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Biomaterial evolution parallels behavioral innovation in the origin of orb-like spider webs

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Todd A. Blackledge¹, Matjaž Kuntner^{2,3,4}, Mohammad Marhabaie¹, Thomas C. Leeper⁵ & Ingi Agnarsson^{3,6}

¹Department of Biology and Integrated Bioscience Program, University of Akron, Akron, Ohio, USA, ²Institute of Biology, Scientific Research Centre, Slovenian Academy of Sciences and Arts, Ljubljana, Slovenia, ³National Museum of Natural History, Smithsonian Institution, Washington, DC, USA, ⁴College of Life Science, Hubei University, Wuhan 430062, Hubei, China, ⁵Department of Chemistry and Integrated Bioscience Program, University of Akron, Akron, Ohio, USA, ⁶Department of Biology, University of Vermont, Burlington, Vermont, USA.

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Correspondence and requests for materials should be addressed to T.A.B. (blackledge@uakron.edu)

Correlated evolution of traits can act synergistically to facilitate organism function. But, what happens when constraints exist on the evolvability of some traits, but not others? The orb web was a key innovation in the origin of >12,000 species of spiders. Orb evolution hinged upon the origin of novel spinning behaviors and innovations in silk material properties. In particular, a new major ampullate spidroin protein (MaSp2) increased silk extensibility and toughness, playing a critical role in how orb webs stop flying insects. Here, we show convergence between pseudo-orb-weaving *Fecenia* and true orb spiders. As in the origin of true orbs, *Fecenia* dragline silk improved significantly compared to relatives. But, *Fecenia* silk lacks the high compliance and extensibility found in true orb spiders, likely due in part to the absence of MaSp2. Our results suggest how constraints limit convergent evolution and provide insight into the evolution of nature's toughest fibers.

Much of Darwin's strongest support for the theory of natural selection came from observations of convergent evolution^{1,2}. When organismal function is determined by interactions among different suites of traits, such as morphology and behavior influencing feeding in stickleback fish or locomotion in *Anolis* lizards, then deterministic evolution should extend to those interactions^{3,4}. However, natural selection only acts upon existing genetic variation, so that it is important to ask "what happens when genetic constraints may resist that facilitation"?

The orb web was a key innovation in the origin of more than 12,000 species of spiders in Orbiculariae^{5–7} – the orb-weaving spiders and descendant taxa such as cobweb spiders. The success of the orb web hinged upon the evolution of two discrete suites of phenotypic traits – novel web spinning behaviors and innovations in the material properties of silks⁸. Aerial orb webs are built by first constructing discrete frames of dragline silk threads and then depositing adhesive silks. The individual behaviors involved in spinning orb webs are well documented⁹ and suggest *no a priori* constraint preventing other taxa from evolving similar web architectures. Yet, substantial phylogenetic evidence argues that orb webs evolved only once^{8–11}. While famous for their gluey adhesive silks, orb webs depend upon a network of dragline silk for support and for the primary work of dissipating the kinetic energy of flying insect prey^{12,13}. All araneomorph spiders produce dragline silk using major ampullate spidroin 1 (MaSp1) proteins¹⁴ that contain varying amounts of crystalline domains. These domains are determined in part by the prevalence of poly-alanine and glycine-alanine repeats, which interlock the proteins, thereby strengthening and stiffening the silk^{15,16}. However, spiders in the orbicularian subclade, the "true" orb spiders, produce dragline silk that is notably tougher, more compliant, and more extensible than other clades¹⁷. These changes are due in part to the origin of a novel protein, MaSp2¹⁸. Proline in the MaSp2 disrupts crystalline intermolecular structures, thereby increasing the compliance and extensibility of the silk¹⁹. Major ampullate silk spun by orbicularian spiders is initially stiff, but then extends greatly under prey impacts, allowing orb webs to arrest prey in midair without the insects ricocheting out of the webs^{12,13}. The resulting fibers are some of the toughest known biological materials^{20,21}, with great biomimetic potential²², but which are also exceedingly difficult to replicate in the laboratory²³.

