Oecanthus*;
- Elaborar cariótipos e caracterizar a morfologia dos cromossomos, com base no índice centromérico;
- Caracterizar a morfologia e descrever o comportamento dos segmentos heterocromáticos dos cromossomos sexuais durante a Prófase I;
- Reconstruir as relações filogenéticas de espécies de *Oecanthus* juntamente com os dados disponíveis em banco de dados públicos.

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CAPÍTULO II

Molecular and cytogenetic markers for inference of phylogenetic relationships in four Neotropical species of *Oecanthus* SERVILLE, 1831 (Orthoptera, Gryllidae)

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ABSTRACT

Orthoptera is a worldwide group and a model organism to different studies, being well studied in the areas of phylogenetics and cytogenetics. In Ensifera the taxa Grylloidea is considered the most basal and compounded with 13 subfamilies. Oecanthinae is one of them, being the genus *Oecanthus* within this group. The genus has 72 species and is poorly studied, even showing interesting variations. In this work we used molecular and cytogenetics approaches to elucidate the phylogenetic relations and to describe the karyotype of *O. lineolatus*, *O. valensis*, *O. pallidus* e *O. pictus*. The phylogenies Bayesian Inference were constructed using the genes COI, 12S, 16S and 18S, and the cytogenetic were realized using conventional and differential staining (C-band); we also include fluorescence *in situ* hybridization (FISH) of 18S rDNA for *O. pictus*. The phylogeny separated the species in Neotropical, Nearctic and Palearctic regions, except for *O. pictus, O. pini*, and *O. antennalis*. In cytogenetics *O. lineolatus* has 2n=20, XY, *O. valensis* and *O. pallidus* have 2n=18, XY, and *O. pictus* 2n=21, X0; and the C-banding showed variations in the large chromosomes; in *O. pictus* two autosome pairs were marked by FISH. The inferred phylogeny and karyotypes do not completely correspond to each other, indicating an independent evolution.

Keywords: Insect, Karyotype, chromosomes, phylogeny, C-banding.

INTRODUCTION

The order Orthoptera has more than 28.000 described species, with worldwide distribution and a variated use as model organism in different areas of study. It is subdivided in the suborders Caelifera (grasshoppers and locusts) and Ensifera (crickets and katydids) (Cigliano et al. 2019). The group Ensifera is well studied phylogenetic, mainly for understanding the bioacoustics mechanism and how it evolved in the group (Alexander 1962; Desutter-Grandcolas 2003; Robillard et al. 2007). In addition, the taxon of Orthoptera is very important by their contribution on studies of cytogenetics, due their large chromosome, low diploid number and variation on the sex determination mechanism (Hewitt 1979; Blackmon et al. 2017).

The Ensifera infraorders are Tettigoniidea and Gryllidea, being the last divided in the superfamilies Grylloidea and Gryllotalpoidae (Desutter-Grandcolas 2003; Gäde et al. 2003;

Zhou et al. 2017; Cigliano et al. 2019). Grylloidea was considered the most basal lineage of ensiferans, which is the first group to diversified, in the early Triassic and continuing during the Mesozoic (Flook et al. 1999; Song et al. 2015). This superfamily comprised of 13 subfamilies, one of them is Oecanthinae, that include the genus *Oecanthodes*, *Viphyus*, *Oecanthus*, *Xabea*, *Neoxabea* and *Paraphasius* (Cigliano et al. 2019).

The individuals of Oecanthinae are commonly known as "tree crickets" as most species of the group are arboreal, but some are found in grasses and shrubs. This taxon differs of others Gryllidae for its slender body, almost horizontal head and very slender posterior legs (Walker 1962). In this subfamily, the genus *Oecanthus* is the most representative with 72 species described and worldwide distribution (Cigliano et al. 2019).

The crickets of *Oecanthus* genus have an omnivorous habit and can be considered beneficial for control and attack insects that damage plants, or harmful by causing damages in developing flowers and fruits (Walker 1962). The Nearctic species cause damage when oviposit in stems of fruit trees and shrubs, being associated with fungi transmission in apple trees (Walker 1962; Eiseman et al. 2010).

According to Liu and contributors (2018) the species of *Oecanthus* from China are a monophyletic group considering the gene Cytochrome c Oxidase subunit I (COI). The inferred tree showed that the first separation occurred between *Oecanthus* ssp. and *Xabea levissima*, from the same subfamily (Liu et al. 2018; Cigliano et al. 2019). Within the genus, *O. antennalis* was the first to diverge followed by the monophyletic group of *O. euryelytra*, composed of *O. longicauda* and *O. similator*. The last two species were considered close phylogenetically, and probably *O. simulator* has been originated from *O. longicauda* group (Liu et al. 2018).

Molecular genes like mitochondrial and nuclear become common in studies of phylogenies and genetics (Zhang and Hewitt 1997). The gene COI is considered the most conserved in animal mitogenome and is widely used to differ species (Folmer et al. 1994). On the other hand, 12S rDNA and 16S rDNA are very informative for subapical and apical levels in phylogenies, giving support to intermediate nodes (Robillard and Desutter-Grandcolas 2006). The nuclear genes usually have a slower evolution when compared with mitochondrial (Lin and Danforth 2003). The 18S rDNA is one of most studied markers in insects, and usually it is used to reconstruct basal level in the phylogenetic tree (Flook and Rowell 1998).

In the genus *Oecanthus* only six species have their karyotype described up to now, and they showed variation in chromosomic number, morphology and mechanism of sex determination (Johnson 1931; Makino 1932; Ohmachi 1935; Kitada 1949; Hewitt 1979; Milach et al. 2016). All these species share an asymmetric karyotype, with one group formed by large

autosomes and the sexual chromosome X, and other with small autosomes and the sexual chromosome Y (when it is present) (Johnson 1931; Makino 1932; Ohmachi 1927, 1935; Kitada 1949; Milach et al. 2016).

Cytogenetic techniques like C-banding are wide used in chromosome studies, being good markers to identify translocations and inversion in the chromosome set (Mansueto 1989; Warchałowska-Śliwa and Maryańska-Nadachowska 1992; Warchalowska-Śliwa et al. 1994). Using the C-band it is possible to detect heterochromatic segments in chromosomes that may vary between species (Vilardi 1986). Besides this kind of banding technique, the use of Fluorescent *in situ* Hybridization (FISH) became an important comparative tool to better understand genomic organization and find chromosomal rearrangements in different organisms (Cabral-de-Mello et al. 2011).

In the present work were used molecular and cytogenetics approaches to elucidate the phylogenetic relations and describe the karyotype of four species of the genus *Oecanthus*. The phylogenies were constructed using the genes COI, 12S, 16S and 18S rDNA in analyses of Bayesian Inference, and the cytogenetic were realized using C-banding for all four species, as well as FISH techniques for *O. pictus* Milach e Zefa, 2015. Thus, the objective of this study was to improve the knowledge of phylogenetic relations and characterized the chromosome of *O. lineolatus* Saussure, 1897, *O. valensis* Milach e Zefa, 2016, *O. pallidus* Zefa, 2012 using conventional and differential staining. The phylogenetic relation of these species was well supported and showed that Neotropical species may be less derivate, and that the karyotypes vary in morphology, sexual determination mechanisms, and in the banding positions. Besides the results indicate an independent evolution of phylogenetic and the chromosome set.

