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ORGANIZAÇÃO CROMOSSÔMICA E MOLECULAR DO DNAr 5S EM DUAS ESPÉCIES DE GAFANHOTOS DO GÊNERO ABRACRIS (ACRIDIDAE/OMMATOLAMPIDINAE)

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Resumo

Os DNAs repetitivos compõem grande porção dos genomas eucariotos e estão organizados em distintos grupos, essencialmente DNAs satélite, microsatélites, minisatélites, elementos de transposição (transposons e retrotransposons) e famílias multigênicas. Estas sequências têm sido úteis nas análises cromossômicas com enfoque em estudos de diversificação cariotípica e de estrutura e evolução dos genomas. Os gafanhotos da família Acrididae representam o grupo com maior diversidade da ordem Orthoptera e apresentam do ponto de vista cromossômico ampla conservação com 2n=23,X0 (macho) na maioria das espécies estudadas. Análises enfocando o entendimento da estrutura das sequências de DNAs repetitivos neste grupo são escassas, e em geral restritas ao mapeamento de algumas famílias multigênicas. Outras sequências repetitivas, tais como DNAs satélites, genes de histonas e DNAr 5S foram realizadas principalmente em espécies de Acridídeos ocorrentes na Europa. No presente trabalho foram isolados e caracterizados do ponto de vista cromossômico e molecular o gene de DNAr 5S e seu espaçador não transcrito (NTS) nas espécies de acridídeos (Ommatolampidinae) *Abracris flavolineata* e *Abracris dilecta*. Estas análises permitiram um aprofundamento no conhecimento da estrutura/evolução desta sequência de DNA repetitivo entre as duas espécies, testando-se os possíveis modelos de evolução para esta sequência, que incluem evolução em concerto, nascimento e morte ou modelo misto.

Palavras-chave: DNAr 5S, NTS, DNAs repetitivos, evolução, genoma.

SUMÁRIO

1. Introdução

1.1 A subfamília Ommatolampidinae, taxonomia e distribuição

Os gafanhotos representantes da superfamília Acridoidea estão representados por mais de 30 famílias, estando algumas distribuídas mundialmente, como por exemplo, Acrididae, Eumastacidae e Pyrgomorphidae, e outras com distribuição exclusivamente neotropical, como por exemplo Ommexechidae, Paulinidae, Proscopiidae, Romaleidae e Tristiridae (http://orthoptera.speciesfile.org/Common/basic/Taxa.aspx?TaxonNameID=1102979, CARBONELL, 1977). A maior diversidade deste grupo é observada na família Acrididae, que possui cerca de 1.100 gêneros com grande amplitude de distribuição ambiental, habitando desde florestas tropicais até regiões semiáridas (AMEDEGNATO, 1974, CARBONELL, 1977, KEVAN, 1982, http://orthoptera.speciesfile.org/Common/basic/Taxa.aspx?TaxonNameID=1102979).

Acrididae é constituída por aproximadamente 25 subfamílias, das quais cinco apresentam ocorrência exclusiva nos neotrópicos, Copiocerinae, Leptysminae, Ommatolampidinae, Proctolabinae e Rhytidochrotinae e cinco apresentam-se distribuídas mundialmente, Acridinae, Cyrtacanthacridinae, Gomphocerinae, Melanoplinae e Oedipodinae (AMENDEGNATO, 1974, CARBONELL, 1977, KEVAN, 1982, http://orthoptera.speciesfile.org/Common/basic/Taxa.aspx?TaxonNameID=1102979). Dentre os gafanhotos com distribuição exclusiva na região neotropical a subfamília Ommatolampidinae é bastante diversificada e constituída por aproximadamente 100 gêneros e 280 espécies (CIGLIANO & LANGE, 1988), e foi inicialmente definida por Amedegnato (1974, 1977). Os representantes desta subfamília estão distribuídos nas américas do sul e central, incluindo o México e as Antilhas e diferenciam-se das outras subfamílias pelo mesonoto não visível, ou visível apenas na região posterior. Ommatolampidinae está subdividida em sete tribos das quais a tribo Abracrini é representada por 21 gêneros e cerca de 90 espécies que se encontram principalmente representadas na America do Sul (AMEDEGNATO, 1974, http://orthoptera.speciesfile.org/Common/basic/Taxa.aspx?TaxonNameID=1102979).

1.2. Classificação e organização genômica de elementos repetitivos de DNA em eucariotos e sua organização cromossômica em gafanhotos

A ampla variação de sequências e de número de cópias de DNA dos genomas eucarióticos tem sido atribuída a diferentes quantidades de DNAs repetitivos nos diversos genomas, que apresentam ampla variabilidade, constituindo distintas famílias que podem representar grande parte da quantidade de DNA das células (CHARLESWORTH et. al., 1994, GREGORY, 2005). Basicamente, os DNAs repetitivos podem estar organizados em tandem (algumas famílias multigênicas, DNAs satélites, microsatélites e minissatélites) ou dispersos nos genomas (transposons e retrotransposons), sendo os elementos de transposição e os DNAs satélites os DNAs repetitivos mais abundantes em eucariotos (MEŠTROVIĆ et. al., 2015). Segundo as hipóteses do DNA egoísta (DOOLITTLE & SAPIENZA, 1980, ORGEL & CRICK, 1980) estas sequências são mantidas no genoma por sua habilidade de colonizar regiões com taxa de recombinação ausente ou reduzida, como por exemplo, centrômeros, telômeros e cromossomos sexuais. Desde o ponto de vista funcional, algumas destas sequências repetitivas estão envolvidos na codificação de importantes proteínas e RNAs que atuam na organização e metabolismo celular. Outras além estão envolvidas na organização e funcionalidade dos centrômeros, telômeros, segregação cromossômica, regulação gênica, reparo e replicação do DNA e até diferenciação de cromossomos sexuais (ANLEITNER & HAYMER, 1992, KRAEMER & SCHMID, 1993, MESSIER et. al., 1996, MARTINS, 2007, SHAPIRO & STERNBERG, 2005, BIÉMONT & VIEIRA, 2006, FESCHOTTE & PRITHAM, 2007).

