

## The Grass was Greener: Repeated Evolution of Specialized Morphologies and Habitat Shifts in Ghost Spiders Following Grassland Expansion in South America

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**Abstract.**—While grasslands, one of Earth’s major biomes, are known for their close evolutionary ties with ungulate grazers, these habitats are also paramount to the origins and diversification of other animals. Within the primarily South American spider subfamily Amaurobioidinae (Anyphaenidae), several species are found living in the continent’s grasslands, with some displaying putative morphological adaptations to dwelling unnoticed in the grass blades. Herein, a dated molecular phylogeny provides the backbone for analyses revealing the ecological and morphological processes behind these spiders’ grassland adaptations. The multiple switches from Patagonian forests to open habitats coincide with the expansion of South America’s grasslands during the Miocene, while the specialized morphology of several grass-dwelling spiders originated at least three independent times and is best described as the result of different selective regimes operating on macroevolutionary timescales. Although grass-adapted lineages evolved towards different peaks in adaptive landscape, they all share one characteristic: an anterior narrowing of the prosoma allowing spiders to extend the first two pairs of legs, thus maintaining a slender resting posture in the grass blade. By combining phylogenetic, morphological, and biogeographic perspectives we disentangle multiple factors determining the evolution of a clade of terrestrial invertebrate predators alongside their biomes. [Amaurobioidinae; anyphaenidae; biogeography; convergence; macroevolution; morphometrics.]

To fully appreciate and understand the evolutionary history of a group of organisms, a holistic approach, considering intrinsic and extrinsic components, is crucial. Changes in the genetic material, morphological traits as well as biotic and abiotic selective pressures interact during the process of diversification of the world’s biota, in an intricate web of adaptation and co-adaptation. The adaptation of organisms to their specific habitats is one of the major driving forces of diversification, often reflected in specific physiological or morphological traits, especially when the association is strict (Wainwright and Reilly 1994). Habitats themselves are spatio-temporally dynamic, responding to changes in the planet’s geomorphology and climate. For example, the grassland biome currently covers an estimated 40% of the Earth’s terrestrial surface (Gibson 2009), and although grasses (Poaceae) are estimated to have existed since the early Cretaceous (Bouchenak-Khelladi et al. 2014), the expansion of the grassland biome did not occur until the Neogene, at different times depending on the continent (Edwards et al. 2010; Strömberg 2011), and probably due to a combination of shifts in climatic CO<sub>2</sub> levels and fire regimes (Osborne 2008). The spread of grasslands has been postulated as the driver for the origin and diversification of

numerous phytophagous insects (e.g., Dietrich 1999; Toussaint et al. 2012) and mammalian groups, including our own genus (e.g., Bobe and Behrensmeyer 2004). Furthermore, well-known cases of co-adaptation and even co-evolution have occurred between grasslands and ungulate grazers (e.g., Stebbins 1981; Jacobs et al. 1999; Bouchenak-Khelladi et al. 2009), although strict co-speciation has not always been the case (Jardine et al. 2012; Palazzi and Barreda 2012; Strömberg et al. 2013). Besides trophic relations, a well-known driver of morphological evolution is the need to deceive the perception of predators or prey by crypsis or mimicry (see Stevens and Merilaita 2009), of which the melanic moths are a textbook example. Many invertebrates match the background color of the leaves or sticks on which they stand, and hide by precisely controlling the posture of the body and legs (Edmunds 1990), thus several spiders visually cryptic on grasses have thin, long bodies (Comstock 1913). For example, in the family Philodromidae, *Tibellus oblongus* evolved an elongated body and dwell within the blades of grasses extending their first two pairs of legs forwards and the rear two backwards (Mikulska 1967); similar characteristics can also be found among species of the crab spider genus *Monaeses* (Dippenaar-Schoeman 1984). These traits have

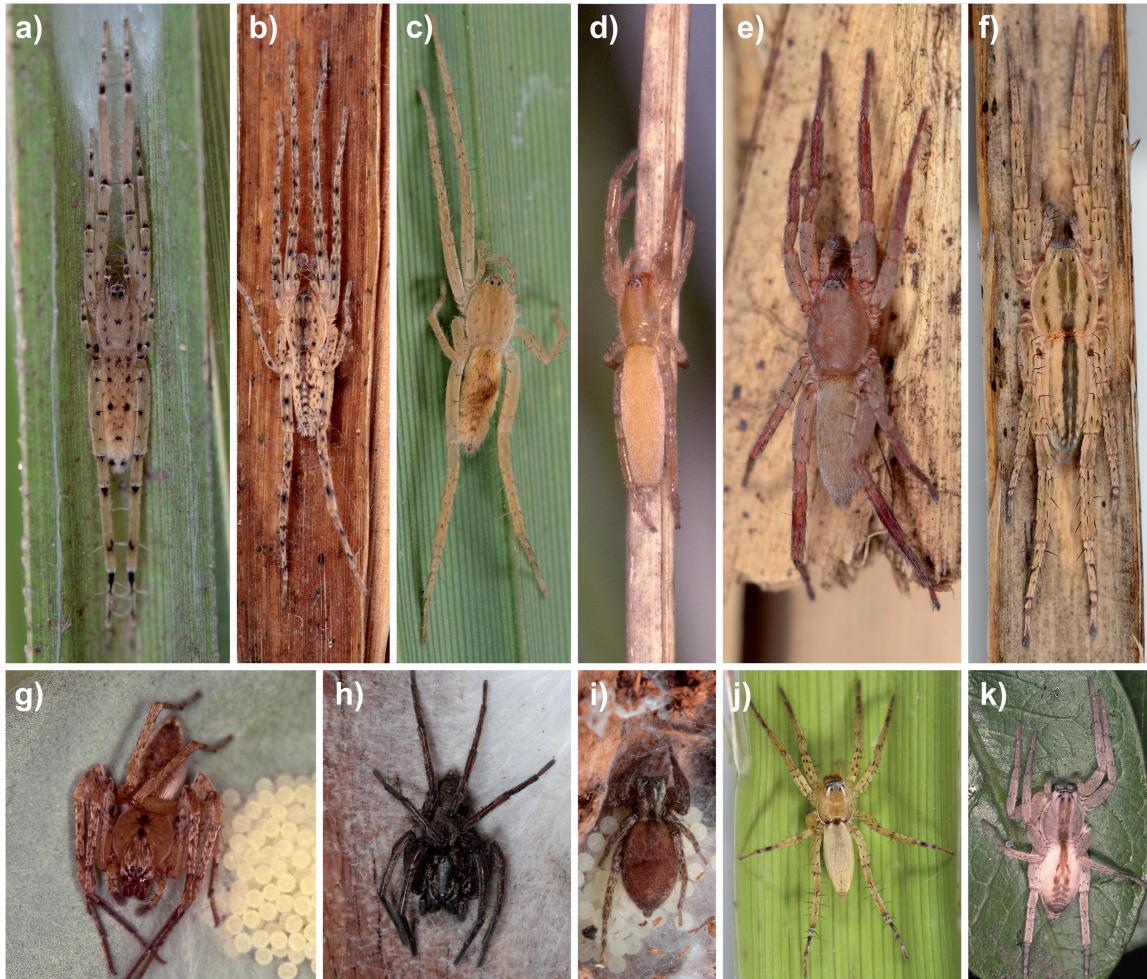


FIGURE 1. Spiders of the tribe Gayennini. The grass-dwelling species (top) have a slender constitution and a morphology that allows the legs to be tightly extended forwards and backwards, or folded at the side. Other species have a stockier constitution (bottom) and different resting positions. a) *Monapia guenoana*. b) *Monapia charrua*. c) *Monapia angusta*. d) *Sanogasta tenuis*. e) *Sanogasta puma*. f) *Arachosia bergi*. g) *Oxysoma saccatum*. h) *Tomopisthes horrendus*. i) *Sanogasta maculosa*. j) *Arachosia proseni*. k) *Arachosia praesignis*.

been interpreted as adaptations for living on vegetation, including grasses.

Grasslands are found on all major landmasses; for example, South America harbors large areas of grass-dominated ecosystems such as the Brazilian *cerrados* and *campos*, and the Argentine *pampas* (Soriano 1991) or lowland plains. Based on studies of fossil pollen records, the Patagonian region of South America, at present characterized by extensive areas of steppe, was covered mainly by forests (Barreda and Palazzesi 2007) until the Miocene (estimates range between 18.5 and 10 million years ago (Ma); Palazzesi and Barreda 2012; Strömberg et al. 2013), when grass-dominated open habitats began expanding. These changes in the floral composition, therefore, altered the availability of food sources and habitats for a large number of vertebrates and invertebrates in the region.

