Research

Cite this article: Foley S, Saranathan V, Piel WH. 2020 The evolution of coloration and opsins in tarantulas. *Proc. R. Soc. B* **287**: 20201688.
<http://dx.doi.org/10.1098/rspb.2020.1688>

Received: 13 July 2020

Accepted: 1 September 2020

Subject Category:

Evolution

Subject Areas:

evolution, ecology

Keywords:

colour evolution, tarantula vision, crypsis, natural selection, sexual selection

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Electronic supplementary material is available online at <https://doi.org/10.6084/m9.figshare.c.5114297>.

The evolution of coloration and opsins in tarantulas

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Tarantulas paradoxically exhibit a diverse palette of vivid coloration despite their crepuscular to nocturnal habits. The evolutionary origin and maintenance of these colours remains mysterious. In this study, we reconstructed the ancestral states of both blue and green coloration in tarantula setae, and tested how these colours correlate with presence of stridulation, urtication and arboreality. Green coloration has probably evolved at least eight times, and blue coloration is probably an ancestral condition that appears to be lost more frequently than gained. While our results indicate that neither colour correlates with the presence of stridulation or urtication, the evolution of green coloration appears to depend upon the presence of arboreality, suggesting that it probably originated for and functions in crypsis through substrate matching among leaves. We also constructed a network of opsin homologues across tarantula transcriptomes. Despite their crepuscular tendencies, tarantulas express a considerable diversity of opsin genes—a finding that contradicts current consensus that tarantulas have poor colour vision on the basis of low opsin diversity. Overall, our findings raise the possibility that blue coloration could have ultimately evolved via sexual selection and perhaps proximately be used in mate choice or predation avoidance due to possible sex differences in mate-searching.

1. Introduction

The large, hirsute spider family Theraphosidae includes some of the most visually striking spiders. Commonly known as tarantulas, these animals are notable for the fact that they are often brightly coloured, with violet, blue, green, red and purple hues in particular, present throughout the family [1,2]. However, a long-standing enigma has been the evolutionary origin and maintenance of such vivid coloration in a group that is almost entirely crepuscular, if not nocturnal [3,4]. The evolution of coloration in tarantulas has previously received only scant attention in the literature (cf. [2]). A well-resolved phylogeny for this group did not exist until recently [5] and previous attempts [2] to reconstruct the ancestral states of blue coloration were based upon a poorly resolved and outdated supertree. Hsiung *et al.* [2] claim that blueness evolved independently among tarantulas on several occasions, but also used vaguely defined criteria to identify blue and green coloration. For instance, no data was provided to validate their colour scorings, some of which appear to be erroneous (e.g. blue coloration was listed as present in *Haplocosmia/Nhandu*, and absent in *Cyrtopholis*; but see electronic supplementary material, table S3).

We aim to verify the findings of Hsiung *et al.* [2] by reconstructing the evolutionary histories of both blue ('blueness') and green ('greenness') integumentary coloration in the setae of tarantulas via quantitative ancestral state reconstructions. We use a robust phylogeny [5,6] and set strict criteria for assigning colour states by calculating just-noticeable difference (JND) values [7] for at least one representative species for each genus represented in our phylogeny.

Table 1. Phylogenetic signal as determined by Blomberg's K and Pagel's λ . Blomberg's K values <1 inform that there is less phylogenetic signal than would be expected under Brownian motion. Pagel's λ informs whether the phylogeny alone is sufficient or not to explain the distribution of traits. Significant p -values ($p \leq 0.05$)

dataset	Blomberg's K		Pagel's λ			
	K	p -value	λ	log likelihood	log likelihood 0	p -value
blue discrete	0.38	0.15	7.21×10^{-05}	-26.69	-26.69	1
blue continuous	0.38	0.17	7.21×10^{-05}	-160.92	-160.92	1
green discrete	0.44	0.04	0.42	-22.33	-24.19	0.05
green continuous	0.46	0.03	0.25	-172.54	-172.79	0.48

In order to address the function of colours in tarantulas, we also constructed data matrices based on prior publications detailing the presence or absence of (i) sound-producing stridulatory setae, (ii) defensive urticating setae, and (iii) arboreal tendencies for each genus in this study, and conducted a series of correlative tests to determine whether any of these traits might inform the function of blue or green coloration in tarantulas. We hypothesize that blue colour should be associated with stridulating/urticating setae, or both, if it either originally evolved or has been exapted (*sensu* [8]) for an aposematic function. However, we posit that green coloration could aid in crypsis and should show an association with arboreality.