A little known spider genus, *Fecenia*, constructs a web, termed a "pseudo-orb", that is spectacularly similar to the true orb webs spun by Orbiculariae (Fig. 1). However, *Fecenia*'s web is considered an example of convergent evolution to true orb webs because of the morphological similarity of *Fecenia* to retrolateral tibial apophysis

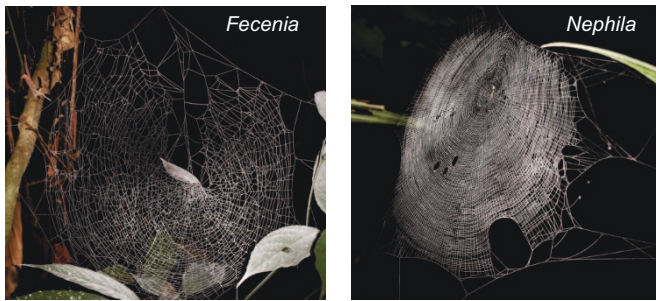


Figure 1 | Comparison of pseudo- and true orb web architectures. *Fecenia* constructs pseudo orb webs that are remarkably similar in architecture to the true orb webs spun by orbicularian spiders such as *Nephila*. Both types of webs consist of radiating support threads spun from major ampullate (MA) silk and overlaid spiral-like arrays of adhesive capture silk. The supporting silk is the focus here because of its dominant role in dissipating prey energy^{12,13}.

(RTA) clade spiders^{24,25} – a group that includes wolf spiders, crab spiders, and jumping spiders as well as many other taxa that often do not construct prey capture webs. If coevolution of complex suites of traits, such as web spinning behaviors and silk biomaterial properties, occurred during the origin of orb webs then the “convergence” hypothesis predicts that *Fecenia* should produce silk with material properties more like that of true orb spiders than its phylogenetic relatives. *Fecenia* silk should be particularly extensible, compliant, and tough. However, all investigations to date of dragline silk in RTA clade show that RTA clade spiders lack the MaSp2 that is critical for shaping the functional properties of orb spider dragline silk^{26,27}. The incorporation of MaSp2 into dragline silk is associated with a dramatic increase in the plasticity of dragline silk properties, particularly its overall extensibility and stiffness after yield²⁸. The end result is that spiders lacking MaSp2 produce dragline silk that occupies only one third of the total performance space defined by interspecific variation in silk properties among Orbiculariae²⁸. Thus, *Fecenia* dragline silk lacks much of the potential variation in mechanical performance that