The spider subfamily Amaurobioidinae (Anyphaenidae), currently with 180 species in 23 genera (World Spider Catalog 2017) is found almost exclusively

in South America, with many of its genera restricted to the Patagonian region (Ramírez 2003). These spiders are active hunters that live inside silken retreats. Several species can be found living in grass-dominated open habitats, and a few remarkable morphological traits facilitate the spiders living and concealing themselves within the folded inner edge of the grass leaves. More specifically, this grass ecomorph has a more slender and elongated body configuration compared with that of all other members of the subfamily (Fig. 1), and adopts a resting posture along the leaf blade with the first two pairs of legs towards the front and two hind pairs towards the back (Fig. 1f); or, in some species, the third pair of legs is very short, and rests tightly folded besides the body (Fig. 1a). Their cryptic posture resembles that of other spiders and insects that live on elongate leaves, complementing their posture with a pattern of dots or lines matching the background (e.g., Mikulská 1967; Dippenaar-Schoeman 1984; Landry 1994).

The amaurobioidine species known to display these specific morphological characteristics belong to three different genera, *Arachosia*, *Monapia*, and *Sanogasta* (Ramírez 1999, 2003; Rubio and Ramírez 2015), and the phylogenetic trees both from morphological (Ramírez 2003) and DNA data (Labarque et al. 2015) indicate that these putative adaptations have most likely arisen independently. The repeated evolution of similar morphologies as a response to similar environmental conditions, such as seen among several well-studied cases of morphological convergence (e.g., Hawaiian *Tetragnatha* spiders: Gillespie 2004; African cichlids: Muschick et al. 2012; Caribbean *Anolis* lizards: Mahler et al. 2013), is an impressive result of evolution under natural selection. Such cases also represent unique natural experiments in which the relative impact of contingency and determinism in evolutionary history can be assessed (Gould 1989; Conway Morris 2003; Turner 2011; Orgogozo 2015).

Amaurobioidinae has been the focus of recent molecular phylogenetic studies in the form of a general, total-evidence study (Labarque et al. 2015) providing insight and confirmation of previous, morphology-based classifications (Ramírez 2003), as well as dated phylogenetic studies focusing on specific genera of the subfamily (Ceccarelli et al. 2016a; Soto et al. 2017). Herein, we present the most extensive sampling of Amaurobioidinae for molecular phylogenetic work to date, as well as the first instance of node age estimates for the whole subfamily. Furthermore, we use the dated molecular phylogeny for character mapping and morphospace analyses, to assess the impact of grassland expansions on morphological adaptations in the subfamily in terms of macroevolutionary mechanisms. In particular, we aim to answer the following general questions. 1) Was the origin of grass-dwelling clades in Amaurobioidinae concurrent with the habitat's expansion in South America? 2) How frequent were the ecological shifts to and from open habitats, including grasslands, throughout the subfamily's evolutionary history?, and 3) did the independent colonization of grasslands result in the repeated origin of similar morphologies? Through the outcomes of this study, it is possible to evaluate the plasticity in terms of adaptation to habitat changes at a macroevolutionary scale in this group of spiders.

## MATERIALS AND METHODS

### *Taxon Sampling*

For the molecular phylogenetic analyses 119 ingroup and 14 outgroup taxa were used. The ingroup taxa belong to 22 described genera and 1 undescribed genus of the subfamily Amaurobioidinae, while the outgroup taxa comprised nine genera belonging to the anyphaenid subfamily Anyphaeninae and one genus (*Elaver*), belonging to the family Clubionidae, to root the trees, following Wheeler et al. (2017). Of these taxa, 38 belonging to the subfamily Amaurobioidinae

(plus one outgroup) were new to this study, while the remainder had been used before (Labarque et al. 2015; Ceccarelli et al. 2016a; Soto et al. 2017). For five of the previously used taxa, part of the DNA data that had been missing was obtained for this study as well. A list of the taxa used here can be found in Supplementary Table S1 of SI File 1 available on Dryad at <http://dx.doi.org/10.5061/dryad.20257>.

For details on sequencing and phylogenetic inferences including node age estimates, readers are referred to the SI File 2 available on Dryad.

### *Habitat and Area Mapping*

Geographic range, habitat type, and ecomorph were assigned as traits for each taxon in the dated phylogeny for further analyses. Because the most notable change in grassland expansions in southern South America occurred in what is today known as the Patagonian region (Palazzi and Barreda 2012; Strömborg et al. 2013), the geographic ranges chosen for the analyses were limited to four categories: the first two, Patagonia (Pg) and non-Patagonian Neotropical (NP) cover the bulk of the diversity (see Fig. 2); Juan Fernández Archipelago (JF) covers the radiation of *Philisca* species (Soto et al. 2017) and the occurrence of *Sanogasta maculosa* "skottsbergi" (Ramírez 2003), and the trans-oceanic distribution in South Africa, Australia, and New Zealand (TO) covers in summary the species of the intertidal genus *Amaurobioides* that were already studied in detail (Ceccarelli et al. 2016a). For Pg, we used the Patagonian Andean region excluding the South American Transition zone as delimited by Morrone (2006), composed by the Argentinean Pg plus Southern and Central Chile (Fig. 2c). This area corresponds well with the distributional limits of many anyphaenid taxa (Ramírez 2003) and other organisms (Morrone 2015).

Similarly, the general habitat type was either chosen as open (Op), referring mainly to grasslands, but also including other open habitats with few or no trees, such as scrublands, marshes, and rocky shores; or forest (Fo), mostly representing broad-leaf forests including foliage of shrubs. Because the two habitat types (Op and Fo) frequently co-exist as patches in many localities, and because some species use both habitats, the two variables (geographic range and habitat type) were treated as independent traits in separate analyses. Each terminal taxon in the maximum clade credibility (mcc) tree from BEAST was assigned the code or codes which best defined the species' geographical range and general habitat type as documented in the taxonomic revisions, the MACN-Ar database (<http://www.gbif.org/dataset/34e892b0-d9c7-11de-b793-b8a03c50a862>) and field notes of author MJR. A detailed habitat analysis is circumscribed to the densely-sampled tribes Amaurobioidini + Gayennini (A+G Clade); the outgroups, including the subfamily Anyphaeninae and the genus *Josa* (sister to A+G Clade) are all non-Patagonian and were sampled more superficially. Finally,

