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Review

Protein families, natural history and biotechnological aspects of spider silk

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ABSTRACT. Spiders are exceptionally diverse and abundant organisms in terrestrial ecosystems and their evolutionary success is certainly related to their capacity to produce different types of silks during their life cycle, making a specialized use on each of them. Presenting particularly tandemly arranged amino acid repeats, silk proteins (spidroins) have mechanical properties superior to most synthetic or natural high-performance fibers, which makes them very promising for biotechnology industry, with putative applications in the production of new biomaterials. During the evolution of spider species, complex behaviors of web production and usage have been coupled with anatomical specialization of spinning glands. Spiders retaining ancestral characters, such as the ones belonging to the Mygalomorph group, present simpler sorts of webs used mainly to build burrows and egg sacs, and their silks are produced by globular undifferentiated spinning glands. In contrast, Araneomorphae spiders have a complex spinning apparatus, presenting up to seven morphologically distinct glands, capable to

produce a more complex set of silk polymers with different degrees of rigidness and elasticity associated with distinct behaviors. Aiming to provide a discussion involving a number of spider silks' biological aspects, in this review we present descriptions of members from each family of spidroin identified from five spider species of the Brazilian biodiversity, and an evolutionary study of them in correlation with the anatomical specialization of glands and spider's spinning behaviors. Due to the biotechnological importance of spider silks for the production of new biomaterials, we also discuss about the new possible technical and biomedical applications of spider silks and the current status of it.

Key words: Spiders; Spidroins; Silk; Biotechnology; Mygalomorph; Araneomorphae

INTRODUCTION

Spiders are exceptionally diverse and abundant in terrestrial ecosystems, and their wide distribution and evolutionary success are related to their capability to produce different types of silks during their life cycle. The variety of silks produced by one species depends on the spider's lifestyle, or alternatively, the spider's lifestyle is highly dependent on the variety of silk proteins they are able to produce. Thus, the evolutionary patterns of web spinning behaviors are essential for understanding spider clades' evolution and diversification. The duplication of genes coding for spidroins (the spider silk proteins, a name originating from an acronym of spider fibroins) along the evolution of the order Araneae has provided these organisms the ablility to conquer new habitats and to acquire new adaptive behaviors.

The most architecturally complex spider webs have evolved within the group of the orb-weaving spiders (Araneoidea and Deinopoidea). Showing an impressive range of designs, spiders' nets are spun from silks mechanically superior to most synthetic or natural high-performance fibers, where they are an effective energy-absorbing trap for flying prey (Blackledge and Hayashi, 2006). Orb-weaving spiders (Araneomorphae: Orbicularia) can have up to six morphologically distinct spinning glands, three of which are responsible for the production of fibers used in the sticky capture silk and radial support threads in the orb-web (Gatesy et al., 2001).

On the other hand, Mygalomorphae spiders are known to retain a higher number of ancestral states, and they are frequently considered more primitive than Araneomorphae ones. Organisms from this clade possess a simpler, undifferentiated spinning apparatus consisting of uniform spigots that lead to 1-3 types of globular silk glands (Palmer, 1985). The suborder Mygalomorphae is the sister group of Araneomorphae and is based on the oldest known spider fossil, *Rosamygale grauvogeli* (Selden and Gall, 1992); the split of the two lineages is estimated to have occurred ca. 240 million years ago (MYA) (Vollrath and Selden, 2007).

Spider silks are predominately composed of proteins containing repetitive motifs encoded by a highly diverse gene family. The function of each member of the spidroin family is mediated by different motifs composed of short amino acid combinations that control dif-

ferent structural and functional aspects of the silk. The mechanics of each functional silk is dependent on these structural modules (Hayashi et al., 1999; Bittencourt et al., 2007). The repeat region of spidroins is thought to be subjected to strong concerted evolution, resulting in difficulties in phylogenetic history inference. Additionally, silk genes have non-repetitive carboxy (C) and amino (N) termini. C-termini are highly conserved and have been repeatedly used for the reconstruction of silk gene family evolution (Garb and Hayashi, 2005; Garb et al., 2007; Bittencourt et al., 2010; Prosdocimi et al., 2011).

The overall phylogenetic pattern suggests a major influence of gene duplication in the evolution of spidroins, aside from other following processes such as gene conversion or intragenic homogenization (Garb and Hayashi, 2005). Gene duplication is known to be responsible for the evolution of new molecular functions in proteins, allowing neo- and subfunctionalization of these molecules (Soskine and Tawfik, 2010), and this is much likely the case for spider silk proteins. 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 Bittencourt et al. (2010), this process problably happened before gland or spinneret differentiation, which is a distinct feature of the derived orb-weaver clades. Prosdocimi et al. (2011) also suggest that the process of gene duplication and differentiation giving rise to new spidroin families probably triggered the anatomical and behavioral evolution of complex patterns of web usage in the Orbiculariae clade.

To better understand the diversification and evolution of spider silks, our group has studied the spinning gland transcriptomes of five different species from the Brazilian biodiversity: i) *Nephilengys cruentata* (Bittencourt et al., 2007), ii) *Parawixia bistriata* (Bittencourt D and Rech EL, unpublished data), iii) *Avicularia juruensis* (Bittencourt et al., 2010), iv) *Gastheracantha cancriformis*, and v) *Actinopus* spp (Prosdocimi et al., 2011). Based on our experience acquired from these previous studies, we aimed to provide discussions of a number of subjects related to the biological systems of spider silks, such as: i) the anatomy of spinning glands and the production of specialized silks; ii) the relevance of the molecular repertoire of genes expressed in these glands, iii) the link between evolution of spidroins and the behavior of web usage, and finally, iv) putative patterns of the gene duplication process giving rise to new spidroins. All discussions centered on the evolution of the great spider suborders Mygalomorph and Araneomorphae since their common ancestor. Here, we show a maximum parsimony phylogenetic analysis involving almost all known sequences for spider silks publicly available to date. We also include silk sequences from *Actinopus* spp (Mygalomorphae) and *G. cancriformis* (Araneomorphae, Orbiculariae), recently identified through transcriptomics experiments performed by 454 pyrosequencing (Prosdocimi et al., 2011). The present study is divided into three major parts: i) the description of spidroin families and their characteristics, ii) the most likely scenario for silk protein evolution and the importance of gene duplication in this process, and iii) the potential biotechnological applications of spider silk.

THE SPIDROIN FAMILIES AND THEIR CHARACTERISTICS

Spiders spin a diverse array of silk fibers predominately composed of repetitive proteins (spidroins) flanked by nonrepetitive, highly conserved N- and C-termini (Hayashi and Lewis, 2000; Garb et al., 2007). The characterization of this gene family has focused on spi-

droins from the Araneoidea clade (~10,000 species, e.g., orb-web and cob-web weavers), a lineage within the infraorder Araneomorphae (true spiders: ~37,000 species), whereas little is known about spidroins from the Araneomorphae sister group, the Mygalomorphae (tarantulas and their kin; ~2,500 species) (Platnick, 2012).

Brazilian biodiversity is unique and one of the richest in the world. It is estimated that there are about one million animal and plant species in the Amazon alone (Soares-Filho et al., 2006), showing the importance of utilizing this vast natural heritage for new genetic discoveries. To better understand sequences and diversity of spidroins, we studied five Brazilian spider species: i) *N. cruentata*, ii) *P. bistriata*, iii) *G. cancriformis*, iv) *A. juruensis*, and v) *Actinopus* spp, the last two being representatives of the Mygalomorphae clade (Bittencourt et al., 2007, 2010; Prosdocimi et al., 2011).

Araneomorphae silks

The orb-web represents an unrivaled feat of natural engineering exquisitely designed to catch flying prey. It is virtually invisible, strong, and extensible. To satisfy this diverse set of requirements, orb-weaving spider species possess a complex spinning apparatus, with up to seven morphologically distinct glands. Six glands are responsible for silk production: i) major ampullate gland, ii) minor ampullate gland, iii) flagelliform gland, iv) cylindrical (tubulliform) gland, v) aciniform gland, and vi) pyriform gland; one extra-gland, vii) the aggregate gland, produces the aqueous silk glue (Vollrath, 1992). Each silk gland has a unique morphology, but the overall organization of these glands is similar among species. According to convention, spidroin gene families and proteins are named according to the gland in which they are mainly expressed, followed by Sp for spidroin and a number referring to the different members of the family (e.g., MaSp1 for major ampullate spidroin 1).

