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A MaSp2-like gene found in the Amazon mygalomorph spider *Avicularia juruensis*

Daniela Bittencourt [a,*], Katharina Dittmar [b], Randolph V. Lewis [c], Elíbio L. Rech [d]

[a] Laboratory of Molecular Biology, EMBRAPA Western Amazon, Brazil
[b] Department of Biological Sciences, SUNY at Buffalo, USA
[c] Department of Molecular Biology, University of Wyoming, USA
[d] Laboratory of Gene Transfer, Biotechnology Unit, EMBRAPA Genetic Resources and Biotechnology, Brazil

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ABSTRACT

Two unique spidroins are present in the silk of the Amazon mygalomorph spider — *Avicularia juruensis* (Theraphosidae), and for the first time the presence and expression of a major ampullate spidroin 2-like in Mygalomorphae are demonstrated. Molecular analysis showed the presence of $(GA)_n$, poly-A and GPGXX motifs in the amino acid sequence of Spidroin 2, the last being a motif described so far only in MaSp2 and Flag spidroins. Phylogenetic analysis confirmed the previously known orthologous silk gene clusters, and placed this gene firmly within the orbicularian MaSp2 clade. Gene tree–species tree reconciliations show a pattern of multiple gene duplication throughout spider silk evolution, and pinpoint the oldest speciation in which MaSps must have been present in spiders on the mygalomorph–araneomorph split, 240 MYA. Therefore, while not refuting orb weaver monophyly, MaSp2s, and major ampullate silks in general cannot be classified as orbicularian synapomorphies, but have to be considered plesiomorphic for Opisthothelae. The evidence presented here challenges the simplified notion that mygalomorphs spin only one kind of silk, and adds to the suite of information suggesting a pattern of early niche diversification between Araneomorphae and Mygalomorphae. Additionally, mygalomorph MaSp2-like might accommodate mechanical demands arising from the arboreal habitat preference of *Avicularia*.

1. Introduction

The visually most elaborate spider webs have evolved within the orb-weaving spiders — the Araneoidea and the Deinopoidea. Their nets have intricate frames and radial support lines, which are spun from silks mechanically superior to most other synthetic or natural high-performance fibers (Blackledge and Hayashi, 2006). Both lineages are highly likely to have evolved from one common ancestor (Garb et al., 2006), which formed part of the infraorder Araneomorphae. However, Mygalomorphae do not build orb webs, and only use their silk for protecting their eggs or lining burrows. Mygalomorphae are the sistergroup to Araneomorphae, and based on the oldest known spider fossil, *Rosamygale grauvogeli* (Selden and Gall, 1992), the split of both lineages is estimated at ca. 240 million years ago (MYA) (Vollrath and Selden, 2007).

Today, most data for spider silk proteins derive from orb-weaving spiders. The function of each spidroin is a result of tandem-arrayed repeats of ensemble motifs composed of smaller amino acid combinations (Gatesy et al., 2001; Hayashi and Lewis, 2000). Additionally, silk genes have non-repetitive, highly conserved carboxy terminal (C-terminal) which have been repeatedly used for the reconstruction of silk gene family evolution (Challis et al., 2006; Garb and Hayashi, 2005). Generally, each different silk is made in distinct glands, which is why silks are commonly classified according to their gland of origin (Vollrath, 1992). Orb-weaving spiders (Araneomorphae: Orbicularia) can have up to seven morphologically distinct glands, and three of them are thought to be responsible for the production of fibers used in the orb web for prey capture; major ampullate glands (MaSp 1 and 2), minor ampullate glands (MiSp) and flagelliforms glands (Flag) (Gatesy et al., 2001). Although MaSp-like silk proteins are known from non-orbicularian spiders (Tian et al., 2004; Rising et al., 2007), the presence of MaSp, MiSp, and Flag at the base of the deinopoid–araneoid divergence is interpreted as support for the single origin of orb-weaving (Garb et al., 2006).

Mygalomorph spiders on the other hand possess an undifferentiated spinning apparatus consisting of uniform spigots that lead to 1–3 types of globular silk glands (Palmer et al., 1982; Palmer, 1985), and a minor fraction of their silks have been characterized. Gatesy et al. (2001) identified a protein from *Euagrus chisoseus* that is different from all other known orb-weaving silks. More recently, six novel spidroins cDNAs from four mygalomorph species were identified (Garb et al., 2007), and like *E. chisoseus* fibroin 1, their proteins are all characterized by a long ensemble repeat (163–183 amino acids). Accordingly to that, it is tacitly assumed that mygalomorph spiders generally do not have silks similar to those used in orb web weaving.

* Corresponding author. Embrapa Amazônia Ocidental – Laboratório de Biologia Molecular, Rodovia AM-10, Km 29, PO Box 319, 69010-970, Manaus, AM, Brazil. Tel.: +55 92 3303 7809.
E-mail address: daniela.bittencourt@cpaa.embrapa.br (D. Bittencourt).

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With more than 39,000 species of spiders described so far (Platnick, 2007), at least 500 of them are found in the Amazon. The Brazilian biodiversity is unique and one of the richest in the world, it is estimated at around one million animal and plant species are present only in the Amazon, which represents half of the species recorded throughout the world (Soares-Filho et al., 2006). In our aim to identify the original "ancestral" silk in a basal mygalomorph spider from the Amazon biodiversity we were able to identify cDNA sequences of silk genes from *Avicularia juruensis* (Theraphosidae). We characterized two expressed spidroin genes. While *Spidroin 1* was the more abundant transcript, and most similar to mygalomorphs spidroins described so far, *Spidroin 2* showed clear similarities to MaSp2 from the orb-weaving araneoid clade. In the light of recent studies on spider evolution, we also investigated the evolutionary history of major ampullate silks in Mygalomorphae, and spiders in general.

2. Materials and methods

2.1. Spider samples

Two mature female *A. juruensis* specimens were obtained with a collecting and export permit (IBAMA/MMA 0128753 BR) from the Amazon Forest native region near Monte Negro, Roraima State (S 10°1740″/W 60°19′ 31″). *A. juruensis* silk glands were dissected under a stereomicroscope and immediately frozen in liquid nitrogen.