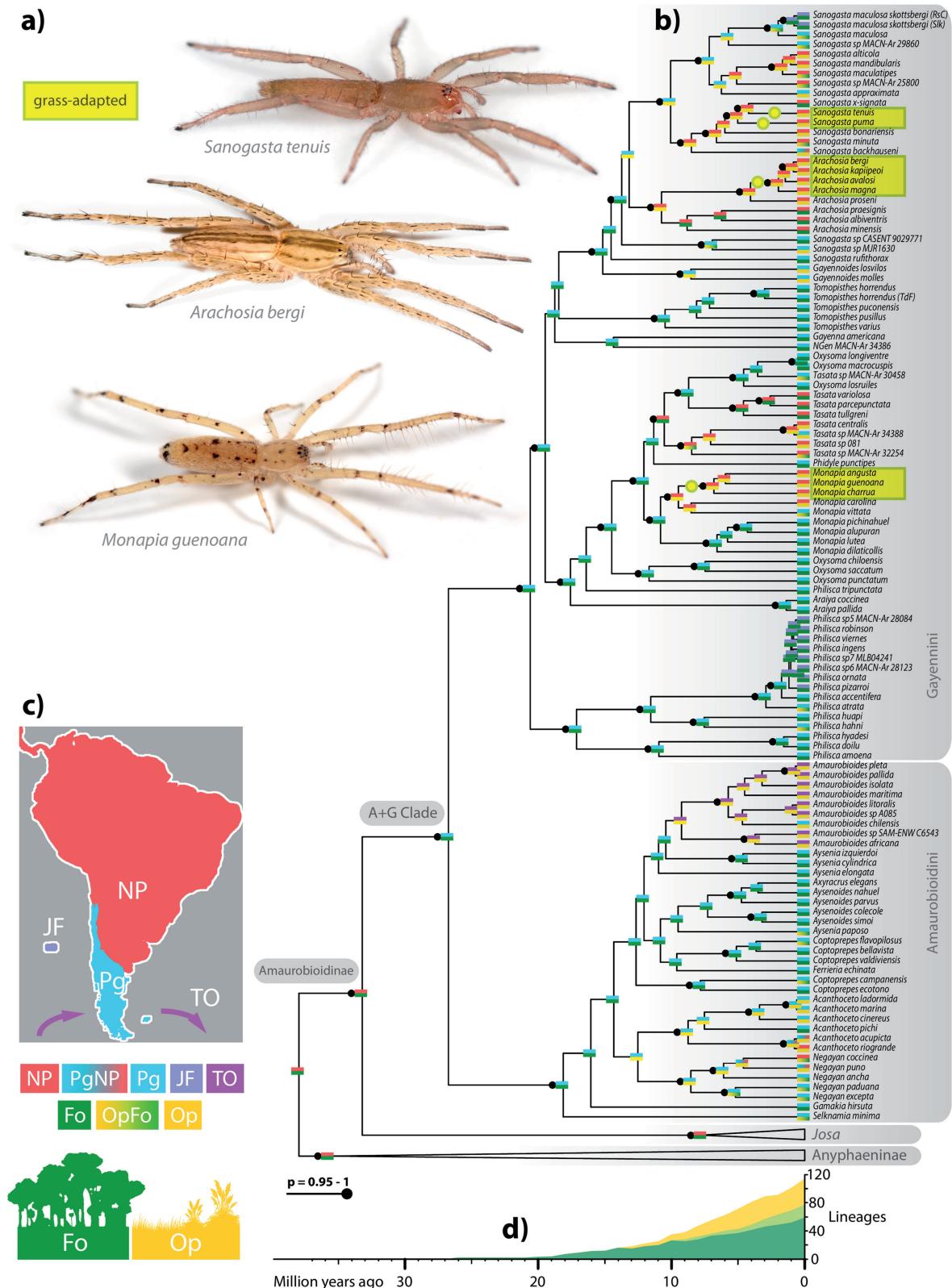


FIGURE 2. Analysis of area and habitat occupancy through time in amaurobioidine spiders. a) Representatives of the three clades exhibiting a grass ecomorph. b) Dated phylogeny with ancestral areas and habitats mapped on nodes (black circles denote nodes with posterior probability  $\geq 0.95$ ). Yellow/light grey circles show the inferred transitions to grass ecomorph with highest posterior probability. c) Regionalization of the main areas of distribution of Amaurobioidinae analyzed in this study. d) Stacked profile of lineages through time, classified by habitat. NP = Non-Patagonian Neotropical; Pg = Patagonian; PgNP = both Pg and NP; JF = Juan Fernandez Archipelago; TO = transoceanic (South Africa, Australia and New Zealand); Fo = broad-leaf forest; Op = open; OpFo = both Op and Fo.

species were coded as being of the grass ecomorph, or lumped into a “generalized morphology” category. As explained in the introduction, all species of the grass ecomorph have pale, elongated bodies, and in the case of *Monapia* and *Sanogasta* are collected exclusively on grasses such as *Coleataenia prionitis*, *Cortaderia selloana*, *Paspalum* spp., *Panicum* spp. (Supplementary Table S4 of SI File 1 available on Dryad). Grass inhabiting *Arachosia* dwell in the same grasses listed above, but are also found in other monocot plants with elongate leaves, such as *Typha angustifolia*, *Eryngium* species, sedges (Cyperaceae), and rushes (Juncaceae).

To test whether the traits are clustered or randomly distributed on the phylogeny (i.e., show phylogenetic signal) Bayesian Tip-Significance testing was carried out on the last 5000 trees from each BEAST run in the program Befi-BaTS v.0.1.1 (Parker et al. 2008). This program uses three statistics, the Association Index (AI; Wang et al. 2001) the Fitch parsimony score (PS), and the Monophyletic Clade (MC) size statistic, to test the significance of phylogenetic signal in the traits against a null distribution in which the characters at the tips are reshuffled 1000 times.

The ancestral trait estimation for areas and habitats were run on the BEAST mcc tree with BioGeoBEARS package v. 0.2.1 (Matzke 2012, 2013) for R v. 3.2.3 (R\_Core\_Team 2015) under three models: Dispersal-Extinction-Cladogenesis (DEC; Ree et al. 2005), DIVALIKE, modified from the DIVA program of Ronquist (1997), and BAYAREALIKE, modified from the BayArea program of Landis et al. (2013) algorithms. These three models factored to six after the addition of the founder-event speciation parameter J, and the best fit model was selected based on the Akaike information criterion (AIC). The BioGeoBEARS analyses were run using a script provided by N. Matzke (<http://phylo.wikidot.com/biogeobears>). The area analysis was stratified into two time periods: before and after 4 Ma, reflecting the emergence of the JF area (Stuessy et al. 1984). Consequently, the “areas allowed” and “dispersal multiplier” input matrices for the BioGeoBEARS run factored in a low likelihood for any occurrence in Juan Fernández before 4 Ma. The input matrices for “distance” and “dispersal multiplier” considered short distances within America, long distance to Juan Fernández, and longer distances to South Africa, Australia and New Zealand (see Supplementary Matrices S1 and S2 of SI File 1 available on Dryad). The ancestral state estimations corresponding to the model with the best fit for the two runs (area and habitat) were analyzed to obtain a profile of area and habitat occupancy through time for the ingroup (A+G Clade); the transitions in habitats and areas were also counted and analyzed for general trends.

To obtain an estimate of the uncertainty of the results, we produced area and habitat analyses for each of 100 trees randomly drawn from the post-burnin posterior sample of the Bayesian analysis in BEAST, using the optimal model selected for the mcc tree in

BioGeoBEARS. The time of transitions and ancestral state estimations for given time slices were calculated by linearly interpolating between the probabilities in dominant states in ancestor and descendant nodes (Supplementary scripts provided in SI File 3 available on Dryad). The expected number of lineages in each state for these given time slices was also calculated, under the assumption that the lineages in each state increased proportionally through time, and compared with the observed (average) numbers from the 100 trees by means of  $\chi^2$  and paired *t*-tests.

For the evolution of ecomorphs, Bayesian stochastic mapping was carried out on 100 randomly selected post-burnin chronograms from BEAST, using the simmap algorithm (Bollback 2006) implemented in the R package *phytools* v. 0.6 (Revell 2012). Ten thousand Markov Chain Monte Carlo (MCMC) generations were run for 100 replicates on each tree under equal rates and the state changes over all stochastic maps summarized. A single stochastic map was also obtained by running 100 replicates, each with 10,000 MCMC generations on the A+G clade of the mcc tree obtained in BEAST, to get a graphical representation of the results.

#### Morphological Macroevolution Analyses

To explore the origin of the putative morphological adaptations associated with the exploitation of grasslands, four morphometric measurements were taken from museum specimens: total carapace length and width (a proxy for slim vs. stocky body), distance between the condyles of the chelicerae (a proxy for the ability to maintain a slender profile while extending the fore legs forward and close to each other), and length of the tibia of the third pair of legs (a proxy for the ability to tightly fold and hide the third legs at the sides of the body). Specimens were measured using a binocular microscope with an ocular micrometer. Given that putative grass-adapted species are restricted to the tribe Gayennini, morphometric data were only gathered for this clade.

With the aim to minimize the effect of sexual dimorphism, only adult females were measured; as is common in spiders, immature anyphaenids throughout all free instars are similar to females in habitus and resting posture, while males have a slender constitution and sometimes cheliceral modifications (Soto and Ramírez 2012). In this way, the measured individuals had similar morphologies to those of the whole life cycle, while bearing the genital characters necessary for taxonomic identification. Whenever possible, multiple female specimens (maximum 5, mean 4.1) per species were measured, and trait values were expressed as the logarithm of the average of individual observations. The historical pattern of morphological change of these characters was studied using a version of the mcc tree pruned down to the 73 members of the tribe Gayennini for which morphological data could be

gathered ([Supplementary Table S5](#) of SI File 1 available on Dryad).