Through the analysis of the spinning gland transcriptomes of the orb-web species *N. cruentata* (Bittencourt et al., 2007; Perry et al., 2010), *P. bistriata* (Bittencourt D and Rech EL, unpublished data), *A. juruensis* (Bittencourt et al., 2010), and *G. cancriformis*, we were able to identify partial sequences for spidroin members from the different spider silk glands (Table 1). However, we could not identify the complete set of the spidroin repertoire in any of the species analyzed. This absence of a number of family members was probably due to the statistical sampling of the transcriptomic analysis. Even in the broadest research published on spider transcriptomics to date (Prosdocimi et al., 2011), in which spinning gland transcriptomes have been sequenced in 454 machines (Margulies et al., 2005), the number of sequences produced was not enough to allow a whole transcriptome sequence analysis. The molecular structure of spidroins is basically formed by the repetition of amino acid motifs organized as a repetitive pattern into a monomer. These monomers are duplicated hundreds to thousands of times to create the natural silk polymer. Based on these sequences, a relatively few amino acid motifs form the basic building blocks of the silk. According to previously described spidroin sequences (Hinman and Lewis, 1992; Hayashi et al., 2004), we were able to identify at least four highly frequent amino acid motifs present in the spidroins studied: poly-alanine (*An*), alternating glycine and alanine $(GA)_n$, amino acid triplets composed of two glycines and a third variable amino acid $(GGX)_n$, and glycine-proline-glycine modules $(GPGXX)_n$, as well as a spacer region. However, these motifs are poorly represented in the Tubuliform (TuSp), Pyriform (PySp) and Aciniform (AcSp) spidroins.

Table 1. Ecological functions of the different orb-web spider silk glands and the repetitive and spacer sequences from the identified *Nephilengys cruentata*, *Parawixia bistriata* and *Gastheracantha cancriformis* spidroins.

Spinning gland	Ecological function	Protein name	Accession No.	Consensus proteins sequence
Major ampullate gland	Frame and radii of the web. Lifeline, outer egg case and prey wrapping	MaSp1	*N. cruentata* (EF638446)	GGAGQGGYGGLGGQGAGQGAGAAAAAA
		MaSp1	*P. bistriata* (GQ275359)	GGLGGQGGLGGSGSQGAGQGGYGQGGAGQGGAA AAAAAA
		MaSp2	*G. cancriformis* (Prosdocimi et al., 2011)	GGYQPGSGQQGPGQQGPGSGGQRGPGSRGPYGPGA AAAAAASA
		MaSp2	*P. bistriata* (GQ275360)	GGYGPGGAGQQGPAAGQQGPGSQGSYGPGAAAAA AAA
Minor ampullate gland	Auxiliary spirals. Outer egg case and prey wrapping	MiSp	*N. cruentata* (EF638449)	Repetitive: GAGAGGAGGFGRGAGAGAGAGAGAAAGAGAGGA GGYGAGQGYGAGAGAGAAAAAGA Spacer: GNAFAQSLSSNLLSSGDFVQMISTTTSTDQAVSVATS VAQNVGNQLGLDANAMNNLLAAVGGYVSSLGGA VADAAAYANAISSAIGNVLANTGSINESTASSAASS AASSVTTTLTSYGPAVFY
		MiSp	*P. bistriata* (GQ275358)	GAGAGGGYGGGYGAGGGAGAGAGAGAGAGAGA GAGRGGAGGYGAGAGAGAGAAAAAAA
Flagelliform gland	Core fibers of capture spiral	Flag	*N. cruentata* (EF638444)	$[GPGGX]_{19}[GGX]_3$TVIEDLDITVNGPGGPITISEELTVG GPGAGGS$[GPGGX_n]_{24}$
		Flag	*P. bistriata* (GQ275357)	GAPGGAGGGVGPGGGAGGTSGGASGSGPVSVSTAVN VGGAGGPGAGGPGAGGVGPGVVGPGGLGGPGGFGG PGGPGGPGGPGAPGGAGGMFGPGGAGGMYGPGGAG GMYGPGGAGRGPGGAGAPGAPGGPGGPGGPGGFGG GAGAGGMVPGGASRGPGGSGPVTVTETVTVGGAGGP GPGGIGGSSGPGAGGAPGGFGGPGGPGGPGGPGGPGG AAGGPGAGGAGPGGSGPATVSSSVTVVGAGGPGGPG AGGIVPGGIYGPGGAGGVVPGGIYGPGGVPSGPGGPG GPVGPGGYGAPGGLGVGILPGTASAGTSGPTTVTEVV SINVSGGQSS
Cylindrical gland	Egg sac	TuSp	*N. cruentata* (EF638445)	TTTTTSASGSQSASQSASSSSASASAFAQQSSASLAA SSSFSQAFASAASASAVGNVAYQLGLSAAQSLGIAN AGALASALAQSVSSVGVGASSSAYANAVAGAVGQF LANQGINTGNASSLASSFSSALSASAAAAQSQSFAQS QAAASAFQQAASQSASQSAAQSGSQSSS
		TuSp	*G. cancriformis* (Prosdocimi et al., 2011)	TTTTSTSGSEAASQSASSSSSQASASSFAQQSSASLATS SFSSAFAAATSLSAVGNVGYQLGLNVANALGLGNAQ ALGAALSQAVSAVGVGASANAYANAISNSVGQFLLG QGILNAANAGSLASSFANALSASAASVAASAASQSAS ASQSAASAFNQAASRSASQSASQSGSKSSSS
Pyriform gland	Attachment disk	PySp	*N. cruentata* (GU062417)	APRPLPAPAPRPLPAPAPRPLPAPLPAPLPRPRPAPIVS QVQQASALQAQSQQSAFAQSQQSSIAQSQQASVAQS QRASVSQSQQSSNAFSSAASFGASSVASSASTYFNSG IVQSSIASSLQSSSALSSIAYGQTTASINDIASAVAGSIA NSIGLSQQTVQSIISQQLASAGSGASAQTLASLISSAV SSLVQQSGSVSAGQEQSISQALSSSISSSLNQLVA
Aciniform gland	Prey wrapping	AcSp	*P. bistriata* (GQ275356)	STALFNSGVLNASNVNTLGSQVVSTLLRGISSTAQGL GLNVDAGSVQSDISSSSSFLSTSSSSTSSSQTTAASTS GFTGASYPGPQVSQPAPFGVGPQPGGALPGFGQVSG AQSALISRIANALGNTATMRAVLRSGVSQQIVSNVV QGAVQALSSSLGIDGNNLARIASQTILRVPAGSDTSA YAQAF

On the basis of physical studies, spider silk motifs adopt a particular secondary structure in the web fiber, as they are responsible for conferring a specific mechanical property to it (Simmons et al., 1996; Parkhe et al., 1997; Hayashi et al., 1999). For instance, *An* and $(GA)_n$ motifs are involved in β-sheet formation and play a crucial role in silk strength (Simmons et al., 1994), both Gly and Ala have a small side chain (H and

CH_3, respectively) that allows a closer, tougher assembly of β-sheets. The major and minor ampullate silks are both very strong and at least one protein (MaSp1 and 2; MiSp1 and 2) in each silk contains the $(GA)_n/An$ β-sheet module (Hinman et al., 2000). The modules $(GGX)_n$ (where X = L, Q, R, or Y, depending on the silk) and $(GPGXX)_n$ (XX representing GA, GS, GY, or QQ) adopt less ordered conformations, most likely 3_{10}-helices and turns, essential for fiber extensibility (Dong et al., 1991; Bram et al., 1997; Hayashi and Lewis, 2001; Rising et al., 2005). Flag silk, produced by the flagelliform gland, can have an elasticity of more than 200%, allowing more than double extension, and has the *GPGXX* module repeated up to 63 times in each repetitive unit (Hayashi and Lewis, 1998). Although the *GPGXX* and *GGX* motifs can be found in Flag and MaSp2, the *GGX* motif is also present in MiSps. Finally, spacer regions found in Flag and MiSp contain amino acids with charged groups and separate the peptide motifs into clusters. Although these may provide organizational areas within silks or surface regions for interactions when a mature fiber is formed, their real function is currently undetermined (Hayashi et al., 1999).