MATERIAL AND METHODS

Samples

Individuals of *O. valensis* were collected in in shrubs and grasses with a sweep net, bordering the highway BR101 alongside to the conservation area “Reserva Natural Vale”, municipality of Linhares, State of Espírito Santo, Brazil (19º05’817’’S, 040º03’116’’W) on July 28, 2012. The specimens of *O. pictus*, *O. pallidus* and *O. lineolatus* were collected in tobacco plantations and on shrubs in the São João da Reserva district, municipality of São Lourenço do Sul, State of Rio Grande do Sul, Brazil (31º17’39.43’’S, 52º09’02.76’’W) in march of 2012.

Molecular and phylogenetic analysis

The DNA extraction was proceed using the hind femora of the specimens and performed with a phenol/ chloroform protocol according to Jowett (1986). The genetic material was amplified using specific *primers* COI, 12S, 16S and 18S rDNA, through the Polymerase Chain Reaction (PCR). The *primers* used were HCO2198 and LCO1490 (Folmer et al. 1994), 12SF and 12SR (Kambhampati 1995), 16SAG and 16SBG (Robillard and Desutter-Grandcolas 2006) and, 18Sa2.0 and 18Sbi (Giribet et al. 1999), for COI, 12S, 16S and 18S, respectively (Table 01).

Table 01 – Primers used for PCR amplification and sequencing, indicating the gene, sequence, temperature for annealing and source of each sequence *primer*.

Marker	Primers	Sequence	Temperature	Author
COI	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	47 a 55ºC	Folmer et al. 1994
	HC02198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		
12S rRNA	12SF	5'-TACTATGTTACGACTTAT-3'	44 a 45ºC	Kambhampati 1995
	12SR	5'-AAACTAGGATTAGATACCC-3		
16S rRNA	16SAG	5'-CGCCTGTTTATCAAAAACATGT-3'	48 a 49ºC	Robillard and Desutter-Grandcolas 2006
	16SBG	5'-AGATCACGTAAGAATTTAATGGTC-3'		
18S rRNA	18S-a2.0	5'-ATGGTTGCAAAGCTGAAAC-3'	59 a 60ºC	Giribet et al. 1999
	18S-bi	5'-GATCCTTCCGCAGGTTCACCTAC-3'		

PCR products were visualized in 1% agarose gel and then purified by EXO-SAP (UAB) enzymatic method for sequencing. The sequencing was performed in both ways, by Macrogen Inc. (Seul, South Korea). Obtained chromatograms were assembled and inspected using Staden Package (Staden 1996). Sequences of the genus *Oecanthus* and outgroup available in *GenBank* were added in the evolutive analysis (Table 02). Sequences generated in this work will be further submitted to *GenBank.* The alignment and edition of sequences were made in the software MEGA X 10.1 (Kumar et al. 2018).

Table 02 – Information of each specie and sequence used for analyzes, indicating the gene and access number of sequences from *GenBank*.

Specie	Gene	Access Number
Oecanthus valensis	12S	Present study
Oecanthus lineolatus	12S	Present study

Oecanthus pictus	12S	Present study
Oecanthus pallidus	12S	Present study
Oecanthus pini	12S	KJ024361.1
Ceuthophilus sp.	12S	KR903978.1
Locusta migratoria	12S	AB497582.1
Oecanthus valensis	16S	Present study
Oecanthus lineolatus	16S	Present study
Oecanthus pictus	16S	Present study
Oecanthus pallidus	16S	Present study
Oecanthus chopardi	16S	KR903784.1
Oecanthus nigricornis	16S	AF514469.1
Ceuthophilus uhleri	16S	AF212056.1
Locusta migratoria	16S	JF932434.1
Oecanthus valensis	18S	Present study
Oecanthus lineolatus	18S	Present study
Oecanthus pictus	18S	Present study
Oecanthus pallidus	18S	Present study
Oecanthus chopardi	18S	KR904148.1
Oecanthus niveus	18S	KM853175.1
Oecanthus nigricornis	18S	AF514514.1
Ceuthophilus maculatus	18S	KY554597.1
Locusta migratoria	18S	AF514580.1
Oecanthus valensis	COI	Present study
Oecanthus lineolatus	COI	Present study
Oecanthus pictus	COI	Present study
Oecanthus nigricornis	COI	KR143926.1
Oecanthus niveus	COI	KM535640.1
Oecanthus fultoni	COI	KR140441.1
Oecanthus quadripunctatus	COI	MG466395.1
Oecanthus celerinictus	COI	KM537641.1
Oecanthus exclamationis	COI	MG436770.1
Oecanthus antennalis	COI	MH893702.1
Oecanthus euryelytra	COI	MH893707.1
Oecanthus longicauda	COI	MH893701.1
Oecanthus oceanicus	COI	MH893718.1
Oecanthus similator	COI	MH893700.1
Oecanthus turanicus	COI	MH893727.1
Oecanthus zhengi	COI	MH893715.1
Oecanthus pellucens	COI	HM422220.1
Ceuthophilus sp.	COI	HQ986388.1
Locusta migratoria	COI	HQ986486.1

For performing hierarchical likelihood ratio tests and calculating approximate AIC values of the nucleotide substitution models were used the software PAUP*4, and then the

evolutive models were calculated in MrModeltest2 (Nylander 2004). For COI the model was GTR+I+G, 12S rDNA was GTR+I, 16S rDNA was GTR+G, and 18S rDNA was SYM+I+G. The tree inference of Bayesian Analysis was running in MrBayes 3.2.6 (Ronquist et al. 2012) on XSEDE in the online platform Cyberinfrastructure for Phylogenetic Research (CIPRES) (http://www.phylo.org/index.php/).

The inference trees for the concatenate data (COI, 12S rDNA, 16S rDNA and 18S rDNA) and nuclear (18S rDNA) were constructed with *O. valensis*, *O. pictus*, *O. lineolatus*, *O. pallidus*, *O. niveus*, *O. chopardi* and *O. nigricornis*, and as outgroup *Ceuthophilus* sp. (Ensifera, Rhaphidophoridae) and *Locusta migratoria* (Caelifera, Acrididae). In the first one, for the analysis we chose to use species that had sequences available for at least two of the genes, the unavailable sequences were calculated as missing data. Both analyses were running for 20 millions generations, sampling every 20,000 generations, the first 25% of samples was discarded as burn-in.

The concatenated tree using only mitochondrial genes was constructed with all the species of Table 02. The unavailable sequences were coded as missing data and *Ceuthophilus* sp. and *Locusta migratoria* were used as outgroup. The analysis was run for 40 millions generations, sampling every 40,000 generations, the first 25% of samples were discarded as burn-in.

Cytogenetic analyses

In the cytogenetic preparations were used: 17 individuals of *O. valensis* (10♂ and 7♀), 25 of *O. pictus* (20♂ and 5♀), four of *O. pallidus* (3♂ and 1♀) and 11 of *O. lineolatus* (8♂ and 3♀). Chromosomes were obtained from testicles follicles of males and medium gut of female and male individuals, previously injected with 0.05% colchicine solution for 5h, and after hypotonized in KCl 0.075 solution for 5-10 minutes, and then fixed in Carnoy I (3 ethylic alcohol: 1 acid glacial acetic acid). The fixed material was squashed on the slide with 45% acetic acid and the chromosomes were stained with 0.5% lacto-acetic orcein.

C-banding technique was performed according to Sumner (1972), which dive the slide in Hydrochloric Acid Solution (0.1N) for 30 minutes at room temperature and rise with distilled water. Then it was treated with 5% Barium Hydroxide at 60ºC for 3 minutes, the slide was washed in HCl 0.2N for 2 minutes and rinsing with distilled water. After, the slide was putted in 2xSSC solution at 60ºC for 45 minutes, washed with distilled water, and stained with 2% Giemsa in Phosphate buffer (pH 6.8) for 10 minutes.