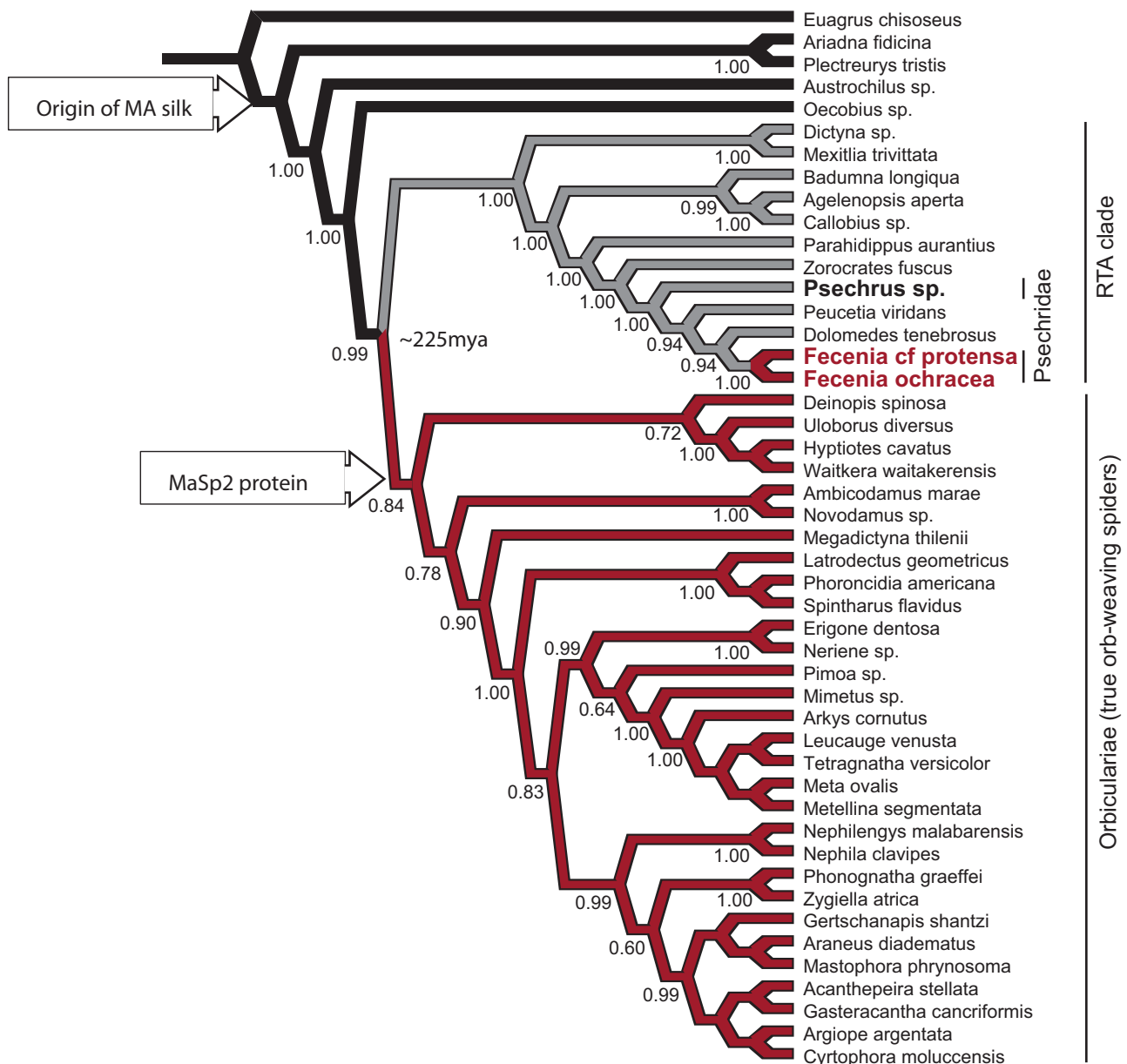


Figure 2 | Phylogenetic placement of *Fecenia*. Pseudo-orb webs are nested deeply within RTA clade, and clearly derived independently of the origin of true orb webs in Orbiculariae. The RTA clade, containing *Fecenia*, diverged from the ancestor of orb-weaving spiders ~225 mya, prior to the known origin of the MaSp2 protein. Posterior probabilities are indicated for most taxa, except where omitted from intra-familial relationships for clarity.