In accordance with other spider silk proteins, TuSp and PySp are highly homogeneous within species. However, their protein sequences show few of the common spidroin amino acid motifs described to be present in MaSp, MiSp or Flag. The TuSp sequence contains repeats of 174 amino acids, largely composed of alanine and serine, forming new motifs such as *Sn*, $(SX)_n$ (X representing Q, L, A, V, or F), and *GX* (X representing Q, N, I, L, A, or V) (Bittencourt et al., 2007). Similarly, PySp also contains large segments composed of repeated amino acids (>200 residues). However, PySp segments are formed by two exclusive repetitive motifs, a glutamine-pair-rich (QQ) sequence such as QQSSVA, and regions of alternating prolines (PXPXP) (see Table 1) (Perry et al., 2010). Like TuSp and PySp, AcSp does not display substantial sequence similarity to any other spidroin previously described, but it is also very homogeneous within species (Hayashi et al., 2004). In Table 1 is shown the *P. bistriata* AcSp consensus repeat that is as much as 189 amino acids in length and highly homogeneous in the protein.

The lack of the usual repeats rich in alanine and/or glycine found in most spider spidroins in TuSp, PySp and AcSp is probably related to their function. Taking advantage of the efficiency of Raman spectromicroscopy in the investigation of micrometer-sized biological samples, Lefevre et al. (2007) determined the secondary structure of proteins in the complete set of glands of the orb-weaving spider *Nephyla clavipes*. According to them, TuSp, PySp and AcSp are folded in their initial state with a predominance of α-helices. However, whereas the cylindrical gland forms a fiber similar to the major ampullate thread, the aciniform and pyriform glands produce fibers dominated by moderately oriented β-sheets and α-helices. Nevertheless, no predicted secondary structures were related to specific motifs in the spidroin sequences.

The nonrepetitive and highly conserved C-terminus regions were also identified in all described *N. cruentata* and *P. bistriata* spidroins. Although we were able to identify the repetitive part and the C-termini only from *G. cancriformis* MaSp2 and TuSp, the C-termini of MiSp, PySp and AcSp were also identified in this species transcriptome. Alignment of the C-terminal amino acid sequences of these proteins showed great sequence conservation, with the more highly conserved sequences among ortholog gene groups more than among paralog genes (Figure 1). Recently, Hagn et al. (2010) provided evidence that the structural

state of this domain is essential for controlled switching between the storage and assembly forms of silk proteins. In addition, it also has a role in the alignment of secondary structural features formed by the repetitive elements in the backbone of spider silk proteins, which is known to be important for the mechanical properties of the fiber. However, while the C-terminal domain is a prerequisite for the formation of continuous silk fibers as opposed to amorphous molecular aggregates, it is the N-terminal domain that senses the pH differences in the glandular lumen and thereby appears to regulate the assembly process *in vivo* (Silvers et al., 2010). Unfortunately, we were unable to identify the N-termini of any spidroin from the spider silk gland transcriptomes analyzed.

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G.cancriformis_Pyri          SGSSLAVSAAQQLTSAAANQRLSKLSNSLRSAVAGG-QVNYGALSSSLAS
G.cancriformis_MiSp          ----------------------SRVSSNIGSFVSGG----PSAISGVIAN
P.bistriata_MiSp             ------------------SGATGRVANSLGAMASGG----INALPGVFSN
P.bistriata_MaSp2            ----------------------SRVSSAVSSLVSSG-PTNSAALTNTISS
P.bistriata_MaSp1            ----------------------SRVSSAVSSLVSSGGPTNSAALSSTISN
N.cruentata_MaSp1            -------------SRLSSPEASSRVSSAVSNLVSSG-PTNSAALSNTISS
G.cancriformis_MaSp2         -------------SRLSSPQAGSRVSSAVSALVASG-PTSPGALSSTISN
N.cruentata_MiSp             -------------SRLSSAQASSRISAAASTLISGG-YLNTSALPSVISD
G.cancriformis_TuSp          ---------SSLASSASSARVSSLAQSIASTISSSGGILNVPTFLNLLSP
N.cruentata_TuSp             ---------SGLASSSATSRVGSLAQSLASALQSSGGTLDVSTFLNLLSP
P.bistriata_Flag             -----------------SARLPSLINGVMSSMQGGG--FNYQNFGNVLSQ
N.cruentata_Flag             -----------------SRVPDLVNGIMRSMQGSG--FNYQMFGNMLSK
G.cancriformis_Acin          -------SPIGLRSGAAVPRIRRLTSSISKAISPYG--VDSNALASTLQE
                                                                 *       : . :

G.cancriformis_PySp          AASQIQSSS-GLSKSEVLVEALLETLAALLDSLSIS----------GSSS
G.cancriformis_MiSp          IFSGVSST--GASYGESVTEALIEVIAMLLHILSNSAIGNVSSAGLENSM
P.bistriata_MiSp             IFSQVSAASGGASGGAVLVQALTEVIALLLHILSSASIGNVSSQGLEGSM
P.bistriata_MaSp2            VVSQISASNPGLSGCDVLIQALLEIVSALVHILGYSSIGQINYDAAAQYA
P.bistriata_MaSp1            VVSQVSASNPGLSGCDVLVQALLEIVSALVHILGSSSIGQVNYNAAGQSA
N.cruentata_MaSp1            VVSQISASNPGLSGCDVLVQALLEVVSALIHILGSSSIGPVNYGSASQST
G.cancriformis_MaSp2         VASQISASNPGLSSCDVLVQALLELVSALVSILASASIGQINYGASGQYA
N.cruentata_MiSp             LFAQVSASSPGVSDSEVLIQVLLEIVSSLIHILSSSSVGQVDFNSVGSSA
G.cancriformis_TuSp          IGSQVISSS-SLGSSEATSQVLLEGISALLQVLNGARVSSVNLANVPNLN
N.cruentata_TuSp             ISTQIQANT-SLNASQAIVQVLLEAVAALLQIINGAQITSVNFGSVSSVN
P.bistriata_Flag             FAT---GSG-TCNSND--INLLMDALFAALHTLSYQGQSSVPTYPSPAAM
N.cruentata_Flag             YAS---GSG-ACNSND--VNVLMDALLAALHCLSSHGSPSPSGSSPTPSAM
G.cancriformis_AcSp          NVSALQSS--GMSSSDAKTEVLFETIAGLLQLLSNTQIRGVNFSTSPSVA
                               :   .     .      : * : :   :  :

G.cancriformis_PySp          SEFAQAVLRAFA-
G.cancriformis_MiSp          ALVQGAVGPLLG-
P.bistriata_MiSp             AIAQQAIGAYAG-
P.bistriata_MaSp2            SLVGQSVAQALA-
P.bistriata_MaSp1            SVVGQSFYQALA-
N.cruentata_MaSp1            QIVGQSVYQALG-
G.cancriformis_MaSp2         SMVGQSVAQALV-
N.cruentata_MiSp             AAVGQSMQVVMG-
G.cancriformis_TuSp          QDLVRALAG----
N.cruentata_TuSp             TALATALAG----
P.bistriata_Flag             SSYSQSVRGCFGY
N.cruentata_Flag             NAYSNSVRRMFQF
G.cancriformis_AcSp          NSVAKSFEYVLA-
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Figure 1. ClustalW alignment of the consensus C-terminal region from different spiders. Amino acids are indicated by one-letter abbreviations from N- to C-terminal.