As famílias multigênicas são um grupo de sequências de DNA (genes) com notável similaridade estrutural e funcional, sendo descendentes de um gene ancestral comum (NEI & ROONEY, 2005). Dentre estas sequências são exemplos bastante conhecidos os genes de RNAs ribossomais (RNAr) representados pelo DNAr maior, o 45S e o DNAr menor, o 5S. O cluster do DNAr 45S corresponde a região organizadora de nucléolos (RON) e consiste de unidade repetitiva de três genes ou regiões codificadoras (18S, 5.8S e 28S) separadas por espaçadores transcritos internos entre estes genes (ITS1 e ITS2) e um espaçador intergênico (IGS). A unidade repetitiva do DNAr 5S consiste de um gene de aproximadamente 120 pares de base (pb) separadas

por um espaçador não transcrito (NTS), este último altamente variável em tamanho e sequência nucleotídica (LONG & DAWID, 1980, MARTINS & GALLETI Jr., 2001, PENDAS et. al., 1994, FERREIRA et. al., 2007), parecendo estar sujeito a rápida evolução (REBORDINOS et. al., 2013).

Em gafanhotos, a heterocromatina constitutiva (HC) apresenta-se bastante conservada quanto a distribuição e localização. A HC ocorre basicamente nas regiões centroméricas dos cromossomos do complemento e em menor escala nas regiões terminais, podendo também ocorrer bandas intersticiais (KING & JOHN 1980, SANTOS et. al., 1983, CABRERO & CAMACHO, 1986, SOUZA & KIDO, 1995, ROCHA et. al., 1997, SOUZA et. al., 2003, ROCHA et. al., 2011, CARVALHO et. al., 2011). Quanto à riqueza de pares de base, blocos de heterocromatina ricos em GC (CMA_3^+), tem sido frequentemente relatada nas poucas espécies estudadas, entretanto os dados ainda são incipientes para o grupo. Estes blocos tem apresentado variação relacionada a tamanho e número de cromossomos que possuem os mesmos, além disso, variações em espécies proximamente relacionadas também têm sido descritas (LORETO & SOUZA, 2000, ROCHA et al., 2004, LORETO et al., 2005, ROCHA et al., 2011).

Em gafanhotos e principalmente na família Acrididae, as análises de marcadores específicos baseados em DNAs repetitivos, tais como por exemplo famílias multigênicas, foram realizadas descrições do número e localização dos clusters de DNAr 45S e 5S, de genes de histona H3 e H4 e dos pequenos RNAs nucleares (RNAsn) (CABRERO & CAMACHO, 2008, CABRERO et al., 2009, CABRAL-DE-MELLO et al., 2011; ANJOS et al., 2015). Estes estudos são restritos as análises cromossômicas revelando localizações conservadas de DNAr para alguns grupos, como por exemplo, proximal ao centrômero na subfamília Gomphocerinae e distal em relação ao centrômero na subfamília Oediponidae em 49 espécies (CABRERO & CAMACHO, 2008). Em relação a genes de H3 e H4, estudos em 35 espécies de Acrididae correspondentes a sete subfamílias distintas revelaram uma localização altamente conservada apresentando um único cluster cromossômico, sendo as vezes a H3-H4 colocalizada na mesma região cromossômica em 11 espécies (CABRERO et. al., 2009), e adicionalmente, o gene de H3 foi localizado intersticialmente no cromossomo 8 e na região proximal do cromossomo B em *Locusta migratória*, e neste mesmo estudo, foi

detectada uma colocalização dos genes de H3 e H4 no cromossomos 8 e também no cromossomo B (TERUEL et. al., 2010).

Adicionalmente, no estudo realizado por Cabral-de-Mello et. al. (2011), foram analisadas 4 espécies pertencentes à família Proscopiidae quanto a localização cromossômica de DNAr 5S e 18S e gene de histona H3. Os genes de DNAr 18S foram mapeados em diferentes cromossomos, enquanto que histona H3 e o DNAr 5S apresentaram-se colocalizados e intercalados na mesma posição cromossômica, sendo esta associação uma possível condição basal para gafanhotos, já que a família Proscopiidae é um grupo basal para Caelifera..

No estudo desenvolvido por Anjos et. al. (2015), o gene de RNAsn U1 envolvido no processo de splicing foi mapeado em 69 espécies de Acridoidea pertencentes às famílias Proscopiidae, Pyrgomorphidae, Ommexechidae, Romaleidae e Acrididae, onde se apresentou conservado, exceto para a família Acrididae, onde foi observada extensa variação em numero e posição cromossômica. As análises genômicas em *Eyprepocnemis plorans* mostram uma associação de RNAsn U1 com outros elementos repetitivos, como por exemplo elementos moveis, sendo esta associação uma possível causa da dispersão deste gene em Acrididae (ANJOS et. al. 2015).

1.3 O DNAr 5S e seu espaçador NTS

A região codificadora de 120 pb do DNAr 5S é caracterizado pela presença de regiões conservadas, envolvidas na transcrição, como box A, elemento intermediário (IE) e box C, além da presença de um motivo poli-T no final dessa região. Para a sequência variável NTS, regiões envolvidas na expressão de gene de RNAr são também encontrados, por exemplo, sequencia TATA-like e sequências de oligonucleotídeos repetidas. Os padrões de evolução molecular do cluster de DNAr 5S (incluindo o NTS) tem sido assunto de distintos estudos e os padrões observados tem sido atribuídos à evolução em concerto e evolução por nascimento e morte, além da aceitação de um modelo misto (FREIRE et. al., 2010, FUJIWARA et. al., 2007, PINHAL et. al., 2011).

Associação de estudos citogenéticos e moleculares sobre a evolução do DNAr 5S em animais tem sido frequentemente reportados em peixe, e menos frequentemente em anfíbios e moluscos, sendo bons marcadores para identificação de espécies, análises

filogenéticas e reconstrução da história evolutiva dos cariótipos. Esses estudos têm revelado alta variabilidade em número e posição de clusters cromossômicos e em sequências distintas de NTS. Tanto em vertebrados (MARTINS & GALETTI Jr., 2001, MARTINS et. al., 2002, WASKO et. al., 2001, RODRIGUES et. al., 2012, MERLO et. al., 2013) como em invertebrados (INSUA et. al., 2001, PERINA et. al., 2011, FREIRE et. al., 2005, VIERNA et. al., 2009, VIZOSO et. al., 2011, VIERNA et. al., 2011) tem sido relatado a presença de dois ou mais tipos de DNAr 5S NTS com diferentes tamanhos devido seus variáveis espaçadores NTS.

