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Molecular phylogeny of the wolf spider subfamily Allocosinae in South America (Araneae: Lycosidae)

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Abstract

The wolf spiders of the subfamily Allocosinae are known for their complex taxonomy, especially in the Neotropical region. Despite previous taxonomic and phylogenetic studies, the diversity and phylogenetic relationships of the subfamily remain largely unknown. This study aims to clarify the evolutionary relationships within South American Allocosinae, hypothesizing a greater diversity than currently recognized and seeking to resolve ambiguities in genus-level classification. We used a combination of mitochondrial and nuclear gene sequences to construct phylogenetic analyses for 73 specimens across 13 species of Allocosinae. Analyses using both maximum likelihood and Bayesian frameworks were conducted to examine internal relationships and phylogenetic structure and to infer a timeline of diversification. Additionally, species delimitation was conducted to identify cryptic lineages. Our results recover the specimens considered to be representatives of the subfamily Allocosinae as a monophyletic group, and identified five major clades. Divergence time estimates suggested Allocosinae originated in the Early Miocene (15–22 million years ago), and underwent significant diversification during the Pleistocene. Species delimitation analysis based on single markers uncovered 24 lineages, indicating potentially overlooked species. Allocosinae has shown to be an interesting group to study incipient speciation processes, ecology of coastal environments and atypical behaviors such as sex role reversal. Knowing and understanding the evolutionary history and relationships within the subfamily is necessary for progress in its study in any field of biology.

Keywords

molecular markers, Neotropics, diversification, divergence times, systematics

1. Introduction

The Neotropical region harbors invaluable biological diversity, which is subject to numerous threats (Myers et al. 2000; Antonelli and Sanmartín 2011; Rull 2011; Ulloa Ulloa et al. 2017; Raven et al. 2020). It is estimated that much of this biodiversity remains unknown despite the efforts of the scientific community to discover and classify it (e.g. Buck 2006; van Nieukerken et al. 2016; Feitosa et al. 2017). Our knowledge deficiency is most noticeable in groups of highly diverse organisms, such as spiders (order Araneae) (Stork 2018), a megadiverse lineage (Coddington and Levi 1991; Wheeler et al. 2017) with more than 52.000 species described (World Spider Catalog 2025). Similarly, within spiders, the most diverse families exhibit significant gaps in knowledge regarding their diversity and evolutionary relationships, which is well exemplified in wolf spiders, Lycosidae (Piacentini and Ramírez 2019).

Lycosidae is one of the most diverse and abundant spider families, comprising at present 135 genera and 2,494 species distributed worldwide (World Spider Catalog 2025). Currently, 10 subfamilies are recognized (Piacentini and Ramírez 2019) and their phylogenetic relationships have been examined using both morphological (Dondale 1986) and molecular data (Murphy et al. 2006; Piacentini and Ramírez 2019). Allocosinae is a subfamily originally proposed by Dondale (1986) to include the genus *Allocosa* Banks, 1901 (from North and Central America) and *Moenkhausiana* Petrunkevitch, 1910 (from South America). Later, Capocasale (1990) subsumed *Moenkhausiana* as a junior synonym of *Allocosa*, and the subfamily remained monotypic for several years thereafter. Piacentini and Ramírez (2019) inferred a phylogenetic framework for Lycosidae using molecular data and recovered Allocosinae as a monophyletic group. In that study they included species previously recognized as Allocosinae, such as *Allocosa funerea* (Hentz, 1844) (type species of the genus *Allocosa*, from North America) and *A. senex* (Mello-Leitão, 1945) from South America, along with other species not previously included in Allocosinae until that publication: "*Arctosa*" *sapiranga* Silva and Lise, 2009, *Pardosa flammula* Mello-Leitão, 1945 (currently *Abaycosa nanica* (Mello-Leitão, 1941), see Laborda et al. 2022) and *Gnatholycosa spinipalpis* Mello-Leitão, 1945, all from South America. Subsequently, based on morphological and molecular studies, other species were also included in Allocosinae, namely "*Paratrochosina*" *amica* (Mello-Leitão, 1941) by Gonnet et al. (2021) and the recently proposed genus *Abaycosa* Laborda et al., 2022, with two species, *A. nanica* (Mello-Leitão, 1941) and *A. paraguensis* (Gertsch and Wallace, 1937). Therefore, as currently delimited, the subfamily contains representatives of five genera. The study of Piacentini and Ramírez (2019) detected several inconsistencies across the taxonomy of Lycosidae, showing that several genera, such as *Arctosa*, *Pardosa*, *Lycosa*, *Hogna*, *Allocosa*, among others, were not recovered as monophyletic.

A prominent example of taxonomic uncertainty in Lycosidae is precisely the genus *Allocosa*, currently including 130 species from the Neotropical, Australian, Ethiopian, Palearctic, Nearctic, and Oriental regions (World Spider Catalog 2025). The type species, *Allocosa funerea* (Hentz, 1844) was described from Alabama, United States of America. Dondale and Redner (1983) reviewed *Allocosa* species from North and Central America and proposed that the genus was restricted to the New World. The non-American species currently in the genus were transferred by Roewer (1955), who did not provide any morphological evidence to support these changes or mostly relied on homoplastic characters within the taxonomy of Lycosidae. Although subsequent revisions have removed several species from *Allocosa*, many others remain to be evaluated. Currently, 23 species of *Allocosa* inhabit the Neotropics, 18 of which have been recorded for South America (World Spider Catalog 2025). The latest taxonomic contributions to the genus in South America included the redescription of three species and the description of a new species from Uruguay and Southern Brazil (Brescovit and Taucare-Ríos 2013; Simó et al. 2017).

The species *Allocosa marindia* Simó, Lise, Pompozzi & Laborda, 2017 and *A. senex* (Mello-Leitão, 1945) inhabit the sandy shores of rivers, lagoons, and of the Atlantic Ocean in Argentina, Brazil, and Uruguay (Capocasale 1990, 2001; Costa 1995; Costa et al. 2006; Simó et al. 2017). They are notable for exhibiting atypical sex roles and sexual size dimorphism (Aisenberg et al. 2007; Aisenberg and Costa 2008). Unlike most spiders, females are smaller than males and they are the more mobile sex, actively seeking males and initiating courtship (Aisenberg et al. 2007, 2023; Aisenberg and Costa 2008; Aisenberg 2014). These two species have served as model organisms for numerous studies in behavior, ecology, phylogeny and phylogeography (Aisenberg 2014; Bidegaray-Batista et al. 2017; Bollatti et al. 2017; Carlozzi et al. 2018; Postiglioni et al. 2019; Albín et al. 2021; Gonnet et al. 2021; Cavassa 2022; Laborda et al. 2022; Aisenberg et al. 2023). Despite these advances, the systematics of the remaining South American Allocosinae needs further in-depth analysis. Many species were proposed based on brief descriptions and, in some cases, are known from only one sex, which, in combination with the suspected incorrect placement of species in other genera or even subfamilies and potential synonyms, create a challenging scenario for the delimitation and identification of species of the group (Laborda et al. 2022). These obstacles, added to their morphological uniformity, especially in genital structures (Simó et al. 2017), makes it necessary to explore other data sources, such as genetic sequences, for their systematic study. Genetic data are valuable in this context, as information generated from various molecular markers provides substantial insight for both species delimitation and phylogenetic inference (Bidegaray-Batista et al. 2014; Wheeler et al. 2017; Macías-Hernández et al. 2020). These approaches are particularly relevant in cases in which speciation processes have occurred recently (Maddison 1997; Knowles and Maddison 2002).

This seems to be the case, for example, of *A. senex* and *A. marindia*, which are sister-species that diverged less than two million years ago during the Pleistocene (Bidegaray-Batista et al. 2017; Postiglioni et al. 2019).