Mechanical properties of *N. cruentata* dragline silk

Major ampullate or dragline silk has been extensively studied for its mechanical properties. It is classified as the strongest silk, responsible for the production of the orb-web frame and the spider's lifeline. Figure 2 shows scanning electron microscopy (SEM) images of *N. cruentata* and *P. bistriata* dragline silks. Often considered as a nanocomposite fiber or natural self-assembling block copolymer, major ampullate silk is composed of two known spidroins MaSp1 and MaSp2 (Hinman and Lewis, 1992). MaSp1 are highly repetitive protein sequences with the amino acid motifs $(GGX)_n$ and *An* responsible for silk strength. The motifs of MaSp2 include *An*-like MaSp1; however, it also contains high amounts of the proline-containing motif $(GPGXX)_n$, which is responsible for fiber elasticity. According to the motifs present in the dragline spidroins, silk features a hierarchical architecture on which a highly organized, densely H-bonded β-sheet nanocrystals are arranged within a semiamorphous protein matrix consisting of 3_{10}-helices and β-turn protein structures (Lefevre et al., 2007; Nova et al., 2010). Although we were unable to determine any molecular structure, atomic force microscopy (AFM) images from *N. cruentata* dragline silk also agree with the predicted fiber nanostructure (Bittencourt, 2007). It clearly shows that dragline silk is a cylindrical fiber formed in some regions by nanofibers arranged obliquely to the longitudinal axis of the fiber (Figure 3). It was also observed that it is composed of regions (nanofibers) with different degrees of hardness (soft regions alternating with more rigid regions), which indicates different molecular interactions between the proteins of the fiber or differences in the composition of the nanofibers.

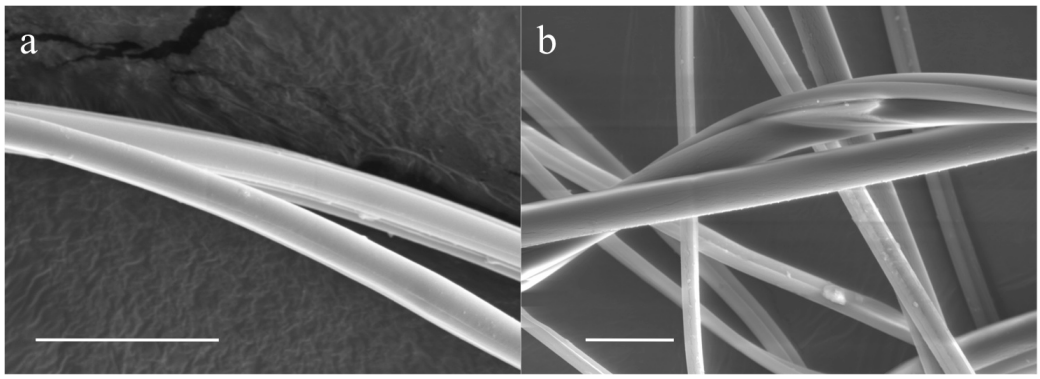

Figure 2. Scanning electron micrographs (SEM) of spider silk dragline. The SEM data in **a.** show the *Nephilengys cruentata* dragline (2000X; scale bar = 20 μm), and in **b.** show the *Parawixia bistriata* dragline (1000X; scale bar = 20 μm).

Ten samples of the dragline silk from two *N. cruentata* spider specimens were also tested on an MTS Synergie 100 (MTS Corporation, Eden Prairie, MN, USA) using a custom-built 10 g load cell (Transducer Techniques, Temecula, CA, USA) as described by Bittencourt (2007) and Brooks et al. (2008). The results obtained by the mechanical testing of *N. cruentata* dragline silk also agrees with data from the AFM, since it has two extreme characteristics, strength and elasticity, attributed to the different molecular structures in

the fiber. According to our results, *N. cruentata* dragline silk showed excellent mechanical properties. Although it is less elastic than *Araneus diadematus* dragline silk, it has similar properties to *Lactrodectus geometricos* dragline silk and appears to be stronger than the fibers from *A. diadematus*, *A. trifasciata* and *N. clavipes* spiders (Table 2). However, *N. cruentata* fiber is even more rigid than all the others. Overall, we conclude that *N. cruentata* dragline silk has mechanical properties similar in order of magnitude to other dragline spider species, including non-orbicularian ones (Blackledge and Hayashi, 2006; Swanson et al., 2006).

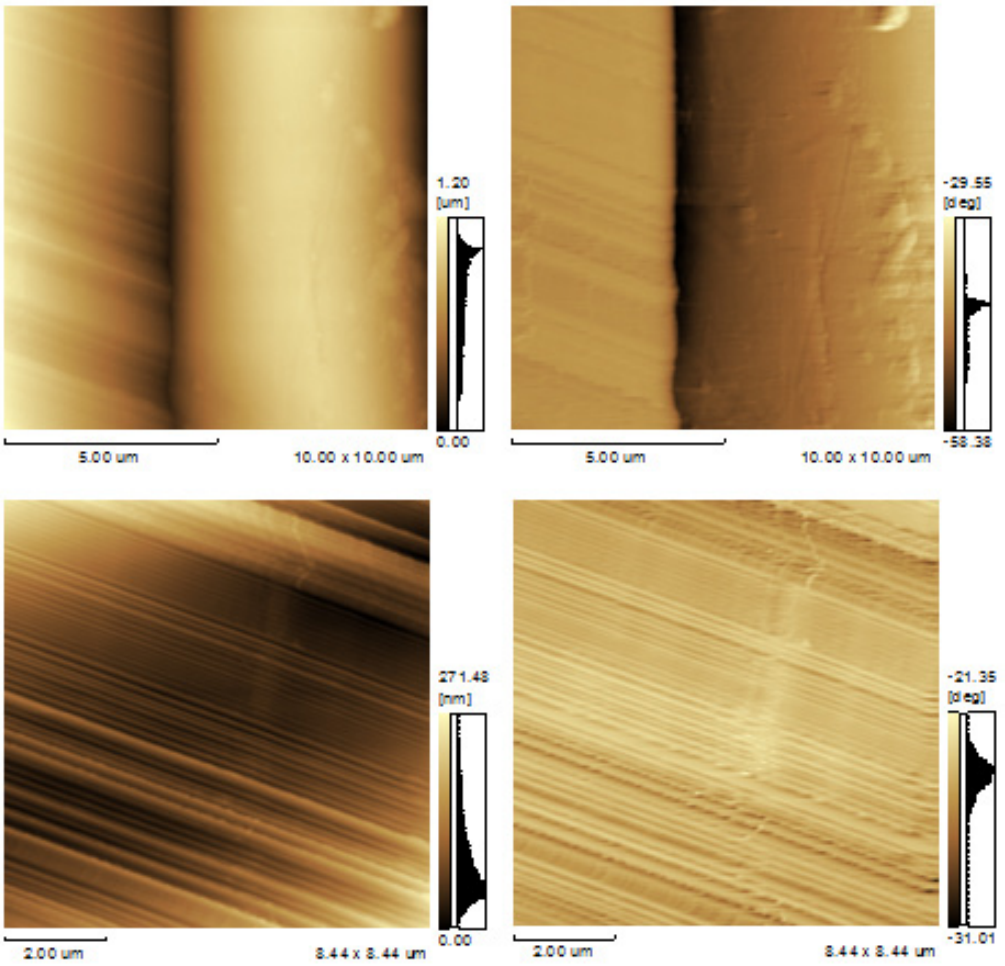

Figure 3. Images obtained from the dragline silk of *Nephilengys cruentata* by atomic force microscopy (Bittencourt, 2007). **A. C.** Topography of the fiber on graphite surface investigated by AFM in contact mode. **B. D.** Images of force modulation at the same topographical regions obtained in **A** and **C**, respectively. The softer fiber regions are shown in black.

Table 2. Mechanical properties of dragline silk form different species of spider.

	Stiffness (GPa)	Strain (GPa)	Stress (%)
Nephilengys cruentata	11.71 ± 5.76	1.40 ± 0.38	27.84 ± 8.26
Nephila clavipes[1]	13.8 ± 0.76	1 ± 0.004	20 ± 1.1
Argiope trifasciata[1]	8.2 ± 0.63	1.2 ± 0.003	23 ± 0.6
Aranaeus diadematus[1]	8.3 ± 0.54	1.06 ± 0.005	29 ± 2.4
Lactrodectus geometricus[2]	12.26 ± 3.91	1.40 ± 0.06	27.13 ± 5.05
Tendon collagen[3]	1.5	0.15	12
Nylon fiber[3]	5	0.95	18
Kevlar 49 fiber[3]	130	3.6	2.7
High-tensile steel[3]	200	1.5	0.8

[1]Swanson et al., 2006; [2]Motriuk-Smith and Lewis, 2004; [3]Gosline et al., 1999.