It is generally thought that tarantulas possess little or no colour vision, with only a weak colour spectrum discrimination [2,9], with Hsiung *et al.* [2] noting that sexual selection as a driving force behind the evolution of tarantula coloration is unlikely. Morehouse *et al.* [10] argue that tarantulas are colour-blind on the basis of finding a low opsin diversity in *Aphonopelma* [10]. We investigate whether this claim holds true for other tarantulas by scoring the presence of opsins from transcriptomic data in 25 tarantula genera. Opsins are specialized, transmembrane proteins belonging to the superfamily of G-protein coupled receptors that are involved in converting light photons to electrochemical signals through a signalling cascade [11]. While the expression of multiple opsin species does not necessarily imply greater colour discrimination, the absence of opsin orthologues could provide support to the hypothesis that tarantulas, broadly speaking, cannot perceive colours.

2. Results

(a) Phylogenetic signal of colour traits

Our phylogeny contains 37 theraphosid genera and 2 outgroups (*Damarchus* sp. and *Linothele* sp.), covering the entire spectrum of theraphosid subfamilies minus Selenogyriinae. Blueness occurred in 25 genera and both outgroups, greenness in 11 genera, stridulation in 25, urtication in 14 and arboreality in 9 (electronic supplementary material, table S3). Only weak phylogenetic signals were detected for blueness and greenness, with Blomberg's K values ranging from 0.38 to 0.46, and λ 's from 0 to 0.42 (table 1). Only four internal nodes in the ancestral state reconstructions of blue had posterior probabilities of greater than or equal to 95%, and no such nodes were recovered in either the green and arboreality reconstructions (figures 1 and 2; electronic supplementary material, figure S1).

The only association between a trait and a colour that displayed a significant p -value is 'greenness depending on arboreality' (0.01, table 2). This informed the need to subsequently conduct dependence tests.

(b) Evolution of blueness

Blueness was probably present at the root of the tree, but was subsequently lost and regained multiple times (figure 1). Of the five gains, three are at terminal nodes in Old World subfamilies, one is relatively shallow (the ancestor to *Orphnaecus* and *Phlogiellus* also in the Old World) and one is at the root of our phylogeny and is largely retained throughout the subfamilies Ornithoctoninae, Poecilotheriinae, Harpactirinae, Theraphosinae, Psalmopoeinae, Aviculariinae and some Ischnocolinae. Blueness is also reported in both of our non-tarantula outgroups (*Linothele* and *Damarchus*), and only two of the included theraphosid subfamilies lack any blue members (Schismatothelinae and Stromatopelminae). Blueness appears to have been lost seven times, with five of those losses occurring at the tips or relatively shallow nodes, especially in Neotropical subfamilies.

(c) Relationship between greenness and arboreality

Both raw and weighted AIC values support an evolutionary scenario where the evolution of greenness depends on the prior evolution of arboreality (table 3). Both arboreality and greenness is reported in six genera, but a further eight genera possess either greenness or arboreality (figure 2). Greenness does not appear to have been lost at any point throughout the evolution of tarantulas, but has been gained at least eight times. Six of these eight gains appear at terminal nodes (tips). Arboreality shows five independent gains, of which two occur at the tips. Full *ancThresh* reconstructions with posterior probabilities and pie charts plotted at every node for arboreality and greenness are reported in electronic supplementary material, figure S1.

(d) Opsin diversity in tarantulas

Our opsin homologue network contains a total of 192 nodes, with each taxon for which data was available being represented by at least one sequence (figure 3; electronic supplementary material, figure S4). While homologues were recovered from some transcriptomes generated from leg-only samples, transcriptomes generated from whole body samples were most successful for recovering a considerable diversity of opsin homologues. Generally, the degree (number of connected edges) of all reference opsins was quite high, with SWS = 57, MWS = 65, RRH = 69, RH1 = 80, RH2B = 91 and RH2A = 107. Many nodes

Figure 1. Blueness mapped onto our phylogeny [5,6] as inferred from the ancestral state reconstructions of the continuous ΔE_{Lab} values (right), with the posterior probabilities of ancestral nodes plotted as pie charts. Left panel shows our conservative ΔE_b mapping, with the discretization of the ΔE_{Lab} values (right) suggesting five gains (single blue bars) and seven losses (double brown bars). Old World subfamilies are shown in red, and New World subfamilies in orange. Pie charts adjacent to taxon names depict the proportions of currently described species in each genus with digital photographs available (black = images found, white = no images), and the proportions of species with images that possess blue coloration (grey = not blue)—see electronic supplementary material, table S7. Blue asterisks denote undescribed species that can be unambiguously attributed to the corresponding genera with blue colour. (Online version in colour.)

demonstrate homology to several opsin types, with some demonstrating homology to all reference opsins. Complete opsin homologue and orthologue data for all species is available in electronic supplementary material, table S6.