First, character selection validation was carried out by testing for differences in the allometric scaling of these between grass-dwelling spiders and the rest, a common method to explore the existence of morphological adaptations in a comparative phylogenetic framework ([Garland and Ives 2000](#)). A phylogenetic generalized least squares regression (PGLS; [Grafen 1989](#); [Martins and Hansen 1997](#)) was performed for all characters against carapace length, which was selected as a proxy for body size. Regressions were performed using R package *caper* ([Orme et al. 2013](#)) excluding species showing putative grassland adaptations, and simultaneously estimating the  $\lambda$  parameter ([Pagel 1999](#)) to account for deviations from a pure Brownian motion (BM), as recommended by [Revell \(2010\)](#). A 95% prediction interval was then calculated for each regression using R scripts provided in [Smaers and Rohlf \(2016\)](#), and data points for grass-dwelling spiders were plotted to see whether they lie within the estimated intervals. Although this procedure does not represent, in a strict sense, a statistical test of deviations from allometric predictions ([Smaers and Rohlf 2016](#)), it was simply used here to determine whether the chosen characters were indeed capturing information relevant to the study of niche-associated morphological differentiation. Furthermore, the interactions among these four characters were explored by visualizing morphological variability and evolution using phylomorphospaces ([Sidlauskas 2008](#)) on phylogenetic principal component axes (pPCA; [Revell 2009](#)).

Using the same dataset, the fit of multiple process-based models of macroevolution were explored in a multivariate framework using R package *mvMORPH* ([Clavel et al. 2015](#)). Such an approach to model phenotypic evolution outperforms commonly employed univariate methods given its capability to take into account the existence of correlations among traits. Furthermore, it allows to circumvent any pre-processing of the data, such as the use of principal component analysis or its phylogenetic variant, transformations that are known—under certain conditions—to introduce biases when used upstream of methods of evolutionary inference ([Revell 2009](#); [Monteiro 2013](#); [Uyeda et al. 2015](#)). However, recent simulation studies have shown that this method suffers from problems of model misspecification as the number of traits ( $p$ ) increases relative to the number of species ( $N$ ) ([Adams 2014](#); [Adams and Collyer 2018](#)). To test whether the  $N:p$  ratio of ~18:1 is enough to guarantee accurate model selection, 200 replicates of 4 dimensional trait matrices were simulated under BM using the empirical tree and phylogenetic variance-covariance matrix. Following [Adams and Collyer \(2018\)](#), a comparison of model fit between BM and Ornstein-Uhlenbeck (OU, see below) models using AIC scores revealed a percentage of model misspecification of only about 2%.

Eight different models were tested, including three models in which morphological change was described

using a single process for the entire tree, and five models with different processes dictating change in different regions of the tree. Among the first class, the fit of the following models were analyzed 1) a BM model, the most common null model of evolution in comparative studies, designed to represent a process of random walk with constant variance (described in detail in [Freckleton et al. 2002](#); [O'Meara et al. 2006](#)); 2) an OU model, which adds to the random walk a tendency to evolve towards an “optimum value”, generally considered to represent a biological scenario of stabilizing selection ([Hansen 1997](#); [Butler and King 2004](#)); and 3) an early burst (EB) model, in which the Brownian rate of morphological change decays exponentially with time ([Blomberg et al. 2003](#)). Furthermore, to explore the degree of morphological convergence shown by clades that independently colonized grasslands, five different multi-OU models were also tested. Such models constitute a convenient representation of evolution towards peaks in a macroevolutionary adaptive landscape ([Ingram and Mahler 2013](#)), and have become increasingly popular to describe cases of adaptive radiations and convergent evolution ([Cressler et al. 2015](#); [O'Meara and Beaulieu 2014](#); [Cooper et al. 2016](#)). All models included a single peak for all species with a general morphology, but differed in the number of peaks used to model the evolution among species of the grass ecomorph, including: 1) a single selective regime for all grass-dwelling species (OU2), 2) two different regimes, one exploited by a single genus and the other by the remaining two (OU3; all three different combinations were explored), and 3) a scenario in which all three grass-dwelling clades exploit different peaks (OU4). These models were specifically designed to test different levels of morphological convergence triggered by the expansion of grasslands, ranging from complete to non-existent ([Supplementary Fig. S1](#) of SI File 4 available on Dryad).

In all cases, measurement errors were incorporated in the estimation process ([Supplementary Table S5](#) of SI File 1 available on Dryad), calculated as squared standard errors of the mean values ([Clavel et al. 2015](#)). For the small number of species coded from single specimens, errors corresponded to the averaged measurement error for each character. All runs were parameterized to assume a stationary distribution for the selective optimum at the root (option `vcv = "randomRoot"` in function `mvOU`), corresponding to a scenario of evolution at a steady state ([Clavel et al. 2015](#)). This assumption is biologically reasonable in our case study, and has been found to stabilize estimates of optimum values ([Cooper et al. 2016](#)). In all cases, regimes were painted on the *mcc* tree using the `make.simmap` function in *phytools*, and the five multi-OU models were fitted to 100 different optimizations derived from stochastic character mappings under equal rates. Furthermore, one simulation was also explored in each of 100 trees randomly drawn from the posterior sample of the Bayesian analysis. In such a way, uncertainty in species trait values ([Silvestro et al. 2015](#)), timing and number

of origins of the grass ecomorph (Huelsenbeck et al. 2003), divergence times and topology (Huelsenbeck et al. 2000) were all contemplated. To avoid problems of overfitting, which are known to impact multi-OU model selection (Boettiger et al. 2012; Ho and Ané 2014), models were compared using weights derived from sample-corrected ACC scores (wAICc; Burnham and Anderson 2002). In case of nested hypotheses, model support was further explored using log-likelihood ratio tests (LRTs). The tempo of the adaptive process was analyzed using phylogenetic half-lives, representing the time needed to move halfway from an ancestral state to an adaptive optimum, which in a multivariate framework are calculated on the eigenvalues of the selection ( $\alpha$ ) matrix (Bartoszek et al. 2012).

## RESULTS

### *Phylogenetic Inference and Node Age Estimates*

The characteristics of the four DNA data matrices used for phylogenetic analyses can be found in Supplementary Table S6 of SI File 1 available on Dryad. The percentage of missing data was 2.25% for the Cytochrome Oxidase I gene fragment (COI), 9.77% for 16S, 5.26% for H3, and 7.52% for 28S. The individual gene trees for each marker (Supplementary Figs. S2–S4 of SI file 4 available on Dryad) vary in their level of resolution, since, as would be expected, the nuclear markers have lower rates of nucleotide substitution (mean rates of substitutions per site per million years of 28S: 0.00243, H3: 0.00498, and mitochondrial loci: 0.0358), therefore resolving only the deeper divergences. The topologies obtained with the concatenated dataset for the Amaurobioidinae taxa in this study (Fig. 2, Supplementary Figs. S2–S8 of SI File 4 available on Dryad) largely agree with the concatenated molecular phylogenetic analyses of previous studies (Labarque et al. 2015; Ceccarelli et al. 2016a; Soto et al. 2017). Relationships for genera vary in the placement of terminals such as the monotypic genera *Selknamia* and *Axyracrus*, always with low support. Most genera are recovered as monophyletic in both Bayesian and maximum likelihood (ML) trees; although certain taxonomic inconsistencies remain, mostly due to complications of previous assignations of species to specific genera (see Soto et al. 2017). Species of *Arachosia* and *Monapia* displaying the grass ecomorph (*A. avalosi*, *A. bergi*, *A. kapiipeoi* and *A. magna*; *M. angusta*, *M. charrua* and *M. guenoana*) form reciprocally monophyletic groups in all estimations. The two species of grass-dwelling *Sanogasta* (*S. puma* and *S. tenuis*) are monophyletic only in the ML tree, although with low bootstrap support, while in the Bayesian analyses *S. x-signata* falls in between, with high support. Furthermore, *Sanogasta* as a whole is probably paraphyletic with regards to *Arachosia*, as already suggested by the morphological evidence (Ramírez 2003). While the lack of resolution for some groups may impede strong

systematic conclusions (e.g., resolving the relationships among certain genera), the three clades of grass ecomorphs are separated from each other by several well supported nodes, thus confirming a scenario of convergent evolution. In order to account for the statistical uncertainty reflected by the low support of some nodes, all further analyses average over a subset of post-burnin trees (see below).