Mygalomorphae silks

The infraorder Mygalomorphae is composed of large, relatively sedentary spiders that primarily dwell in silk-lined burrows (Hedin and Bond, 2006). Mygalomorphs include the tarantulas and funnel-web and bird-eating spiders. Mygalomorphs have a comparatively undifferentiated spinning apparatus consisting of uniform spigots that lead to 1-3 types of globular silk glands (Palmer, 1985). Unlike Araneomorph spiders that have evolved a multitude of task-specific fiber types, Mygalomorphs appear to draw upon a small number of generalized silks for different purposes. They do not build orb-webs, and use their silk for a variety of ecological functions, including burrow construction and covering, as well as egg protection (Coyle, 1986). Figure 4 shows SEM image of *A. juruensis* silk. The reduced level of gene family expansion in Mygalomorphs is consistent with the relative uniformity of their silk glands in morphology and function. By contrast, Araneomorphs have experienced a greater diversification of spidroins in parallel with the functional specialization of their spinning apparatus (Vollrath and Selden, 2007).

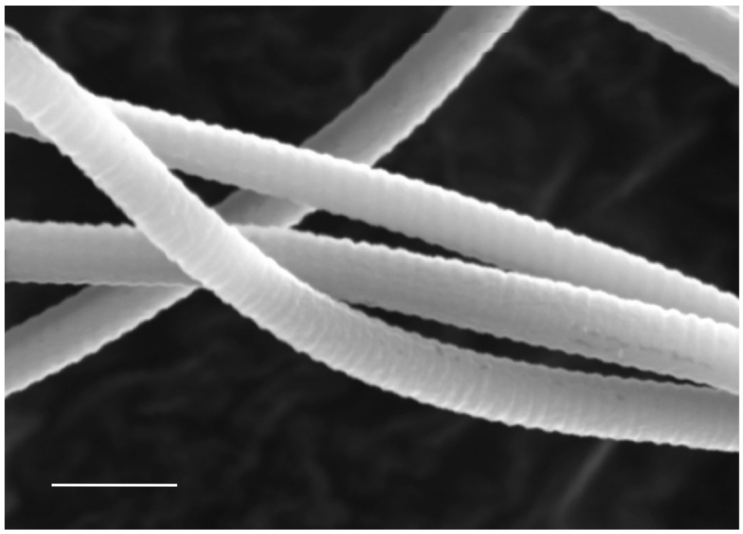

Figure 4. Scanning electron micrograph from *Avicularia juruensis* silk (10,000X; scale bar = 2 μm).

Within and across species, Mygalomorph spidroins are compositionally similar. Even these more basic silks still show a repetitive nature, but the consensus repeat is frequently much longer. Like Mygalomorph spidroins previously described (Garb et al., 2007), *A. juruensis* Spidroin 1 tandem arrays consist of repetitive units of ~180 amino acids, composed of a complex mixture of serine- and alanine-rich sequences, including a string of threonines (Figure 5A) (Bittencourt et al., 2010). Alanine-rich regions are found in a wide variety of spider silks. Threonine, however, is a rare amino acid in araneoid silks, but it may have important implications in Mygalomorph spidroin functions. Although we were unable to identify the repetitive part of *Actinopus* spp spidroin (Prosdocimi et al., 2011), alignment of the C-terminal region of *A. juruensis* and *Actinopus* spp spidoins with fibroins from four different Mygalomorph species showed high sequence conservation (Figure 5B). The high degree of conservation of both C-termini and the repeats in Mygalomorph spidroins suggest that the modular architecture of spiders' spidroins may have been inherited from a repetitive, ancestral sequence, and its maintenance through concerted evolution has persisted since the Mygalomorph/Araneomorph split (240 MYA) (Garb et al., 2007).

A. *Avicularia juruensis* Spidroin 1 sequence

```
YSLASSIASAASSSASSAAAAASSSSAAAGAAAASEAAASAAATSTTTTTSTSRAAAAASAAAAASASGAA
GAAGAASAASAASASSSLQQSLGSALAQSSSFAAAFAQASSAASAAAIAYALAQTVANQIGFSSYSSAFAR
AASSAVYSIGGLASASAYAFAFASAFSQVLSNYGLLNINNA
```

B. C-terminal alignment

```
Euagrus_Fibroin           SGLL-------SPAANQRIASLIPLILSAISP--NGVNFGVIGSNIASLA
Aptostichus_Fibroin1      DGLL-------SSSASERISAIILPLVSALSP--TGVNFSEIGNIILSLI
Bothriocyrtum_Fibroin2    TGLT-------SEAAKERIASIIPSLLSAISP--NEFDAVLLSDSLASLI
Bothriocyrtum_Fibroin1    SGLT-------SEAAKERISSIIASLLSAKSS--QDFNALLLSNILPSLI
Bothriocyrtum_Fibroin3    ADLT-------SPAANQRITSIIPILRSGISP--KGFDASLLADSLSSLI
Aptostichus_Fibroin2      SGLS-------SPAAIRRIDSLIPLLFSSASS--NNLSASFLSNVLATSV
Aliatypus_Fibroin1        SGLLSSPYGSVSISVENRIISLISSILSEFISIESAFNYSSFAKKLAFLA
A.juruensis_Sp1           -PLFVLP----SNSATERISSMVSSLLSAVSS--NGLDASSFGDTIASLV
Actinopus_ssp._Sp         ----------------PLRPGMPSMLSLVSS--GSVNSAAFHSIIASLI
                                            :.. :...: *   .     ..   :.. :

Euagrus_Fibroin           SQISQSGGGIAASQAFTQALLELVAAFIQVLSSAQIGAVSSSSASAGATA
Aptostichus_Fibroin1      SKISGSCVGLSPSQTFSEALLEVIIALMQILSSAKVTTVSTS---ASGTA
Bothriocyrtum_Fibroin2    SQISQSGSGLSTSQIAMEALLEVLAGCMEILSSSNVGAASVSS--SRASS
Bothriocyrtum_Fibroin1    SKISQRASGLSPTEMVTEALLEVLAGCMEILSSFNVGAQSISS--SRTSS
Bothriocyrtum_Fibroin3    SEISQSASELSASDVLTEALLELVSAFLQILSS-DAGSVGISS--STAFS
Aptostichus_Fibroin2      SQISEGSSGLSATQIIIEALFELISGLMHILTSAHFDAVSRAT--SSATA
Aliatypus_Fibroin1        SEISVSNPGLSASEVISEVLLETVTALIHILASSQVGSVSTAD--LSSVS
A.juruensis_Sp1           SQISVNNSDLSSSQVLLEALLEILSGMVQILSYAEVGTVNTKT--VSSTS
Actinopus_ssp._Sp         FAISQSATDLSRTEVFVEALLEMISALIQLLCHARIEDVISSS----TSS
                            **.......:: ::    :.*:* : . :.:*               :

Euagrus_Fibroin           NAFAQSLSSAFAG-----
Aptostichus_Fibroin1      RSLAQSLSSAMAG-----
Bothriocyrtum_Fibroin2    NALVQSISNAFSGLNAAA
Bothriocyrtum_Fibroin1    NALVQSISNQFSGLNAAA
Bothriocyrtum_Fibroin3    NALAQSVSNAFYGLNTVA
Aptostichus_Fibroin2      SALANSLSTAFSGVNNIA
Aliatypus_Fibroin1        RAFAQSFAQAFAHQ----
A.juruensis_Sp1           AAVAQAISSAFSGNQNS-
Actinopus_ssp._Sp         NAVALAVSSGLLG-----
                           :.. :.:   :
```

Figure 5. A. Amino acid sequence from *Avicularia 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 between the C-terminal region from *A. juruensis* and *Actinopus* spp spidroins (EU652181 and Prosdocimi et al., 2011, respectively) with fibroins from four different mygalomorph species: *Euagrus chisoseus* (ABW80568); *Aptostichus* sp (ABW80562 and ABW80564); *Bothriocyrtum californicum* (ABW80565, ABW80566 and ABW80567); *Aliatypus plutonis* (ABW80562).