The phylogenetic relationships among opsin families in tarantulas are shown in electronic supplementary material, figure S2. It is largely consistent with results from other invertebrate studies [12], but differs in that peropsins (RRH) emerge as the most derived in our case, where they emerge as the most basal in Biscontin *et al.* [12]. Full sequence alignments (MSA) of tarantula opsins are available in electronic supplementary material, figure S3. Generally, even at positions where individual amino acids vary in each MSA, they demonstrate functional conservation.

3. Discussion

(a) Blueness is probably ancestral to Theraphosidae

As seen in figure 1, the outgroup taxa are blue. This polarity, along with the posterior probabilities on the ancestral-most nodes, suggests that the earliest tarantulas were probably blue. This early origin of blueness appears to be retained in the present-day members of the New World subfamilies

Theraphosinae, Aviculariinae and Psalmopoeinae (and at least some of the taxonomically unresolved Ischnocolinae), with four losses distributed mostly at the tips or shallower nodes. Blueness is even retained throughout some Old World subfamilies, namely Ornithoctoninae, Poecilotheriinae and Harpactirinae, while it has been lost twice in older clades and re-evolved four times at the tips or shallower nodes.

While Hsiung *et al.* [2] also recovered blueness as ancestral, our estimates of the frequency and pattern of evolution differ. Hsiung *et al.* [2] suggested that blueness evolved at least eight independent times in tarantulas, and that the true number of gains could be much greater still. Hsiung *et al.* [2] supported their claim that blueness evolved many times independently in part by arguing that distinctly different nanostructures have converged to produce the same colour. By comparison, our results (figure 1) indicate that blueness has evolved at most five times, and was lost more often than it was gained (seven times). Interestingly, of the five gains reported here, all occur in Old World groups (three tips), and five of our seven losses are in New World groups at the tips or relatively shallow nodes.

Hsiung *et al.* [2] further characterized the photonic nanostructures within the setae of some blue theraphosid tarantulas as being either amorphous spongy or multi-layer,

Figure 2. Mirror topologies showing the distribution of greenness (left) and arboreality (right). Posterior probabilities for select (for clarity) ancestral nodes are shown as pie charts. Trait gains are represented by single green bars. In the left panel, trait values and branch shading refer to the ancestral state reconstructions of continuous $\Delta E a$ values. The ancestral posterior probabilities of continuous $\Delta E Lab$ values of relevant nodes that were used to discretize the greenness trait are also shown. Greenness is gained eight times, primarily at the tips with no loss. Arboreality is gained five times, and is never lost. Pie charts adjacent to taxon names depict the proportions of currently described species in each genus with digital photographs available (black = images found, white = no images), and the proportions of species with images that possess green coloration (grey = no available images)—see electronic supplementary material, table S7. Green asterisk denotes undescribed species that can be unambiguously attributed to the corresponding genera with green colour. (Online version in colour.)

and concluded that these distinct nanostructures are a product of convergent evolution to produce similar blue colours. They argued against sexual selection as the driver behind blue coloration because typically sexual selection causes colour divergence, not convergence, at least in sympatry [13,14], and because tarantula mating (while poorly understood) is thought to rely more on vibratory and chemical elements than visual cues. However, the TEM data presented by Hsiung *et al.* [2], together with precise, bulk 3D SAXS structural information from taxa in both Old World (Ornithoctoninae and Poecilotheriinae) and New World (Psalmopoeinae) theraphosid subfamilies [15], suggest there is only one fundamental type of photonic nanostructure in tarantula setae—a perforated lamellar (i.e. a multilayer nanostructure

with random perforations, with varying degrees of spatial disorder, as observed in other arthropods [15–18]).