In this study, we integrate mitochondrial and nuclear gene information from a broad sampling of taxa to infer species relationships and estimate the timeline of diversification of Allocosinae in South America. We further use genetic evidence to delimit potential overlooked lineages since we hypothesize that the species diversity in the subfamily is larger than presently known.

2. Materials and methods

2.1. Data collection and voucher specimens

The data collected for the present study includes material from collections and collecting field trips. Voucher specimens of DNA extraction or morphological study are deposited in the following collections: Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay (FCE-Ar, M. Simó), Museo Argentino de Ciencias Naturales "Bernardino Rivadavia", Buenos Aires. Aires, Argentina (MACN-Ar, M. Ramírez), Laboratório Especial de Coleções Zoológicas, Instituto Butantan, São Paulo, Brazil (IBSP, A.D. Brescovit), Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil (MCTP, R. Teixeira), Laboratorio de Biología Reproductiva y Evolución, Cátedra de Diversidad Animal I, Universidad Nacional de Córdoba, Córdoba, Argentina (LABRE-Ar, M. Izquierdo), California Academy of Sciences, San Francisco, California, USA (CASENT, L. Esposito). Information and photographs of type material were obtained from the following institutions: The Natural History Museum, London, England (J. Beccaloni), Museo de Biología de la Universidad Central de Venezuela, Caracas, Venezuela (E. Guerrero), Muséum National d´Histoire Naturelle, Paris, France (C. Rollard), Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil (A. B. Kury), Museum of Comparative Zoology, Harvard University, Cambridge, USA (G. Giribet), Museum Wiesbaden, Wiesbaden, Germany

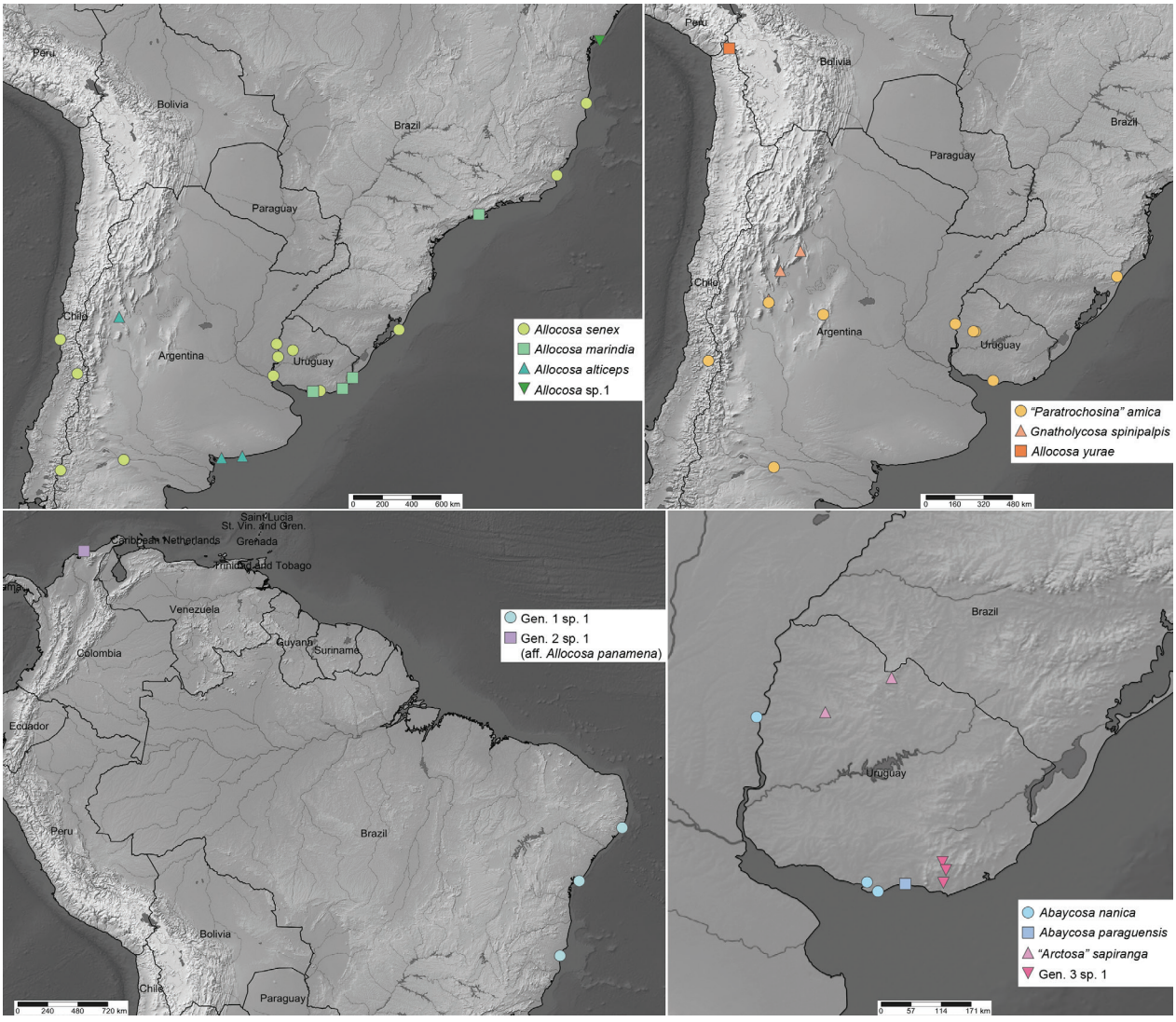

Figure 1. Map showing the sampling localities of Allocosinae specimens from South America included in the phylogenetic analyses.

(S. Kridlo), National Museum, Dublin, Ireland (P. Viscardi), Naturmuseum Senckenberg, Frankfurt, Germany (P. Jäger), Peabody Museum of Natural History, Yale, USA (R. J. Pupedis), Royal Belgian Institute of Natural Sciences, Brussels, Belgium (F. Trus), Swedish Museum of Natural History, Stockholm, Sweden (J. Stigenberg).

Field work for this study was carried out in: Uruguay (Clemente Estable Biological Research Institute in Montevideo, Melilla in Montevideo, and Montes del Queguay Protected Area in Paysandú); Argentina (Ischigualasto Provincial Park in San Juan, Lanín National Park in Neuquén, and El Palmar National Park in Entre Ríos); Chile (Río Clarillo National Park in Santiago) and Brazil (Parque das Dunas in Salvador, Bahia, and Pró-Mata Reserve in São Francisco de Paula, Rio Grande do Sul). The collecting sites were selected based on previous records, and new ones aimed to cover the largest area and diversity of environments.

Male and female genitalia were examined under stereomicroscopes (Nikon SMZ 10 and SMZ 745), and specimens were identified by comparing them with images of type specimens, if available, or with original descriptions and taxonomic revisions. Maps were made with SimpleMappr (http://www.simplemappr.net) (Fig. 1). When coordinates were not specified in the original labels, localities were georeferenced using Google Maps, and in those cases provided between brackets in the lists of examined material.