A second spidroin named Spidroin 2 was also identified, where it is produced in the silk glands of *A. juruensis*. Similar to fibroins produced by the Lepidoptera moth larvae *Bombyx mori* (Bombycidae) (Bm-Fhc) and by the Embiopteran *Antipaluria urichi* (Collin et al., 2009), Spidroin 2 amino acid composition indicated that glycine, alanine and serine are the most abundant residues, representing ~75% of the protein composition. Since neither of these species is closely related to *A. juruensis*, these similarities can be assumed to be of convergent origin, and putatively related to functional constraints (Bittencourt et al., 2010). The Spidorin 2 repetitive sequence is largely composed of a rare motif present in the spidroins from the non-orb-web-weaving spiders *Kukulcania hibernalis* (MaSp2, AAT08434) and *Agelenopsis aperta* (MaSp1, AAT08436), the poly-GS motif. Interestingly, we have found the $(GA)_n$ motif in the repetitive amino acid composition of Spidroin 2, and a small number of the *An* and *GPGXX* motifs close to its C-terminal region (Figure 6A). All of them are very important motifs in the composition of orb-web-weaving spider fibroins, the last one being a motif described so far only in MaSp2 and flagelliform spidroins. Since the motifs found in orb-weavers are poorly represented in the translated products of Mygalomorph spidroins (Garb et al., 2007), Spidroin 2 presents a completely new pattern in its amino acid sequence when compared to other silk proteins described from Mygalomorphae spiders. The C-terminal amino acids of Spidroin 2 are also very similar to those of Aranaeomorphae spider MaSp2, showing sequence similarities of 83 and 86% for *Argiope amoema* and *A. trifasciata* MaSp2, respectively (Figure 6B). Even the orb-weaver genes' preference for adenine and thymine as the third nucleotide encoding for one amino acid is similar in *Spidroin 2*. Phylogenetic analysis performed with the spidroin C-terminus of 36 spider species also positioned Spidroin 2 within orbicularian MaSp2 cluster. According to these molecular characteristics, Spidroin 2 was considered to be an MaSp2-like spidroin (Bittencourt et al., 2010).

A. *Avicularia juruensis* Spidroin 2 sequence

GSGSGSGSGAGSGGGSGAGSGSGSGAGAGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGARSGSGSG
SGSGAGSGSGSGSGSGAGAGSGSGSGSGAGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGAGSGSGSGRGA
GSGSGSGSGSGAGSGSGSGSGRGAGSGSGSGSGSGAGAGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSG
AGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGSGSGSGSGSGAGAGSGSGSCR
KDAGGHDGGYGKKLGFEFGTPAAAAVTLGPGAGQQGPGGAGQQGPGGQGPYGPVASAAAAVAGGYGPGALP
QGPARQGPSGPVSSAPVASAAAARLSSPQAS

B. C-terminal alignment

```
A.amoema_MaSp2          SRLSSPQASSRVSSAVSSLVSSGPTNPAALSNAMSSVVSQVSASNPGLSG
A.trifasciata_MaSp2     SRLSSPQASSRVSSAVSTLVSSGPTNPASLSNAISSVVSQVSSSNPGLSG
A.juruensis_Sp2         ARLSSPQASSRVSSAFFSLVSSGPTSPGALSNAISSVVSQVSASNPGLSG
                        :**************. :*******.*.:****:********:*******

A.amoema_MaSp2          CDVLVQALLEIVSALVHILGSSSIGQINYAASSQYAQMVGQSVAQALA-
A.trifasciata_MaSp2     CDVLVQALLEIVSALVHILGSSSIGQINYAASSQYAQLVGQSLTQALG-
A.juruensis_Sp2         CDVLVQALLEIVS--------------------QYASLVGQSVNQALRY
                        ************                    ***.:****: ***
```

Figure 6. A. Amino acid sequence from *Avicularia juruensis* Spidroin 2. Amino acids are indicated by one-letter abbreviations. Motifs are represented by: blue - poly-GS; green - poly-A, and red - $(GA)_n$ and *GPGXX*. **B.** ClustalW alignment between the C-terminal region from *A. juruensis* spidroin 2 (EU652184), *Argiope amoema* and *A. trifasciata* MaSp2 (AAR13813 and AAK30596, respectively).

The presence of an MaSp2-like spidroin in *A. juruensis* silk is probably related to its web spinning behaviors and habitat. The Amazon *Avicularia* spp are arboreal "tarantula" spiders (Stradling, 1994). They use the foliage of trees or bushes to build their nest in crevices of trees, holding them together with silk, preventing the blade from fully expanding, and producing a silk tube web. 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 brood sacs. Major ampullate silks are noted for their unique combination of high strength, stiffness and toughness in situations of uniaxial tension, and could accommodate the continuous pull resulting from diverging leaf margins (Bittencourt et al., 2010).

THE EVOLUTION OF SILK PROTEINS

As we have already pointed out, spiders are exceptionally diverse and abundant organisms, and the spectacular innovation in silk use is considered to be the major contributor to the evolutionary diversification of the group (Vollrath and Selden, 2007; Blackledge et al., 2009). According to recent analyses of spider silk evolution (Bittencourt et al., 2010; Garb et al., 2010), gene duplication had a major influence in it, aside from other following processes such as gene conversion or intragenic homogenization (Gatesy et al., 2001). Indeed, gene duplication may underlie the origin of many or even most novel genes and hence represents an important process for functional innovation along evolutionary time (Kaessmann, 2010).

Araneomorph orb-weaving spiders - the Araneoidea and the Deinopoidea - are unique in synthesizing many different kinds of silk, and using these polymers for a variety of ecological functions throughout their lives, particularly to make prey-catching webs. The Mygalomorphae, the sister group to the Araneomorphae, however, do not build orb-webs, and only use their silk for lining burrows or to build egg sacs, contrary to the mechanically demanding tasks of orb-weaver silks. This may be the reason why we do not know much about Mygalomorph spidroins, but also why they are often regarded as "primitive" spiders (Coddington and Levi, 1991). It is assumed that Mygalomorph spiders possess only "ancestral-like" silks (phylogenetically basal), completely different from those used in orb-web-weaving.

However, a recent study from our group has shown the presence of an MaSp2-like sequence in the Mygalomorphae spider *A. juruenses* (Bittencourt et al., 2010), a spidroin formerly considered an orbicularian synapomorphy (Garb and Hayashi, 2005), providing evidence that spidroin paralogs occurred prior to the divergence of Mygalomorph and Araneomorph spiders, in a period estimated at 240 MYA (Vollrath and Selden, 2007).

Figure 7 shows a phylogenetic tree of different spider silks from several species, including *A. juruensis* spidroins and the spidroins from other Brazilian spiders studied by our group. Since the repetitive part of spidroins is highly variable, spidroin evolutionary relationships have been determined from their non-repetitive C-terminal domains. The phylogeny was constructed by maximum parsimony methods using the Nei's MEGA software and bootstrap replicates.

Our results suggest that most spidroins form ortholog clusters according to their functions. Thus, the gene family for each spidroin is confirmed by both sequence similarity and evolutionary analysis. However, some spidroin gene families have shown one or more sequences out of the main cluster for their family, showing evidence of putative polyphyletic patterns. Garb et al. (2010), using phylogenetic analyses of the conserved N- and C-terminal domains, also observed the non-monophyly for at least MaSp sequences, and pointed that out as a reflec-

tion of the repetitive sequence diversity in orbicularian species (areneoid + denopoid). This result may also be interpreted as a case of parallel genetic evolution, where changes in homologous genes in different species evolve independently to generate particular specialized rules during development (Stern and Orgogozo, 2009). However, these non-monophyletic patterns will be better evaluated as soon as a wider range of spidroin sequences become available from different species in distantly related spider families. Nevertheless, in accordance with previous researchs, most of the spidroins analyzed were correctly associated with others with similar annotations in the tree produced (Garb et al., 2007; Bittencourt et al., 2010).