Moreover, it is well appreciated that certain tarantula species exhibit sexual dimorphism, including age-related dimorphism across several genera (e.g. *Haplopelma*, *Xenesthis* [2,19], *Psalmopoeus* [20], *Aphonopelma* [21] and many Aviculariinae [22]). Penultimate males frequently display vivid metallic colorations, and having examined a mature *Pamphobeteus antinous* male with vivid iridescent blue bristle ornamentation on the legs and pedipalps, Simonis *et al.* [23] proposed that such coloration might play a role in sexual signalling. These observations taken together suggest it is premature to preclude a role for sexual selection in the origin and evolution of blue tarantula coloration.

Figure 3. Network of opsin sequences in tarantulas. Thick, black edges are cases where all three tools predict the homology, and the connected nodes can be considered orthologous. Thin, black edges connect nodes when any two tools agree on the homology. Grey edges connect nodes when only a single tool agrees on the homology. Reference opsins are arranged in increasing order of wavelength sensitivities. The presence of blue and/or green colour in each genus (see electronic supplementary material, figure S4) is indicated. Reference opsins are represented by hexagonal nodes, whole body samples by circles, and leg-only samples by triangles. (Online version in colour.)

Table 2. Correlative tests as derived via Pagel's test. p -values indicate that blueness does not appear to have any correlation with any traits tested, and greenness is only correlated when a model where it depends upon arboreality is selected. Significant p -values ($p \leq 0.05$)

colour	trait	likelihood ratio	p -value
blue	stridulation	2.82	0.59
	urtication	1.02	0.90
	arboreality	3.42	0.49
	depends on arboreality	3.42	0.18
green	stridulation	4.0	0.40
	urtication	9.09	0.06
	arboreality	9.14	0.06
	depends on arboreality	8.69	0.01

Alternatively, given that upon maturity many male tarantulas will leave their retreats and wander in search of females [24–26], it is plausible that different selective pressures other than sexual selection could be driving sexual dimorphism. For instance, certain colours are either aposematic or cryptic under moonlight [27,28], and therefore useful to the wandering male but not to the fossorial female.

(b) Does arboreality precede the evolution of greenness?

Blomberg's K values for greenness (table 1) support the hypothesis that green taxa tend to cluster together in our phylogeny. Despite the small K values, the significant p -values imply that closely related taxa are probably more similar in their greenness than random pairs chosen from across the phylogeny. However, Pagel's λ analyses indicate

Table 3. AICs and weighted AICs (relative probabilities) to assess four different evolutionary scenarios for greenness, where models of different evolutionary relationships are tested in each case. A scenario where greenness depends on the prior evolution of arboreality is supported. Significant p -values ($p \leq 0.05$)

dependency	AICs	weighted AICs
independent	81.47	0.03
arboreality depends on colour	77.77	0.19
colour depends on arboreality	75.13	0.72
interdependent	80.32	0.054

that the phylogeny alone cannot explain the observed patterns. When both measures are considered together, it is reasonable to infer that it is slightly more probable to find greenness clustered among closely related taxa, but the overall structure of the topology fails to explain why this is the case. This observation is generally consistent with figure 2. One can observe that if a given genus exhibits either arboreality or greenness, then its close relatives also tend to exhibit one or both traits.

In agreement with Hsiung *et al.* [2], green coloration was found to be rare in our phylogeny. However, our ancestral state reconstruction (figure 2) has revealed a previously undocumented relationship between greenness and arboreality. Of the 11 green genera in this study, six taxa are arboreal. Both traits appear to have evolved within the genus of *Poecilotheria* (i.e. emerged as tip states in this analysis). For *Avicularia*, *Caribena* and *Ybyrapora*, both greenness and arboreality are shown to emerge in the common ancestor to the three. However, in the cases of *Stromatopelma* and *Tapinauchenius*, arboreality is reported in the immediate ancestors of these genera, but greenness is only reported as a tip-state in each—arboreality preceded the evolution of greenness. Our

dependency tests (table 3) support an evolutionary scenario where evolving greenness depends on the *a priori* presence of arboreality. We hypothesize that this is the case for *Stromatopelma* and *Tapinauchenius*, with some caveats (see below). We cannot conclusively say whether this is true for either *Poecilotheria* or the subfamily Aviculariinae (*Avicularia*, *Caribena* and *Ybyrapora*), but the addition of further taxa at the species level could assist in determining whether greenness or arboreality emerged first in these clades.