The phase of meiosis and mitosis were selected and photographed with digital camera Nikon S3200, in the Olympus CX21 optic microscope. The centromeric index (ci) was calculated according to Levan et al. (1964). The karyotypes assembly were made using Adobe Photoshop CC 2015 program.

The Fluorescence *in situ* Hybridization (FISH) was performed according to Bertocchi et al. (2018), with modifications. The 18S rDNA fragments were amplified by PCR using nuclear DNA *primers*, 18Sa2.0 and 18Sbi (Table 01), in *O. pictus*. The fragments were labeled with biotin-dUTP by PCR and detected with Cy3 dye (GE Healthcare). The slides were analyzed and photographed in the ZEISS Axiophot microscope using ZEN BLUE edition software, and photos edition were realized in Adobe Photoshop CC 2015.

RESULTS

Molecular analyzes

In the tree of the 18S rDNA generated by Bayesian inference (BI) the group *Oecanthus* spp. shows to be monophyletic, and the first specie to split is *O. lineolatus* being the sister specie for the others. After that, *O. pictus* and *O. pallidus* separate, and the last clade is formed by *O. chopardi*, *O. valensis*, *O. nigricornis* and *O. niveus*. The posterior probabilities are high for almost all relations, being lower for *O. nigricornis* and *O. niveus* close relation (Figure 01).

The BI tree using concatenated data (COI, 12S rDNA, 16S rDNA, and 18S rDNA) was also monophyletic, being the last clade to be inferred the same as in the tree with 18S rDNA, formed by *O. chopardi*, *O. valensis*, *O. nigricornis* and *O. niveus*. However, there are variations in the position of *O. lineolatus*, *O. pictus* and *O. pallidus*, the first species to diverged were *O. lineolatus* and *O. pallidus*, and after that, *O. pictus* separated. The posterior probabilities indicated in the nodes were high, indicating a well-defined relationship between the species (Figure 02).

In the consensus mitochondrial tree (COI, 12S rDNA, and 16S rDNA) the monophyly was not recovered, but it is possible infer the phylogenetic relation of *O. lineolatus*, *O. pallidus*, *O. valensis* and *O. pictus* with the other species. The first species to diverge were *O. pallidus* and *O. lineolatus*, and then we have the separation of *O. pictus* and *O. pini*. After, occur the formation of two clades, being *O. antennalis* the sister taxa of both. One clade is formed with Nearctic species and *O. valensis*, and the other with species from Palearctic Region. In this inference the higher posterior probabilities were found in the basal nodes, and in the nodes of

Palearctic species. Besides that, the close relations between *O. turanicus* and *O. pellucens*, and *O. smilator* and *O. longicauda* were well supported. For the position of *O. pictus*, *O. pini*, and *O. antennalis* they did not cluster together with the well separated groups, it may occur due to the lack of data for the species of the genus (Figure 03a).

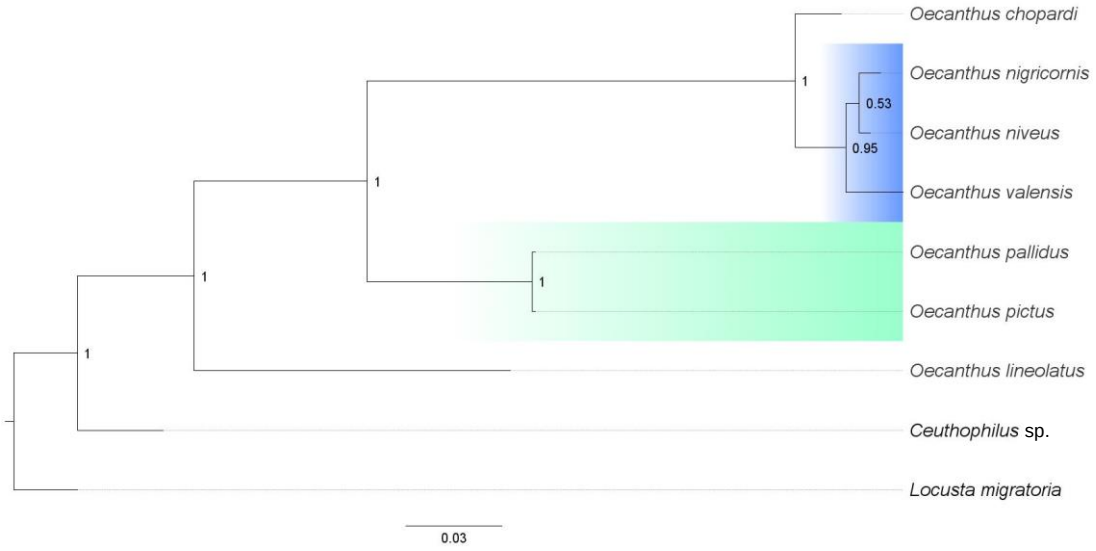

Figure 01 – Consensus Bayesian Inference tree of 18S rDNA with *O. chopardi*, *O. valensis*, *O. nigricornis*, *O. niveus*, *O. pallidus*, *O. pictus* and *O. lineolatus*, and *Ceuthophilus* sp. and *Locusta migratoria* as outgroups. In blue is indicated the close relation between Nearctic species and *O. valensis*; and in green the relation of two Neotropical species.

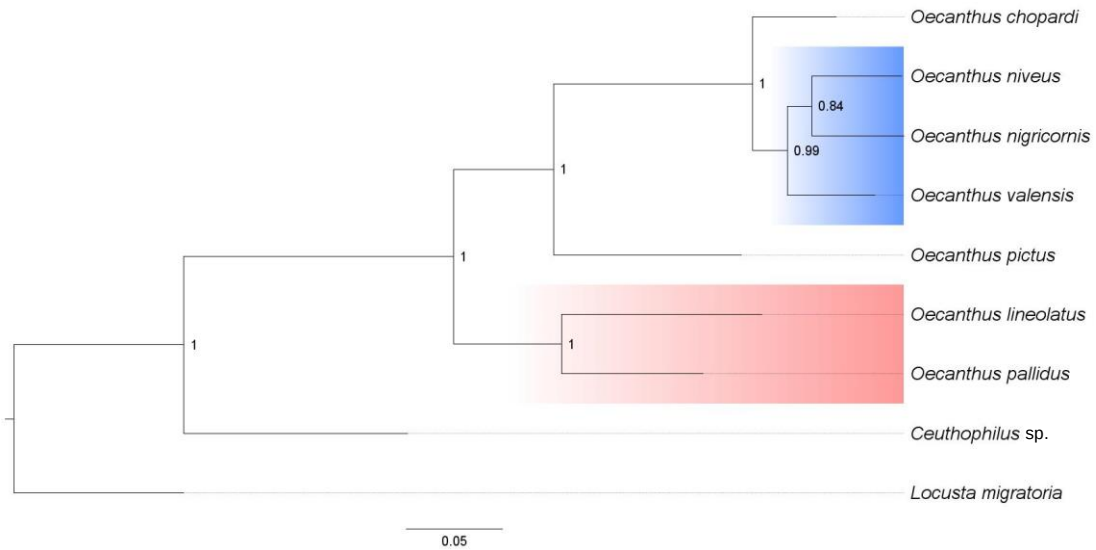

Figure 02 – Consensus Bayesian Inference tree of concatenated data (COI, 12S rDNA, 16S rDNA, and 18S rDNA), with *O. chopardi*, *O. valensis*, *O. nigricornis* and *O. niveus*, *O. pallidus*, *O. pictus* and *O. lineolatus*, and *Ceuthophilus* sp. and *Locusta migratoria* as outgroups. In blue is indicated the close relation between Nearctic species and *O. valensis*; and in red the relation of two Neotropical species.