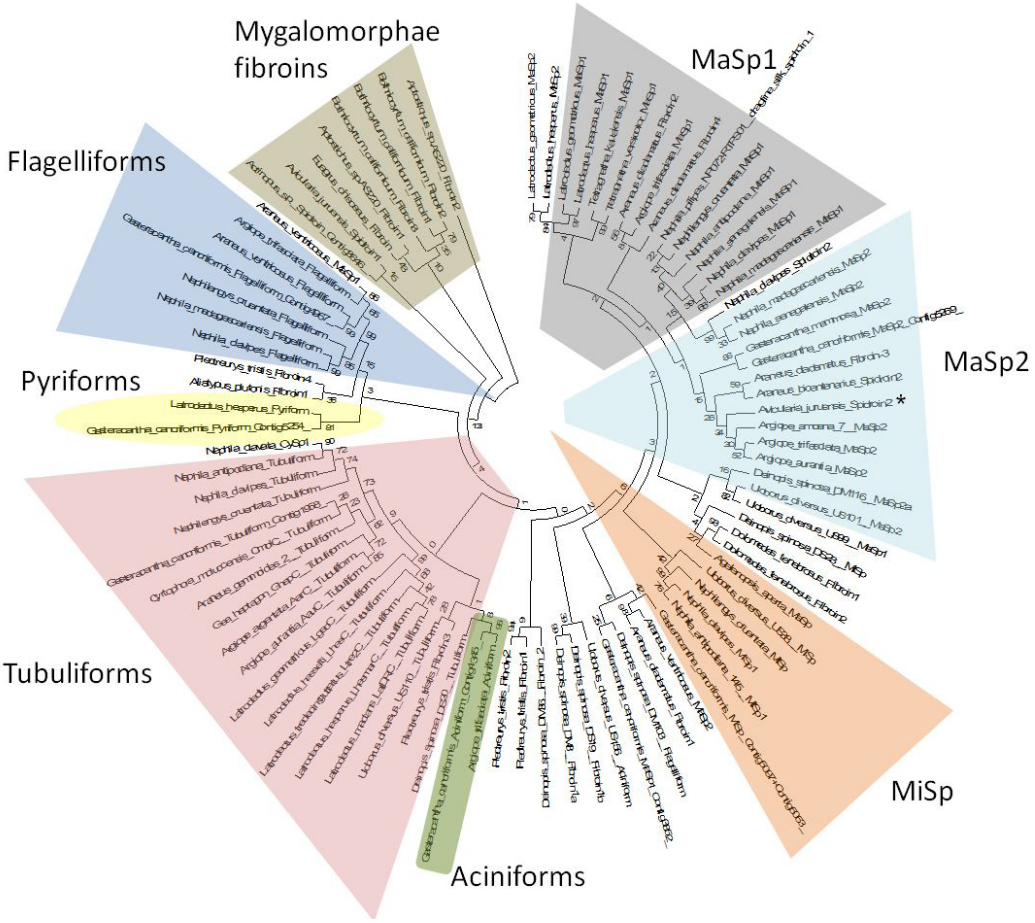

Figure 7. Phylogenetic tree of spider silk proteins (Prosdocimi et al., 2011).

Here, the maximum parsimony tree obtained for spider silk proteins demonstrates a closer link between the i) MaSp1, MaSp2, and MiSp families, such as for ii) pyriform and flagelliform spidroins and finally a closer evolutionary origin between iii) aciniform and tubuliform proteins. However, we have only a weak support for these relationships, and when we consider the basis of each ortholog group, we are not highly confident in unequivocally sup-

porting these ancestral hypotheses. Although the same ancestral group relationships were seen when we built minimum evolution and neighbor-joining trees (data not shown), we could not determine precisely which group gave rise to others by gene duplication, since the bootstrap values were always inconclusive. On the other hand, these possible evolutionary relationships make sense if one considers the ecological functions of these proteins. MaSps and MiSps are structural silks for orb-webs, Flag and PySp form gluey silks, and AcSp and TuSp are both used for the production of protective sacs for prey and eggs. Of course, this also alludes to functional convergence at the molecular level, and it appears that we will need more data to correctly support common gene ancestry for these silk protein ortholog groups. Yet, most of the sequences used in the phylogeny consist of partial sequences obtained from transcriptome analysis, and genomic data would be absolutely necessary to trace a high-quality evolutionary signal of the natural history leading to the evolution of these specialized proteins.

The present phylogenetic analysis also indicates that Mygalomorph Spidroin 2 from *A. juruensis* clustered together within orbicularian MaSp2 sequences. The Spidroin 2 sequence analysis allowed the clear identification of common motifs previously described only in orbicularian MaSp2 repetitive sequences (Bittencourt et al., 2010). This motif-signature observation is also supported by Garb et al. (2007), who showed the occurrence of spidroin paralogs prior to the divergence of Mygalomorph and Araneomorph spiders.

The overall result contests the glandular affiliation hypothesis of Hayashi and Lewis (1998), which proposes that spidroins evolved in association with the glands where they are primarily expressed. If we consider MaSps as being the most ancestral of specialized spidroins, being the ones produced by non-differentiated glands of Mygalomorphs together with some fibroins, it is conceivable that MaSps were the first spidroins to putatively direct the evolution of the morphological simple spinning gland into an anatomical diverse and specialized set of sericigene glands. The finding of this MaSp2 in Mygalomorphae also points out that putative gene duplication producing new spidroin families probably began to occur prior to the anatomical specialization of glands, which is a distinct feature of the derived orb-weaver clades. We therefore suggest an evolutionary model on which a new spidroin gene appears first in the genome and it may need a specialized compartment to produce mainly itself as a specialized protein. The anatomical subdivision of the spinning glands in orbicularian spiders suggests that each spidroin needs a very specific condition to be produced and correctly assembled or folded, this condition being particular to each protein and provided by each new anatomical sac (gland). Since the evolutionary pattern of gland split has happened through the course of spider evolution, this is a testable hypothesis, and it is now necessary to search for new spidroin sequences through the study of key spiders to validate this hypothesis. Thus, the sequence of these spiders' genome or transcriptome may help in future assessment of evolutionary relationship between gene duplication, gland differentiation and behavioral analysis to provide a clear view of the biology of web-spider systems. Furthermore, it will also be important to explain why and how a single new protein molecule can trigger the anatomical specialization of such a complex organ, making it capable to produce mainly one single sort of these highly specialized molecules.

Interestingly, we have shown that a family of proteases (from the Meprin A family) has been extensively duplicated in the orbicularian clade since its split from Mygalomorph, and that they are also highly expressed in the spinning gland of both spider clades (Prosdocimi et al., 2011). While the Mygalomorph spider *Actinopus* spp has 6 genes annotated as Meprin A

metalloproteases in its transcriptome, the orbicularian *G. cancriformis* has more than 60 members of this same family, when we evaluate its spinning gland-transcribed genes. We believe that the evolution of these proteases is also somehow linked to the evolution of spidroins, and to the spinning gland anatomical development. We can speculate that some members of these proteases may act in some sort of specific assembly or disassembly of web polymers produced from different spidroins in different glands. Once again, genomic information will be crucial to allow further cloning and some sort of interactome of double-hybrid experiments to test the interaction between spidroin mixed fibers and specific metalloproteases.

The early notions that gene duplication provides a significant reservoir for the emergence of genes and hence phenotypic adaptation agree with spider silk evolution. Spider silks are made from different spidroin molecules and are used in different ecological and behavioral contexts. Gene duplication is an important mechanism for using a single ancestral sequence to generate two (or more) functionally divergent products (neofunctionalization), and it has been historically viewed as the predominant mechanism for functional innovation during evolution (Ohno, 1970). However, the probability of fixation and maintenance of duplicates depends on many variables, including population sizes and selection regimes experienced by the corresponding genes. Neofunctionalization is context-dependent and may require multiple mutations. Mutations that are neutral in a particular environment can be positively selected by new environments or by epistatic interactions with subsequent mutations (Levasseur and Pontarotti, 2011). Accordingly, it is conceivable that the functional basis for different silks may be shaped by natural selection and probably arose at the outset of the Mygalomorph-Araneomorph radiation into their separate ecological niches, allowing further neofunctionalization and subfunctionalization of the spidroin genes.

