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

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**A B S T R A C T**

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 Mygalomorphs are demonstrated. Molecular analysis showed the presence of (GA)n, poly-A and GPGXX 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: Orbidaria) 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-orbidarian spiders (Tian et al., 2004; Rising et al., 2007), the presence of MaSp, MiSp, and Flag at the base of the deinopoid–araneomorph 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 *Eugagus chisoseus* that is different from all other known orb-weaving silks. More recently, several 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.

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, Mygalomorphs 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, *Rosamigale 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: Orbidaria) 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-orbidarian spiders (Tian et al., 2004; Rising et al., 2007), the presence of MaSp, MiSp, and Flag at the base of the deinopoid–araneomorph 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 *Eugagus chisoseus* that is different from all other known orb-weaving silks. More recently, several 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.
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°17′40″, 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 kb 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 (A/p) 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 repeated conditions: initial template denaturation was set for 2 min at 94 °C, 35 repeated cycles were 30s at 94 °C, 1 min at 56 °C and 30s 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’): AJSp1f: GTGTCCGAGTTTCTTCATTGG, AJSp1rev: CCACGACGCAGGAGTT-

GAAC, AJSp2fwd: GGACACACAGCCAAAGCCAC and AJSp2rev: CGAAGACGTTTACAGATTGCCC.

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); 4 (ABD61590), 6 (ABD61589)], Dolomedes tenebrosus [1 (AAK30598); 2 (AAK30599)], Argiope aurantia [2 (AAK30592); 3 (AAX45292)], Argiope trifasciata [1 (AAK30959); 2 (AAK30956); 4 (AAK30953); 5 (AAR83925)], Argiope amoeona [2 (AAR13813)].

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 \(3 \times 10^6\) generations, implementing GTR + I + \(\Gamma\), 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 (pP) 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 v2.1 (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

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**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).
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 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 exclusively 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 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 GX (where X represents a small subset of amino acids), and GPXX motifs (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 araneid 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 silk spider proteins (data not shown).

Alignment between the C-terminal region from Spidroin 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 GPXX 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 Embiotopera 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.
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. Sequence alignment abbreviations are the same as in Fig. 2b. Abbreviations of spider species used in this figure are abbreviated: *A. juruensis* (EU652184); *A. amoena* (AAR13813); *A. trigata* (AAK30601); *A. urichi* (Antipaluria); *A. aurantia* (AAK30592); *A. bicentenarius* (AAC04503); *L. hesperus* (AAY28936); *L. geometricus* (AAK30603); *N. clavipes* (AAT75315); *N. madagascariensis* (AAZ15322); *G. mammosa* (AAK30594); *U. diversus* (ABD61599); *A. ventricosus* (AAN85281); *A. diadematus* (AAC47010 and AAC47011).

Table 1

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<thead>
<tr>
<th>Codon</th>
<th>Frequency (%)</th>
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<tr>
<td>Ala</td>
<td>10</td>
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<tr>
<td>GCA</td>
<td>14</td>
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<tr>
<td>GCT</td>
<td>21</td>
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<td>CTT</td>
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<td>GCC</td>
<td>39</td>
</tr>
<tr>
<td>GTC</td>
<td>43</td>
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Codon frequencies for most abundant amino acids in *A. juruensis* silk proteins.
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 synapomorph (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 + T 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). Spidroin 1 groups with the previously isolated mygalomorph E. chiosseus 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 1 (Garb and Hayashi, 2005). 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).

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 MaSp 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 MaSp5, is contested.

In fact, reconciling the species tree with the silk gene tree does just that; it pinpoints the oldest speciation in which MaSp5 must have been present on the mygalomorph–araneomorph split, 240 MYA (Vollrath and Selden, 2007) (Fig. 5b). Therefore, while not refuting orb weaver monophyly, MaSp5s, and major amputate 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 amputate 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 Mygalomorphe. 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.

References