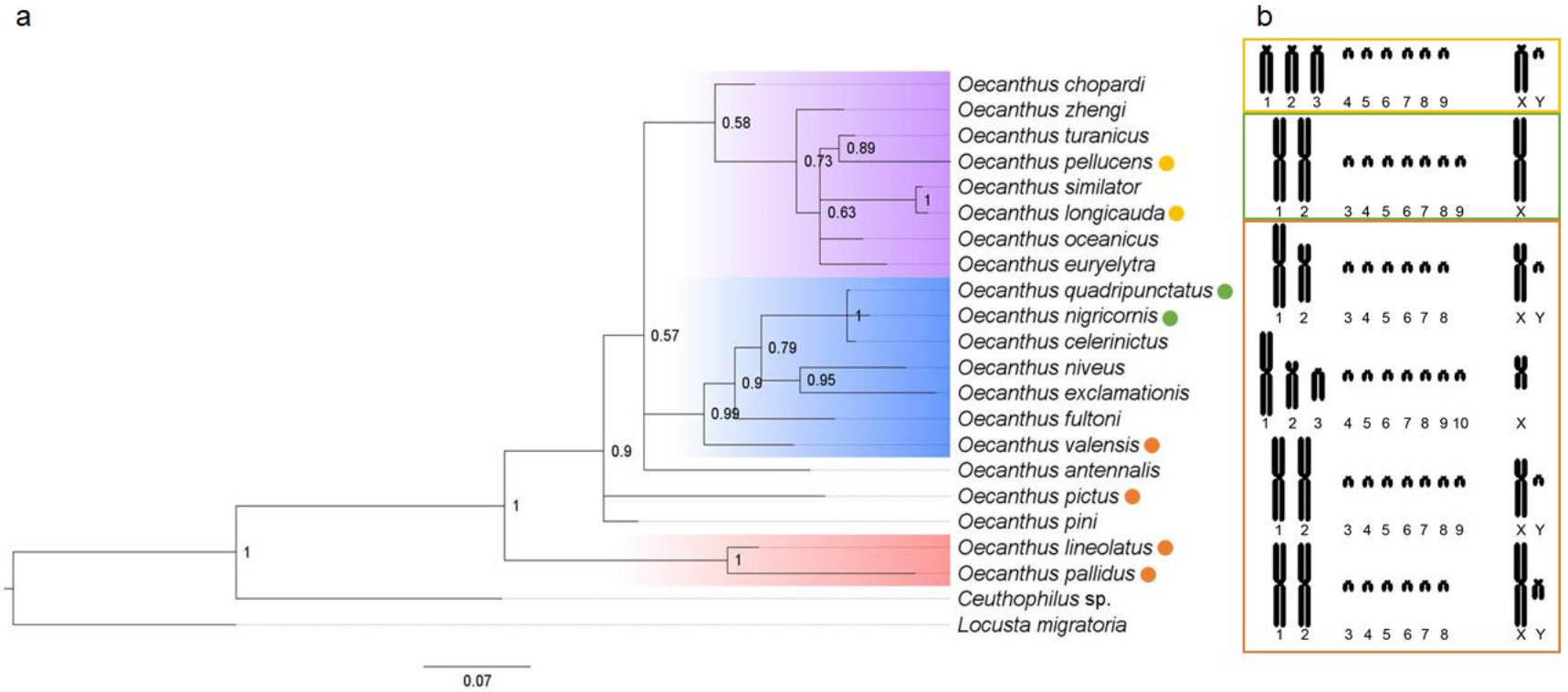

Figure 03 – a) Bayesian Inference using mitochondrial concatenated data (COI, 12S rDNA, and 18S rDNA), which the purple represented the Palearctic clade, blue Nearctic clade, and red the Neotropical. *Oecanthus* pictus, *O. antennalis*, and *O. pini* were not highlighted because their position may be not right due lack of data. The circles indicate the species with described karyotype; b) Schemes of karyotypes according with the description of *O. pellucens* by Hewitt (1979); *O. longicauda* by Makino (1932); *O. nigricornis* by Johnson (1931); and *O. quadripunctatus* by Beaudry (1973); and karyotype of *O. valensis*, *O. pictus*, *O. lineolatus*, and *O. pallidus*. The karyotype dispositions are respective with the position on the phylogenetic tree, in the yellow square are the Palearctic karyotypes, green the Nearctic karyotypes, and in orange the Neotropical.

Cytogenetics

The species *O. lineolatus* shows a diploid number of 2n=18+XY♂=20 and 2n=18+XX♀=20, with two pairs of large metacentric autosomes (ic=40,9; ic=42,10), pair two with a secondary constriction, and seven pairs of small chromosomes (Figure 03b, 04). The sexual chromosome X is a large submetacentric (ic=31,25) and the Y is one of smallest chromosomes (Figure 04). During meiosis I the sexual chromosomes behave as bivalents, forming chiasma in prophase I, positing together in the equatorial plate in metaphase I, and each one migrating for different poles of the cell in anaphase I. In diplotene it is possible observe elastic constrictions, that may correspond with the second constrictions in pair two (Figure 05 a-c, 06).

The individuals of *O. valensis* have 2n=16+XY♂=18 and 2n=16+XX♀=18, with two pairs of large meta or submetacentric autosomes (ic=48; ic=36,84), and six pair of small chromosomes (Figure 03b, 04). The sexual system is formed with a large submetacentric X (ic=25,92) and a small Y, and during meiosis they behave like bivalents. It is possible observe a gradual increasing of heterochromatinization of XL and Y segments in prophase I (Figure 05 d-f, 07). In some divisions were observed the presence of a B chromosome and in two individuals were found the formation of chromatin bridges in anaphase/telophase II (Figure: 04, 08).

Oecanthus pallidus shows 2n=16+XY♂=18 and 2n=16+XX♀=18, with two pairs of large metacentric (ic=47,8; ic=47,36) and six pairs of small autosomes chromosomes. The X chromosome is a large metacentric (ic=41,17) and the Y is a small subtelocentric (ic=20) (Figure 03b, 04). In diplotene I, sexual chromosomes behavior as bivalents being positive heteropicnotic, with a heterochromatic segment between them, that corresponds to the arm XR of chromosome X (Figure 05 g-I, 9).

In specimens of *O. pictus*, the diploid number is 2n=20+X0♂=21 and 2n=20+XX♀=22, with three pairs of large autosomes, one metacentric (ic=45), one subtelocentric (ic=16,66), and one acrocentric (ic=7,69), and seven pairs of small autosomes (Figure 03b, 04). The chromosome X is a large metacentric (ic=45,45) and during metaphase I behaves as a univalent, migrating for one of the poles of cell. In diplotene I the sexual chromosome shows positive heteropicnose in comparison with autosomes (Figure 05 j-m).

The C-banding pattern showed that, in all the species, the small chromosomes are acrocentric with a paracentromeric C-band in one of the extremities, and the chromosome X has a high heterochromatinization. Besides, it was found variations in the large autosomes, like in *O. lineolatus* where the chromosomes 2 exhibited a heterochromatic block in secondary constriction (Figure 10a). For *O. valensis* there is an interstitial band in the chromosome 1 and a heterochromatic block in autosome 2 (Figure 10b). *Oecanthus pallidus* has a C-band in the telomeric region of chromosome 1 and a heterochromatic block in the arm of pair 2, and the chromosome Y is heterochromatic (Figure 10c). Analysis of C banding in *O. pictus* showed a high heterochromatinization in the three large chromosomes (Figure 10d); the FISH analysis was performed in this species and showed clusters of 18S rDNA in two pairs of small autosomes, and the chromosome X was not marked (Figure 11).