The evolutionary appearance and specialization of new spidroins may be correlated with the rise of specific behaviors associated with the use of a given silk and that may have happened even before the anatomical specialization of glands. However, since anatomical specialization did occur, it strongly suggests that the complete anatomical specialization was truly necessary for the correct production and assembly of new spidroin polymers. However, behavior, with its great flexibility and complexity, allows the animal to take advantage of novel opportunities as they arise far quicker than morphology can. Highly variable circumstances as well as specific conditions that persist briefly lead to a fast adaptable behavior, with prospects far beyond the ability of morphology to exploit, with its long, averaging time frames. Being able to take optimal advantage of environmental conditions has obvious rewards in terms of fitness, both physical and genetic (Vollrath and Selden, 2007). Web spinning behaviors were critical for orb-webs to evolve, and although the evolution of extreme stereotypy in spinning behaviors appears to have been a crucial prerequisite for the transition from substrate defined sheet webs to architecturally defined aerial orbs, subsequent loss of that stereotypy may then allow continued diversification of web shape and thus occupation of novel niches (Blackledge et al., 2009). Indeed, Sensenig et al. (2010) tested whether material quality and behavior (web design) co-evolved to fine-tune web function, and found a dominant pattern of material and behavioral coevolution where evolutionary shifts to larger body sizes, a common result of fecundity selection in spiders, is repeatedly accompanied by improved web performance because of changes in both silk material and web-spinning behaviors.

Despite the improved understanding of spidroin evolutionary relationships and how evolutionary shifts in web production are likely related to the diversification of major spider radiations (Garb et al., 2007; Vollrath and Selden, 2007; Blackledge et al., 2009; Bittencourt

et al., 2010; Garb et al., 2010), much about spider silk evolution is still to be elucidated. One point is how the highly diverged spider silk paralogs arose despite their incredible homogeneity of repeats within some spidroins from different spider species (Bittencourt et al., 2007; Garb et al., 2007)? More intriguing is how those motifs evolved convergently to supply the demanding high-performing mechanical tasks of silk fibers (Hayashi et al., 1999; Hayashi and Lewis, 2000; Swanson et al., 2006)? Surely these hypothesized gene duplications and neofunctionalization should also be accompanied by analysis of the differences in regulatory sequences related to the specific transcription of silk mRNAs and also confirmed by sequence analysis of the whole genomic content of spidroin genes. As an example, recent study has demonstrated that there are multiple genomic copies of the MaSp1 dragline silk gene in *Latrodectus* sp spiders (Ayoub and Hayashi, 2008). All theories presented here will be probably better understood after the complete sequencing of a number of spider genomes.

SYNTHETIC BIOLOGY AS A TOOL FOR THE PRODUCTION OF SILK-BASED MATERIALS

Nature has always served as an inspiration for human thinking in material and design. This also applies to spider silk. Spider silk is a biopolymer with high tensile strength and elasticity, leading to a toughness that is two to three times that of synthetic fibers available (Vollrath, 2000). Natural spider silk is also antimicrobial, hypoallergenic and completely biodegradable (Gellynck et al., 2008), properties that are of great interest for new technical and biomedical applications. Since the composition and design of spider silks allow their recombinant production, synthetic biology has been used to create artificial spider silk molecules designed for specific biotechnological applications (Scheibel, 2004; Spiess et al., 2010). Aiming to produce novel biodegradable materials that recapitulate the behavior of natural spider silks, we were able to design *de novo* proteins and produce synthetic spider-like fibers based on MaSp2 from *P. bistriata* (Rech, 2011). Moreover, it has been proven that it is possible to assemble spider silk proteins not only as a linear thread, but also as films, capsules, nanofibrils, and nanovesicles (Scheibel, 2005; Bogush et al., 2009; Krishnaji et al., 2011), also demonstrating that engineered silk proteins have a huge potential as materials for tissue engineering and guided tissue repair in biomedical applications. The major limitation for spider silk production is the inability of protein production systems to assemble the recombinant spider silk into fibers. To overcome this limitation, Teulé and co-workers (2012) showed that silkworms can be engineered to express a chimeric silkworm-spider silk gene producing composite silk fibers with improved mechanical properties

The great potential use of engineered silk proteins as nanowires or biosensors was also demonstrated by Huang and Sun (2010). Using the identified functional native domains from spider flagelliform silk protein and the Ca^{2+}-binding domain of the lipase *Lip A* from *Serratia marcescens*, they designed a newly sequenced *eD2* by "hiding" the ion binding sequence in the silk structure sequence. In water, *eD2* formed uniform spherical agglomerates with a β-spiral structure. Triggered by Ca^{2+}, *eD2* formed nanofibers with higher compliance and thermal stability. The specialties of this novel peptide design were also demonstrated by changing the pH, using other metal ions, and mutating the model sequence.

More recently, Lammel et al. (2011) using the recombinantly produced engineered spider silk protein *eADF4*(*C16*), mimicking the known amino acid sequence of the natural

spider silk protein *ADF4* from the European garden spider *A. diadematus* (Scheibel, 2004), proved its potential use for sustained controlled delivery of positively charged and sufficiently hydrophobic drug molecules. According to them, the major advantage of the system is that *eADF4(C16)* particles can be produced and loaded within an all-aqueous process under ambient conditions, which is extremely important considering encapsulation of labile compounds and the biocompatibility of the product. The load and release mechanisms of spider silk particles begins with the attachment of drug molecules to silk by electrostatic forces, and after particle surface saturation, low molecular weight drugs start to diffuse into the biopolymer matrix, and interact with the matrix via attractive hydrophobic and electrostatic interactions. With time, drug molecules are slowly released from the surface leading to constant release rates at physiologic conditions (37°C, pH 7.4). So far, this is the most successful attempt to prove the applicability of such particles as drug carriers.

Finally, the advances in synthetic biology have made it possible to produce recombinant spider silk proteins as well as to engineer silk genes, and therefore, proteins with properties tailored for specific requirements. Although many advances have been made in this field, much is still to be done. For instance, the whole process of recombinant spider silk synthesis is not well controlled. The lack of full comprehension of these processing steps has limited the ability to spin-reconstituted silk solutions into fibers with properties comparable to those of native spiders (Omenetto and Kaplan, 2010). Further studies related to the natural spinning process in spider silk glands will probably provide new insights about the regulation of such spider features. Maybe a good starting point is to analyze the possible interaction between spidroin molecules and specific metalloproteases, a family of proteases found to be highly expressed in the silk glands of *Actinopus* spp and *G. cancriformis* spiders (Prosdocimi et al., 2011). Due to their high number of transcripts, it is quite probable that they may have some influence on spider silk assembly. Nevertheless, spider silk has proven to be suitable for several technical purposes, and its biomedical application does not seem to be far from reality.

CONCLUSIONS

Spider webs have attracted human attention for centuries. Spider silks are remarkable not only for their exceptional material properties but also for their production, processing, and evolution. Orb-web spiders are able to produce several kinds of spidroins responsible for the production of a specific silk with particular mechanical properties. The silk proteins are produced by one of the six specialized sericiginal glands that comprise the spinning apparatus of the orb-web-weaving spiders. According to our data, spider silks probably evolved through a major influence of gene duplication that began prior to the anatomical specialization of glands, probably at the outset of the Mygalomorph-Araneomorph radiation (240 MYA). Once a new spidroin gene appears in the genome and is maintained through natural selection due to its survival advantage to the spider, the anatomical specialization of glands are then needed for the correct production and assembly of a new spidroin polymer, and of course, to take advantage of its full-mechanical potential. It is conceivable that the functional basis for new silks to be maintained in the genome is closely related to positive aspects involving the spinning behavior of spiders, and it is likely related to the diversification of spiders in different environments. However, only a wider sampling of spidroin sequences and the complete sequencing of different spiders' genomes will allow a mature gain in our understanding about the evolutionary

relationships among spidroins, and how the evolution of such molecules might have driven anatomical and ecological developments.

The modular nature of spider silks together with their mechanical properties have also stirred interest in synthetic spider silk production. Spider silks have been proven capable of generating a wide range of materials such as hydrogels, fibers, sponges, films, and other forms. Moreover, through synthetic biology, genetically engineered silks can lead to new materials and technological platforms with suitable mechanical, morphological, and structural features for a number of applications. Although a wide range of silk-based material can be envisioned for several technical and biomedical purposes, their production and spinning processes must be improved for the correct assembly of synthetic spidroins, aiming at the production of a synthetic fiber with similar or even improved mechanical properties of natural spider silks. Finally, the applicability of engineered spider silk particles as drug carriers has been recently demonstrated by Lammel et al. (2011), again providing evidence of the striking properties of these molecules and opening a vast field to explore: the biomedical application of silk-based materials.

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