There are five genera for which greenness is reported without arboreality. Of those five, neither *Aphonopelma* nor *Phormictopus* have any close arboreal relatives. However, *Ephebopus*, *Pterinochilus* and *Harpactira* are all sister to arboreal clades that do contain green members. The case of *Ephebopus* is particularly interesting. It is the only fossorial member of the subfamily Psalmopoeinae, which includes the above-mentioned *Tapinauchenius*, and greenness is only restricted to a small abdominal patch in our representative species (*E. cyanognathus*). West *et al.* [29] reported that certain *Ephebopus* species may colonize arboreal domains; for example, early instars of *E. murinus* have been observed creating tube webs in bromeliads, and *E. rufescens* can be found in hollow rocks and tree-holes. The study also noted, however, that *Ephebopus* is primarily a fossorial genus, and even those species that display arboreal tendencies are more often found in burrows, which informed our treatment of the genus as non-arboreal. Within the context of our results, it is plausible to speculate that, as a genus whose members contain localized patches of green along with fossorial species that occasionally display arboreal tendencies, *Ephebopus* may represent a taxon that is in the process of evolving an increasingly arboreal lifestyle, concomitant with the emergence of greenness and the crypsis that it may confer.

Four genera are listed as arboreal and non-green. *Heteroscodra*'s closest relative, *Stromatopelma*, is both arboreal and green, and the sister clade to the pair also contains green members (*Pterinochilus* and *Harpactira*). *Cyriopagopus* forms a clade with *Poecilotheria* (a genus that is both arboreal and green) and *Haplopelma* (fossorial and non-green). For *Psalmopoeus*, the ΔE^* value for this genus was calculated to be 53 (electronic supplementary material, table S3), which was very close to the cut-off (less than 50) required to be considered green. Its closest relatives in this study all contain green members.

(c) Opsin diversity and evolution in tarantulas

Our results document that much of the same suite of opsins as in jumping spiders are likewise expressed in a wide array of tarantula species. In particular, both homologues and orthologues to short-wavelength and medium-wavelength sensitive opsins that can detect UV/blues and greens [30] were found in all whole-body transcriptomes, except for *Hysteroocrates* sp., *Lasiadora parahybana*, *Pterinochilus chordatus*, and the diplurid *Linothele* sp. Morehouse *et al.* [10] reported that *Aphonopelma* only possesses RH2A and UV-sensitive short-wavelength opsins. While we acknowledge that some of our tarantula opsin sequences show homology to many types of reference opsins, our results nonetheless suggest that Morehouse *et al.*'s observation underestimates true opsin diversity among tarantulas when more broadly sampled. All subfamilies possess the full complement of typical arthropod opsins. Given that jumping spiders are known

to discriminate colours, including reds, greens and blues [30], we cannot discount the possibility that tarantulas are able to perceive colours based on our results (figure 3). While Dahl & Granda [9] noted that *Aphonopelma chalcodes* lack the neurological response complexities seen in colour discriminating eyes, we encourage future psychophysical and behavioural studies to see if this reported inability is broadly representative of tarantulas. Lack of colour discrimination on the basis of low opsin diversity (e.g. [2]) can no longer be used to rule out inter- and intra-sexual communication as a function of blue coloration in tarantulas. However, given that blueness is lost more frequently than it is gained, we suggest that it is perhaps under weak selection or has been exapted for crypsis or aposematic signalling under twilight or moonlight conditions [27,28].

4. Conclusion

Our research offers novel insights into the evolutionary origin and potential functions of blue and green coloration in tarantulas. We report that blueness is an ancestral condition for tarantulas and was probably present among the groups' earliest representatives. This finding leaves open the possibility that blueness may have originally evolved for the purposes of sexual signalling, and may continue to serve in this role given that most tarantulas still possess a considerable diversity of arthropod opsins. On the other hand, greenness has probably evolved at least eight times, primarily at the tips, and does not appear to have been lost at any point in the evolution of tarantulas. We provide support for a conditional relationship where evolving greenness probably depends on the prior presence of arboreality, and compare the ancestral state reconstructions of both greenness and arboreality to further support this idea. We hope that future work will include comparative studies on the visual perception and putative mate-choice experiments in tarantulas to better understand the selective pressures involved in the origin and maintenance of vivid coloration in this enigmatic group.