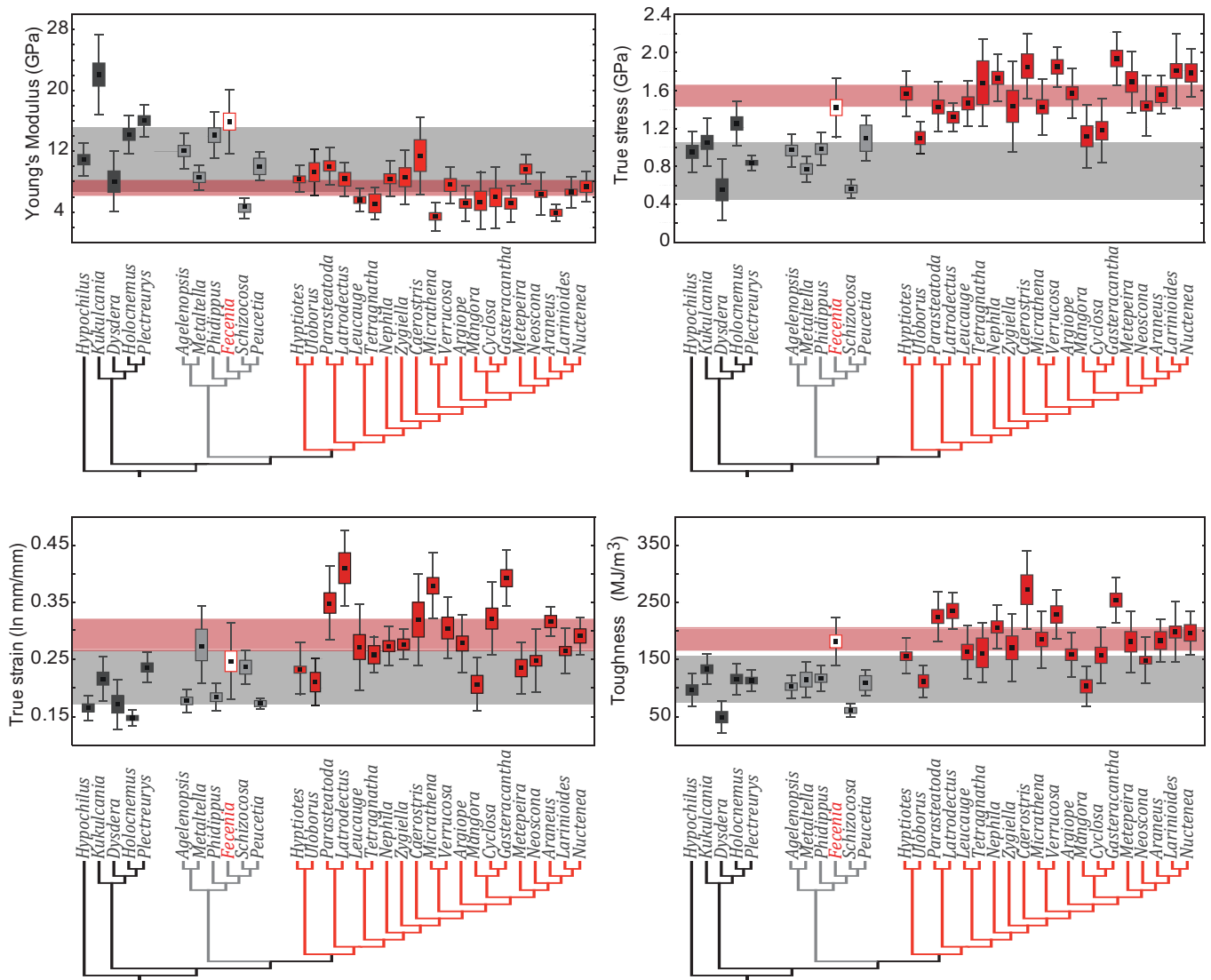


Figure 3 | Major ampullate silk material properties across the spider phylogeny^{17,49}. The performance of *Fecenia* silk is compared to basal taxa of spiders (Haplogynae and Austrochiloidea in black), its own clade (RTA clade in gray) and true orb spiders (Orbiculariae in red). Boxes represent \pm SEM and tails represent \pm SD for individual species. The bands represent the 95% confidence for mean performance of silk from species in the RTA clade (gray) and Orbiculariae (red). *Fecenia* shares the relatively high stiffness and low extensibility characteristic of non-orb-weaving spiders, but outperforms taxa outside Orbiculariae in strength and toughness.

natural selection could act upon to evolve the high extensibility, and hence also toughness, that underlies orb web function. Here, we first use molecular data to test the convergent evolution hypothesis (see also²⁹). We then characterize the material properties of *Fecenia* dragline silk and place them in a comparative framework with orbicularian, RTA clade, and outgroup species of spiders.

Results

Our analysis clearly shows the pseudo-orb web's independent origin from true orbs. The phylogenetic analysis recovered a monophyletic Orbiculariae sister to a monophyletic RTA clade (Fig. 2), together forming the morphologically recognized “canoe tapetum” clade³⁰. *Fecenia* nests deep within the RTA clade (Fig. 2; see also²⁹).

We found that *Fecenia*'s major ampullate silk is both tougher and stronger than major ampullate silk produced by its RTA clade relatives (toughness and true stress respectively in Fig. 3 and Table S1; $P = 0.0001$ in post-hoc comparisons for both parameters). This supports the hypothesis that the evolution of aerial web-spinning behaviors is accompanied by strong selection on silk biomaterial properties in spiders. However, *Fecenia*'s silk resembles that of its

phylogenetic relatives in RTA clade in being both stiff and non-extensible (Young's modulus and true strain respectively in Fig. 3 and Table S1; $P = 0.15$ and $P = 0.051$ respectively in post-hoc comparisons).

Table 1 compares the amino acid composition of major ampullate silk from *Fecenia* webs to published compositions for a variety of Orbiculariae, including *Latrodectus* which spins an evolutionarily derived three-dimensional cobweb. *Fecenia* major ampullate silk contains (mean \pm SD) $3.3 \pm 0.3\%$ proline. *Fecenia*'s major ampullate silk also contrasts with orbicularian taxa in its relatively lower glycine content and higher content of asparagine/aspartic acid, serine, tyrosine and valine. These differences are consistent with cDNA data that show MaSp composition of orbicularian taxa to be more homogeneous, dominated by ~ 5 common amino acids, compared to other taxa^{26,31}.