2.2. cDNA library construction and gene screening

After homogenization of the gland tissue from both spiders, total RNA was extracted using a TRIzol kit (Invitrogen, USA) following the manufacturer's recommendations. The Oligotex kit (Qiagen, Germany) was used for mRNA isolation according to the technical manual. The yield, purity and quality of total RNA were determined by spectrophotometry and agarose gel electrophoresis. The cDNA was synthesized using the SuperScript II Plasmid System (Invitrogen, USA). After transformation of electrocompetent *Escherichia coli* DH5α (Sambrook and Russell, 2001), the cDNA library was amplified and then plated on selective media. The cDNA clones were then picked and transferred to 96 well plates. Plasmid cDNA isolated by alkaline lysis was sequenced and quantified (Sambrook and Russell, 2001). Sequencing reactions were performed using the Big Dye chemistry (Applied Biosystems, USA) on an ABI 3700 DNA sequencer, following the manufacturer's instructions, using the T7 and T6 promoter primers. The resulting chromatograms were directly transferred to a central data base similar to the one described by Telles et al. (2001) for processing and analysis. Several combinations of restriction enzymes were used to characterize the size of the inserts. Clones with inserts longer than 1.5 kbp were treated with exonuclease III (Erase-a-Base kit, Promega, USA) and used in a nested deletion strategy for sequencing. Base calling and quality assignment of individual bases were done through the use of Phred (Ewing and Green, 1998; Ewing et al., 1998). Ribosomal poly(A) tails, low-quality sequences, vector and adapter regions were removed as described by Telles and da Silva (2001). In order to verify the positive clones found in the library, silk gland total RNA was subjected three times to RT-PCR. *Superscript* II (Invitrogen, USA) was used in the reactions following the manufacturer's instructions. Polymerase chain reaction (PCR) analyses were conducted using Taq Polymerase (Invitrogen, USA) under the following conditions: initial template denaturation was set for 2 min at 94 °C, 35 repeated cycles were 30 s at 94 °C, 1 min at 56 °C and 30 s at 68 °C, the final extension at 72 °C for 10 min. The oligonucleotides used were designed according to the C-terminal sequence of the spider silk cDNA positive clones. The respective forward and reverse primers used for each spider silk cDNA were (5′ to 3′): AJSp1fwd: TGTCCGCAGTTTCTTCCAATGG, AJSp1rev: CCACAGCAGCGGAAGTTGAAC, AJSp2fwd: GGACCAGCACGCCAAGGACC and AJSp2rev: CGAAGAGCTTGATTTACAGATTGGCC .

2.3. Phylogenetic analyses

Nucleotide sequences were edited using Sequencher 4.8 (Gene Codes, Ann Arbor, Michigan) and submitted for BLAST searches. Phylogenetic relationships of the spidroins reported here (GenBank accession # EU652181 and EU652184) were analyzed with publicly available data. From the Araneomorphae we included *Plectreurys tristis* [1 (AAK30610); 2 (AAK30611); 3 (AAK30612); 4 (AAK30613)], *Deinopsis spinosa* [1b (ABD61592); 1a (ABD61591); 2a (ABD61593); 2b (ABD61594); 3 (AAY28934.1); 4 (ABD61590), 6 (ABD61589)], *Dolomedes tenebrosus* [1 (AAK30598); 2 (AAK30599)], *Argiope aurantia* [2 (AAK30592); 3 (AAX45292)], *Argiope trifasciata* [1 (AAK30595); 2 (AAK30596); 4 (AAK30593); 5 (AAR83925)], *Argiope amoena* [2 (AAR13813)], *Argiope argentata* [3 (AAY28932)], *Latrodectus hasselti* [3 (AAY28941)], *Latrodectus hesperus* [1(AAY28935); 2 (ABD66603); 3 (AAY28931)], *Latrodectus geometricus* [1 (AAK30602), 2 (AAK30604), 3 (AAY28940)], *Latrodectus mactans* [3 (AAY28938)], *Latrodectus tredecimguttatus* [3 (AY953078)], *Cyrtophora moluccensis* [3 (AAY28944)], *Psechrus sinensis* [1 (AAV48939)], *Octonoba varians* [1 (AAV48931)], *Uloborus diversus* [1 (ABD61596), 2 (ABD61599), 3 (AAY28933), 5 (ABD61598), 6 (ABD61597)], *Araneus diadematus* [1 (AAC47008), 2 (AAC47009), 3 (AAC47010), 4 (AAC47011)], *Araneus ventricosus* [1 (AAN85280), 2 (AAN85281), 4 (ABK00016)], *Araneus bicentenarius* [2 (AAC04503)], *Araneus gemmoides* [3 (AAX45294)], *Gea heptagon* [3 (AAY28943)], *Gasteracantha mammosa* [2 (AAK30601)], *Agelenopsis aperta* [1 (AAT08436)], *Nephila clavipes* [1 (AAT75312), 2 (AAT75315), 3 (AAX45295), 4 (AAF36089), 6 (AAC14589)], *Nephila clavata* [3 (BAE54450)], *Nephila antipodiana* [1 (ABC72644), 3 (AAY90151), 6 (ABC72645)], *Nephila madagascarensis* [1 (AAK30606), 2 (AAK30607), 4 (AAF36092)], *Nephila pilipes* [1 (AAV48946)], *Nephila senegalensis* [1 (AAK30608), 2 (AAK30609)], *Nephilengys cruentata* [1 (EF638446), 3 (EF638445), 4 (EF638444), 6 (EF638447)], *Tetragnatha versicolor* [1 (AAK30615)], and *Tetragnatha kauaiensis* [1 (AAK30614)]; where 1 stands for MaSp1, 2 for MaSp2, 3 for Tubuliform spidroins (TuSp), 4 for Flag spidroins, 5 for Aciniform spidroins (AcSp) and 6 for MiSp. From the Mygalomorphae were used: *Macrothele holsti* [1 (AAV48940)], and *E. chisoseus* [1 (AAK30600)].