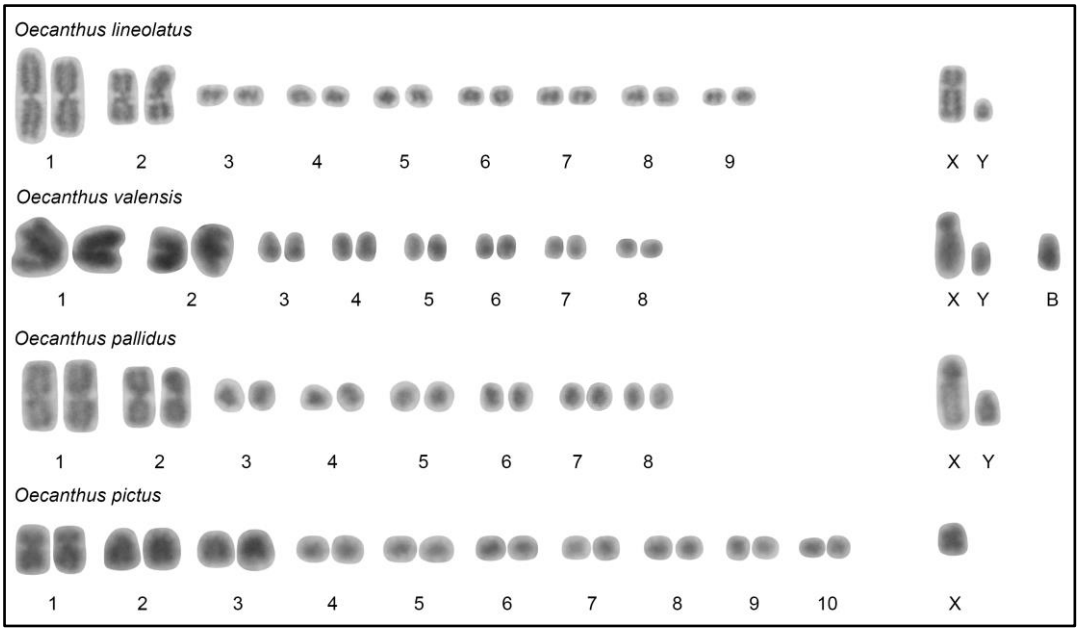

Figure 04 – Male mitotic karyotypes of *O. lineolatus*, *O. valensis*, *O. pallidus* and *O. pictus.*

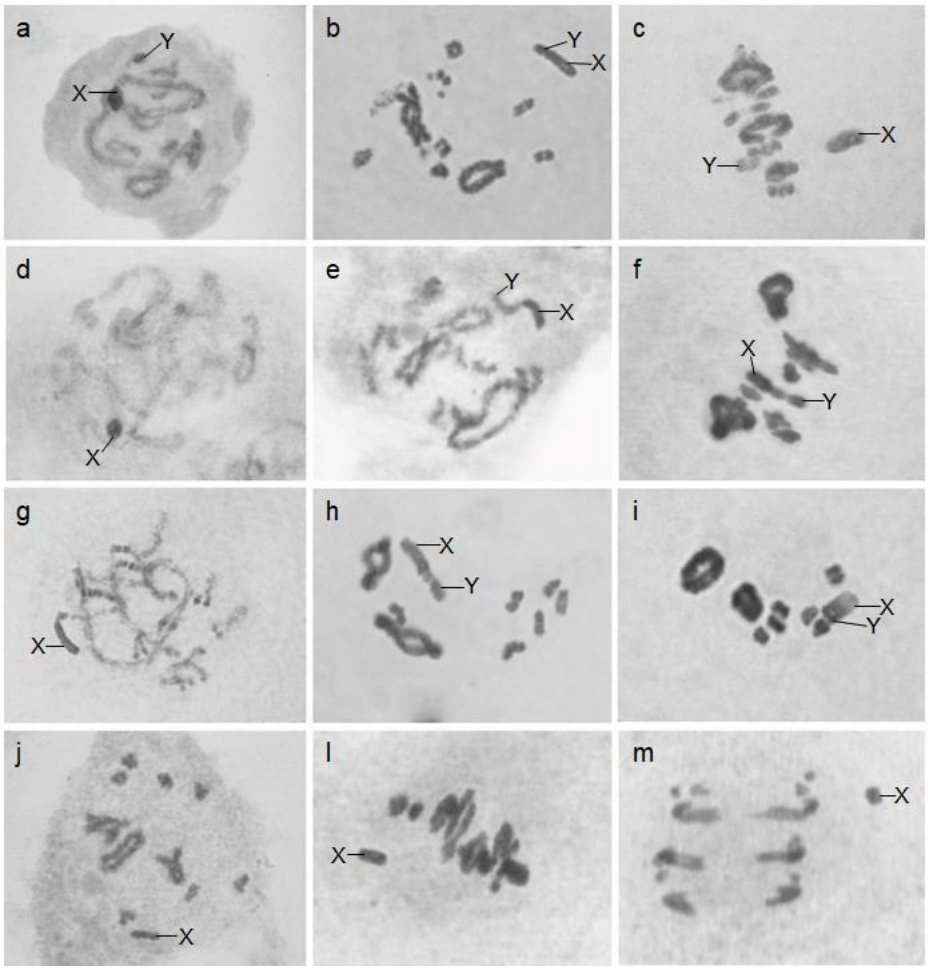

Figure 05 – Meiotic phases of male individuals showing the behavior of the sexual chromosome; a-c) *O. lineolatus* during Pachytene (a), Diplotene (b), Metaphase I (c); d-f) *O. valensis* during Zygotene (d), Diplotene (e), and Metaphase I (f); g-i) *O. pallidus* during Pachytene (g), Diplotene (h), and Metaphase I (i); and j-m) *O. pictus* during Diplotene (j), Metaphase I (l), and Anaphase I (m).

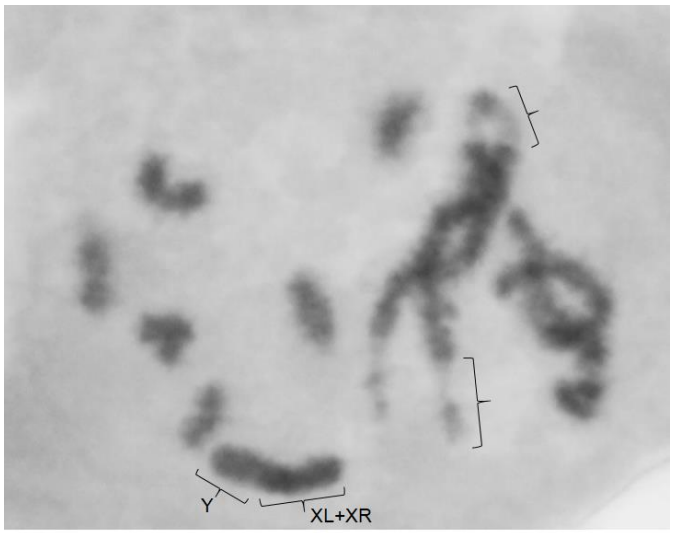

Figure 06 – Diplotene phase of *O. lineolatus*, highlighting the connection between XL+XR and Y, without a negative heteropicnotic region. One of the pairs of large autosomes shows elastic constrictions in the terminal region of their arms ({).