Discussion

Fecenia's placement deep within RTA clade shows that the orb-like architectures of their webs evolved convergently with true orb webs. The origin of the orb web placed new demands on the function of silk



Table 1 | Amino acid composition of the major ampullate silk of Orbiculariae spiders compared to *Fecenia*. All values are mean percentage of the total of all amino acids

	<i>Fecenia</i> ¹	<i>Latrodectus hesperus</i> ²	<i>Nephila clavipes</i>				<i>Araneus diadematus</i> ⁵
			Lombardi & Kaplan ³	Arcidiacono ⁴	<i>Argiope aurantia</i> ³	<i>Neoscona domiciliorum</i> ³	
Alanine	21.1	28.5	21.1	27.5	22.2	18	17.60
Arginine	2.3	2.5	7.6	2.4	2.9	0.6	0.5
Asparagine/ Aspartic acid	7.3	1.5	2.5	1.1	1.6	0.6	1.04
Cysteine	0.2	-	0.1	0.1	0.3	0.7	-
Glutamine/ Glutamic acid	11.1	10.9	9.2	10.3	11.1	10	11.50
Glycine	16.2	39.9	37.1	42.2	34.7	38	37.20
Isoleucine	4.1	1	0.9	0.5	0.8	0.5	0.63
Leucine	4.1	1	3.8	4.5	4.2	1.2	1.27
Lysine	1.2	-	0.5	0.3	0.5	0.2	0.54
Methionine	0.3	-	0.4	0.3	0.3	0.2	-
Phenylalanine	2.3	0.7	-	0.4	-	-	0.45
Proline	3.3	2.5	4.3	1.7	6.4	11.2	15.77
Serine	14.4	3.7	4.5	3.6	5.1	6.8	7.40
Threonine	2.9	1.5	1.7	0.5	0.8	0.9	0.9
Tyrosine	1.2	5.3	2.9	3.7	3.8	3.7	3.92
Valine	7.4	1	1.8	0.8	1.5	0.7	1.15

¹This study.

²La Mattina, C. et al. Spider minor ampullate silk proteins are constituents of prey wrapping silk in the cob weaver *Latrodectus hesperus*. *Biochemistry* **47**, 4692–4700 (2008).

³Lombardi, S. J. & Kaplan, D. L. The amino acid composition of major ampullate gland silk (dragline) of *Nephila clavipes* (Araneae, Tetragnathidae). *J. Arachnol.* **18**, 297–306 (1990).

⁴Arcidiacono, S., Mello, C., Kaplan, D., Cheley, S. & Bayley, H. Purification and characterization of recombinant spider silk expressed in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **49**, 31–38 (1998).

⁵Anderson, S. O. Amino acid composition of spider silks. *Comparative Biochemistry and Physiology* **35**, 705–711 (1970). - indicates either that the amino acid wasn't detected or that data were not provided.

threads. In contrast to plesiomorphic web types, orb webs target primarily flying insects that transfer tremendous amounts of kinetic energy to the webs, which must be dissipated quickly by the silk. Orb webs accomplish this feat through the very tough major ampullate silk in the radial threads of the webs. Energy is dissipated by the high damping capacity of the MA silk³². Moreover, the substantial softening of MA silk after yield, coupled with its high extensibility and strain hardening, help to localize damage during impact and ensure robust performance despite possible defects¹². Thus, a suite of bio-mechanical traits interact with web spinning behaviors in optimizing orb web function.

If coevolution of complex suites of traits, such as web spinning behaviors and the material properties of spider silks, occurred during the origin of orb webs then *Fecenia* should produce silk with material properties more like that of true orb spiders than its phylogenetic relatives. We show that *Fecenia*'s MA silk is both tougher and stronger than MA silk produced by its RTA clade relatives (Fig. 3), as predicted by the “coevolution” hypothesis. However, *Fecenia*'s silk resembles that of its phylogenetic relatives in being both stiff and non-extensible. Compared to Orbiculariae, *Fecenia* MA silk likely achieves similarly high toughness through mechanisms that enhance inter-molecular bonding, and hence improve stiffness and strength.