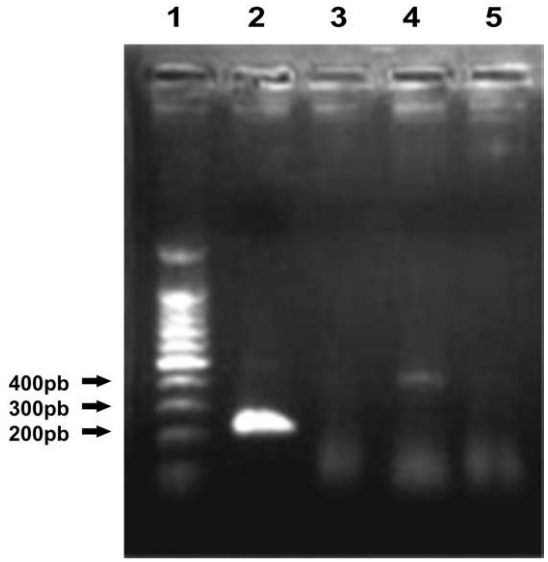

Fig. 1. RT-PCR was performed using total RNA from *A. juruensis* silk gland. 1, 100pb DNA ladder (Promega, USA); 2, Spidroin 1 cDNA (219 bp); 3, Spidroin 1 negative control; 4, Spidroin 2 cDNA (356 bp); 5, Spidroin 2 negative control. The oligonucleotides used in the PCR are described in the Materials and methods.

Spidroins' C-terminal were translated into their respective amino acid sequences, and aligned using the accuracy oriented G-Ins-i algorithm, which utilizes global pairwise alignment information, as implemented in MAFFT 5.861 [gap opening penalty = 3] (Katoh et al., 2005). Sequences were then back-translated to their respective nucleotide alignments (Biopython script), and subjected to phylogenetic analyses by Maximum Parsimony (MP), Maximum Likelihood (ML) [PAUP* v4.0b10 (Swofford, 2002)], and Bayesian Markov Chain Monte Carlo [MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001)] approaches. Heuristic MP searches were conducted with TBR branch swapping and 10,000 random sequence additions (RSA), [gaps = 5th state]. ML analysis was performed using the best-fit model of evolution as estimated by Modeltest v3.7 (Posada and Buckley, 2004), combined with 100 replicates of RSA. Bootstrap support (BP) was evaluated by 1000 pseudoreplicates/100 RSA, and 100 pseudoreplicates/1 RSA for MP, and ML, respectively. Bayesian analysis included three runs at 3×10^6 generations, implementing GTR + I + Γ, each with four chains (temperature: 0.15), and random starting trees; sampling frequency was 1000. All sample points prior to reaching stationary were discarded as burn-in. The posterior probabilities for individual clades obtained from separate analyses were compared for congruence and then combined and summarized on a majority-rule consensus tree. Clade posterior probabilities (p*P*) in the 95 percentile were taken as supportive of a topological relationship. Consistent with previous work and NOTUNG analyses (below) (Challis et al., 2006; Garb and Hayashi, 2005) trees were rooted to mygalomorph silk sequences.

The relative contributions of gene duplications, and their topological placement versus speciation events, were tested in NOTUNG v.2.1 (Durand et al., 2006) by reconciling gene trees with species trees (species tree according to Ayoub et al. (2007), [edge weights = ML − BP, duplication = 2.0, loss cost = 0.5]). Additionally, different roots were tested by optimizing the number of duplications (D) and losses (L), and upper and lowest bounds of duplications were evaluated. Presence [1] and absence [0], (ambiguous = missing) of MaSps was traced in

a. Consensus repeat

***Avicularia juruensis* Spidroin 1**

```
YSLASSIASAASSSASSAAAAASSSSAAAGAAAASEAAASAAATSTTTTTSTSRAAAAASAAAAASASGAAGAAGAA
SAASAAASASSSLQQSLGSALAQSSSFAAAFAQASSAASAAAIAYALAQTVANQIGFSSYSSAFARAASSAVYSIGGL
ASASAYAFAFASAFSQVLSNYGLLNINNA
```

***Bothriocyrtichus californicum* Fibroin 1**

```
AAAAAGSAISSSAAAASAAAFSSSLSRALIYRLQQNQDFIYTFNSIETSDAARAITYSSALAAANAIGAGSSASQAV
AFAAAKAAEGVPIRSSSYAYAEAITNAITPHFLASHLVNSANVDAFASDFTSSFASATASAASASAAAASSAASAAS
AATTTASSSTSTSTTTTSS
```

***Aptostichus* sp. Fibroin 1**

```
TGASSAAAGASHGAASAGGAFDFTRALFSELLESKIFSATTANFHSSTTASETLYATILKCLRELHFRYDECLQGAT
EVVSDLSVVSVGSGVHNYARVIAAFFERFFRKHDIPLSANYASRVAQYLISAMGSSVASADASSSSGAAATSSSTSG
ASASSISSTTASSSS
```

***Aliatypus plutonis* Fibroin 1**

```
SAAADASAAATASAASRSSANAAASAFAQSFSSILLESGYFCSIFGSSISSSYAAAIASAASRAAAESNGYTTHAYA
CAKAVASAVERVTSGADAYAYAQAISDALSHALLYTGRLNTANANSLASAFAYAFANAAAQASASSASAGAASASGA
ASASGAGSASGAASSSSTIITTKSASA
```

***Euagrus chisoseus* Fibroin 1**

```
SAAAGAASSASASAAASAFSSAFISALLGFSQFNSVFGSITSASLGLGIAANAVQSGLASLGLGAAASAAASAVANA
GLNGSGSYAYATAIASAIGNALLGAGFLTAGNASQIAASVASAVASSASAAAAAASSSAAAAGASSAAGAASSSSTT
TTTSTSSSAAAAAAAAAAAASASGASSASAAASASAAATAFSSALIGDLLGIGVFGNTFGSIGSASAASSIASAAAQA
ALSGLGLSYLASAGASAVASAVAGVGVGAGAYAYAYAIANAFASILANTGLLSVSSAASVASSVASAIATSVSSSSA
AAAASASAAAAAAASASAASSASASSSASAAAAAGA
```