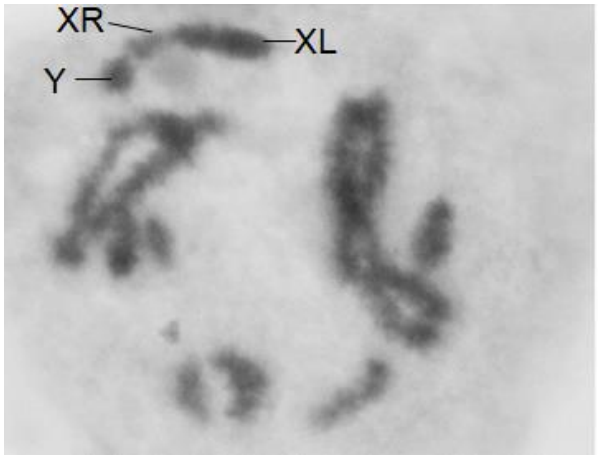

Figure 07 – In the Diplotene of *O. valensis* (A) there is a negative heteropicnotic region, between XL and Y segments.

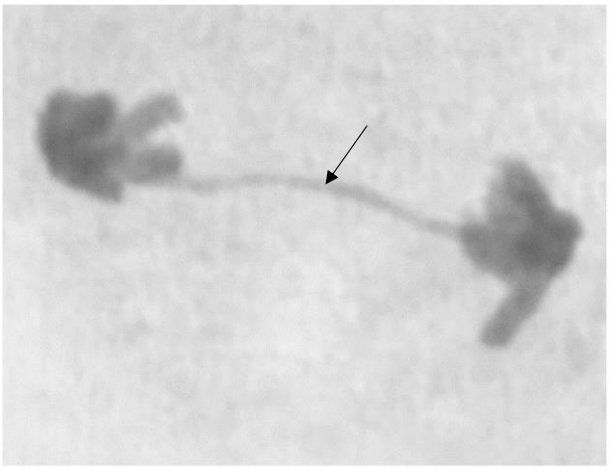

Figure 08 – Chromatin bridge (arrow) analyzed in one individual of *O. valensis* during Anaphase II.

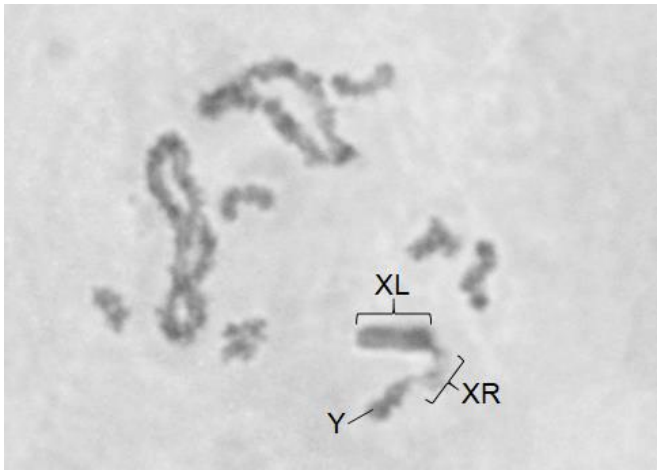

Figure 09 – Diplotene phase of *O. pallidus*, highlighting the isopicnotic segment that correspond to XR arm, between segments XL and Y.

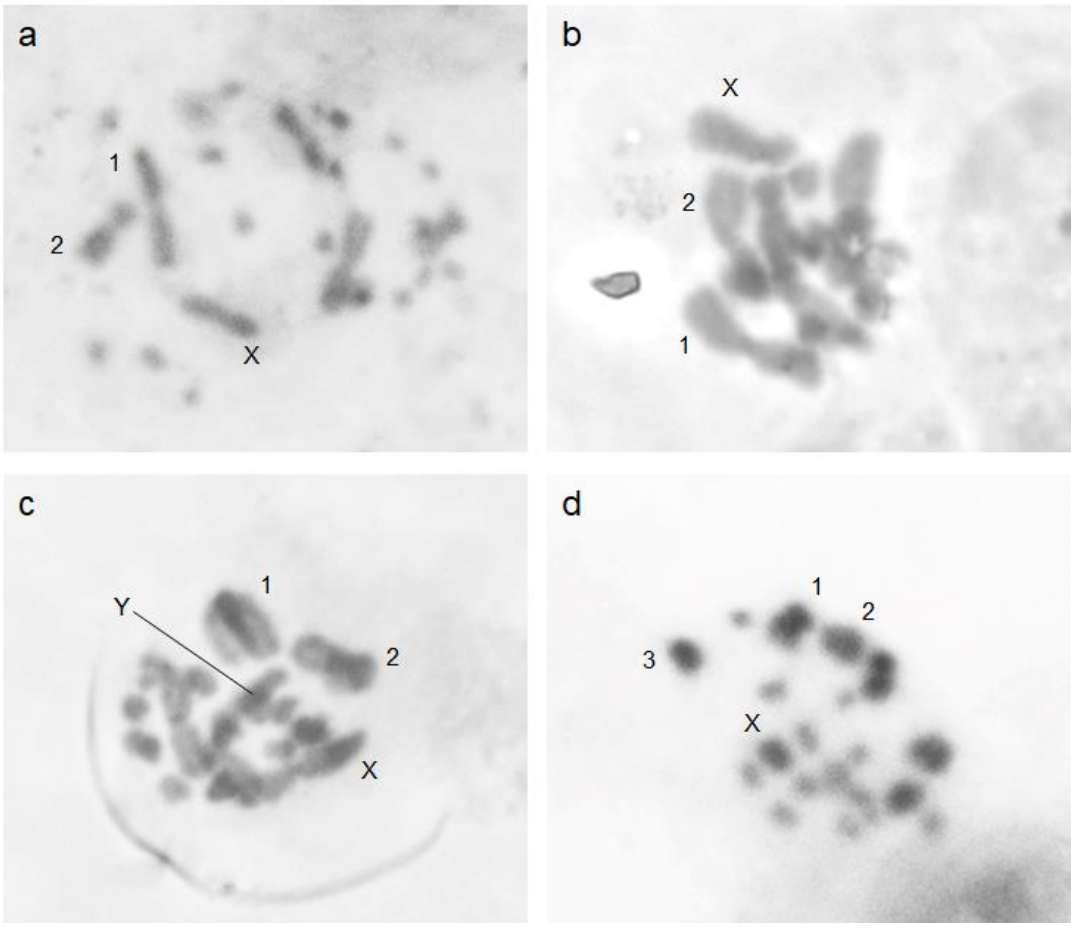

Figure 10 – C-banding during Mitotic Metaphase of a females of *O. lineolatus* (A) and *O. valensis* (B), and in male of *O. pallidus* (C) and *O. pictus* (D). Indicating the large chromosome of the karyotype and chromosome X.