Em gafanhotos, o conhecimento da organização do DNAr 5S é baseado somente em estudos cromossômicos, com mapeamento de regiões codificadoras em várias espécies, como por exemplo, as espécies pertencentes às família Proscopiidae, Acrididae e Romaleidae (CABRAL-DE-MELLO, et. al., 2010., CABRAL-DE-MELLO et. al. 2011, PALACIOS-GIMENEZ, et. al. 2013, BUENO et. al., 2013., ANJOS et. al., 2013). Esses estudos tem identificado uma extensa variedade em número e posição desses marcadores, embora a ocorrência de dois sítios (um bivalente) é uma situação comum (CABRAL-DE-MELLO et. al., 2011). Adicionalmente, a co-localização do gene de DNAr 5S com outras sequências, tal como do gene de histona H3, foi também reportado (CABRAL-DE-MELLO et. al., 2011).

1.4 Mapeamento cromossômico de DNAr 5S e seu espaçador NTS

Quanto ao mapeamento físico cromossômico de DNAs repetitivos, a sequência de DNAr (5S) apresentam ampla variabilidade (CABRERO & CAMACHO, 2008, CABRAL-DE-MELLO et. al., 2011, BUENO et. al., 2013, CALADO et. al., 2014, SUPIWONG et. al., 2014). Em vertebrados, como anfíbios e peixes, tem sido relatado a maioria dos mapeamentos físico dos cromossomos envolvendo a localização dos dois tipos de DNAr 5S, diferenciados devido a variação de sequências e do tamanho da região NTS de cada um (DA SILVA et. al., 2011, RODRIGUES et. al., 2012).

Em invertebrados, poucos estudos envolvendo mapeamento físico cromossômico de DNAr 5S e seu espaçador NTS foram relatados (INSUA et. al., 1999, INSUA et. al., 2001, PELLICCIA et. al., 1998). Os estudos com grupos de invertebrados envolvendo mapeamento físico de DNAr 5S e seu espaçador NTS são

escassos quando comparados à vertebrados. Para insetos, destaca-se a ausência de dados em relação ao mapeamento físico de DNAr 5S e seu espaçador NTS, evidenciando o quanto é necessário estudos a cerca deste assunto.

O uso de elementos repetitivos de DNA descritos acima tem se mostrado uma ferramenta esclarecedora para diversas questões, desde o entendimento da estrutura de diferentes regiões cromossômicas, tais como centrômeros e telômeros, até análises relacionadas à diversificação cariotípica, incluindo cromossomos autossômicos e origem e evolução de cromossomos sexuais e supernumerários. Além disso, o mapeamento físico cromossômico do genoma tem contribuído no entendimento da estrutura e evolução dos genomas eucariotos, principalmente em relação aos DNAs repetitivos.

2. Objetivos

2.1. Objetivo geral

Neste estudo foram utilizadas duas espécies de gafanhotos, *Abracris flavolineata* e *Abracris dilecta* (Acrididae: Ommatolampidinae) para entender os padrões de diversificação de DNAr 5S e seu espaçador tanto do ponto de vista molecular como cromossômico.

2.2. Objetivos específicos

- Isolar através da PCR e sequênciar o DNAr 5S e seu espaçador NTS de duas espécies de gafanhotos do gênero *Abracris*, buscando entender os padrões de diversificação molecular dos mesmos do ponto de vista intra- e interespecífico;
- Mapear os distintos tipos de DNAr 5S+NTS nos cromossomos de duas espécies de *Abracris*, buscando entender sua dinâmica nos cariótipos e possível relação com os processos de diversificação molecular das sequências.

3. Materiais e Métodos

3.1 Animais, obtenção de cromossomos e extração de DNA

Machos adultos de *Abracris flavolineata* (65 indivíduos) e *A. dilecta* (18 indivíduos) foram coletados em Missiones, Posadas/Argentina e Rio Claro, São Paulo/Brasil com autorização do ICMBio SISBIO (número de processo 16009-1). Os cromossomos foram obtidos dos folículos de testículos ou do ceco gástrico seguindo o protocolo descrito por Castillo et al. (2011). Animais inteiros foram armazenados em 100% de etanol no freezer a -20ºC destinado ao uso de extração de DNA seguindo o protocolo de Sambrook & Russel (2001).

3.2 Amplificação por PCR, clonagem e análise de sequências

O DNAr 5S e a região NTS foram obtidos por PCR usando os primers Sca5Sf e Sca5Sr. A mistura de PCR foi constituída de um volume total de 25 μl que sendo submetida a ciclos de 94ºC durante 5 minutos iniciais, 30 ciclos de 94ºC durante 30 segundos, 55°C durante 30 segundos e 72ºC durante 80 segundos seguidos por uma extensão final à 72ºC durante 5 minutos.

O produto de PCR foi separado em gel de agarose 1% e as bandas de DNA obtidas foram purificadas usando o Wizard Genomic DNA Purification Kit (Promega) de acordo com as recomendações do fabricante. O produto de PCR purificado foi então ligado ao plasmídeo pGEM-T (Promega, Madison, WI, USA), que posteriormente foram inseridos em células competentes DH5 de *Escherichia coli.* Clones positivos foram sequenciados pelo sequenciador de DNA automático ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA) com Dynamic Terminator Cycle Sequencing Kit (Applied Biosystems).

3.3 Análise das sequências

A qualidade das sequências foi determinada usando o software Geneious (DRUMMOND et. al., 2009). As sequências consenso foram submetidas a pesquisa mediante BLAST (ALTSCHUL et. al., 1990) no NCBI. Para todas as sequencias a

regiões correspondentes a os primers foram descartadas. Analises estadísticos de polimorfismos de DNA foram feitos no software DnaSP v.5.10.01 (LIBRADO & ROZAS, 2009). Os analises filogenético e moleculares foram realizados usando MEGA v.5 (TAMURA et. al., 2011).