2.2. DNA extraction, amplification, and sequencing

Specimens sequenced (N=73) included museum and fresh, field-collected material. DNA was extracted from the left legs of the specimens (two or four, depending on the size of the specimen) using the DNeasy Tissue Kit (Qiagen), following the manufacturer's instructions. For collection specimens, which had not been kept in appropriate conditions to preserve the DNA, the QIAamp DNA Micro Kit (Qiagen) was used following the manufacturer's instructions. The complete specimens were immersed in the lysis buffer after performing a puncture of the carapace to expose internal tissue. DNA was quantified, and the purity was determined by spectrophotometry using a ND 1000 NanoDrop (Thermo Scientific). The DNA extraction codes of all the specimens used, as well as their sex and locality data are shown in Table S1. The selection of molecular markers was made based on the information available in the literature and public genetic sequence databases (GenBank). Fragments of six genes were amplified using the PCR; the mitochondrial cytochrome oxidase c subunit 1 (*cox1*), 12S rRNA (*12S*) and NADH dehydrogenase subunit 1 (*nad1*); and the nuclear histone H3 (*h3*), histone H4 (*h4*) and 28S rRNA (*28S*). Standard spider primers and protocols were used for each gene (Wheeler et al. 2017; Planas et al. 2013). The following primer pairs were used, *cox1*: C1-J-1490 (Folmer et al. 1994) and C1-N-2662 or Porricosa-R1 (Laborda et al. 2022); *12S*: SR-J-14233 (Simon et al. 1994) and SR-N-14503 (Croom et al. 1991); *28S*: 28S "O" and 28S "C" (Hedin and Maddison 2001), *nad1*: TL-1-N-12718 (Hedin 1997) and M510 (Murphy et al. 2006); *h3*: H3F and H3R (Colgan et al. 1998); *h4*: H4F2er and H4F2s (Pineau et al. 2005). PCR reactions were carried out following the protocol for Taq DNA Polymerase using Standard Taq Buffer (M0273, New England Biolabs Inc.). The PCR conditions were as follows: initial denaturation at 95°C for 3 min; followed by 35 cycles at 95°C for 30 s, from 42° to 58°C for 45 s (depending on primers), and extension at 68°C for 45 s; with a final extension step at 68°C for 5 min. For the *cox1* and *nad1* gene fragments, successful amplification was achieved with an annealing temperature at 45°C, for *12S* at 42°C, for *28S* at 58°C, while for *h3* and *h4* at 48°C. The products obtained by PCR were visualized by 1% agarose gel electrophoresis and purified with FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I enzymes (Thermo Fisher Scientific). PCR products were sequenced in both directions using the sequencing service of Macrogen, Seoul, Korea. DNA sequences were edited and aligned using the trial version of the Geneious program (Kearse et al. 2012). In addition to the sequences obtained in this study, the sequences published in Piacentini and Ramírez (2019) and deposited in the GenBank and BOLDSYSTEMS repositories were used (Table S2). These sequences come from a broad sampling of Lycosidae, so they were used as out-groups in the analysis to provide an extensive context for the subfamily Allocosinae.

2.3. Matrices, alignment, and substitution models of evolution

Based on the molecular markers obtained in this study and those previously published in Piacentini and Ramírez (2019), Gonnet et al. (2021), and Laborda et al. (2022), a concatenated matrix was constructed, including representatives of other currently recognized Lycosidae subfamilies to test the monophyly of Allocosinae. This matrix (M1) concatenates the sequences of five molecular markers (*cox1*, *nad1*, *h3*, *12S*, and *28S*) including 154 taxa, rooted in *Cupiennius* spp. A second matrix (M2, 93 taxa, rooted in *Aglaoctenus lagotis* (Holmberg, 1876)) was built by reducing the outgroups to focus on resolving the internal relationships of Allocosinae. These matrices were analyzed using maximum likelihood (ML) and Bayesian inference (BI) (see below). A third matrix (M3) was constructed keeping only the Allocosinae specimens (86 taxa, rooted in "*Arctosa*" *sapiranga*, the taxon considered as a sister group to the rest of Allocosinae in previous analyses, see Piacentini and Ramírez 2019), removing the *28S* marker (due to lack of data on terminals and low genetic variation) and adding the *h4* marker sequences (see Table S1), and analyzed only under Bayesian inference. In a complementary analysis, public access sequences (GenBank and BoldSystems) from *cox1* of four North American *Allocosa* species (*A. funerea*, *A. noctuabunda*, *A. parva*

and *A. absoluta*) were added to M2 to create a fourth matrix (M4).

The alignment of cytochrome oxidase c subunit 1 (*cox1*), NADH dehydrogenase subunit I (*nad1*), histone H3 (*h3*), and histone H4 (*h4*) was trivial since no insertions or deletions were inferred. The *12S* and *28S* alignments were made with MAFFT (Katoh and Standley 2013) using the L-INS-i algorithm. Gappy fragments of uncertain positional homology were removed with Gblocks 0.91b (Castresana 2000). PartitionFinder2 was used to select the partition schemes and molecular evolution models that best fit the data (Lanfear et al. 2016). These analyses were based in two initial partition schemes, by genes (*cox1* / *12S* / *nad1* / *h3* / *28S*) and by genes and 1st, 2nd and 3rd codon positions of the coding genes (*cox1*_p1 / *cox1*_p2 / *cox1*_p3 / *nad1*_p1 / *nad1*_p2 / *nad1*_p3 / *h3*_p1 / *h*3_p2 / *h3*_p3/ *12S*/ *28S*), hereafter called P1 and P2, respectively. The ML and BI analysis were performed based on the result indicated by PartitionFinder2.

2.4. Phylogenetic analysis

2.4.1. Concatenated gene matrices approach

Phylogenetic inferences were performed using the concatenation approach of gene fragments with maximum likelihood analysis and Bayesian inference using RAxML v.8.2.12 (Stamatakis 2006) on the CIPRES platform (Miller et al. 2010) and MrBayes v.3.1.2 (Ronquist et al. 2012), respectively. For the MrBayes analysis, two independent runs of 10 million generations were conducted, each using six Markov Chain Monte Carlo (MCMC) chains and sampling every 1000 generations. The program TRACER v.1.7.2 (Drummond and Rambaut 2007) was used to verify that the Markov chains had reached stationarity and convergence by examining the effective sample size (ESS), and to determine the appropriate burn-in generations. RAxML analyses were conducted using independent GTR+G substitution models for each partition based on the results of PartitionFinder2. The rapid bootstrapping algorithm was used for clade support estimation, and the best-scoring Maximum Likelihood (ML) tree was searched for in a single run. The majority-rule tree criterion was employed to automatically halt bootstrapping. The information from the sampled trees was summarized with TreeAnnotator v2.4.7 (Bouckaert et al. 2014). The graphic editing of the resulting trees was performed using FigTree v1.4.4. For phylogenetic analyses, three support states are recognized according to the following thresholds: supported, with bootstrap > 80%, BI pp > 0.95; recovered but not supported, with bootstrap < 80%, BI pp < 0.95; not recovered.

2.4.2. Species tree

For the estimation of species trees and divergence times (see below), a multi-species coalescent analysis was conducted on the gene matrices used in M3, including both mitochondrial and nuclear gene datasets, using the *BEAST (Heled and Drummond 2010) in BEAST v.2.6.3 (Drummond et al. 2006; Drummond and Rambaut 2007). Since this method requires a priori assignment of individuals to lineages or species, we first conducted species delimitation analyses (GMYC and STACEY; see below) to assign individuals to independent evolutionary lineages. Four runs of 40 million generations were performed, with sampling every 4000 generations.