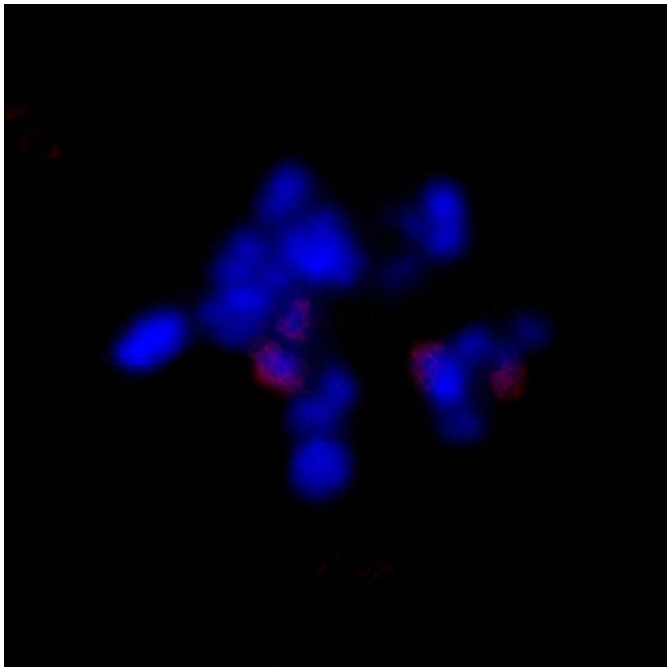

Figure 11 – FISH analysis of the 18S rDNA in Mitotic Metaphase mitotic division in male of *O. pictus*, showing two pairs of autosomes hybridized with the probe.

DISCUSSION

The studies using interdisciplinary approaches have been increasing, the combination of methodologies like morphology, cytogenetics, molecular analysis, and others, permit robust results about species systematics (Hemp et al. 2013; Kretschmer et al. 2018; Steiner et al. 2018). When a multidisciplinary methodology is used, information about the species is generated in different areas of study, thus being able to evaluate whether the evolutionary patterns in each area corroborate (Steiner et al. 2018). In Orthoptera phylogenies studies generally use morphological characters, bioacoustics, and molecular dada in analysis (Alexander, 1962; Sharov, 1971; Desutter-Grandcolas, 2003; Robillard et al, 2007). However, cytogenetics is a well-studied subject for the group, generating a lot of information about karyotype evolution, but little inserted in phylogenetic analyzes (Hewitt 1979; Blackmon et al. 2017).

The phylogenetic tree inference showed the separation between species from the Palearctic and Nearctic Region, and they showed a closer relationship when compared with the Neotropical group, which differ from the subfamily Eneopterinae, that formed only two groups, the Australian Eurepini and the other with remained species, being Australia considered their place of origin (Vicente et al. 2017). *Oecanthus valensis* was always placed close into the Nearctic species group, indicating a close relationship. On the other hand, *O. lineolatus*, *O. pallidus* and *O. pictus* were always inferred to be less derived, being sister groups to others.

In the concatenated data *O. lineolatus* and *O. pallidus* are closely related and in the 18S phylogeny the close relation was between *O. pictus* and *O. pallidus*. However, we observed a variation in the relations between *O. pictus*, *O. lineolatus*, and *O. pallidus*, but in each tree their position has a high support. Analysis comparing the gene 18S between orthopterans showed that Grylloidea had sequences extremely diverged from the other species of the order (Flook et al. 1999). Then this gene may be more variable when comparing to others, and consequently, cause some variation in the phylogenetic relations.

The mitochondrial phylogeny did not recovery the monophyly of *O. euryelytra* group, but the Palearctic species were grouped together and *O. antennalis* was external to them (Liu et al. 2018). In this clade the close phylogenetic relation between *O. longicauda* and *O. simulator* was recovered, being *O. simulator*, probably, evolved from a group of *O. longicauda* (Liu et al. 2018).

The karyotypes of the species of *Oecanthus* are poorly investigated, with data available only for nine species of 72 described for the group. Despite that there are interesting

chromosome variations, not only in number, but also in morphology and sex mechanism. The diploid number on the genus range in the male from 2n=18 to 21 (*O. valensis* and *O. pictus*), and it can have X0 or XY as sex system (Milach et al. 2016).

The cytogenetic studies with *Oecanthus* comprise species that occur in three bioregions, Palearctic, Nearctic and Neotropical (Cigliano et al. 2019). We described for the first time the karyotypes of *O. pallidus*, *O. lineolatus*, and *O. pictus*, all of them from the Neotropical Region. In addition, this was the first time that banding techniques were used in the genus (Johnson 1931; Makino 1932; Beaudry 1973; Hewitt 1979; You et al. 2007; Milach et al. 2016). It was observed that the sexual mechanism and diploid number were the same for *O. valensis* and *O. pallidus*, with 2n=18, XY, and *O. lineolatus* has the same mechanism but different diploid number, 2n=20, XY, and the most distinct karyotype was of *O. pictus*, with 2n=21, X0.

Considering the six species of *Oecanthus* already studied, three were Palearctic, two Nearctic and, one Neotropical. The Palearctic species *O. longicauda*, *O. indicus*, and *O. pellucens* presents three pairs of autosomes, all of them large and acrocentric, and six pair of dot-like small autosomes (Ohmachi 1927, 1935; Makino 1932; Kitada 1949; Montalenti et al. 1965; Hewitt 1979). The Nearctic, *O. quadripunctatus* and *O. nigricornis* shows two pairs of large metacentric autosomes, and seven pairs of small acrocentric autosomes (Johnson 1931; Ohmachi 1935; Kitada 1949; Beaudry 1973). Our results for *O. valensis* were congruent with Milach et al. (2016) study. The sexual mechanisms are XY for *O. valensis*, *O. longicauda*, and *O. pellucens* (Ohmachi 1927, 1935; Makino 1932; Kitada 1949; Hewitt 1979; Milach et al. 2016); and X0 for both Nearctic species and *O. indicus* (Table 03) (Johnson 1931; Ohmachi 1935; Kitada 1949; Beaudry 1973).

The species *O. valensis*, *O. lineolatus*, and *O. pallidus* has the same XY sex mechanism like in Palearctic specie, with exception of *O. indicus*. While the large autosomes showed similar morphology to the Nearctic species. In the case of *O. pictus* the sex mechanism is X0, like in *O. indicus* and both Nearctic species; in the other hand the karyotype is formed with three large chromosomes as well as in the Palearctic (Table 03) (Ohmachi 1927; Johnson 1931; Montalenti et al. 1965; Hewitt 1979; Milach et al. 2016).

In the species of *O. valensis* and *O. pellucens* was seen the occurrence of one B chromosome, in both it was a small size and similar with chromosome Y (Hewitt 1979; Milach et al. 2016). In Orthoptera supernumeraries are well studied in grasshoppers, in which such chromosomes are diagnostic markers of species (Hewitt 1979). The origin of Bs is currently unclear, but some studies showed similarities of the DNA between the supernumerary and autosomes (Camacho et al. 2000; Grzywacz et al. 2017). *Oecanthus valensis* present meiotic

nuclei with a chromatic bridge during Anaphase II. It was also observed in other species of Orthoptera, usually being related with chromosomal rearranges (Warchalowska-Śliwa et al. 2005; Zefa et al. 2014a).

The C-banding in our results showed different patterns in the large chromosomes of each specie, thought all the individuals had paracentromeric band in some chromosomes, the same pattern was observed in other orthopterans, like in the genus *Gryllus* sp. (Yoshimura 2005). In *O. lineolatus* a large heterochromatic block was seen in the secondary constriction of autosome 2, like it was observed in *Gryllus assimilis* and *Eneoptera surinamensis* (Palacios-Gimenez et al. 2015). For *O. pallidus* there was a telomeric C-band in the larger autosomes 1, which differ from *G. assimilis* and species of *Paracinipe* sp., that showed terminal bands in medium and small chromosomes (Palacios-Gimenez et al. 2015; Buleu et al. 2019).