5. Methods

(a) Phylogeny construction

The phylogeny published by Foley *et al.* [5], based on 2460 orthologous genes, was used as a constraint tree for this study, but taxa were reduced to the genus level for ease of assigning character states in subsequent steps. The R package 'PASTIS' [31] facilitated the inclusion of a further 11 genera that lack sequence data by constraining their clade memberships as determined by previous studies (electronic supplementary material, table S1). Branch lengths for these additional taxa were estimated by MrBayes v. 3.2 [32], which ran under a birth-death model for 100 000 generations, with sampling every 160 generations and the burnin set to 25%.

(b) Colour survey

Photographs corresponding to representative species of each genus in the phylogeny were primarily acquired from arachnologist Rick C. West's photographic archive (<http://birdspiders.com>), but other sources included Gaia Biological (<http://thespidershop.co.uk>), Nicky Bay (<http://nickybay.com>) and the published literature. A list of representative species per genus, as well as the source for each photograph used in this study, can be found in electronic

supplementary material, table S2. These photographs were analysed with ImageJ v. 1.52.

Each photo was individually examined with the 'Colour Inspector 3D' ImageJ plugin and visualized in the CIELAB colour space. The 'bluest' regions of each photo were characterized by greater negative b values, and the 'greenest' regions by greater negative a values. These bluest and greenest regions of each photo were then cropped and reloaded into ImageJ as new images, regardless of the location of the colour. The 'Colour Transformer' plugin was used to convert each of these images into a stacked CIELAB image. The 'Measure' command was used on each frame to obtain mean L , a and b values for each taxon, as well as for six reference leaf images. The Lab values for the six leaves were averaged and used as reference green colour values, and the Lab values for the taxon with the greatest $-b$ value (i.e. the 'bluest' species, *Haplopelma lividum*) was chosen to be our reference for blue values. We also obtained Lab values for the jumping spider, *Maratus splendens*, as a potential blue reference [33]. However, their $-b$ values were less extreme than our chosen reference, *Haplopelma lividum*.

Using the conservative International Commission on Illumination (CIE) metric for colour discrimination, ΔE 76 [34], four separate Euclidean difference values were then calculated for each taxon in order to quantify the perceived colour difference of each taxon relative to the reference colour values: 1. ΔE Lab against the blue reference, 2. Δb against the blue reference, 3. ΔE Lab against the green reference, and 4. Δa against the green reference. Values 1 and 3 informed our continuous colour dataset, whereas values 2 and 4 were discretized, with any taxon scoring less than 50 [34] being scored as possessing the corresponding colour (i.e. '1'; for values less than 50 indicate colours are more similar than opposite). Values 2 and 4 also served to correct for varying light intensities across images, as only the actual colour values were considered in these cases without the influence of L affecting further calculations. Other methods were considered for colour discrimination, but they were ultimately deemed too permissive. For example, the same discriminatory criteria under ΔE 2000 [35] would result in all but a single species in our phylogeny being considered blue. All measurements and instances where our colour scoring differs from Hsiung *et al.* [2] are noted in electronic supplementary material, table S3.

(c) Habitat and defensive trait survey

A discrete data matrix was constructed based on the presence or absence of the following traits: Urticating setae were marked as present in genera from the subfamilies Theraphosinae [36] and Aviculariinae [22], as well as the genus *Epebopus* [37,38]. Stridulatory setae were marked as present in *Chilobrachys*, *Coremiocnemis*, *Haplocosmia*, *Lyrognathus*, *Orphnaecus*, *Phlogiellus*, *Poecilotheria*, *Selenocosmia* [39], *Acanthoscurria*, *Brachypelma*, *Cyrtopholis*, *Lasiadora*, *Nhandu*, *Pamphobeteus*, *Phormictopus* [40], *Cyriopagopus*, *Haplopelma*, *Harpactira*, *Hysterochrates*, *Monocentropus*, *Pelinobius*, *Psalmopoeus* [41], *Bumba* [42], *Pterinochilus* [43] and *Thrigmopoeus* [44]. Lastly, arboreal tendencies were marked as present in *Avicularia*, *Caribena*, *Heteroscodra*, *Poecilotheria*, *Psalmopoeus*, *Stromatopelma*, *Tapinauchenius* [45], *Cyriopagopus* [46] and *Ybyrapora* because its members have historically been treated within *Avicularia*. All data is available in electronic supplementary material, table S3.