The node age estimates obtained using fossil calibrations are similar to those based on the COI rate prior (tree not shown), as well as node ages from the previous studies focused on *Philisca* (Soto et al. 2017) and *Amaurobioides* (Ceccarelli et al. 2016a) (Supplementary Table S7 of SI File 1 available on Dryad), corroborating the node age estimates from the previous studies with the dates obtained here (Supplementary Fig. S4 of SI File 4 available on Dryad). In particular, the estimated dates of dispersal and speciation for the endemic species of the volcanic JF postdate the origin of the islands. We added two *Philisca* species not studied by Soto et al. (2017), as well as specimens of *Sanogasta maculosa* from Robinson Crusoe and Serlkirk islands; these last are denoted *Sanogasta maculosa "skottbergi"*, because *Sanogasta skottbergi* described from the archipelago is currently considered a synonym of *S. maculosa* (Ramírez 2003). Comparing with the previous studies, our more extensive sampling provides better estimates for the whole subfamily and most of its genera (Fig. 2). Greater accuracy in node age estimates is also expected for the genera with putative grass-adapted species, i.e., *Arachosia*, *Monapia* and *Sanogasta*, as these were also sampled more intensively.

With regards to whether the three traits (geographic range, habitat and ecomorph) were clustered or randomly distributed throughout the phylogeny, both the AI and PS metrics detected significant phylogenetic signal in all cases (all  $P < 0.001$ ). Accordingly, all states showed non-random distributions according to the MC statistic. However, phylogenetic signal for geographic range was found to be mostly driven by the non-Patagonian areas (MC NP:  $P < 0.001$ ; MC Pg:  $P < 0.05$ ), while grass-dwelling spiders contributed most of the clustering in the case of ecomorph (MC grass:  $P < 0.001$ ; MC other:  $P < 0.05$ ). Contributions were more balanced for habitat type (MC open:  $P < 0.01$ ; MC forest:  $P < 0.05$ ). Therefore, all the traits studied here are to some extent maintained through common descent, with the strongest phylogenetic signal found in species from non-Patagonian areas and those exhibiting a grass ecomorph.

### *Habitat and Area Mapping*

The model selected by AIC for the area and habitat analyses with BioGeoBEARS was DEC+J, concentrating 96% and 90% of the AIC weights respectively (Supplementary Table S8 of SI File 1 available on Dryad). The ancestral state estimations (Fig. 2, Supplementary Figs. S9 and S10 of SI File 4 available on Dryad) and lineage profiles through time (Fig. 3) were thus calculated for the selected model.

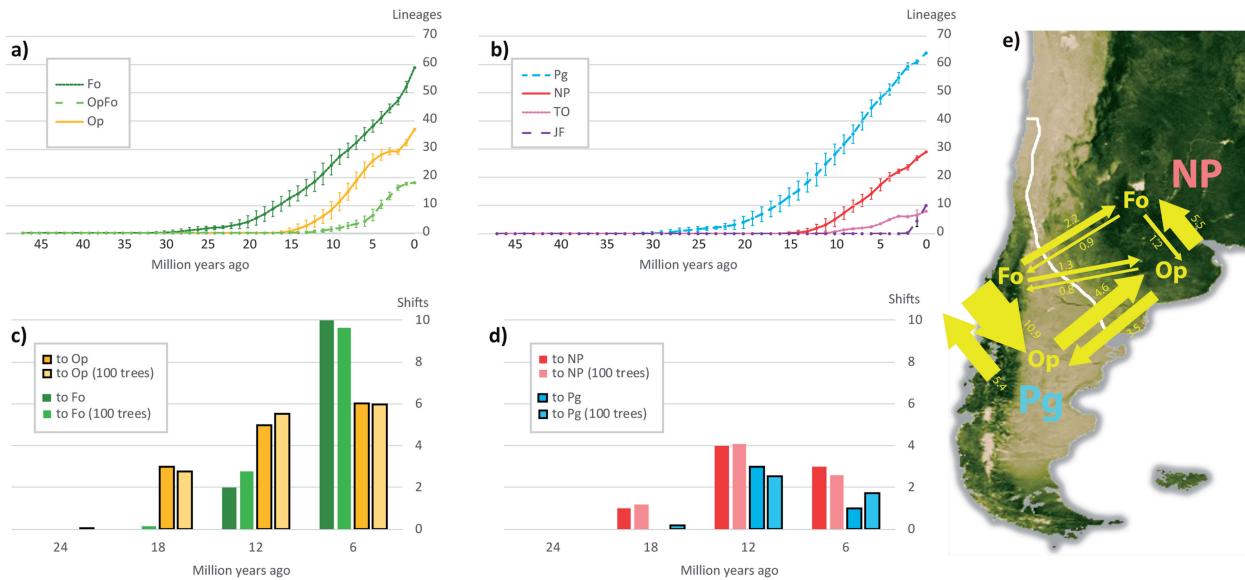


FIGURE 3. Analyses of ancestral habitats and areas for the A+G clade of amauroboidine spiders, summarizing runs of 100 randomly selected post-burnin trees from the Bayesian analysis in BEAST. a) Count of lineages through time, by habitat (mean  $\pm$  standard deviation). b) Count of lineages through time, by area. c) Habitat shifts grouped in time periods. d) Area shifts grouped in time periods, focusing on Patagonia, and the Neotropics. e) Shifts by combination of habitat and geographic area in South America (average over 100 Bayesian trees) summarized on a vegetation index map; arrow thickness is proportional to estimated number of shifts (image by Reto Stockli, NASA's Earth Observatory Group, using data provided by the MODIS Land Science Team).

The ancestor of the subfamily Amaurobioidinae is likely to have arisen from a forest-dwelling Neotropical non-Patagonian lineage; although the outgroup sampling is sparse, no species of *Josa* or Anyphaeninae live in Pg, and most live in forest habitats. The most recent common ancestor of the A+G Clade colonized the Patagonian forests about 27 Ma ago (22.01–32 Ma 95% Highest Posterior Density, HPD), where it diversified. Up to approximately 14 Ma ago the increase in diversity was accounted for exclusively by forest-dwelling lineages in Pg (Fig. 3a and b). From that point on starts the increase of lineages in the open habitats (Figs. 2d, 3a) and in Neotropical regions outside Pg (Fig. 3b).

Comparing the observed lineage state occupancies (based on 100 randomly-selected post-burnin chronograms from the BEAST analysis) with lineage state occupancies expected if the proportions had been the same throughout history, the mean overall state occupancy in open habitat and forest-dwellers is significantly different ( $\chi^2_{(66)} = 114.672, P < 0.001$ ; see Supplementary Fig. S11a of SI File 4 available on Dryad), driven by the differences observed before 10 Ma ( $\chi^2_{(44)} = 87.67, P < 0.001$ ), while there was no significant difference from 10 Ma to the present ( $\chi^2_{(20)} = 27.0, P = 0.135$ ). This result reflects the occupancy of the open habitat, which is only significantly lower than expected before 10 Ma ( $t$ -test  $t = 4.996, P < 0.001$ ; Supplementary Fig. S11b of SI File 4 available on Dryad), while the forest occupancy is higher than expected by chance throughout the history of the spiders ( $t = -5.407, P < 0.001$ ; Supplementary Fig. S11b of SI File 4 available on Dryad).

on Dryad). Similarly, there is a significant difference between observed and expected state occupancies through time in Pg ( $t = -5.627, P < 0.001$ ), whereas state occupancy in non-Patagonian areas was significantly lower than expected before 10 Ma ( $t = 3.907, P < 0.001$ ; Supplementary Fig. S12 of SI File 4 available on Dryad).

The overall temporal pattern is that of an initial source of lineages coming from the Patagonian forests, as shown in the earlier start of shifts from forest to open habitats (Fig. 3c), and from Patagonian to NP areas (Fig. 3d). The process becomes dynamic shortly after, with a gradual increase in the reverse transitions near recent times, especially in habitat shifts. The relatively high number of backward shifts in recent times from open to forest habitats is mainly due to generalist species that have come to use both habitats (Supplementary Table S9 of SI File 1 available on Dryad). In comparison, the backward shifts to Pg are very few (Supplementary Table S10 of SI File 1 available on Dryad), and were found to be significantly fewer than the number of movements out of this region (mean  $\pm$  standard deviation of 100 random posterior BEAST trees, Pg  $\rightarrow$  NP and Pg  $\rightarrow$  PgNP =  $7.88 \pm 0.72$ , NP  $\rightarrow$  Pg and NP  $\rightarrow$  PgNP =  $4.49 \pm 1.00, t = -20.707, P < 0.001$ ).