3.4 Hibridização in situ fluorescente

A hibridização *in situ* fluorescente foi realizada seguindo o protocolo proposto por Pinkel et. al. (1986) com as modificações indicadas por Cabral-de-Mello et. al. (2010). As amostras para DNAr 5S tipo I foi tratada com digoxigenina-11-dUTP (Roche) e o DNAr 5S tipo II com biotina-14-dATP (Invitrogen) através de PCR e nick translation, respectivamente. Amostras tratadas com digoxigenina-11-dUTP foram detectadas usando anti-digoxigenina-rodamina (Roche), e amostras tratadas com biotina-14-dATP foram detectadas usando streptavidina, alexa fluor 488 conjugado (Invitrogen). As preparações foram contrastadas usando DAPI e montada usando Vectashield (Vector, Burlingame, CA, USA). Os cromossomos e os sinais da FISH foram observados com microscópio Olympus BX61 equipado com uma lâmpada fluorescente e filtros apropriados. As fotografias foram registradas usando a câmera digital DP70 resfriada. As imagens foram otimizadas com brilho e contraste usando o software Adobe Photoshop CS2.

4. Resultados e discussão

Os dados obtidos neste trabalho estão apresentados em formato de manuscrito a ser submetido a revista "Molecular Genetics and Genomics" (anexo 1). O mesmo já encontra-se em fase final de correção para submissão.

5. Conclusões gerais

Os dados obtidos indicam distinta organização com ocorrência de duas classes de DNAr 5S nas espécies de gafanhoto estudadas, assim como tem sido amplamente descrito em outros grupos animais, principalmente vertebrados. Além disso, é marcante a possível influência da organização cromossômica das distintas unidades de DNAr 5S em sua evolução. Isto tem como consequência (i) a ocorrência dos mecanismos de nascimento e morte, com geração de pseudogenes, e (ii) processo de evolução concertada que causa homogeneização entre as repetições das duas diferentes unidades de 5S. Para a unidade maior de DNAr 5S em ambas as espécies a presença de sequências similares a elementos de transposição sugerem a possível influência desta outra classe de DNA repetitivo na dispersão cromossômica observada para este marcador.

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7. ANEXO 1: The 5S rDNA in two *Abracris* grasshoppers (Ommatolampidinae: Acrididae): molecular and chromosomal organization

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Short running title: 5S rDNA in the *Abracris* grasshoppers

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Abstract

In higher Eukaryotes the 5S ribossomal DNA (rDNA) sequences are repeated units mostly tandemly arrayed. Each unit is composed by a conserved coding region and by a variable non-transcribed spacer sequence (NTS). This multigene family is subject of a highly evolutionary dynamics at molecular and chromosomal points to view, mainly for NTS. Among grasshoppers this multigene family was studied under chromosomal analysis being established the number and position of clusters in about 50 species, but few molecular information to understand the patterns of evolution was obtained. In order to identify the evolutionary dynamics of 5S rDNA in grasshopper genomes here we integrated data from chromosomal and sequencing analysis in two related species, i.e. *Abracris dilecta* and *A. flavolineata*. In each species two arrays of 5S rDNA were identified, consisting of units with 850 and 130 bp for *A. dilecta* and 880 and 130 bp for *A. flavolineata*. The larger sequences (named type-I) contained the entire 5S rDNA gene, while for the smaller (named type-II) only 42 bp corresponded to the gene sequence ant it was considered a pseudogene. The type-I fragments contained the internal control region (ICR), i.e. A box, intermediate element (IE) and C box and the poly-T motif was evidenced in the beginning of NTS region, suggesting functionality. Moreover in the NST of type-I partial transposable elements were identified, that could influenced in the type-I chromosomal dispersion. Some differences for type-I were noticed between the species while type-II were identical, suggesting the pseudogenization in a common ancestor. In the tree obtained using neighbor joining analysis the type-II grouped together and the type-I were not grouped for both species. At intraspecific level these sequences presented distinct degree of variability, depending of the region, i.e. 5S rDNA gene or NTS region. Fluorescent *in situ* hybridization

(FISH) identified two clusters (one bivalent) of type-II, while the type-I occurred in multiple copies in distinct chromosomes. Our data suggest that the distinct organization for 5S rDNA noticed in other animal groups is also present among grasshoppers and in *Abracris* the evolution of this multigene family is influenced by the chromosomal distribution and number of clusters, resulting in concerted evolution and birth-and-death depending of the unit.

Key-words: FISH, genome, multigene Family, non-trascribed spacer, repetitive DNAs

Introduction

A multigene family is a group of multiple genes descendent from a common ancestor presenting similar sequences and functional role (Nei and Rooney 2005). The ribossomal RNA (rRNA) genes in higher Eukaryotes are represented by two distinct multigene families constituted by hundreds to thousands copies tandemly arrayed occupying one or more chromosomal loci. The 45S ribosomal DNA (rDNA) comprise three distinct genes separated each other by the internal transcribed spacer (ITS), coding for the 18S, 5.8S and 28S rRNAs. While the 5S rDNA is formed by multiple repeats of a 120 bp sequence highly conserved separated each other by non-transcribed spacer called NTS, that is subject of rapid evolution presenting variability in size and nucleotide composition (reviewed by Long and Dawid 1980). Based in the similarity between the 5S rDNA sequences traditionally some studies propose that this multigene family is subject of concerted evolution, a model in which multiple copies of a sequence are homogenized (Ney and Rooney 2005). On the contrary, other studies have proposed that in some genomes the 5S rDNA could also evolves through birth-and-death evolution or in a mixed effects of concerted and birth-and-death evolution (see for

example Rooney and Ward 2005; Úbeda-Manzanaro et al. 2010; Perina et al. 2011; Pinhal et al. 2011; Vierna et al. 2011, 2013; Vizoso et al. 2011; Merlo et al. 2013).

Cytogenetic or molecular studies aiming the understand 5S rDNA genome organization and evolution were frequently performed in fish (Martins and Galetti Jr 2001; Wasko et al. 2001; Martins et al. 2002; Rebordinos et al. 2013), and in a lesser extend in other animals, as for example amphibians (Rodrigues et al. 2012), crustaceans (Perina et al. 2011) and mollusks (Vierna et al. 2011). These studies pointed that the 5S rDNA and its NTS are good markers for species identification, phylogenetic analyses, karyotype ongoing reconstruction and to understanding genome evolution. High variability in number of chromosomal clusters has been noticed for 5S rDNA with few or multiple clusters per genome, besides the occurrence of scattered copies (Little and Braaten 1989). The linkage of 5S rDNA+NTS with other types of sequences, like other multigene families, transposable elements (TEs) and microsatellites has also been reported (Drouin and Moniz de Sá 1995; Cross and Rebordinos 2005; Merlo et al. 2013; Anjos et al. 2015).