2.4.3. Molecular species delimitation

The GMYC method (Generalized Mixed Yule Coalescent) was used to delimit evolutionarily independent lineages based on data from a single locus (Fujisawa and Barraclough 2013). For this analysis, an ultrametric tree was previously constructed using BEAST v.2.6.3 from the mitochondrial genes (*cox1*, *nad1* and *12S)* (subsampling M3), with four runs of 40 million generations, sampling every 4000 generations. The analysis was conducted using a strict clock with the mean rate arbitrarily fixed at 1 and a coalescent constant size demographic model as tree prior. Additionally, the multi-species Bayesian coalescent model implemented in the STACEY package (Jones 2017) for BEAST v.2.6.3 (Bouckaert et al. 2019) was employed. This method co-estimates gene and species trees, or minimal clusters, alongside species delimitation. The analyses included mitochondrial and nuclear gene datasets (subsampling from matrix M3). Each independent evolutionary lineage recovered with the GMYC analyses was considered a priori as a minimal cluster for the STACEY analyses. One individual of each lineage, with both the mitochondrial and nuclear genes sequenced, was selected and included in the analyses. Substitution model parameters were specified for each gene fragment, with mitochondrial trees and clock models linked. A strict molecular clock model was applied to each partition. The parameters *bdcGrowthRate* and *popPriorScale* were set with a log-normal distribution, and the relative priors *DeathRateSpecies* and *collapseWeight* were set with a uniform (0–1) distribution. Ten independent runs of 40 million generations were performed, sampling every 4000 generations. The results were analyzed using the SpeciesDelimationAnalyser (speciesDA.jar package available at http://www.indriid.com/software.html).

2.4.4. Divergence time estimation

Diversification times were estimated using mitochondrial and nuclear gene datasets under a concatenated approach and a species-tree approach in BEAST and *BEAST, respectively. Analyses were performed using a partition scheme by genes (P1), with the best model selected by Partitionfinder. In BEAST we used a relaxed clock model, independent nucleotide substitution model for each gene and an independent molecular clock for each gene. In *BEAST we used strict clock models and an independent nucleotide substitution model for each

gene. We concatenated mitochondrial genes and used a single molecular clock. Independent molecular clocks were specified for each nuclear gene. The Yule speciation process was specified as prior. Absolute divergence times were estimated based on the substitution rates estimated in Piacentini and Ramírez (2019) and Bidegaray-Batista and Arnedo (2011), which were incorporated as priors on the different partition clocks. Eight independent runs of 40 million generations were conducted, sampling every 4000 generations. The runs were combined using LogCombiner v.2.6.3 (Drummond and Rambaut 2007), applying a burn-in rate of 10%.

3. Results

3.1. DNA extraction, amplification, and sequencing

Sequences of 73 specimens were obtained (Table S1): *cox1* (71 sequences), *nad1* (69 sequences), *12S* (73 sequences), *h3* (70 sequences) and *h4* (71 sequences). Sequences of the *28S* marker showed multiple overlapping peaks and were discarded from downstream analyses. The *cox1* alignment was 1,278 bp long, *nad1* at 615 bp,

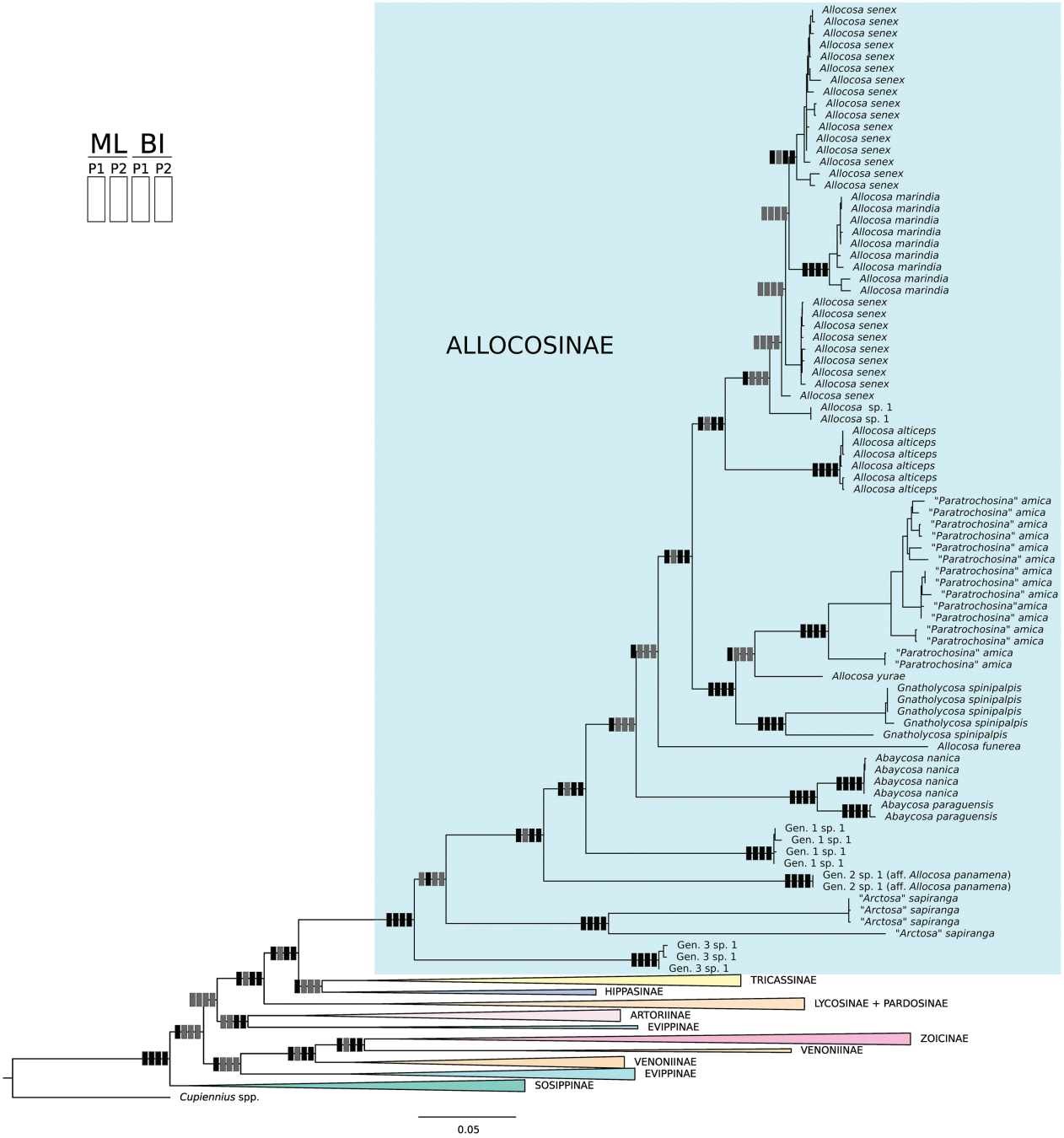

Figure 2. Topology of the tree obtained from Maximum Likelihood (ML) analysis under a scheme of partition by genes P1 (concatenation of *cox1*, *nad1*, *h3*, *12S*, and *28S* genes), including Allocosinae representatives and other currently recognized Lycosidae subfamilies. Bars on nodes indicate support for Maximum likelihood (ML) and Bayesian inference (BI). Black: ML bootstrap > 80%, BI pp > 0.95; gray: recovered clade with support below the indicated limit; white: node not recovered in the analysis.

h3 at 384 bp, and *h4* at 159 bp. The alignment of the *12S* fragment resulted in 430 bp and 191 bp after being analyzed in Gblocks.

3.2. Phylogenetic analysis

3.2.1. Concatenated matrices

The PartitionFinder2 identified a partition scheme by gene, with *12S* and *nad1* combined in a single partition as the preferred option (P1). When the starting partition was defined by codon, the preferred scheme was: *cox1_p1*, *cox1_p3*, *nad1_p3*, *h3_p1*, and *h3_p2*, while *cox1_p2* and *nad1_p2*, *12S* and *nad1_p1*, *28S*, and *h3_p3* were combined (P2). The best substitution models selected were: GTR+I+G for *cox1*, *12S*, and *nad1*; TVMef+I+G for *h3*; and TrN+G for *28S* under P1. For P2, the models were GTR+G for the first positions of *cox1*, *nad1*, and *12S*; K81uf+I+G for the second positions of *cox1* and *nad1*; HKY+G for the third position of *cox1* and *nad1*; SYM+I+G for the first position of *h3*; JC for the second position of *h3*; and TVM+G for the third position of *h3* and *28S*.