A dissection of the number of transitions by combinations of habitat and geographic area (averaged over 100 Bayesian trees; Fig. 3e, Supplementary Tables S11 and S12 of SI File 1 available on Dryad) reveals a strong asymmetry in habitat changes within Pg (10 shifts Op  $\rightarrow$  Fo, vs. 5 Fo  $\rightarrow$  Op) compared with the ones that occurred in the non-Patagonian Neotropics (1 vs. 5, respectively). It is also evident that more events of

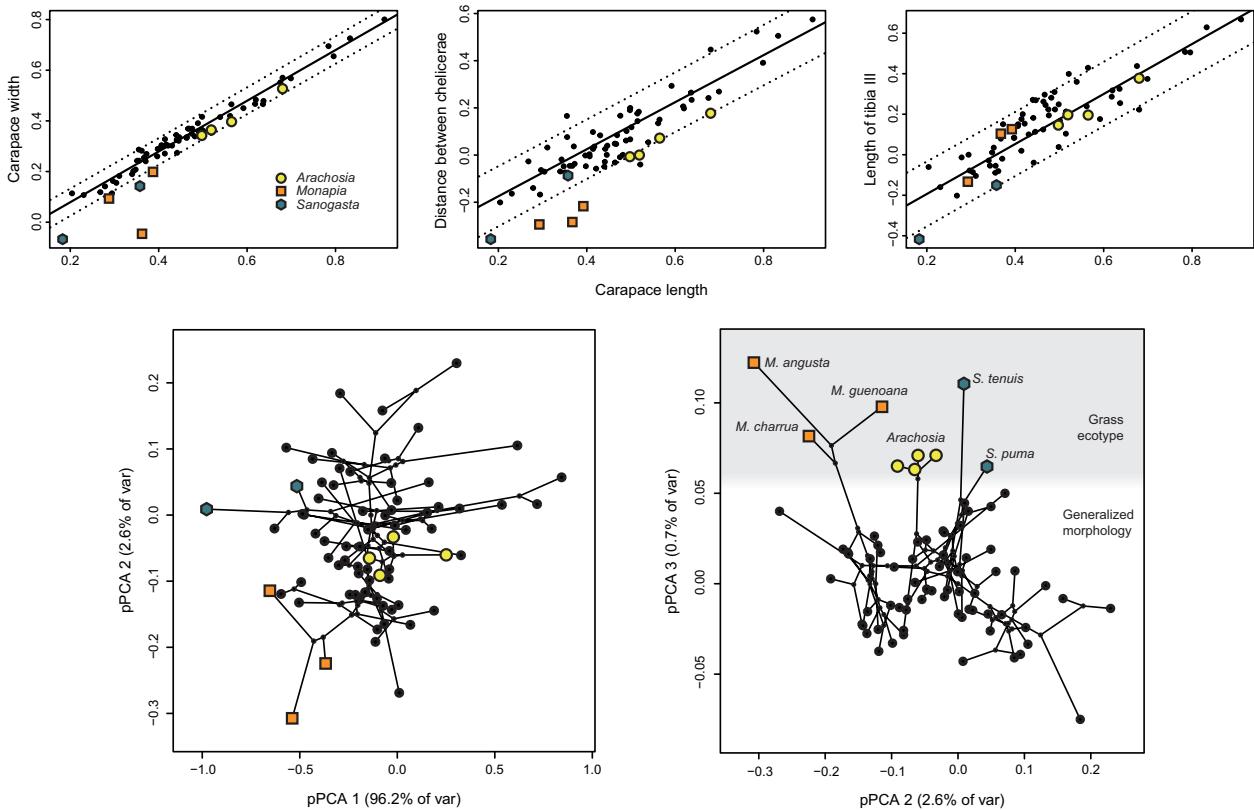


FIGURE 4. Variability among morphometric traits associated with the colonization of grasslands. Top, allometric scaling of traits, using carapace length as proxy for body size. Values are expressed as the logarithm of the species mean. Solid lines correspond to a PGLS $\lambda$  regression for species of general morphology (black circles), dotted lines correspond to the 95% prediction interval (all  $P < 10^{-16}$ ; from left to right:  $R^2 = 0.98, 0.91, 0.93$ ;  $\lambda = 0.512, 0.836, 0.918$ ). At least one grass-dwelling species (other shapes) lies outside the expected interval in all cases. Bottom, phylogenetic spaces on phylogenetic PCA axes, built using the same four traits. Axis pPCA 1 captures differences in size, with low values to the left. The rest represent differences in body proportions (see Supplementary Table S13 of SI File 1 available on Dryad).

dispersal between these biogeographic regions occurred in open habitats, rather than to/from forests.

#### Origin of Grass-Adapted Species

Based on the results of this study, clades showing putative adaptations to grasslands arose from ancestors already living in open habitats in the NP region. Bayesian stochastic mapping on 100 random BEAST trees and the mcc tree (100 replicates in both cases) detected an average of 4.44 and 4.38 state changes across the phylogenies, respectively. These changes were divided as an average of 3.98 (100 trees) and 3.99 (mcc tree) transitions from a general morphology to a grass ecomorph and 0.46 (100 trees) and 0.39 (mcc tree) transitions in the opposite direction. In terms of integers this translates as four transitions to a specialized morphology and zero reversals (see Supplementary Fig. S13 of SI File 4 available on Dryad), although in a few simulations one or more transitions from grass ecomorph to general morphology were detected, most of them apparently accounted for by the ambiguity introduced by the inclusion of *S. x-signata* inside the clade containing the grass-dwelling *Sanogasta*.

In the case of *Arachosia*, the genus' most recent common ancestor (MRCA) is estimated to have diverged

10.75 Ma (8.14–13.6 Ma, 95% HPD) and probably inhabited open habitats, while the grass ecomorph species diversified approximately 1.98 Ma (1.16–2.92, 95% HPD). At ca. 4.07 Ma (2.58–5.8, 95% HPD), the MRCA of these species and their sister-group, *Arachosia proseni* (Fig. 1j), diverged; *A. proseni* being a rather flat and greenish species that inhabits bromeliad-like *Eryngium* species. The second case of putative grass-adaptation is found in *Sanogasta*; the clade containing the two grass ecomorph species *Sanogasta tenuis* and *S. puma* diverged approximately 5 Ma (3.4–6.84, 95% HPD), while their ancestors inhabited open areas from approximately 8.51 Ma (6.36–10.89, 95% HPD). Lastly, while the group of *Monapia* using open habitats traces back to ca. 9.49 Ma (7.12–12.01, 95% HPD), the clade containing the grass ecomorph *Monapia* species diversified at about the same time as the grass ecomorph *Sanogasta* species, around 6.82 Ma (4.70–9.09, 95% HPD).

#### Morphological Macroevolution Analyses

Several aspects of the morphology of grass ecomorph spiders were found to diverge from the allometric expectations based on data from the remaining amauropioidines (Fig. 4, top). Most notably perhaps is

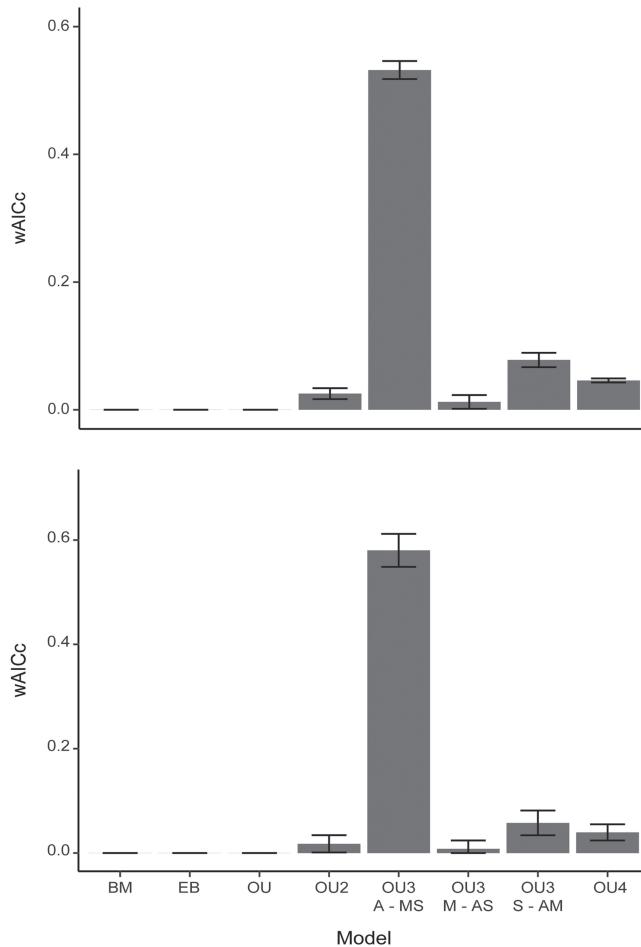


FIGURE 5. Histograms of the relative support for each of the eight models of macroevolution tested. Support was measured as AICc weights in 100 replicates of stochastic character mapping histories on the MCC tree (top) and in one replicate in 100 randomly drawn topologies from the posterior sample of trees (bottom). In case of OU3 models, capital letters stand for grass-dwelling genera (A = *Arachosia*; M = *Monapia*; S = *Sanogasta*), and genera on the same side of the dash were modelled using the same selective regime. Values represent as median  $\pm$  standard error.