b. C-terminal alignment

```
Fibroin1 Apt. sp   DGLLSS-------SASERISAIILPLVSALS--PTGVNFSEIGNIILSLISKISGSCVGL51
Fibroin2 Apt. sp   SGLSSP-------AAIRRIDSLIPLLFSSAS--SNNLSASFLSNVLATSVSQISEGSSGL51
Fibroin1 E. chi.   SGLLSP-------AANQRIASLIPLILSAIS--PNGVNFGVIGSNIASLASQISQSGGGI51
Fibroin1 B. cal.   SGLTSE-------AAKERISSIIASLLSAKS--SQDFNALLLSNILPSLISKISQRASGL51
Fibroin2 B. cal.   TGLTSE-------AAKERIASIIPSLLSAIS--PNEFDAVLLSDSLASLISQISQSGSGL51
Fibroin3 B. cal.   ADLTSP-------AANQRITSIIPILRSGIS--PKGFDASLLADSLSSLISEISQSASEL51
Spidroin1 A. jur.  QGLLPPLFVLPSNSATERISSMVSSLLSAVS--SNGLDASSFGDTIASLVSQISVNNSDL58
Fibroin1 A. plu.   SGLLSSPYGSVSISVENRIISLISSILSEFISIESAFNYSSFAKKLAFLASEISVSNPGL60

Fibroin1 Apt. sp   SPSQTFSEALLEVIIALMQILSSAKVTTVSTS---ASGTARSLAQSLSSAMAG-----101
Fibroin2 Apt. sp   SATQIIIEALFELISGLMHILTSAHFDAVSRAT--SSATASALANSLSTAFSGVNNIA107
Fibroin1 E. chi.   AASQAFTQALLELVAAFIQVLSSAQIGAVSSSSASAGATANAFAQSLSSAFAG-----104
Fibroin1 B. cal    SPTEMVTEALLEVLAGCMEILSSFNVGAQSISS--SRTSSNALVQSISNQFSGLNAAA107
Fibroin2 B. cal.   STSQIAMEALLEVLAGCMEILSSSNVGAASVSS--SRASSNALVQSISNAFSGLNAAA107
Fibroin3 B. cal.   SASDVLTEALLELVSAFLQILSS-DAGSVGISS--STAFSNALAQSVSNAFYGLNTVA106
Spidroin1 A. jur.  SSSQVLLEALLEILSGMVQILSYAEVGTVNTKT--VSSTSAAVAQAISSAFSGNQNS-113
Fibroin1 A. plu.   SASEVISEVLLETVTALIHILASSQVGSVSTAD--LSSVSRAFAQSFAQAFAHQ----112
```

Fig. 2. a. Consensus repeats from different mygalomorph spidroins, including *A. juruensis* Spidroin 1. Amino acids are indicated by one letter abbreviations. Motifs are represented by: blue – poly-S, green – poly-A, and red – threonine strings. b. ClustalW alignment of C-terminal amino acid sequences. Amino acids are indicated by one letter abbreviations and numbered from N- to C-terminal. Hyphens indicate gaps introduced to obtain the best alignment. According to the color: red - Small aa (small + hydrophobic (incl.aromatic -Y)), blue - Acidic aa, magenta - Basic aa, green - Hydroxyl + Amine + Basic – Q. Abbreviations of spider species used in this figure and GenBank accessions (from top to bottom): *Apt.*, *Aptostichus* sp. (ABW80562 and ABW80564); *E. chi.*, *Euagrus chisoseus* (ABW80568); *B. cal.*, *Bothriocyrtum californicum* (ABW80565, ABW80566 and ABW80567); *A. jur*, *Avicularia juruensis* (EU652181); *A. plu.*, *Aliatypus plutonis* (ABW80562).

MacClade 4.08 under ACCTRAN and DELTRAN on the species tree (Maddison and Maddison, 2006).

3. Results and discussion

Despite the presence of only one undifferentiated silk gland in *A. juruensis* spider, we were able to cluster the thirty-four positive clones found in the cDNA library into two different fibroin groups. The most abundant transcript was called *Spidroin 1* (3154 bp) and the silk cDNA translating a different spidroin was named *Spidroin 2* (1874 bp) (see Supplementary material). All sequenced cDNA clones were incomplete toward the 5′ end and like others spidroin sequences their translation featured repeated amino acid units followed by a non-repetitive C-terminal. Although it is not common, different silk proteins produced by the same gland were also found in web weavers spiders with specialized glands. MaSp1 was identified being produced by Tubuliform glands from the spiders *A. diadematus* and *L. hesperus* (Guerette et al., 1996; Garb and Hayashi, 2005). It was also showed that two proteins can be present in the same silk like MaSp1 and MaSp2 in the dragline silk (Xu and Lewis, 1990), suggesting that the combination of proteins must have functional applications regarding the mechanical qualities of the silk. In order to confirm the expression of both spidroins in *A. juruensis* silk gland, RT-PCR was performed using primers flanking the C-terminal region of *Spidroin 1* (219 bp) and *Spidroin 2* (356 bp) transcripts (Fig. 1).

In orb weavers, silk protein repeats are known to be composed of four simple amino acid motifs (poly-alanine (A), glycine and alanine (GA)n, two glycines and a third variable amino acid GGX (where X represents a small subset of amino acids), and GPGX(X)n (P — proline). However, these motifs are poorly represented in the translated products of *Spidroin 1* found in *A. juruensis* library. Like mygalomorph spidroins described previously (Gatesy et al., 2001; Garb et al., 2007), Spidroin 1 tandem arrays consist of repetitive units of ~180 amino acids, composed of a complex mixture of serine and alanine rich sequence including a string of threonines (Fig. 2a). Alanine rich regions are found in wide variety of spider silks. Threonine however, is a rare amino acid in araneoid silks, but might have important implications in mygalomorph spidroin function. BLASTX (Altschul et al., 1997) searches showed that Spidroin 1 repetitive sequence is also similar to the tubuliform silk protein 1 (BAE54450) from the spider *N. clavata*, although the C-terminal region showed high sequence conservation in comparison with different spider silk proteins (data not shown). Alignment between the C-terminal region from Spidoin 1 and the fibroins from four different mygalomorph species showed identities up to 46% (Fig. 2b). Three putatively orthologous transcripts were found for *Spidroin 1* gene (*Spidroin 1A* — 2 clones, *1B* — 9 clones and *1C* — 17 clones), their translation showed high similarity between their repeats and C-terminal regions with few amino acid substitutions and deletions (Fig. 3). Five other clones containing only the repetitive sequence of Spidroin 1 were also identified. The greater number of clones representing *Spidroin 1* in comparison with *Spidroin 2* gene (only one clone), suggests that this gene might be highly expressed in *A. juruensis* silk gland.