The ML and BI analyses with M1 and M2 supported the monophyly of Allocosinae and its close relationship to representatives of the Tricassinae and Hippasinae subfamilies (Figs 2, 3). The topology suggests that Gen. 3 sp.1 is the sister group of the remaining Allocosinae, though this relationship lacks support in all cases. The trees from BI and ML analyses on M2 (Fig. 3) revealed several major lineages inside Allocosinae: *Allocosa*, *Abaycosa*, and three undescribed genera (Gen. 1 from northeastern Brazil, Gen. 2 from northern Colombia and Gen. 3 from Uruguay), as well as "*Arctosa*" *sapiranga*. Gen. 2 sp. 1 (aff. *Allocosa panamena*) was recovered as

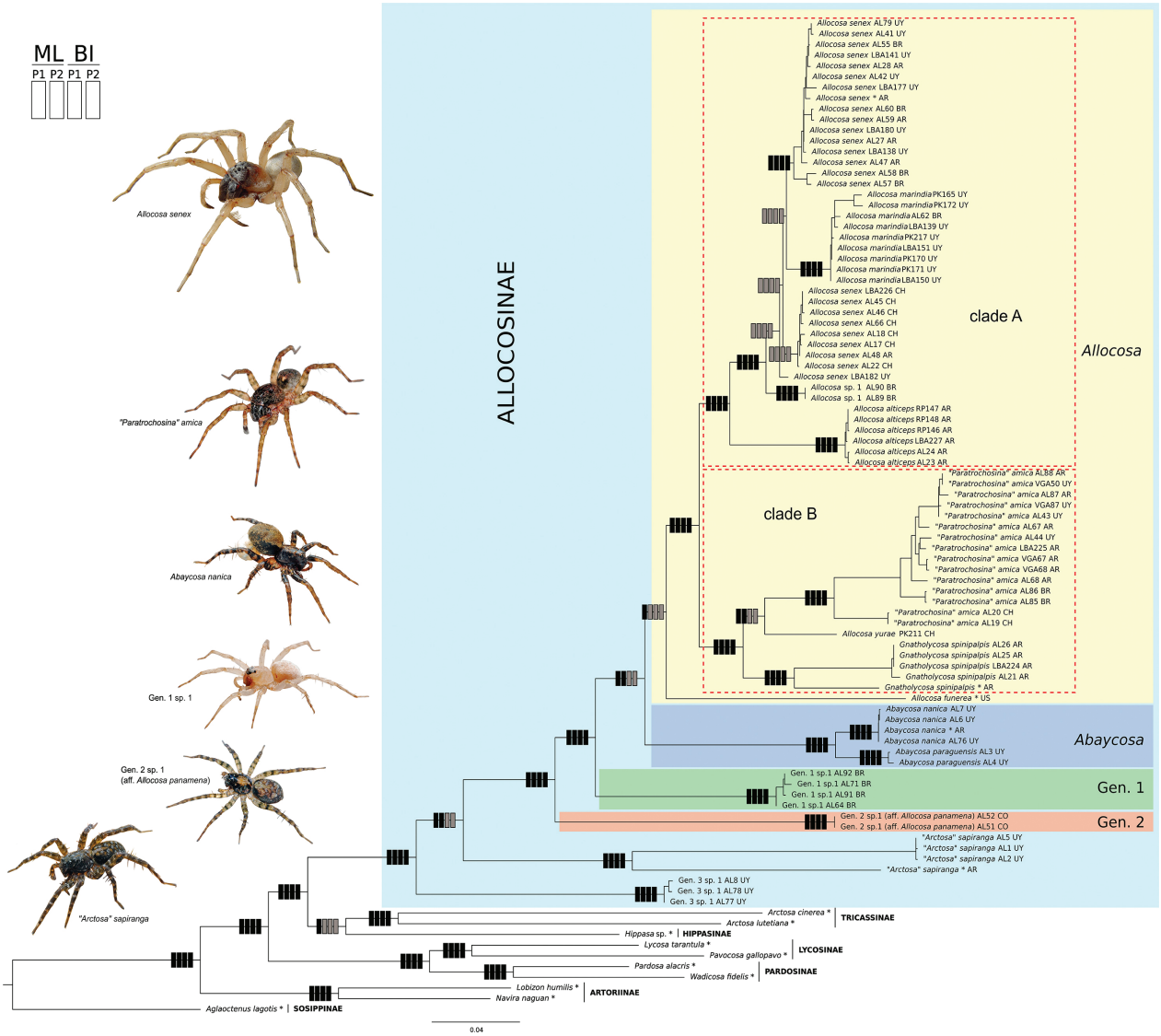

Figure 3. Topology of the tree obtained from the Maximum Likelihood (ML) analysis under a scheme of partition by genes P1 (concatenation of *cox1*, *nad1*, *h3*, *12S*, and *28S* genes), including Allocosinae representatives and some specimens of the currently recognized Lycosidae subfamilies. Bars on nodes indicate support for ML analysis and Bayesian inference (BI). Black: ML bootstrap > 80%, BI pp > 0.95; gray: recovered clade with support below the indicated limit; white: node not recovered in the analysis. AR Argentina; BR Brasil; CH Chile; CO Colombia; UY Uruguay.

a sister group to the rest of the subfamily except "*A*". *sapiranga*, while Gen. 1 sp. 1 appeared as a sister group to *Allocosa*+*Abaycosa* in all analyses. The relationship between *Allocosa* and *Abaycosa* receive support in the ML analyses (Fig. 3). The genus *Abaycosa* was recovered with support in all analyses, comprising two species, *A. nanica* and *A. paraguensis*, which are reciprocally monophyletic. The genus *Allocosa* is recovered with support only in ML under P1 (Fig. 2, 3), placing *Allocosa funerea* as sister to clades A and B. A complementary analysis including the *cox1* gene fragment for *Allocosa* species from North America (Fig. S1), recovered *A. funerea* together with the other North American species, they form a supported clade, and this group is recovered as sister to the clades A and B. These two clades are also supported, clade A includes species adapted to sandy coastal environments, namely *Allocosa senex*, *A. marindia*, *A. alticeps*, and *A.* sp. 1. The monophyly of *A. alticeps* and *A. marindia* was supported across all the analyses. Conversely, *Allocosa senex* was recovered as paraphyletic since it included *A. marindia*. The different lineages of *Allocosa senex* were not supported, except for the group including specimens from Argentina (including El Palmar national park, a site very close to the type locality), Southern Brazil, and Uruguay. The remaining *A. senex* specimens formed an unsupported