The extensibility and non-linear behavior of Orbiculariae MA silk was facilitated by the origin of MaSp2 because proline residues fold the protein backbone into molecular “nanosprings” that disrupt intermolecular bonding in the amorphous regions of the silk^{19,33,34}. This increases the mobility of the proteins when strained and is critical for decelerating prey while also optimizing the dissipation of impact energy within discrete regions of the orbs^{12,13}. Much of the interspecific variation among Orbiculariae in MA silk properties, such as extensibility and energy damping, correlates with differences among species in the proline composition of the silk^{33,35} – determined by the amino acid sequence of MaSp2 and its expression level. The low proline content in threads from the webs of *Fecenia* is consistent with its MA silk containing mostly MaSp1. Lack of MaSp2 is further supported because cDNA from all RTA clade taxa examined to date lack proline-rich MaSp2^{26,27}, with one possible exception -

Euprosthenoops expressed a MaSp2-like sequence with some isolated proline residues³⁶. Many structures induced by amino acid sequence, such as helical glycine repeats and crystalline poly-alanine regions, clearly play critical roles in determining MA silk's remarkable properties^{16,20,37}. Yet, proline plays a dominant role in determining the elasticity of many fibrous proteins³⁴. Moreover, the evolution of new amino acid sequences in silk proteins is quite rare among spiders, compared to changes in expression levels of existing proteins^{38,39} or the effects of concerted evolution on amino acid sequences^{26,31,40,41}. We therefore argue that *Fecenia*, and perhaps all RTA clade taxa, face substantial phylogenetic constraint on the evolvability of their MA silk properties.

Orbiculariae is a remarkably speciose and diverse clade, while *Fecenia* is species-poor and restricted to habitats in South East Asia²⁹. Why are true orb spiders so successful compared to pseudo orb-weaving *Fecenia*? We speculate that constraints on web function imposed by lack of MaSp2 are important, although many other factors likely help to explain *Fecenia*'s low species richness. For instance, *Fecenia* uses cribellate adhesive silk in its capture threads⁸, a primitive and behaviorally costly type of silk utilized by few true orb spiders. However, while cribellate Orbiculariae are also species-poor^{5,8}, the materials properties of their MA silk are clearly more like other orbicularians than like *Fecenia* (e.g. *Hyptiotes* and *Uloborus* in Fig. 3). Thus, the type of adhesive silk used in a web does not itself inherently constrain the evolution of MA silk properties. Behavioral differences may also contribute because the radiating threads in *Fecenia* webs are not as regularly spaced as in true orbs⁴². *Fecenia* also does not produce a complete capture spiral, instead spinning discrete bands of capture silk in segments that zigzag back and forth across the web surface²⁹. Finally, the two core fibers of the capture silk are broadly separated in *Fecenia*²⁹. However, orb webs are generally robust to minor perturbations in the shape of the capture spiral¹² and often include substantial zig-zagging, rather than spiraling, of the capture silk^{43–45}. *Fecenia* is also younger than Orbiculariae (Fig. 2). Yet, changes in the expression levels of MA silk genes allow even relatively young clades within Orbiculariae to show large variation in silk performance (e.g. within *Argiope*⁴⁶ and between *Argiope* and its



sister clade *Cyrtophora*^{33,35}) so that age also appears insufficient to explain constraint on the success of pseudo orb-weaving spiders. Instead, many orb spiders depend upon rare, but very large insects for the bulk of their calories^{47,48}. Dissipating the high kinetic energy of these insects is a formidable challenge for orb webs⁴⁹. Our investigation shows that *Fecenia* MA silk lacks many aspects of mechanical performance that are essential for energy dissipation by true orb webs, therefore suggesting that *Fecenia* may have reduced ability to capture large or fast-moving prey. Furthermore, the absence of MaSp2 proteins likely limited the evolution of *Fecenia*'s MA silk properties during the origin of the pseudo orb. Thus, our study adds to growing evidence that the evolution of innovations in silk production, such as the origin of novel silk proteins like MaSp2, plays a critical role in determining patterns of spider diversification^{5,6,50}.

Methods

We collected *Fecenia cf protensa* from MacRie, Singapore, *Fecenia ochracea* from New Britain, Papua New Guinea, and one unidentified species from the morphologically related, confamilial genus *Psechrus*, collected from Pulau Ubin, Singapore, and preserved them in 100% ethanol. Vouchers are deposited in the invertebrate collection of the University of Vermont. We then used standard DNA extraction, amplification and sequencing methods to sequence partial fragments for two mitochondrial (16 S, COI) and four nuclear (18 S, 28 S, H3, wingless) loci, resulting in ~4600 bp of data. We added these sequences to the preferred alignment of the orbicularian phylogenetic analysis of Blackledge et al.⁸ by hand to minimize change to the existing data sets, using Mesquite⁵¹. We used jModeltest v0.1.1^{52,53} to select the best model of nucleotide substitution for each gene, and position for the protein coding genes COI and H3, and then used Bayesian inference to analyze the matrices. This resulted in a total of 10 data partitions. The resulting models were: (GTR + Γ + I for COI^{1st}, COI^{2nd}, 16 S, 18 S, 28 S, H3^{3rd}; GTR + Γ for H3^{1st} and Wingless; HYK + Γ + I for COI^{3rd}, and JC for H3^{2nd}). We ran the MC³ (Metropolis coupled Markov chain Monte Carlo) chain in MrBayes V3.1.2⁵⁴ for 10,000,000 generations while sampling every 1000 generations. The first 50% of the trees were discarded as burnin, after stationarity was reached.