Spidroin 2 showed a completely new pattern in its amino acid sequence in comparison with other silk proteins described from Mygalomorphae spiders. Spidroin 2 amino acid composition indicates that glycine, alanine and serine are the most abundant residues, representing ~75% of the protein composition. So far, glycine has been poorly represented in mygalomorph spidroins (Garb et al, 2007). Although we found the $(GA)_n$ motif in the repetitive amino acid composition of Spidroin 2, an important motif in the composition of orb web weaver spider fibroins, we also found in a larger number a poly-GS motif (S, serine), a rare motif present in the spidroins from the non-orb web weaver spiders *Kukulcania hibernalis* (MaSp2, AAT08434) and *A. aperta* (MaSp1, AAT08436). Interestingly, we also found poly-A and GPGXX motifs close to the C-terminal region of Spidroin 2, being the last one a motif described so far only in MaSp2 and Flag spidroins (Fig. 4a). The Spidroin 2 repetitive sequence is also very similar to those present in the fibroins produced by the Lepidoptera moth larvae *Bombyx mori* (Bombycidae) (*Bm-Fhc*) and by the Embiopteran *Antipaluria urichi*, also

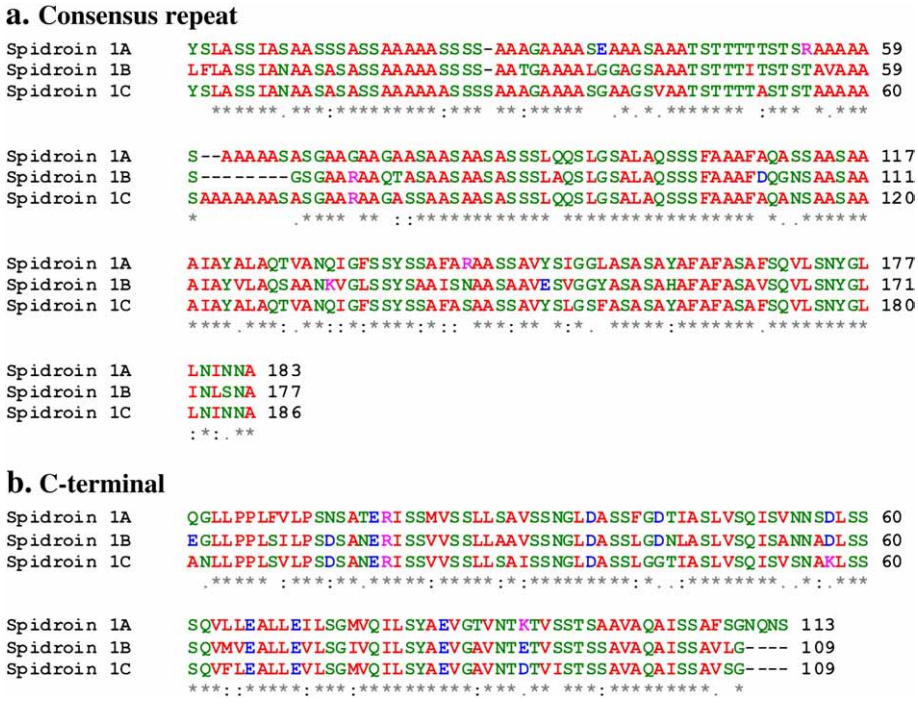

Fig. 3. ClustalW alignment of amino acid sequences of *A. juruensis*' Spidroin 1 proteins from their three orthologous transcripts (1A, 1B and 1C). a. Consensus repeats amino acid alignment, and b. C-terminal amino acid alignment. Amino acids are indicated by one letter abbreviations and numbered from N- to C-terminal. Hyphens indicate gaps introduced to obtain the best alignment. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, and "." means that semi-conserved substitutions are observed. According to the color: red - Small aa (small + hydrophobic (incl.aromatic -Y)), blue - Acidic aa, magenta - Basic aa, green - Hydroxyl + Amine + Basic - Q).

a. Spidroin 2 sequence

```
...GSGSGSGSGAGSGGGSGAGSGSGSGAGAGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGARSG
SGSGSGSGAGSGSGSGSGSGAGAGSGSGSGSGAGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGAGSGSG
SGRGAGSGSGSGSGSGAGSGSGSGSGRGAGSGSGSGSGSGAGAGSGSGSGSGAGSGSGSGSGSGAGSGSG
SGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGAG
SGSGSCRKDAGGHDGGYGKKLGFEFGTPAAAAVTLGPGAGQQGPGGAGQQGPGGQGPYGPVASAAAAVAG
GYGPGALPQGPARQGPSGPVSSAPVASAAAARLSSPQASSRVSSAFFSLVSSGPTSPGALSNAISSVVSQ
VSASNPGLSGCDVLVQALLEIVSALVSILASSSIGQINYGASAQYASLVGQSVNQALRY*
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b. Consensus repeat

```
GAG(A/S)GSGSGSGS                                  Avicularia
GAGSGSGAGSGSGAGSGAGSGSGAGSGSGA                    Antipaluria
GAGSGAASGAGSGAGAGSGAGAGSGAGAGS                    Bombyx
```

c. C-terminal alignment

```
Spidroin2 A.jur ARLSSPQASSRVSSAFFSLVSSGPTSPGALSNAISSVVSQVSASNPGLSGCDVLVQALLE60
MaSp2a D.spi    SRMSTPGSGSRISNAVSNILSSGVSSSSGLSNAISNISSSISASNPGLSGCDVLVQVLLE60
MaSp2 A.amo     SRLSSPQASSRVSSAVSSLVSSGPTNPAALSNAMSSVVSQVSASNPGLSGCDVLVQALLE60
MaSp2 A.tri     SRLSSPQASSRVSSAVSTLVSSGPTNPASLSNAISSVVSQVSSSNPGLSGCDVLVQALLE60
MaSp2 A.aur     SRLSSPQASSRVSSAVSTLVSSGPTNPAALSNAISSVVSQVSASNPGLSGCDVLVQALLE60
MaSp2 A.bic     SRLSSSAASSRVSSAVSSLVSSGPTTPAALSNTISSAVSQISASNPGLSGCDVLVQALLE60
MaSp2 L.hes     SALSSPTTHARISSHASTLLSSGPTNAAALSNVISNAVSQVSASNPGSSSCDVLVQALLE60
MaSp2 L.geo     SALSSPTTHARISSHASTLLSSGPTNSAAISNVISNAVSQVSASNPGSSSCDVLVQALLE60
MaSp2 N.cla     SRLASPDSGARVASAVSNLVSSGPTSSAALSSVISNAVSQIGASNPGLSGCDVLIQALLE60
MaSp2 N.mad     SRLASPDSGARVASAVSNLVSSGPTSSAALSSVISNAVSQIGASNPGLSGCDVLIQALLE60
MaSp2 G.mam     SRLSSPQAGARVSSAVSALVASGPTSPAAVSSAISNVASQISASNPGLSGCDVLVQALLE60
MaSp2 U.div     SRLNSPASTSRVASAVSSLASAGAPSVGSLSSVISSLSSSVSASNPGLSGCELLTQVLLE60
MaSp2 A.ven     NRLSSSGAANRVSSNVAAIASGG---AAALPNVMSNIYSGVLGS--GVSSSEALIQALLE55
ADF-3 A.dia     SRLSSPAASSRVSSAVSSLVSSGPTKHAALSNTISSVVSQVSASNPGLSGCDVLVQALLE60
ADF-4 A.dia     SVYLRLQPRLEVSSAVSSLVSSGPTNGAAVSGALNSLVSQISASNPGLSGCDALVQALLE60