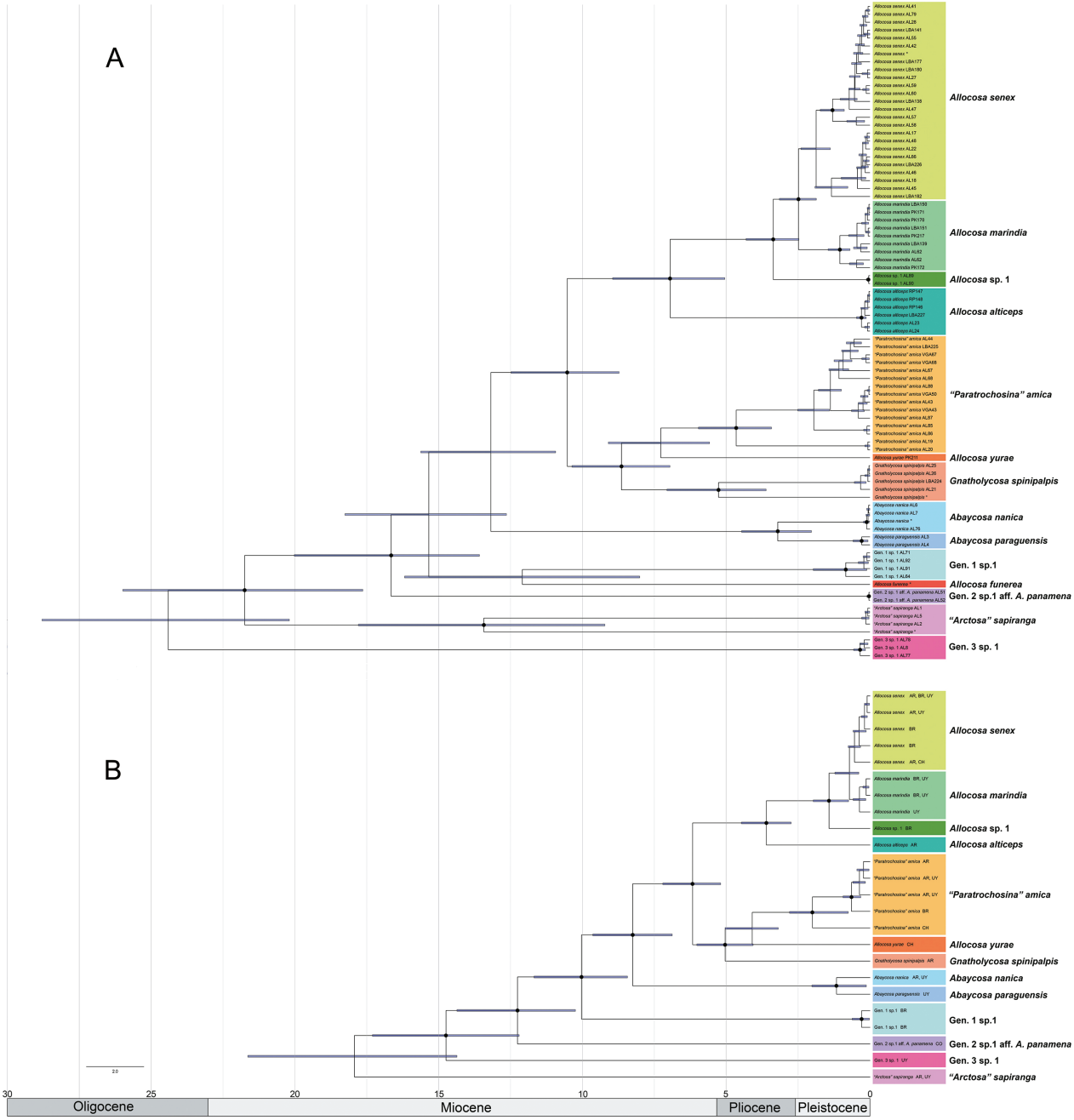

Figure 4. Chronogram obtained from the Bayesian inference analysis based on an approximation of concatenated gene tree (A) including *cox1*, *nad1*, *h3*, *12S*, and *28S* genes, and species tree (B) including *cox1*, *nad1*, *12S*, *h3*, and *h4* genes. The bars on the nodes indicate the 95% plus top of the posterior density range. Nodes with a black dot indicate posterior probability > 0.95. AR Argentina; BR Brasil; CH Chile; CO Colombia; UY Uruguay.

group comprising individuals from Chile and Southwestern Argentina. A sister group to *A. senex* and *A. marindia* includes two specimens from Northern Brazil (Bahia), belonging to an undescribed species (Fig. 3). Clade B was supported in all analyses and comprised the species *Gnatholycosa spinipalpis*, *Allocosa yurae*, and "*Paratrochosina*" *amica*.

3.2.2. Species delimitation and species tree

The GMYC species delimitation analysis (based on mitochondrial genes) identified 26 independent evolving entities (Fig. S2). When running STACEY, incorporating all nuclear and mitochondrial gene fragments (M3), 24 lineages, also identified in GMYC analyses, were recovered with a 92% probability (Fig. 4B). These analyses suggest a greater number of lineages than the 13 initially recognized based on morphology, subdividing *A. senex* into five lineages (four of which include specimens from Argentina, Brazil, and Uruguay, and one from Chile and Southern Argentina), *A. marindia* into three lineages (two from Uruguay and one from Brazil), "*P*". *amica* into five lineages (three from Argentina and Uruguay, one from Southern Brazil, and one from Chile), and Gen. 1 sp. 1 into two lineages (both from Northern Brazil).

The topology of the species tree in Figure 4B shows "*Arctosa*" *sapiranga* and Gen. 3 sp.1 as sister groups to the rest of the Allocosinae. Gen. 2 sp. 1 (aff. *Allocosa panamena*) is positioned as a sister to the remainder of the subfamily. Gen. 1 sp. 1 and *Abaycosa* are recovered with high support, maintaining the same positions observed in previous analyses. The genus *Allocosa* is also recovered with high support, represented by clades A and B. Within clade A, *Allocosa alticeps* is strongly supported as a sister to the other species, while *Allocosa* sp. 1 is identified as a sister to *A. marindia* + *A. senex*, albeit without support. In this topology, *Allocosa senex* is divided into two groups: one consisting of specimens from Brazil, Argentina, and Uruguay, including those from a site very close to the type locality of the species, and a second group comprising specimens from Chile and southern Argentina.

3.2.3. Divergence time estimation

The two approaches, concatenated matrix and species tree, inferred different timelines (Fig. 4A, B). However, the confidence interval of the divergence time estimates was considerably wide and frequently overlapped. The concatenated matrix tree traces back the origin of Allocosinae at about 24.4 Ma, while it was 18 Ma in the species tree. Regarding the internal structure, the divergence of Gen. 2 sp. 1 (aff. *Allocosa panamena*) is estimated in 16.6 and 12.2 Ma for the concatenated and species tree, respectively; *Abaycosa* + *Allocosa* diverged from Gen. 1 sp. 1 15.3 and 10 Ma.; and *Allocosa* diverged from *Abaycosa* in 13.2 and 8.2 Ma. The diversification of the clade B dates back to 8.6 and 5 Ma, and for the clade A to 6.9 and 3.6 Ma according to the concatenated matrix and species tree approaches, respectively.

4. Discussion

4.1. Phylogeny of South American Allocosinae spiders

In this phylogenetic study, which included mitochondrial and nuclear genes, we recover the specimens considered to be representatives of the subfamily Allocosinae as a monophyletic group, and propose a new hypothesis for the phylogenetic relationships within the subfamily (Figs 2, 3, 4). Although the monophyly of the subfamily was previously reported by Piacentini and Ramírez (2019), Gonnet et al. (2021), and Laborda et al. (2022), this study included a more comprehensive taxonomic sampling, especially for South American species (13) (Fig. 1). It should be noted that the taxonomy currently does not reflect the monophyly of Allocosinae. Many species currently classified in *Allocosa* are not related to the type species *Allocosa funerea* or to other genera in Allocosinae, and are therefore incorrectly placed in the subfamily. Allocosinae also contain a member of a genus currently classified in Tricassinae ("*Arctosa*" *sapiranga*). Until these nomenclatural problems are resolved, the subfamily cannot be considered truly monophyletic.

The resulting tree placed Tricassinae + Hippasinae as a sister group to Allocosinae, consistent with findings by Gonnet et al. (2021), but differing from Piacentini and Ramírez (2019), who found Lycosinae + Pardosinae as sister group to Allocosinae. The remaining internal relationships within Lycosidae, specifically between the other subfamilies, exhibited differences from previous studies (Piacentini and Ramírez 2019; Gonnet et al. 2021). However, these variations occurred in unsupported groups, and further data will be required to confirm those relationships.