the reduced distance separating the chelicerae, the only trait that is found to have arisen in at least some members of all three grass-dwelling clades. On the other hand, members of the genera *Monapia* and *Sanogasta* seem to have convergently acquired a reduced body size with a slender carapace morphology, while the length of the third pair of legs seems to be beyond the predicted interval only for grass-dwelling species of *Sanogasta*. The exploration of this dataset with pPCA reveals a similar pattern (Fig. 4, bottom). The morphology acquired by different clades specialized to life on grasses seems to show some component of convergence, as revealed especially by pPCA axis 3 (Fig. 4, bottom right) which shows the clustering of all three clades and a clear distinction with respect to all other members of the A+G clade. Species with higher values of pPCA axis 3 tend to have narrower and elongated bodies, with a shorter third pair of legs and a reduced distance

between the chelicerae (loadings matrix can be found in Supplementary Table S13 of SI File 1 available on Dryad). This specialized morphology evolved independently in each one of the three lineages from ancestors that exhibited a generalist morphology. However, it is also evident from the remaining axes that grass-dwelling clades have explored partially different regions of morphospace.

The relative support for the explored models of macroevolution are shown in Fig. 5. Overall, there was a relatively strong support for a 3 peak multi-OU model, favored across multiple trait histories on the MCC tree (Fig. 5, top) and multiple topologies sampled from the posterior distribution (Fig. 5, bottom). This macroevolutionary scenario (OU3 A-MS) models the history of morphological change in the tribe Gayennini using three multivariate adaptive peaks, with one peak assigned to all species displaying a generalist morphology, one occupied by grass-dwelling species of *Arachosia*, and a third one convergently encountered by grass-dwelling species of *Monapia* and *Sanogasta*. The difference in support between this model and the second most supported one (OU3 S-AM, also a multi-OU model with three peaks), was higher than the threshold value of 4 (Burnham and Anderson 2002) generally considered to represent a strong difference in support (average  $\Delta$ AICc = 4.03 for replicates on the MCC tree, 4.22 for those in the sample of posterior topologies). Moreover, across all 200 replicates explored, we found that the addition of a third adaptive regime significantly improved the fit of the model 87% of the times, while the addition of a fourth one only resulted in a significant increase in fit 11% of the times (in both cases,  $P < 0.05$  for LRT with four degrees of freedom). This further confirms that the repeated colonization of grasslands by spiders of the tribe Gayennini constitutes a scenario of partial convergence, with two different grassland-associated morphologies, one of which has evolved multiple times.

A summary of the inferred optimal values for each trait (i.e., the  $\theta$  parameters of the multi-OU model) for the three adaptive peaks can be found in Fig. 6. The morphological evolution of grass-dwelling *Monapia* and *Sanogasta* is best modelled as having suffered a tendency towards body size reduction, narrowing of the carapace and strong reduction of the distance between chelicerae, as well as a mild decrease in the length of the third pair of legs (although the confidence interval on this trait is very large). On the other hand, the adaptive peak explored by grass-dwelling *Arachosia* differs much less from the ancestral peak, and seems to entail a slight tendency towards body size increase and, most notably, a strong reduction in the distance between chelicerae. This last attribute therefore characterizes all three clades that have specialized in the exploitation of grassland niches.

Most of the temporal dynamics of this adaptive process seems to have occurred at a relatively fast pace, with average phylogenetic half-lives of 0.71, 1.32, 2.97, and 131.03, for each of the four eigendimensions of the  $\alpha$  matrix (Supplementary Fig. S10 of the SI File 4 available on Dryad). The first of these dimensions entails

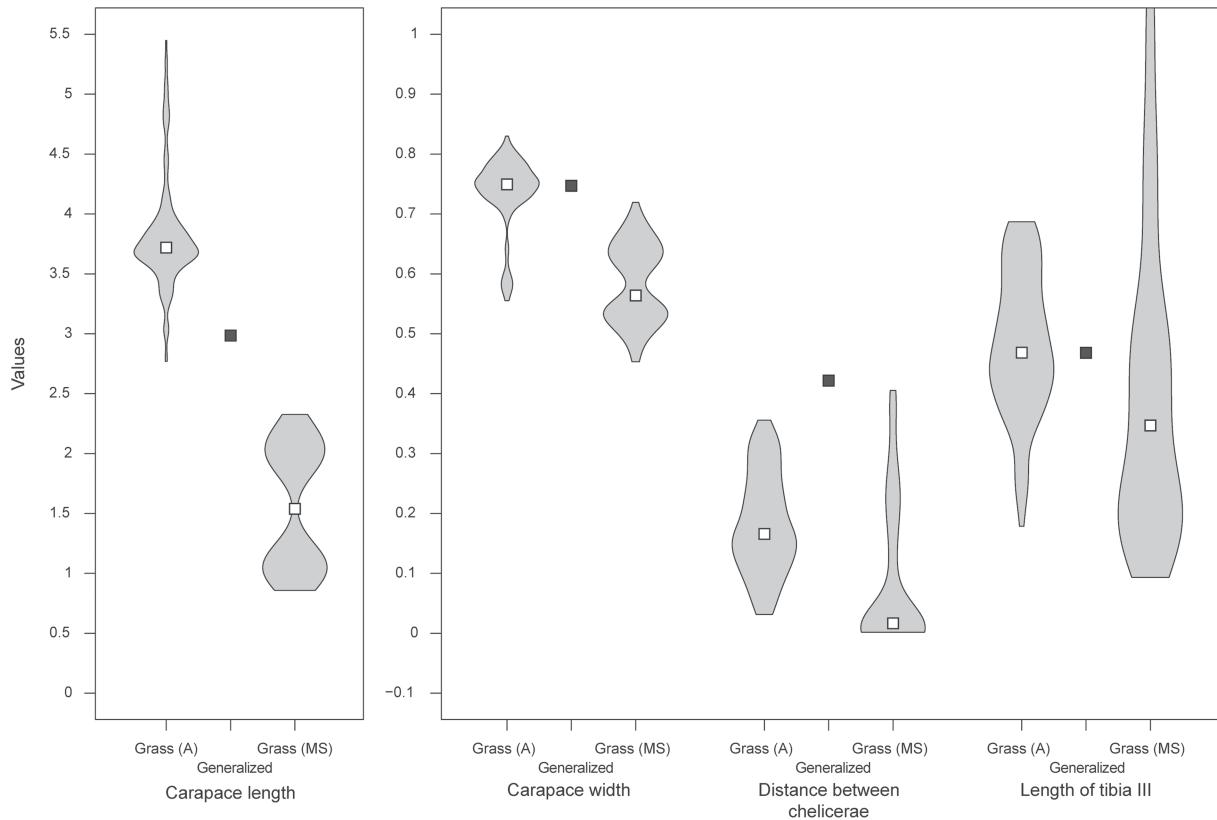


FIGURE 6. Inferred optimum values ( $\theta$  parameters) for all traits in 200 replicates of the OU3 A-MS model. Values can also be interpreted as the approximate locations of the peaks in a multivariate adaptive landscape. Violin plots show the probability densities of the estimates after excluding the most extreme 5% of results, rectangles represent median values. Body size optima are shown in mm, other values are expressed as the ratio of each trait to body size, so as to represent inferred differences in proportions. Given the way the replicates were parameterized, uncertainty for the ancestral selective regime is minimal and not shown. Peaks for grass-dwelling clades are identified using the same codes for genera as in Fig. 5.

the relative reduction of all traits with respect to size (the same pattern as seen in pPCA axis 3), while the second one can be interpreted as changes in overall size (Supplementary Fig. S15 of the SI File 4 and Table S14 of SI File 1 available on Dryad).