Spidroin2 A.jur IVS--------------------QYASLVGQ-SVNQALRY79
MaSp2a D.spi    VISALVHILGSASVGQVG--SSPQNAQMVAANAVANAFS-97
MaSp2 A.amo     IVSALVHILGSSSIGQINYAASSQYAQMVGQ-SVAQALA-98
MaSp2 A.tri     IVSALVHILGSSSIGQINYAASSQYAQLVGQ-SLTQALG-98
MaSp2 A.aur     LVSALVHILGSSSIGQINYAAS----------------82
MaSp2 A.bic     VVSALVHILGSSSVGQINYGASAQYAQMV----------89
MaSp2 L.hes     IITALISILDSSSVGQVNYGSSGQYAQIVGQ-SMQQAMG-98
MaSp2 L.geo     LITALISIVDSSNIGQVNYGSSGQYAQMVG----------90
MaSp2 N.cla     IVSACVTILSSSSIGQVNYGAASQFAQVVGQ-SVLSAF--97
MaSp2 N.mad     IVSACVTILSSSSIGQVNYGAA----------------82
MaSp2 G.mam     IVSALVSILSSASIGQINYGASGQYAAMI----------89
MaSp2 U.div     VVSALVALLGSARVGPVDVSSSQQYAGLVSS-AIAQAL--97
MaSp2 A.ven     VISALMHVLGSASIGNVSSAGLDSTLNVVQN-AVSQYAG-93
ADF-3 A.dia     VVSALVSILGSSSIGQINYGASAQYTQMVGQ-SVAQALA-98
ADF-4 A.dia     LVSALVAILSSASIGQVNVSSVSQSTQMISQ-ALS-----94
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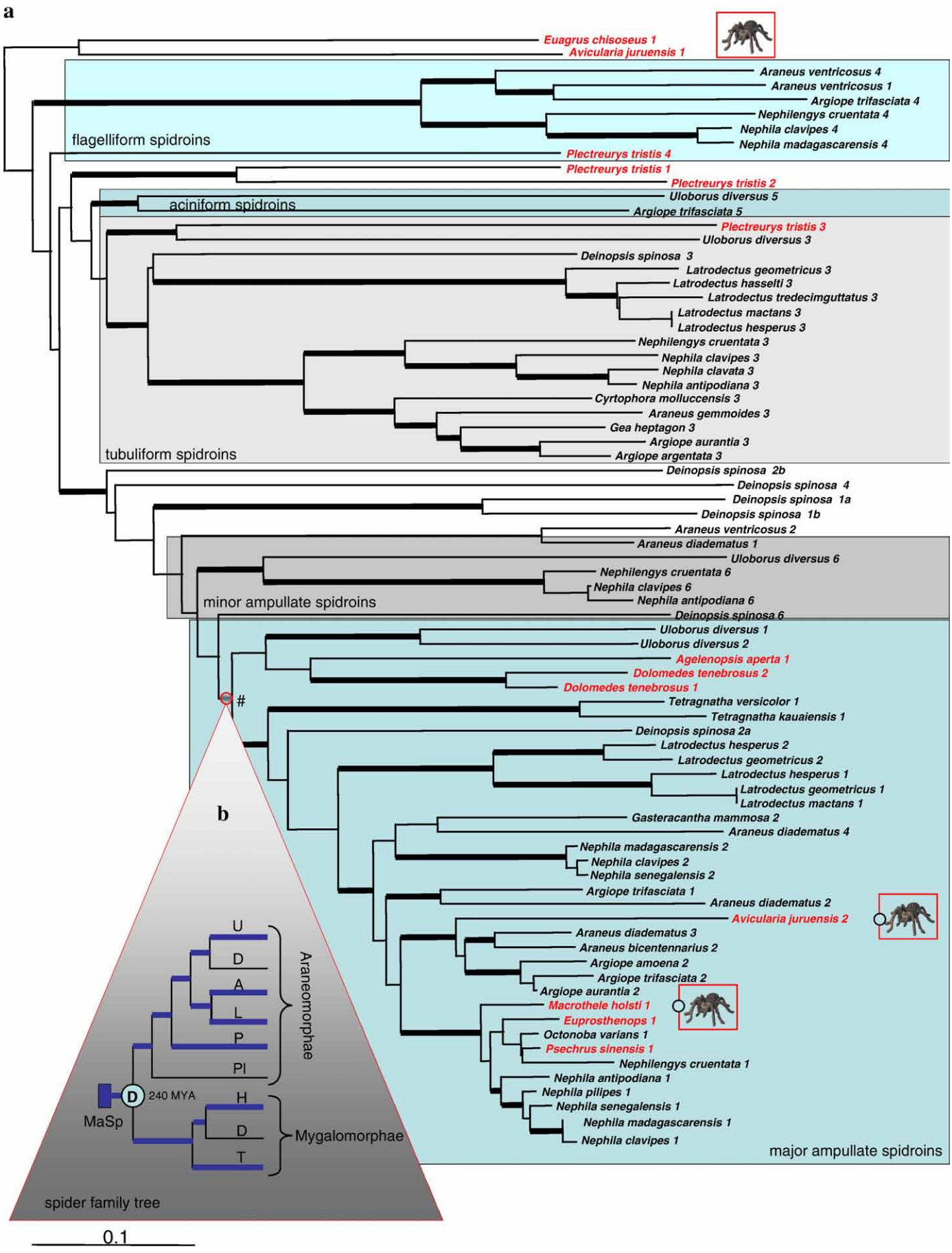
Fig. 4. a. Amino acid sequence from *A. juruensis* Spidroin 2. The C-terminal is in bold and motifs are represented by: blue - poly-GS (a motif present in non-orb web weaver spiders spidroins), green - poly-A (a motif found in various spider silks), and red - $(GA)_n$ and GPGXX (important motifs in the composition of orb web weaver spider spidroins). b. Ensemble repeats from Spidroin 2 (*Avicularia*), *B. mori* fibroin heavy chain (*Bombyx*), and the fibroin from the Embiopteran *A. urichi* (*Antipaluria*). c. ClustalW alignment of C-terminal amino acid sequences from *A. juruensis* Spidroin 2 with different spiders MaSp2. Sequences alignment abbreviations are the same as in Fig. 2b. Abbreviations of spider species used in this figure and GenBank accessions (from top to bottom): *A. jur*, *Avicularia juruensis* (EU652184); *D. spi*, *Deinopis spinosa* (ABD61593); *A. amo*, *Argiope amoena* (AAR13813); *A. tri*, *Argiope trifasciata* (AAK30596); *A. aur*, *Argiope aurantia* (AAK30592); *A. bic*, *Araneus bicentenarius* (AAC04503); *L. hes*, *Latrodectus hesperus* (AAY28936); *L. geo*, *Latrodectus geometricus* (AAK30603); *N. cla*, *Nephila clavipes* (AAT75315); *N. mad*, *Nephila inaurata madagascariensis* (AAZ15322); *G. mam*, *Gasteracantha mammosa* (AAK30601); *U. div*, *Uloborus diversus* (ABD61599); *A. ven*, *Araneus ventricosus* (AAN85281); *A. dia*, *A. diadematus* (AAC47010 and AAC47011).