Piacentini and Ramírez (2019) included "*Arctosa*" *sapiranga* within Allocosinae, positioning it as a sister group to the rest of the subfamily. They also noted the morphological differences between "*A*". *sapiranga* and other Allocosinae species, sharing few morphological characters such as the presence of a long, triangular hyaline conductor (Piacentini, pers. obs.). Our phylogenetic analysis confirmed the position of "*A*". *sapiranga*. A similar pattern was recovered for Gen. 3 sp.1, which, while morphologically distinct from other Allocosinae species, shares specific characteristics, particularly in genital morphology with "*A*". *sapiranga* (Piacentini and Laborda, pers. obs.). Recently, Paredes-Munguia et al. (2024) reviewed the Neotropical species of *Arctosa*, including "*A*". *sapiranga*, and highlighted the challenge of assigning these species to a specific subfamily, as they exhibit traits of both Tricassinae and Allocosinae. However, due to a greater number of diagnostic elements, they leaned toward placement in Tricassinae. The characters shared by "*A*". *sapiranga* with Tricassinae may be plesiomorphic. This is supported by the results obtained by Piacentini and Ramírez (2019), who recovered *Arctosa* as a polyphyletic group. This seems to indicate that it is a "tailor's drawer" genus lacking real synapomorphies that

group the species. According to these authors' results, the genus would be placed in Tricassinae given the position in which they recovered *Arctosa cinerea* (the type species of the genus). Therefore, those species recovered in other subfamilies, such as "*A*". *sapiranga*, would be incorrectly placed in the genus.

Our results supported Gen. 2 sp. 1 (aff. *Allocosa panamena*) as the sister group to the remaining Allocosinae, except "*A*". *sapiranga* and Gen. 3 sp.1 (Figs 2, 3). Specimens of this group were collected from a sandy streambank in a jungle environment on the Colombian Caribbean coast. This habitat and distribution align with what Dondale and Redner (1983) reported for *Allocosa panamena* Chamberlin, 1925. It is also morphologically similar, being probably congeneric with *A. panamena* but not conspecific, since it has differences in the genital structures that suggest it is an undescribed species. Gen. 2 sp. 1 (aff. *Allocosa panamena*) shares traits with *A. funerea* (and other North American *Allocosa* species), such as small body size and a glabrous, shiny prosoma. Phylogenetic analyses did not reflect these morphological affinities, as *Allocosa funerea* was not closely related to Gen. 2 sp. 1 (aff. *Allocosa panamena*).

The undescribed Gen. 1 sp. 1 consists of four specimens from Bahia, Brazil, specifically from sandy coastal environments, dune fields and associated vegetation. This species was included in the size dimorphism analysis by Aisenberg et al. (2023) (referred to in this study as Allocosinae sp. 6 'Bahia'), where it was reported that females in this species are larger than males. Another species (not included in the analysis) also found in Northern Brazil showed similar morphological characteristics of genitalia, suggesting that it is congeneric with Gen. 1 sp. 1, and would be an undescribed genus with two species.

Representatives of the genus *Abaycosa* were supported as monophyletic, in the same position obtained by Piacentini and Ramírez (2019) (as *Pardosa flammula*) and Laborda et al. (2022). The two known species of the genus *A. nanica* and *A. paraguensis* are included in the analysis. These are small species distributed in the central area of South America and are very abundant in anthropic environments Laborda et al. (2022).

The genus *Allocosa* was also recovered as monophyletic in the tree topologies generated by ML and BI analyses; however, it showed support only in the ML analysis under a gene-based partition scheme (P1) (Figs 2, 3). The nomenclatural status of *Allocosa* is tied to the inclusion of *Allocosa funerea*, the type species of the genus. The position of *A. funerea* in our analysis is congruent with the findings of Piacentini and Ramírez (2019), who positioned it as the sister group to *Allocosa senex* + *Gnatholycosa spinipalpis*. In an additional analysis (Fig. S1), a monophyletic group composed of *A. funerea*, *A. noctuabunda*, *A. parva*, and *A. absoluta* was recovered in the same position as *A. funerea* in other trees, as a sister group to the South American *Allocosa* species. Although this analysis used only the *cox1* molecular marker available for *A. noctuabunda*, *A. parva*, and *A. absoluta*, it supports a group of North American *Allocosa* species. For these reasons, the boundaries of *Allocosa* genus are not entirely clear, as it may constitute a single genus or potentially three distinct ones. Clades A and B could indeed represent different genera than *Allocosa*. Based on the taxonomic history of the species they include, the names *Glieschiella* for clade A and *Gnatholycosa* for clade B are available for naming these putative genera (see World Spider Catalog 2025). *Allocosa senex*, *A. alticeps*, *A. marindia*, and *Allocosa* sp. 1 are recovered, forming clade A. The species in this group, studied by Simó et al. (2017), display distinct morphological and ecological traits. They are burrowing species adapted to sandy substrates, with specialized macrosetae on their pedipalps, structures involved in burrow digging, as well as well-developed spinnerets that they use to cover their burrows with silk (Aisenberg et al. 2010; Albín et al. 2018; Foelix et al. 2017). *Allocosa senex* is recovered in the topology as paraphyletic, due to the position of *A. marindia* (Figs 2, 3). It could be interpreted that these two species are synonyms, however, there is a large amount of independent evidence that points to morphological, ecological and behavioral differences between these two species (Aisenberg and Costa 2008; Aisenberg et al. 2010; Aisenberg and González 2011; Aisenberg et al. 2011; Bidegaray-Batista et al. 2017; Simó et al. 2017; Cavassa et al. 2022). A possible scenario is that the specimens included in the analysis and identified as *A. senex* belong to more than one species. A supported group includes specimens from Brazil, Uruguay, and Argentina (Figs 3, 4), with two individuals (AL27 and AL28) collected very near the type locality of the species (El Palmar National Park in Entre Ríos), which could be considered *Allocosa senex* s.str. The remaining specimens of *A. senex* could constitute different species. For example, another group of *A. senex*, comprising specimens from Chile and Southwestern Argentina, was recovered without support in the topology (Figs 3, 4A). These specimens were analyzed separately from *A. senex* in Aisenberg et al. (2023) (Chilean specimens: Allocosinae sp. 3 'Coquimbo'; Southwestern Argentina specimens: Allocosinae sp. 4 'Lanin'), in which atypical sexual size dimorphism and sex roles were reported. Clade B is also monophyletic and sister clade to clade A. It comprises specimens from *Gnatholycosa spinipalpis*, *Allocosa yurae*, and "*Paratrochosina*" *amica*. Species in this group have a broad, elevated head region and prominent chelicerae, especially in males. *G. spinipalpis* and *A. yurae* are associated with foothill habitats in Argentina and Chile (Mello-Leitão 1940; Brescovit and Taucare-Ríos 2013), whereas "*P*". *amica* is a generalist species found in grasslands and widely distributed across Southern South America (Gonnet et al. 2021).