Among grasshoppers the knowledge of 5S rDNA was primarily obtained from chromosomal analysis. Until now the cytogenetic mapping through fluorescent *in situ* hybridization (FISH) was performed in about 50 species belonging to distinct families Acrididae (Cabral-de-Melo et al. 2011a, Palacios-Gimenez et al. 2013, 2015), Romaleidae (Anjos et al. 2013; Neto et al. 2013) and Proscopiidae (Cabral-de-Mello et al. 2011b). On the other hand, few molecular studies aiming to understand the organization and evolution of 5S rDNA were performed. Here we used two congeneric grasshopper species, *Abracris dilecta* and *A. flavolineata* (Acrididae: Ommatolampidinae) as subject to understand the patterns of diversification of 5S rDNA. Two distinct kinds of 5S rDNA units in each species were recognized and their

nucleotide sequence were characterized and used as probes for chromosomal mapping through FISH. The results of molecular and chromosomal data are discussed to shed light of possible mechanism involved in the 5S rDNA+NTS, increasing the knowledge about evolution of multigene families among grasshoppers.

Materials and Methods

Animals, chromosome obtaining and DNA extraction

Male and female adults from *Abracris dilecta* (08 individuals) were collected in Missiones, Posadas, Argentina and from *A. flavolineata* (12 individuals) were collected in Rio Claro, São Paulo, Brazil. The chromosomes were obtained from testis or from gastric caecum following the protocol described by Castillo et al. (2011). Entire animals were stored in 100% ethanol in freezer -20oC until the use for DNA extraction following the protocol proposed by Sambrook and Russel (2001).

5S rDNA isolation, cloning and sequence analysis

The 5S rDNA and NTS region were obtained through PCR using degenerated divergent primers designed using as reference the alignment of 5S rDNA gene sequences from insects, 5S-NTS-F 5'TACCGGTTCTCGTCCGATCAC and 5S-NTS-R 5'TACAGCGTGCTATGGCCGTTG. The PCR was carried out using 10xPCR Rxn Buffer, 0.2 mM MgCL2, 0.16 mM dNTPs, 2 mM each primer, 1U of *Taq* Platinum DNA Polymerase (Invitrogen, San Diego, CA, USA) and 50-100 ng/µl template DNA. The mix was subjected an initial denaturation at 94 ºC (5 min) and 30 cycles at 94 ºC (30s), 55 ºC (30s), and 72 ºC (80s), plus final extension at 72 ºC for 5 min. The PCR product were separated in 1% agarose gel and the DNA bands were purified using the kit Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corp., The Epigenetics

Company, USA) according to manufacturer's recommendations. The purified PCR products were ligated to the plasmid pGEM-T (Promega, Madison, WI, USA), and the recombinant constructs were used to transform DH5α *Escherichia coli* competent cells. Positive clones were sequenced using an ABI Prism 3100 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a Dynamic Terminator Cycle Sequencing Kit (Applied Biosystems).

Sequence analysis

The quality of the sequences was determined using the Geneious 4.8.5 software (Drummond et al. 2009). The consensus sequences were subjected to BLAST (Altschul et al. 1990) searches on the NCBI website (http://www.ncbi.nlm.nih.gov/blast) and, as expected, were recognized as 5S rRNA genes, plus NTS regions. The sequences were deposited into the NCBI database under the following accession numbers: XXX0000 and XXX1111. For DNA sequence analyses the basic sequence statistics were computed with the program DnaSP v.5.10.01 (Librado and Rozas 2009). Phylogenetic and molecular evolutionary relationships among sequences were inferred by neighbor-joining (NJ) tree using the implemented option in MEGA5 (Tamura et al. 2011) and the proportion of nucleotide differences (*p* distance).

Fluorescent* in situ *hybridization

The Fluorescent *in situ* hybridization were performed following the protocol proposed by Pinkel et al. (1986) with the modifications indicated by Cabral-de-Mello et al (2010). The probes were labeled with digoxigenin-11-dUTP (Roche) or biotin-14-

dATP (Invitrogen) through PCR and nick translation, respectively. Probes labeled with digoxigenin-11-dUTP were detected using anti-digoxigenin-rhodamine (Roche), and probes labeled with biotin-14-dATP were detected using streptavidin, alexa fluor 488 conjugate (Invitrogen). The preparations were counterstained using 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) and mounted using Vectashield (Vector, Burlingame, CA, USA). The chromosomes and FISH signals were observed using an Olympus microscope BX61 equipped with a fluorescence lamp and appropriate filters. The photographs were recorded using a DP70 cooled digital camera. The images were merged and optimized for brightness and contrast using Adobe Photoshop CS2 software.

Results

Electrophoresis of 5S rDNA PCR amplification revealed two fragments distinct in sizes for each species. The larger fragments were approximately 850 bp for *A. dilecta* 880 bp for *A. flavolineata*, while the small ones were approximately 130 bp in both species. The larger fragments were named type-I and the smaller type-II and these fragments will be referred as Ad-type-I and Ad-type-II for *A. dilecta* and Af-type-I and Af-type-II for *A. flavolineata*. The consensus sequences from a total of 23 clones, being 11 from type-I (six from *A. dilecta* and 5 from *A. flavolineata*) and 12 from type-II (five from *A. dilecta* and seven from *A. flavolineata*) were obtained for analysis. The comparison of the PCR fragments with the 5S rDNA coding region from *A. flavolineata* previously isolated (Gene bank access number KC936996) confirmed the isolation of entire 5S rDNA units or parts of them. For the larger fragments the entire 5S rDNA 120 bp gene was recognized for all clones, on the other hand, the smaller fragments contained invariably only 41 bp of 5S rDNA gene was recognized and they were

considered pseudogenes. The remaining base pairs for the type-I and type-II sequences in each species were considered NTS regions (Figure 1). The comparison between the large and small fragments confirmed that the small ones harbor only 41 bp of 5S rDNA gene, corresponding to the initial region of the gene.