We also collected silk from the pseudo-radii of *Fecenia* webs and characterized its properties using a Nano Bionix testing system. We then constructed the broadest data set of material properties for naturally produced spider dragline silks to compare *Fecenia* silk to both its relatives and true orb spiders. *Fecenia* specimens were collected in Jiangfeng, Hainan, China and brought into the laboratory where they were housed in 20 cm diameter, 20 cm high cages with removable sides. Small leaves were placed in the cages for the construction of retreats. Spiders were then allowed to construct webs for ~2 days before silk was collected for testing. We collected silk from the pseudo-radii of *Fecenia* webs using standard protocols that are well-established for orb spiders⁴⁹. In total, 65 samples of silk from 14 webs spun by a total of nine different spiders were obtained from regions of webs lacking capture spiral onto cardboard mounts and secured with cyanoacrylate adhesive. We then tested the 10–12 mm long samples using a Nano Bionix UTM (Agilent Technologies) at a strain rate of 0.10/s. We compared the data to two existing data sets on the material properties of MA silk from spiders. Swanson et al.⁵⁵ provides the phylogenetically broadest dataset on the properties of MA silk. Most of the silk in this study was collected from naturally spun silk structures, such as draglines and webs. However, silk from the orb spider taxa were collected not from webs, but rather through forcible silking of restrained spiders, which alters the alignment of silk proteins such that the resulting fibers are unusually stiff compared to naturally spun silk in webs. Therefore, we substituted data from Sensenig et al.⁴⁹ because it provides the most diverse dataset on the properties of major ampullate silk from actual radii in naturally spun orb webs. Silk in both of these studies was collected using techniques similar to our own and was tested using a Nano Bionix under similar conditions.

For each species, we used individual spiders or webs as the primary sampling unit. We then constructed 95% confidence intervals for the mean performance of silk for two major clades – the RTA clade, which is the phylogenetic “home” of *Fecenia*, and the orbicularian clade, with whom *Fecenia* has converged behaviorally. We also used Tukey's HSD tests to make post-hoc comparisons of the performance of *Fecenia* silk to non-Orbicularia.

Finally, we analyzed the amino acid composition of the MA silk produced by *Fecenia* to test for proline content. Because proline is largely confined to the MaSp2 locus in Orbicularia²⁶, the percentage of proline in MA silk is a strong indicator of the presence of MaSp2 and its proportion relative to MaSp1³⁵. The vapor phase hydrolyzation and amino acid analysis were performed using an established protocol described by Smith (2003)³⁶. Briefly, we collected four 5–10 cm samples of frame and radial threads from *Fecenia* webs. The samples were transferred to the bottoms of 6×50 mm glass tubes that were vacuum-sealed into a glass vial containing 300 μ l of 6 N HCl. We then hydrolyzed the samples in this vial-in-vial setup under the vapor of 6 N HCl at 112°C for 20 hr. Then, the inner sample tubes were removed, dried under vacuum, and free amines derivatized with an aminoquinolyl-NHS compound (AQC) using a Waters AccQ Tag kit (Milford, MA). The derivatized amino-acids were separated by the 3.9×150 mm C18 column from the AccQ Tag kit using an ÄKTAPurifier HPLC system. Chromatographs were analyzed and peak areas

integrated using Unicorn 5.20 software and compared to identically derivatized amino-acids standards run under the same conditions.

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Author contributions

T.A.B., M.K. and I.A. designed the experiment. M.K. and I.A. collected the spiders. T.A.B. collected and analyzed the silk data. M.M. and T.C.L. collected and analyzed the amino acid composition data. T.A.B. sequenced DNA and I.A. conducted the phylogenetic analysis. T.A.B., M.K. and I.A. wrote the manuscript.

Additional information

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