## DISCUSSION

The global expansion of grasslands during the Miocene has impacted numerous animal taxa. For grass-specialized herbivores, the increase in their food source presented an ecological and evolutionary benefit; this is especially true for insect groups such as the leafhoppers and stem-borers, many of which have specialized in feeding on grasses (Dietrich 1999; Zahniser and Dietrich 2010; Toussaint et al. 2012) and whose diversification is temporally congruent with grassland expansions. With the increase in grassland herbivores and new habitats to exploit, predators such as falcons also underwent time-congruent diversification in the late Miocene (Fuchs et al. 2015). Similarly, the predators among arthropods such as spiders are expected to exploit the abundant insect biomass found in grasslands (Tscharntke and Greiler 1995). Based on the results of this study, the habitat

changes from forest to open habitats in amaurobiodine spiders occurred during the mid- to late Miocene, before which (ca. 10 Ma) there were proportionally fewer lineages than expected in open habitats. These dates are congruent with the increased expansion of the South American grassland biome, especially in Pg (Barreda and Palazzi 2007; Palazzi and Barreda 2012) suggesting that, as open habitats started expanding, amaurobiodine spiders readily occupied the newly available niches. The node age estimates obtained in this study are plausible, based on the similar ages obtained from previous studies with different taxon sampling (Ceccarelli et al. 2016a; Soto et al. 2017) as well as geological age corroboration with the volcanic JF (Stuessy et al. 1984) and the age of the MRCA of the *Sanogasta* and *Philisca* species endemic to those islands. These two colonization events of Juan Fernández, plus the trans-oceanic dispersal of *Amaurobioides* probably by oceanic drift with the Antarctic circumpolar current (Ceccarelli et al. 2016a) are evidence for the long-distance dispersal ability of some amaurobiodine spiders, yet overall, a strong historical component is still preserved in their geographic distribution. While the Amaurobioidinae are probably of NP origin, the

A+G clade originated and diversified in forested Patagonian habitats. South American orogeny must also be considered as an important factor for the diversification and movement of species, and for the evolution of habitats; for example, during the last 10 Ma the Andean uplift also had a profound effect on structuring both Patagonian and other South American fauna (e.g., Sersic et al. 2011; Ceccarelli et al. 2016b). Based on our study, the interchanges of fauna between Pg and the non-Patagonian Neotropics occurred predominantly in open habitats. This is consistent with the prediction that biome shifts are more probable over larger shared boundaries (Donoghue and Edwards 2014), since the boundary between these biogeographic areas is constituted by open habitats, with the forested areas now separated by ample expanses of arid lands (Fig. 3e). It is also noteworthy that the initial flow of species out of Patagonian forests became dynamic shortly after the expansion of grasslands, with a backflow of lineages returning to Pg, or expanding back to forest habitats.

Some species of Amaurobioidinae seem especially suited for life on grasses due to morphologies that evolved at least three independent times among lineages already inhabiting open ecosystems in the non-Patagonian Neotropics. Although grasses are common in the understory of forests, grass-dwelling lineages originated exclusively in open habitats, likely a consequence of the difference in prey biomass harbored by grasslands compared with forest grasses. The traits involved in this specialization are all related with the need to fit tightly on a single blade of grass and minimize visual cues to predators, moving and building retreats in the narrow spaces between grass blades, and might therefore be considered morphological adaptations to the peculiar shape and disposition of the graminean leaves. The association between these morphologies and the strict exploitation of grasslands, their departure from the allometric scaling shown by close relatives living in different habitats, as well as their inferred origin only after grass-dominated ecosystems spread all warrant their recognition as adaptations to grasslands. Many shifts from forest to open habitats are inferred to have occurred at about the same time as grasslands were expanding, which in conjunction with the strong phylogenetic signal found in the habitat traits, suggest that there is an intermediate degree of niche conservatism (see Wiens et al. 2010). On the other hand, the strict adaptation to living on grasses is a much more conservative trait, and in at least two cases seems to have originated only long after the colonization of open habitats. The MRCA of grassland-adapted *Arachosia* and *Sanogasta* is inferred to have already lived in open habitats some 13 Ma, while adaptations to grasslands are unlikely to have originated any time before 6 Ma; in *Monapia* the delay is less marked (about 10 Ma for shift to open habitat and 7 Ma for grass-adaptation). This delay in the start of the process of grassland specialization might indicate that the specific microhabitats employed by

grass-dwelling ghost spiders—grasslands dominated by large grass species in regions of high humidity and mild temperatures—did not become common until later in the history of the region. The current paleoenvironmental reconstructions in the region (e.g., Barreda et al. 2007) are still too coarse to trace the origin of this specific environment. Nonetheless, based on our results, grassland ecomorphs originated very rapidly once the selective process was underway. The half-lives for the first two eigendimensions, which seem to capture the transition from general morphology to grass ecomorph, as well as changes in overall size, are on the timescale of approximately 1 Ma. This is a relatively short amount of time compared with the results of other studies inferring selective processes on macroevolutionary timescales (Uyeda et al. 2011; Hansen 2012; Toljagić et al. 2018), although the difference in generation times between taxa must also be considered. This rapid adaptation is even more remarkable considering the complexity of the morphological changes involved, which likely involved the accommodation of multiple constraints and trade-offs. For example, it has been shown that the slender abdomen of ant-mimicking spiders increases their resemblance to their model ants at the expense of smaller egg clutches compared with other, stockier salticid spiders (see Cushing 1997).

The expansion of grasslands in South America resulted in an increase in complexity of the macroevolutionary adaptive landscape of amaurobioidine spiders, setting them off on previously unexplored evolutionary pathways. The morphology of two of the three genera of grass-adapted spiders is best modelled as the result of evolution under the same selective regime, characterized by body size reduction, narrowing of the carapace, strong reduction of the distance between chelicerae, as well as a mild decrease in the length of the third pair of legs. This can therefore be considered an instance of adaptive morphological convergence driven by habitat, a thoroughly-studied phenomenon in evolutionary biology (e.g., Gillespie 2004; Losos 2011; Muschick et al. 2012; Thiele 2012). Nonetheless, the evolution of a second grassland ecomorph shows that not all evolutionary trajectories associated with grassland specialization have resulted in the same end-product. Grass-adapted species of *Arachosia* have evolved towards a different adaptive peak, characterized by a longer body and a reduced distance between chelicerae. The fact that replicated events of adaptation to similar environmental conditions fail to converge is considered evidence of the importance of historical contingency in evolutionary processes (Seehausen 2007; Blount et al. 2008; Young et al. 2009). On the other hand, although there is strong statistical support for grassland-adapted amaurobioidine spiders evolving under two different selective regimes, they are both characterized by a common trend: a reduction of the distance between the chelicerae. This slender configuration at the anterior

region of the prosoma, which originated at least three independent times, seems to be a necessary condition for the successful colonization of grasslands. At least at this level, adaptation has followed an entirely deterministic route.

In conclusion, based on the results of our analyses, it is clear that the evolutionary history of Amaurobioidinae has been shaped by recurrent colonization of open habitats, followed by events of diversification and adaptation. Even considering some of the shortcomings of the phylogenies upon which these conclusions are based, such as poor resolution in some clades, the nodes corresponding to grass-adapted clades are all well-supported. Furthermore, sampling over a subset of posterior trees allowed us to test these hypotheses in light of alternative topologies. We can therefore conclude that these spiders are a group of organisms for which the expansion of grasslands in South America presented an ecological and evolutionary opportunity. The evolution of amaurobiodine spiders shows a traceable history of convergent, yet diverse morphological adaptations in response to changes in the environment, the colonization of new habitats and regions and a dynamic flow of lineages. We have shown here that a combination of phylogenetic, morphological, and biogeographic analyses helps to untangle and understand the multiple factors and levels affecting the evolution of organisms and the biomes they inhabit.

#### SUPPLEMENTARY MATERIAL

Data available from the Dryad Digital Repository:  
<http://dx.doi.org/10.5061/dryad.20257>.

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