The type-I fragments contained the internal control region (ICR), i.e. A box, intermediate element (IE) and C box. The poly-T motif was evidenced in the beginning of NTS region for Ad-type-I and Af-type-I. These elements were not noticed in the type-II fragments (Figure 1). The NTS regions varied between 588 pb and 591 bp for Ad-type-I and between 707 bp and 732 bp for Af-type-I, and these differences were related to base deletions or insertions. For Ad-type-II and Af-type-II the NTS were identical in size for all clones, being 89 bp long (Table 1). The search for similarity with other repetitive elements using transposable elements database from RepBase (http://www.girinst.org/repbase/) using CENSOR software identified gypsy and SINE (Short Interspersed Nuclear Element) fragments for Ad-type-I and of a gypsy fragment for Af-type-I (Figure 2a). For the Ad-type-II and Af-type-II NTS no repetitive sequences associated were noticed.

The FISH analysis using as probes of type-I and type-II sequences of both species resulted in distinct patterns of chromosomal organization for these elements. While the type-I was located in multiple chromosomal loci the type-II was placed only in two chromosomes, corresponding to one bivalent. In *A. dilecta* the type-I sequence was placed in chromosomes 1, 11 and X, proximally, terminally and interstitially located, respectively. For *A. flavolineata* the type-I sequence was located in pairs 1, 2 and 5, presenting terminal, interstitial and proximal location. The type-II sequence was placed proximally in pair 11 of *A. dilecta* and interstitially in pair 2 of *A. flavolineata* (Figure 2b). For *A. flavolineata* no signal were recognized in the B chromosome.

The NTS of type-I sequences were highly differentiated at interspecific level, while the NTS from type-II were remarkably conserved (Figure 1). These patterns were also evidenced through neighbor joining analysis, reinforcing the occurrence of two classes of 5S rDNA in both species, that are interespecifically divergent for type-I and well conserved for type-II (Figure 3). At intraspecific point to view we noticed distinct nucleotide variability depending of the species, sequence (type-I or type-II), and the specific region, i.e. 5S rDNA or NTS. Considering the entire 5S rDNA unit the type-I was most variable than type-II sequences, but considering each region, the 5S rDNA and NTS separately distinct patterns emerged (Table 1).

Discussion

The organization of distinct classes of 5S rDNA at molecular and/or chromosomal point to view is widely known in vertebrates, mainly fish (see for example Martins and Galetti 2001; Pinhal et al. 2011; Merlo et al. 2012a,b, 2013), an in lesser extent in other groups it was also characterized, like in amphibians (Rodrigues et al. 2012), crustaceans (Perina et al. 2011), mollusks (Vierna et al. 2011). The detection of at least two distinct units of 5S rDNA as observed in the two species studied here were frequently reported, like in some fish (Martins et al. 2001), *Engystomops* (Rodrigues et al. 2012) and *Xenopus* (Harper et al. 1983) amphibians, crustaceans, mollusks and mammals (Jensen and Frederiksen 2000; Vierna et al. 2011). According to Pinhal et al. (2011) the accumulated data in teleost fish and elasmobranches fish suggest that the presence of two distinct classes of 5S rDNA is a general trend. Moreover in fish more 5S rDNA variants up to ten in *Diplodus sargus* (Merlo et al. 2013) were also observed, as also in some filamentous fungi with eight variants (Rooney and Ward 2005) and *Pollicipes* crustacean species with seven types considering a group of three

species (Perina et al. 2011). A remarkable pattern is that frequently the units isolated in some species present the entire 5S rRNA gene region or a significant part, differing from *Abracris* species, in which one of the units lost significant part of 5S rRNA gene. It was the case of type-II unit that putatively after duplication diverged from ancestral sequence, being pseudogenized due to the deletion of a large gene region, conserving only a short initial part of the 5S rRNA ancestral gene. Interestingly, the occurrence of this truncated pseudogene with high similarity in the genome of both *Abracris* species suggests an origin for this sequence in the common ancestor.

The occurrence of ICs that function as internal promoters and downstream T-rich region located at the 3'end of the putative coding region in the type-I sequences suggest that they may have potential transcription, representing the functional unit in the genome of *A. dilecta* and *A. flavolineata*. On the contrary the type-II unit is certainly a pseudogene sequence due its short size. Truncated sequences were also reported in species from fish (Martins et al. 2002), filamentous fungi (Rooney and Ward 2005) and human (Nederby-Nielsen et al. 1993). This pattern observed here and in other animal groups, i.e. occurrence of pseudogenes, is a strong evidence of birth-and-death evolutionary process (Rooney and Ward 2005), and it was commonly observed, as in stingrays (Pinhal et al. 2011), *Pollicipes* crustaceans (Perina et al. 2011) and razor shells (Vierna et al. 2011). In this process the number of a given sequence in the genome could be increased due to duplication and the duplicated copies accumulate divergence, causing pseudogenization. On the other hand, presumably concerted evolution mechanism that led to the homogenization of copies, is also operating in 5S rDNA evolution. Some authors have documented the both evolutionary mechanisms acting in the same genome (Pinhal et al. 2011). Apparently the mixed evolution is applicable for 5S rDNA of *Abracris* species, that present remarkable divergence between the two 5S

rDNA unit types probably caused by birth-and-death mechanism, but the sequence for each unit type share homology, being probably resultant from concerted evolution.

Our FISH data suggests that at least some 5S rDNA sequences from distinct units are in tandem organized, allowing the detection of evident blocks in distinct chromosomal regions, but the occurrence of dispersed repeats could not be ruled out. The distinct chromosomal location, including distinct chromosomes, for the clusters of the two types of 5S rDNA units certainly contributes for the accumulation of differences between them and the in tandem organization facilitates the independent homogenization process in each cluster, causing independent evolution through concerted fashion. This was also suggested for distinct fish species in which two distinct classes of 5S rDNA were detected and evolve separately due different chromosomal location (Martins and Galetti Jr 2001). Although, divergent organization with different 5S rDNA types evolving in the same chromosome was also documented, in in the Cichlid fish *Oreochromis niloticus* (Martins et al. 2002).

It is noticeable that the type-II sequences were less variable, considering the nucleotide diversity, when compared with type-I, in both cases, considering the entire regions and the 5S rRNA gene and NTS region. Due to the putative functionality of type-I sequences it was expected a higher conservation than to type-II, but in the *Abracris* species two main aspects should be considered that could explain the inversed pattern, (i) the type-I is much more larger than type-II allowing the accumulation of more mutations and (ii) the type-I is located in both species in multiple loci located in distinct chromosomes, that could facilitate the accumulation of differential mutations between them, favoring diversification.