The 18S rDNA cluster was found in two autosome pairs, being unusual for crickets, but common for grasshoppers, like in *Paracinipe crassicornis*, *P. tarudantica*, and *P. parvulus* (Buleu et al. 2019). The superfamily Grylloidea showed some variations, like in *Cycloptiloides americanos*, the signals were revealed from almost all chromosomes of the karyotype, with exception of pair 5 and chromosome X_2 (Palacios-Gimenez and Cabral-de-Mello 2015). On the other hand, *G. assimilis* and *E. surinamensis* revealed the 18S signal only in pair 1, and it was co-localized in the arm with a secondary constriction (Palacios-Gimenez et al. 2015).

We believe that, the species *O. valensis* and *O. pallidus* (Neotropical Region) show derivative chromosome set, since for the karyotype evolution is expected that occurs a reduction on chromosome number, due fusion between chromosomes be more common than fission (Baker and Bickham 1980; Hemp et al. 2013). As it was observed in the genus *Saga* sp. (Tettigoniidae) where the modern karyotype is considered very different from the ancestral in number and morphology, and it is related with chromosomal inversions and/or chromosomes fusions (Warchalowska-Śliwa et al. 2009).

In Orthoptera, the sex system considered less derivate is the X0, and variations in this mechanism are more derivate (White 1978; Hewitt 1979; Blackmon et al. 2016). These variations may occur by rearrangements between the X chromosome and autosomes, and originated different mechanism, such as the neo-XY in *O. valensis* (Milach et al. 2016), X_1X_2Y found in *E. surinamensis* (Palacios-Gimenez et al. 2015), and X_1X_20 mechanism in *C. americanus* and *Endecous ubajarensis* (White 1951; Palacios-Gimenez and Cabral-de-Mello 2015; Zefa et al. 2014b).

It is supposed that after speciation the karyotypes suffer independent arrangements and modification, probably due different genetic and environmental factors that may increase rates

of chromosome mutation (Baker and Bickham 1980). Thus, the inferred molecular phylogeny and karyotypes in the present work do not completely correspond to each other, indicating independent evolutionary patterns. (Baker and Bickham 1980). Cytogenetic studies showed that in a few orders the chromosomal events could agree with phylogenetic reconstructions (Kretschmer et al. 2018). As well it was found in the genus *Aerotegmina* sp. (Tettigoniidae), where the karyotype appeared to contradict the molecular and morphology data (Hemp et al. 2013).

The present work was the first to use banding techniques and FISH in karyotypes of *Oecanthus*, and to inference the relationship of this group, using individuals from different Bioregions. This genus showed karyotype with independent evolution, when compared with the data from molecular phylogeny, but there are many species to be included in analysis. Thus, future cytogenetic and molecular studies involving more species of *Oecanthus* and related genera are needed, in order to comprehend the chromosome and group evolution.

Table 03 – Karyotype information of *Oecanthus* reviewed and studied in the present work, according with the disposable data, indicating the bioregions, species name, diploid number (2n), sex determination system, chromosome set morphology, and the author that described the karyotype.

Region	Specie	2n	Sexual system	Morphology	Authors
Palearctic	*O. longicauda*	20	XX/XY	- 6 large acrocentric - 12 dot like - X large acrocentric - Y dot like	Ohmachi 1927
Nearctic	*O. nigricornis*	19	XX/X0	- 4 large metacentric - 14 dot like - X large metacentric	Johnson 1931
Nearctic	*O. quadripunctatus*	19	XX/X0	- 4 large metacentric - 14 dot like - X large metacentric	Johnson 1931
Palearctic	*O. pellucens*	20	XX/XY	- 6 large acrocentric - 12 dot like - X large acrocentric - Y dot like	Montalenti et al. 1965
Palearctic	*O. indicus*	19	XX/X0	- 6 large acrocentric - 12 dot like - X large acrocentric	Hewitt 1979
Neotropical	*O. valensis*	18	XX/XY	- 4 large Meta/submetacentric - 12 small acrocentric - X large submetacentric - Y dot like	Milach et al. 2016 and present study
Neotropical	*O. pictus*	21	XX/X0	- 2 large metacentric - 2 large subtelocentric - 2 large acrocentric - 14 small acrocentric - X large metacentric	Present study
Neotropical	*O. pallidus*	18	XX/XY	- 4 large metacentric	Present study

				- 12 small acrocentric - X large metacentric - Y small subtelocetric	
Neotropical	*O. lineolatus*	20	XX/XY	- 4 large metacentric - 14 small acrocentric - X large submetacentric - Y dot like	Present study

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Author Contribution Statement

AF and MD conceived and designed research. EZ made the species samples available. NB and TO contributed with new methods and techniques. AF made phylogenetic and slides analysis. AF wrote the manuscript. All authors analyzed data, read and approved manuscript.

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CAPÍTULO III

CONSIDERAÇÕES FINAIS

Este trabalho visou o estudo filogenético e citogenético de quatro espécies do gênero *Oecanthus*, sendo estas *O. valensis*, *O. pallidus*, *O. lineolatus* e *O. pictus*. Filogeneticamente as relações dessas espécies com os dados disponíveis na literatura foram bem suportadas pelas análises, e mostrou que possivelmente as espécies Neotropicais sejam menos derivadas do que as que ocorrem nas Regiões Neártica e Paleártica. Nas análises citogenéticas as espécies mostraram variações cromossômicas quanto ao número, morfologia e mecanismo sexual, e foram observadas diferenças quanto às marcações utilizando técnicas de bandeamento C.

As análises filogenéticas mostraram alto suporte dos ramos, e apesar de se formarem politomias na reconstrução de genes mitocondriais, foi possível observar padrões interessantes que poderão ser melhor abordados em trabalhos futuros. Além disso, os dados moleculares gerados incrementam o conhecimento genético do gênero, acrescentando dados de espécies Neotropicais, o que não existia até então. Além disso, o aumento de estudos moleculares com outras espécies do gênero permitirá esclarecimentos sobre a história evolutiva do grupo.

O gênero *Oecanthus* apresentava seis cariótipos descritos das 72 espécies taxonomicamente válidas, sendo com este trabalho três novos conjuntos cromossômicos foram descritos. Porém, são poucos os dados gerados para o grupo, mesmo apresentando variações cromossômicas importantes relacionadas à evolução cariotípica. Também é importante ressaltar a necessidade de reanálises de algumas espécies com descrições antigas, e a dificuldade de gerar dados citogenéticos decorrente do tamanho pequeno dos cromossomos. Tornando assim, as informações geradas neste trabalho de grande importância para o estudo evolutivo dos cariótipos do grupo.

Neste trabalho foram utilizadas análises citogenéticas e filogenéticas para melhor compreensão das relações entre as espécies do gênero *Oecanthus*. As relações filogenéticas e a descrição dos cariótipos das espécies, até o momento, indicam que estes caracteres estão derivando de forma independente. Eles estão mostrando um padrão filogenético de separação clara entre grupos Neárticos, Paleárticos e Neotropicais, enquanto os padrões cromossômicos são diferentes, em relação a morfologia e mecanismo de determinação do sexo.

Portanto, a continuidade deste trabalho poderá auxiliar na compreensão dos processos evolutivos que ocorrem nessas espécies, principalmente com relação aos rearranjos cromossômicos. A falta de dados gerados para o gênero dificulta as análises atuais, gerando

gaps nos sequenciamentos e podendo levar a distorções ou erros posicionais nas reconstruções filogenéticas. Além disso, a utilização de análises integrativas permitirá um suporte maior para as futuras filogenias, e informações importantes quanto a história biogeográfica do grupo.

ANEXO I

NORMAS DE SUBMISSÃO DE ARTIGO

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