(d) Ancestral state reconstructions and correlative tests

Phylogenetic signal of discrete blue, discrete green, Δb values against the blue reference, and Δa values against the green reference were assessed using both Blomberg's K [47] and Pagel's λ [48]. A set of functions in the R package 'phytools' [49] facilitated ancestral state reconstructions and correlative tests. The ancestral

states for discrete blue, discrete green and arboreality were separately estimated using 10 million generations of Bayesian MCMC with the *ancThresh()* function [50], and the posterior probabilities were plotted at ancestral nodes as pie charts. We coded the traits as present at any internal nodes whose corresponding posterior probabilities were greater than or equal to 66%, and made note of any nodes whose corresponding posterior probabilities were greater than or equal to 95%. The JND (continuous) colour data was then traced through the phylogeny using the *contMap()* function. The *ancThresh* pie charts corresponding to ancestral nodes whose posterior probabilities indicated that a trait was present were subsequently overlaid onto the corresponding *contMap* outputs for ease of interpretation.

Pagel's [51] models of correlated evolution were applied to our discrete data to estimate the relationships between the presence of blue or green and habitat and defensive traits using the *fitPagel()* function. Additionally, sets of AIC values were generated with *fitPagel* to estimate more specific relationships between colours and arboreality (i.e. whether either trait depends on the other, or whether the traits are independent/interdependent). The *fitDiscrete()* function was used in each case.

(e) Generating an opsin homologue network

We assembled publicly available tarantula transcriptomes for each genus in our phylogeny that had data available (data sources in electronic supplementary material, table S4). We then acquired candidate opsin sequences from six different types of opsin proteins that other spiders are known to possess [10]: middle-wavelength-sensitive rhodopsin-like-1, 2A and 2B (RH1, RH2A and RH2B respectively), medium-wave-sensitive opsin-1 (MWS), short-wave-sensitive opsin-1 (SWS), and retinal pigment epithelial RPE-specific rhodopsin (RRH). Opsin accession numbers are available in electronic supplementary material, table S5. These six representative sequences served as queries for BLASTP [52] searches against our transcriptome set. Only hits with e -values greater than 10^{-7} were discarded, with a view towards treating these hits as a tentative list of tarantula opsin homologues.

Homology was inferred between our candidate opsin sequences and our reference opsin set for each species using three different tools: SwiftOrtho [53], FastOrtho [http://enews.patricbrc.org/] and OrthoFinder [54]. SwiftOrtho and FastOrtho were run with e -values of 10^{-40} for homology searching to reduce potential false positives while OrthoFinder was run using default settings. Per the 'moderate' criteria from the Alliance of Genome Resources [55], hits may be considered orthologous if three or more of the twelve tools in their suite converge upon that result. Hence, any hits which are recovered by all of the above-mentioned tools can be considered orthologous. Results from each of these programs were pooled, and loaded into Cytoscape [56] for network visualization.

The reference opsins, along with sequences corresponding to nodes that only showed a degree of 1 and were considered orthologous were then aligned using MAFFT [57]. This was to ensure that only nodes showing unambiguous orthology to a single reference opsin would be retained. Alignment served as an input for RAXML v. 8.2.11 [58], which generated a maximum-likelihood tree.

Data accessibility. All data used in this study can be found in this article and in the electronic supplementary material.

Authors' contributions. S.F. and V.S. designed the study. S.F. compiled the data matrices. V.S. and S.F. coded and performed the phylogenetic analyses of coloration in R. S.F. generated the transcriptomic results with input from V.S. and W.H.P. S.F. and V.S. analysed the results. S.F. and V.S. wrote the manuscript with input from W.H.P.

Competing interests. The authors declare no competing interests, financial or otherwise.

Funding. The computational results reported in this study were possible thanks to a Yale-NUS College Shared Instrumentation Grant no. (IG15-SI101) to V.S. and W.H.P. V.S. acknowledges support from Yale-NUS start-up funds (grant no. R-607-261-182-121) and a Singapore NRF CRP Award (CRP20-2017-0004). W.H.P. acknowledges support from the South East Asian Biodiversity Genomics

(SEABIG) centre, the Singapore Ministry of Education (grant no. MOE2016-T2-2-137), and by Yale-NUS College (grant nos. IG14-SI002 and R-607-265-052-121).

Acknowledgements. We thank Nate Morehouse and Megan Porter for kindly sharing their tarantula opsin sequence data. We are grateful to two anonymous reviewers for their helpful comments.

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