The occurrence of gypsy and/or SINE transposable elements fragments in the NTS of type-I sequences in *Abracris* species could be involved their spreading to

distinct chromosomes, favoring the variability noticed for these sequences. Other cases of transposable elements association with 5S rDNA units mediating sequence dynamism was also documented in the fish *Diplodus sargus*. At chromosomal point to view in the fish *Gymnotus paraguensis* the multiplication of 5S rDNA to 19 chromosomal pairs was attributed to the ptesence of the transposable element Tc1-like in the NTS (da Silva et al. 2011).

Finally our data reveals that the distinct organization for 5S rDNA noticed in other animal groups is also present among grasshoppers. Moreover it is highlighted the impact of chromosomal organization and association with other repetitive sequences, like TEs, in the evolutionary history of 5S rDNA in *Abracris* grasshoppers, leading to distinct fates, i.e. high variability or conservation, resultant of concerted evolution or birth-and-death. Among other grasshoppers higher chromosomal variability for 5S rDNA gene was noticed, with species presenting multiple clusters, including clusters in all chromosome pairs, which predicts possible association with TEs (Cabral-de-Mello et al. 2011), like for *Abracris* species. The diversification of clusters number could be followed by higher sequence variability and some species of grasshoppers serve as good models to test this aspect.

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Figure legends

Figure 1. Alignments of monomeric consensus units of type-I and type-II sequences of *A. dilecta* and *A.flavolineata*. The 5S rDNA gene or pseudogene regions are indicated and the rest of the sequence corresponds to the NTS regions. Note for type-I the difference in size and variability of the sequence between the species and the complete similarity for the type-II. In the type-I the ICR are also indicated.

Figure 2. (a) Schematic representation of the two 5S rDNA units observed in *A. dilecta* and *A. flavolineata*. In NTS region of type-I units the regions with similarity with TEs are indicated; (b) Chromosomal mapping of 5S rDNA units in metaphase I chromosomes of *A. dilecta* and female mitotic metaphase of *A. flavolineata*. Note the single bivalent bearing the type-II and the multiple signals for type-I. Chromosomes bearing signals are indicated and the arrowhead shows the B chromosome. Bar = 5μm.

Figure 3. Neighbor-joining tree infered from 5S rDNA units from *A. Dilecta* and *A. flavolineata*. Type I unit (green) and type-II (red). Note the grouping of type-II sequences, while the type-I is species specific.

Tables

Table 1. Polymorphism by 5S rDNA units regions in *Abracris* species.

	Region	**size**	**n**	**m**	**s**	**h**	**π**	**sd**
Type I								
A.dilecta	entire	713 to 714	6	85	83	6	0.04922	0.00983
	5S rDNA	120	6	9	9	3	0.02500	0.01414
	NTS	588 to 591	6	76	74	6	0.05417	0.00991
A.flavolineata	entire	858	5	39	37	5	0.02236	0.00379
	5S rDNA	120	5	13	12	5	0.05083	0.01308
	NTS	707-732	5	26	25	5	0.01750	0.00291
Type II								
A.dilecta	entire	130	5	4	4	5	0.01385	0.00296

	5S rDNA	41	5	1	1	2	0.00976	0.00579
	NTS	89	5	1	1	2	0.00833	0.00494
A.flavolineata	entire	130	7	6	6	3	0.02198	0.00639
	5S rDNA	41	7	0	0	1	0.00000	0.00000
	NTS	89	7	6	6	3	0.0321	0.00933

n= number of sequences; m= number of mutations; s= number of polimorphyc sites; h= number of haplotypes; π= nucleotide variability; sd= standard deviation.


Type-I
putative functional gene
A box
IE
C box
Consensus identity
A. dilecta
A. flavolineata
Consensus identity
A. dilecta
A. flavolineata
Consensus identity
A. dilecta
A. flavolineata
Type-II
pseudogene
Consensus identity
A. dilecta
A. flavolineata

Figure 1

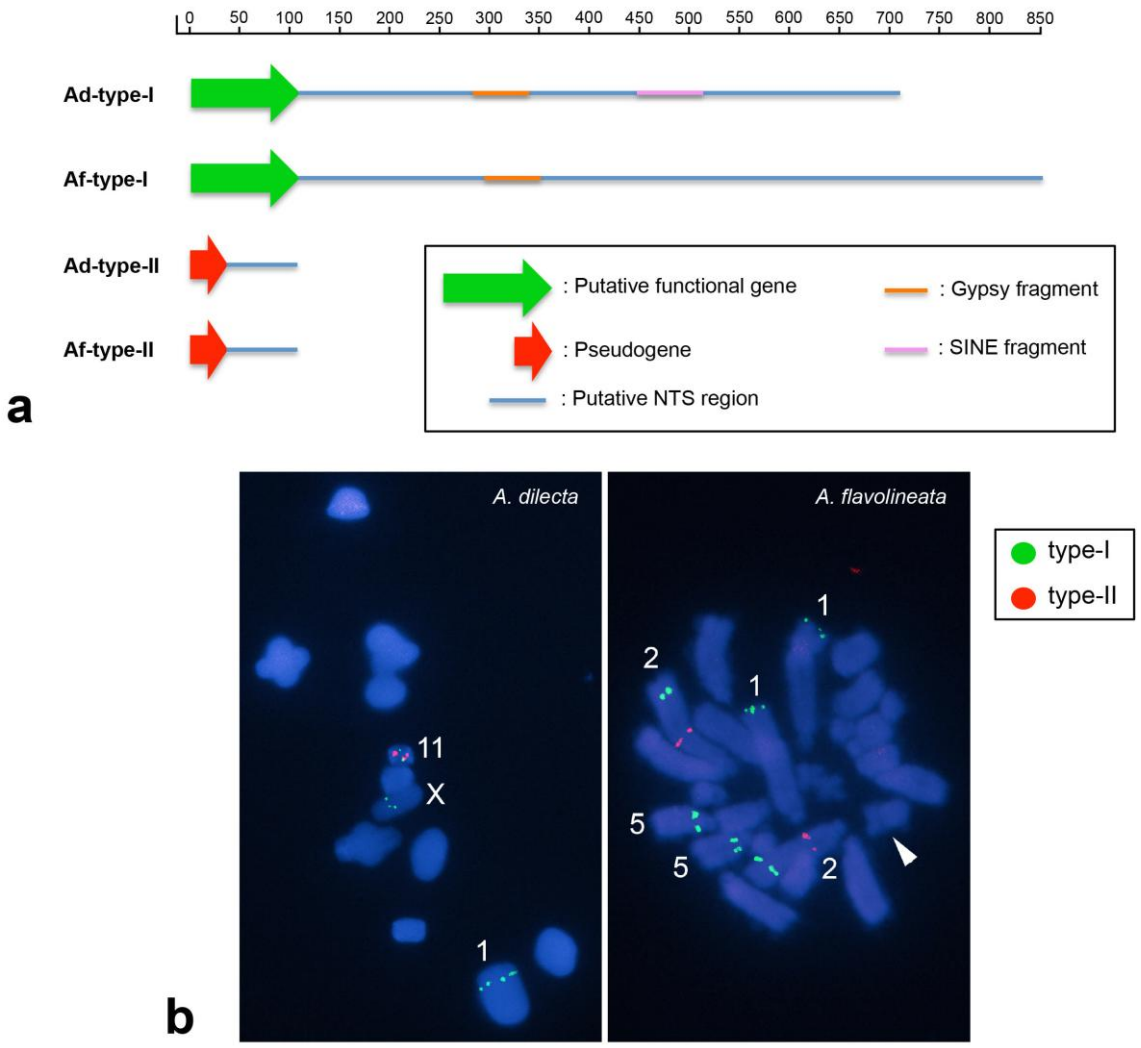
0 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850
Ad-type-I
Af-type-I
Ad-type-II
Af-type-II
: Putative functional gene
: Pseudogene
: Putative NTS region
: Gypsy fragment
: SINE fragment
a
A. dilecta
A. flavolineata
type-I
type-II
11
X
1
2
1
1
5
5
2
b