4.2. Species complexes: overlooked diversity

Species delimitation analyses identified 24 independent evolutionary lineages, about twice the number of morphology-based lineages. It should be noted that these methods delimit the population structure, so the different lineages found do not necessarily correspond to different species, and independent evidence is important to consid-

er. Within the species *Allocosa senex*, five lineages were identified. As already noted, a recovered lineage, which includes specimens collected near the type locality, is considered to be *Allocosa senex* s.str. The remaining lineages have a wide distribution in South America. Some of these lineages, as a group comprising specimens from Chile and Southwestern Argentina (Figs 3, 4A), could be a different species to *A. senex* due to the great geographical distance and marked environmental differences. However, they are not morphologically distinct enough to consider these groups as distinct species. Species delimitation analyses suggest that they could be overlooked species, but further phylogeographical, ecological and behavioral studies are needed to test this hypothesis. Three lineages were identified within *Allocosa marindia*, two in Uruguay and one in Brazil, indicating a distribution along the Atlantic coast extending to São Paulo, Brazil. However, the morphological data do not support these as distinct species. *Allocosa* sp. 1 was recovered with support, including two specimens from Bahia, Brazil, which were collected in a dune environment and that have the characteristic morphological adaptations to the sandy habitats of clade A. This species was referenced as *Allocosa* cf. *senex* in Aisenberg et al. (2023), where it was reported to exhibit sexual size dimorphism reversal, suggesting possible sex role reversal. In "*Paratrochosina*" *amica*, five lineages were recognized. In this widely distributed species, specimens from distant locations formed distinct lineages; two specimens from Río Clarillo National Park in Chile were recovered as a sister group to the remaining specimens. In turn, specimens from Pró-Mata, Brazil formed a sister group to a lineage that includes specimens from Uruguay and Argentina. This latter lineage includes specimens collected near the type locality in Córdoba, Argentina, which may represent "*Paratrochosina*" *amica* s.str. Finally, two lineages were recognized in Gen. 1 sp. 1. These specimens do not exhibit notable morphological differences and were collected in similar environments at nearby locations in Brazil. However, sympatry of morphologically similar Allocosinae species has been reported in other regions (Aisenberg and Costa 2008; Laborda et al. 2022), so the presence of two lineages remains a possibility, and population-level analysis may reveal overlooked diversity.

4.3. Diversification timeline

The two approaches used to estimate divergence times, the concatenated matrix and the species tree, differed slightly, with estimates from the concatenated gene tree being slightly older. However, the confidence intervals for node age estimates are relatively broad, and in some cases, they display areas of overlap (Fig. 4 A and B). This aligns with the findings of McCormack et al. (2011), who noted that concatenated tree approach methods can significantly overestimate divergence times because they do not account for genetic divergence before speciation.

The diversification of the subfamily begins in the Oligocene and Miocene, but many clades diversified more recently in time, in the Pliocene and Pleistocene. For example, within clade A, the divergence of *Allocosa alticeps* from *A. senex* + *A. marindia* was estimated at 7 Ma and 3 Ma (according to the concatenated matrix and species tree, respectively). The estimate from the species tree aligns with Postiglioni et al. (2019), who placed this divergence at 2.45 Ma. Similarly, the sister species *A. senex* and *A. marindia* were estimated to have diverged at 2.5 Ma and 0.9 Ma (according to the concatenated matrix and species tree, respectively). The species tree estimates agree with Bidegaray-Batista et al. (2017) and Postiglioni et al. (2019), who indicated that this divergence occurred less than one million years ago. The diversification of clade A in the Pleistocene may be related to intense climatic fluctuations and geological changes that contributed to the modification of the landscape of the area where the species inhabit. Some of these changes include the increase in the areas of sandbanks, dunes and other sandy soil environments (Clapperton 1993; Carignano 1999; Mon and Gutierrez 2009; Nascimento et al. 2013; Turchetto-Zolet et al. 2013). Species in group A can be considered specialists in this type of environment, since they only inhabit sandy soil systems and have morphological, ecological and physiological adaptations to live there (Costa 1995; Costa et al. 2006; Aisenberg et al. 2010; Foelix et al. 2017; Simó et al. 2017; Albín et al. 2018). Therefore, it is expected that they have expanded and diversified in the subcontinent due to the expansion of their habitable environments.

4.4. Future perspectives

Our phylogenetic framework provides insights into the diversification and taxonomy of South American Allocosinae. While some lineages, such as *Abaycosa* and Gen. 1, were consistently recovered with strong support across all analyses, others, particularly *Allocosa* and its internal structure, are ambiguously supported. Consequently, future studies will have to focus on resolving these specific groups. Future analyses will have to include a better representation of the North American fauna, both in terms of species and genes, to assess the stability of the genus *Allocosa* and to better define the boundaries and members of this group. Given the recent divergence times observed within the Clades A and B and the reported independent evolutionary lineages, analyzing thousands of informative molecular markers, such as single nucleotide polymorphisms (SNPs), together with ecological, morphometric, and behavioral studies will be necessary to infer species boundaries.

Here we present a phylogenetic hypothesis of the subfamily with a broad sampling of internal taxa. The main lineages that comprise Allocosinae are recognized, confirming the position of previously recognized species in the subfamily but also showing a still unknown diversity with possible new genera and species. The results presented here will be a starting point for future taxonomic contributions and an evolutionary frame of reference for ecological, genetics and behavioral studies that are already being developed in this group.

5. Declarations

Authors' contributions. Álvaro Laborda: Conceptualization, Formal Analyses, Investigation, Methodology, Data curation, Writing- Original draft preparation. – Miguel Simó: Conceptualization, Supervision, Investigation, Writing- Reviewing and Editing. – Luis N. Piacentini: Investigation, Writing- Reviewing and Editing. – Antonio D. Brescovit: Investigation, Writing- Reviewing and Editing. – Carolina Beloso: Investigation; Writing- Reviewing and Editing. – Anita Aisenberg: Conceptualization, Investigation, Project Administration, Resources, Funding Acquisition, Writing- Reviewing and Editing. – Miquel A. Arnedo: Investigation, Writing- Reviewing and Editing. – Martín J. Ramírez: Investigation, Writing- Reviewing and Editing. – Leticia Bidegaray-Batista: Conceptualization, Supervision, Investigation, Methodology, Project Administration, Resources, Funding Acquisition, Writing- Reviewing and Editing.

Conflict of interest. The authors do not have any conflict of interest to declare.

Data availability statement. The molecular data newly generated for this study is available in GenBank. Accession numbers: *cox1*: PV719874–PV719937; *nad1*: PV763390–PV763445; *12S*: PV789152–PV789216; *h3*: PV775783–PV775844; *h4*: PV775845–PV775912.

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Supplementary Material 1

Figures S1, S2

Authors: Laborda Á, Simó M, Piacentini LN, Brescovit AD, Beloso C, Aisenberg A, Arnedo MA, Ramírez MJ, Bidegaray-Batista L (2025)

Data type: .zip

Explanation notes: Figure S1. Topology of the tree obtained from the Maximum Likelihood (ML) analysis under a scheme of partition by genes P1 (concatenation of *cox1*, *nad1*, *h3*, *12S*, and *28S* genes), including Allocosinae representatives and some specimens of the XX currently recognized Lycosidae subfamilies. Bars on nodes indicate support for Maximum Likelihood (ML) and Bayesian (BI). Black: ML bootstrap > 80%, BI pp > 0.95; gray: recovered clade with support below the indicated limit; white: node not recovered in the analysis. — **Figure S2.** Lineages indicated by GMYC species delimitation analysis. AR Argentina; BR Brasil; CH Chile; CO Colombia; UY Uruguay.

Link: https://doi.org/10.3897/asp.83.e152943.suppl1

Supplementary Material 2

Tables S1, S2

Authors: Laborda Á, Simó M, Piacentini LN, Brescovit AD, Beloso C, Aisenberg A, Arnedo MA, Ramírez MJ, Bidegaray-Batista L (2025)

Data type: .zip

Explanation notes: Table S1. List of Allocosinae (Lycosidae) specimens used for the extraction of genetic material. Reference codes corresponding to GenBank. — **Table S2.** List of specimens used in phylogenetic analyses. Data published in Piacentini and Ramírez (2019), except for the sequences used in the complementary analysis which are indicated with an asterisk (data available for public use). Reference codes corresponding to GenBank or BOLDSYSTEM.

Link: https://doi.org/10.3897/asp.83.e152943.suppl2