rich in glycine, alanine, and serine (Zhou et al., 2000; Collin et al., 2009) (Fig. 4b). Since neither of these species is closely related to *A. juruensis*, these similarities can be assumed to be of convergent origin, and related to functional constraints.

Another interesting feature is that the C-terminal region of Spidroin 2 is very similar to that from Araneamorphae spiders MaSp2. Alignment between the amino acid sequences of the C-terminal region from *A. juruensis* Spidroin 2 with different spiders MaSp2 showed identity values of 86% for *A. amoena* and 83% for *A. trifasciata* (Fig. 4c). Even the codon bias preference for *Spidroin 2*, a feature related to the repetitive nature of the silk gene transcript composition (Candelas et al., 1990; Hinman and Lewis, 1992), was similar to those from orb weaver spider genes, which are known to have a highly preference for adenine (A) and thymine (T) as the third nucleotide encoding for one amino acid (Hinman and Lewis, 1992; Tian et al., 2004; Bittencourt et al., 2007). In *Spidroin 2* the wobble base to encode glycine was GGA/T in 82% of the cases, for serine the preference for A and T was high as 91%, and alanine codons also used A or T in the wobble position in 86% of the cases. On the other hand, as for

Table 1
Codon frequencies for most abundant amino acids in *A. juruensis* silk proteins.

AmAcid	Codon	Frequency (%)		AmAcid	Codon	Frequency (%)	
		Spidroin 1	Spidroin 2			Spidroin 1	Spidroin 2
Ala	GCG	10	06	Gly	GGC	10	14
Ala	GCA	45	61	Ser	AGT	06	34
Ala	GCT	22	25	Ser	AGC	12	03
Ala	GCC	23	07	Ser	TCG	13	02
Gly	GGG	13	04	Ser	TCA	18	41
Gly	GGA	33	39	Ser	TCT	32	16
Gly	GGT	44	43	Ser	TCC	19	03


a
Euagrus chisoseus 1
Avicularia juruensis 1
flagelliform spidroins
Araneus ventricosus 4
Araneus ventricosus 1
Argiope trifasciata 4
Nephilengys cruentata 4
Nephila clavipes 4
Nephila madagascarensis 4
Plectreurys tristis 4
Plectreurys tristis 1
Plectreurys tristis 2
Uloborus diversus 5
aciniform spidroins
Argiope trifasciata 5
Plectreurys tristis 3
Uloborus diversus 3
Deinopsis spinosa 3
Latrodectus geometricus 3
Latrodectus hasselti 3
Latrodectus tredecimguttatus 3
Latrodectus mactans 3
Latrodectus hesperus 3
Nephilengys cruentata 3
Nephila clavipes 3
Nephila clavata 3
Nephila antipodiana 3
Cyrtophora molluccensis 3
Araneus gemmoides 3
Gea heptagon 3
Argiope aurantia 3
Argiope argentata 3
tubuliform spidroins
Deinopsis spinosa 2b
Deinopsis spinosa 4
Deinopsis spinosa 1a
Deinopsis spinosa 1b
Araneus ventricosus 2
Araneus diadematus 1
Uloborus diversus 6
Nephilengys cruentata 6
Nephila clavipes 6
Nephila antipodiana 6
minor ampullate spidroins
Deinopsis spinosa 6
Uloborus diversus 1
Uloborus diversus 2
Agelenopsis aperta 1
Dolomedes tenebrosus 2
Dolomedes tenebrosus 1
#
Tetragnatha versicolor 1
Tetragnatha kauaiensis 1
Deinopsis spinosa 2a
Latrodectus hesperus 2
Latrodectus geometricus 2
Latrodectus hesperus 1
Latrodectus geometricus 1
Latrodectus mactans 1
Gasteracantha mammosa 2
Araneus diadematus 4
Nephila madagascarensis 2
Nephila clavipes 2
Nephila senegalensis 2
Argiope trifasciata 1
Araneus diadematus 2
Avicularia juruensis 2
Araneus diadematus 3
Araneus bicentennarius 2
Argiope amoena 2
Argiope trifasciata 2
Argiope aurantia 2
Macrothele holsti 1
Euprosthenops 1
Octonoba varians 1
Psechrus sinensis 1
Nephilengys cruentata 1
Nephila antipodiana 1
Nephila pilipes 1
Nephila senegalensis 1
Nephila madagascarensis 1
Nephila clavipes 1
major ampullate spidroins
b
U
D
A
L
P
PI
Araneomorphae
D
240 MYA
MaSp
H
D
T
Mygalomorphae
spider family tree
0.1

others mygalomorphs fibroin genes (Gatesy et al., 2001; Garb et al., 2007), *Spidroin 1* codons for the most prevalent amino acids in its protein sequence, alanine and serine, are only moderately biased toward A and T, with values of 67% and 56% respectively (Table 1).

Based on these molecular characteristics the Spidroin 2 from *A. juruensis* was classified as a Masp2-like spidroin, a spidroin considered so far as an orbicularian synapomorphie (Garb and Hayashi, 2005).