Figure 2

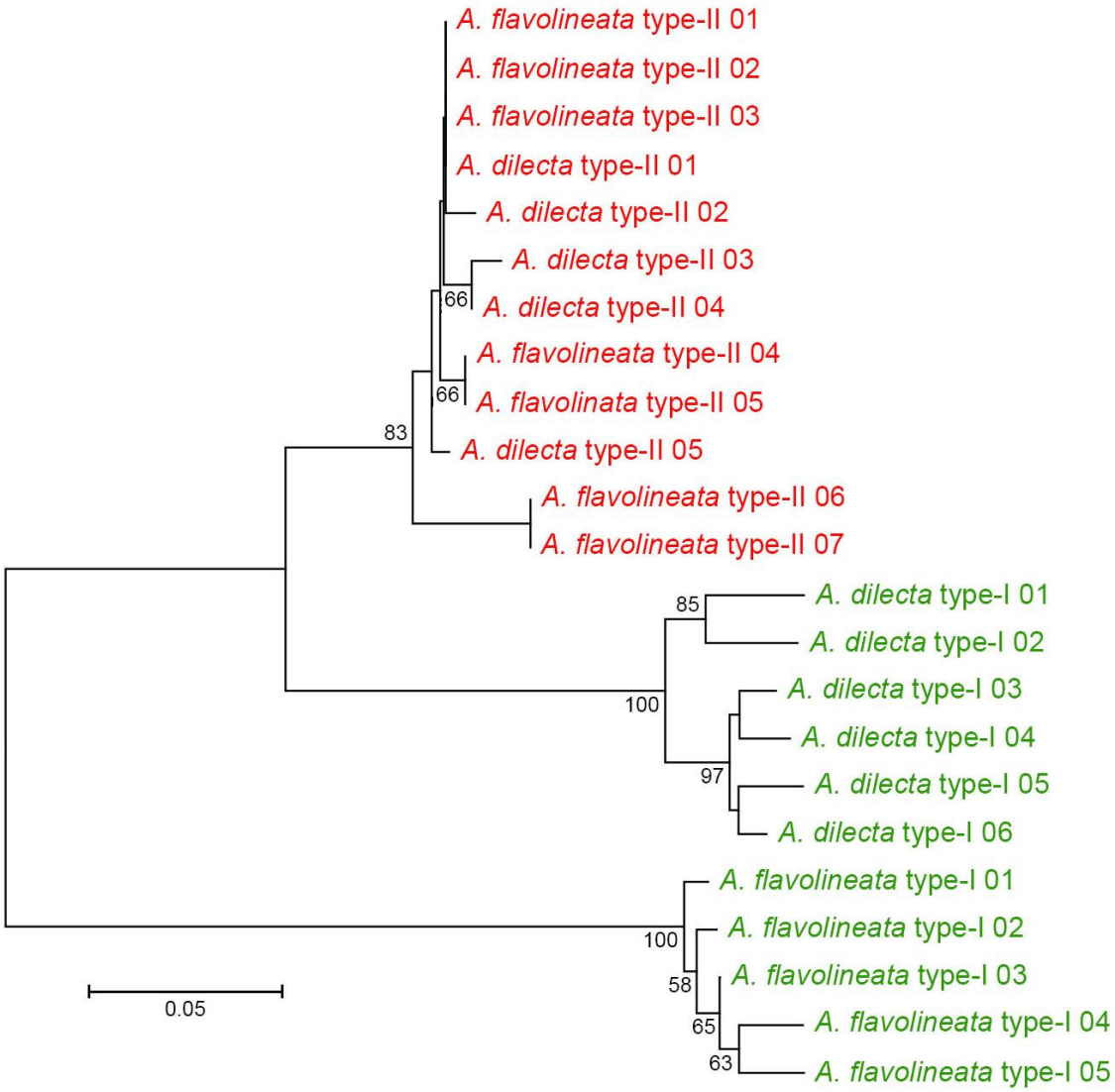
A. flavolineata type-II 01
A. flavolineata type-II 02
A. flavolineata type-II 03
A. dilecta type-II 01
A. dilecta type-II 02
A. dilecta type-II 03
66
A. dilecta type-II 04
A. flavolineata type-II 04
66
A. flavolinata type-II 05
83
A. dilecta type-II 05
A. flavolineata type-II 06
A. flavolineata type-II 07
85
A. dilecta type-I 01
A. dilecta type-I 02
100
A. dilecta type-I 03
A. dilecta type-I 04
97
A. dilecta type-I 05
A. dilecta type-I 06
A. flavolineata type-I 01
100
A. flavolineata type-I 02
58
A. flavolineata type-I 03
65
A. flavolineata type-I 04
63
A. flavolineata type-I 05
0.05

Figure 3