To validate these findings, phylogenetic analyses involving 78 spidroins C-terminal sequences from 36 spider species, including *A. juruensis*, were conducted. The sequences are representative of all known functional spidroins, Flag (orb web capture spiral [4]), AcSp (prey wrapping, sperm web, decoration [5]), TuSp (egg case construction [3]), MiSp (temporary orb web spiral [6]), and MaSp1 and 2 (frame, dragline, radii [1,2, respectively]) (Fig. 5a). Aligned sequences resulted in a 366 bp length, with 312 parsimony informative characters. Analyses [MP, ML, Bayesian] reached similar overall topologies, but differed slightly in the position of individual termini. The MP analyses resulted in 25 most parsimonious trees (length: 4074, CI: 0.43, RI: 0.598).

Strict consensus collapses all basal nodes, and BP indicates lack of support on these nodes. Maximum likelihood analysis (presented), implementing the GTR + I+Γ model (AIC Criterion), converged on one tree (−LnL = −7671.60611). The consensus tree of all three Bayesian runs of 11,500 samples resulted in a similar topology to the ML tree.

Consistent with previous results, most sequences form orthologous clusters according to function, interspersed with termini that do not seem to belong to either functional group (Garb and Hayashi, 2005; Challis et al., 2006) (Fig. 5a). Spidroin 1 groups with the previously isolated mygalomorph *E. chisoseus* fibroin 1 (Gatesy et al., 2001). This implies high conservation of both the C-terminal and the repeats in mygalomorph spidroins over a long evolutionary time. Therefore, consistent with previous arguments regarding stabilizing selection towards motif conservation within the orb-weaving clade (Gatesy et al., 2001; Challis et al., 2006), we suggest similar processes at work in mygalomorph spidroin evolution. Indeed, according to Garb et al. (2007) the modular architecture of spiders spidroins and its maintenance through concerted evolution have persisted since the mygalomorph/araneomorph split (>240 MYA).

More importantly, however, *A. juruensis* Spidroin 2 clearly nests within orbicularian MaSp2 sequences, and is positioned in the same cluster encompassing a C-terminal sequence from another mygalomorph spider – *M. holsti* – which was classified as a MaSp1-like silk (Tai et al., 2004). Although the MaSp1-like silk from *M. holsti* were considered to be a probable PCR contaminant from *N. pilipes* (Garb et al., 2007), the Spidroin 2 clearly shows common motifs present in orbicularian MaSp2 (Fig. 4a and c). Considering previous phylogenetic analysis from the C-terminal region of the spidroin gene family, where ortholog genes cluster together rather than by species (Garb and Hayashi, 2005; Challis et al., 2006; Garb et al., 2006, Bittencourt et al, 2007), the Spidroin 2 was confirmed to be a MaSp2-like spidroin.

From an evolutionary perspective, the most likely reasoning is that mygalomorph MaSp-like silks are the survivors of gene duplications (Garb and Hayashi, 2005), which would imply that the origin of orb weaver MaSps should be placed to a time point before the origin of Orbicularia. Our results confirm the occurrence of spidroin paralogs prior to the divergence of mygalomorph and araneomorph spiders as suggested by Garb et al. (2007). However, the presupposition that some spidroin paralogs (e.g. Flag, MiSp1, MaSp1 and MaSp2) are associated with silk glands that are restricted to particular araneomorph lineages (Garb et al., 2007), at least for MaSps, is contested.

In fact, reconciling the species tree with the silk gene tree does just that; it pinpoints the oldest speciation in which MaSps must have been present on the mygalomorph–araneomorph split, 240 MYA (Vollrath and Selden, 2007) (Fig. 5b). Therefore, while not refuting orb weaver monophyly, MaSp2s, and major ampullate silks in general cannot be classified as orbicularian synapomorphies (Garb and Hayashi, 2005), but have to be considered plesiomorphic for Opisthothelae.

The overall phylogenetic pattern attests to a major influence of gene duplication in silk evolution, aside from other following processes such as gene conversion or intragenic homogenization (Guerette et al., 1996; Gatesy et al., 2001; Garb and Hayashi, 2005). Given the apparent importance of gene duplication for the evolution of new biological functions, this makes sense for spider silks. It is conceivable that the functional basis for different silks arose at the outset of the mygalomorph–araneomorph radiation into their separate ecological niches. According to our data, this process likely happened before gland or spinneret differentiation, which is a distinct feature of the derived orb weaver clades.

Theraphosidae are renowned for containing the world's largest spiders, and unlike other members of the family, the Amazon *Avicularia* spp. prefer an arboreal habitat (Stradling, 1994). They often "nest" in the foliage of trees or bushes, holding large leaves together with silk, preventing the lamina from fully expanding, and producing a silk lined tube. This type of shelter certainly exceeds the usual structural and mechanical demands on mygalomorph spider silk, which is otherwise only used to line burrows or produce brooding sacs. Major ampullate silks are noted for their unique combination of high strength, stiffness and toughness in situation of uniaxial tension (Kaplan et al., 1992; Spooner et al., 2005), and would accommodate the continuous pull resulting from diverging leaf margins. Indeed, according to Blackledge et al. (2009) discovering the pattern of evolution of web spinning behaviors is essential for understanding spider diversification.

It remains unclear at this point whether the presence of a MaSp2-like spidroin is a common feature to all mature specimens of the genus *Avicularia*, or the unique survivor of an otherwise lost gene in Theraphosidae, or Mygalomorphae. Only a wider sampling will provide an answer to this question, and in the future more emphasis should be placed on sampling mygalomorph and araneomorph non-orbicularian taxa to increase our understanding of spidroin evolution in the context of ecology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpb.2010.01.005.

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Fig. 5. a. ML phylogram (lnL = −7671.60611). Bold branches = BP > 75. Red Branches = Non-orbicularian taxa, black = Orbicularia. # = MaSp node estimated to be result of duplication (among 23 others, not shown). b. Spider family tree using ML data from Ayoub et al. 2007. U = Uloboridae, D = Deinopidae, A = Araneidae, L = Latrodectidae, P = Pisauridae, Pl = Plectreuridae, T = Theraphosidae, D = Dipluridae, H = Hexathelidae). D = Lower bound of duplication at mygalomorph–araneomorph split, date from Vollrath and Selden, 2007. Blue branches: Presence of MaSPs.

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