Global distribution and richness of *Armillaria* and related species inferred from public databases and amplicon sequencing datasets

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ABSTRACT

Armillaria is a globally distributed fungal genus most notably recognized as economically important plant pathogens that are found predominantly in forest and agronomic systems. The genus *sensu lato* has more recently received attention for its role in woody plant decomposition and in mycorrhizal symbiosis with specific plants. Previous phylogenetic analyses suggest that around 50 species are recognized globally. Despite this previous work, no studies have analyzed the global species richness and distribution of the genus using data derived from fungal community sequencing datasets or barcoding initiatives. To assess the global diversity and species richness of *Armillaria*, we mined publicly available sequencing datasets derived from numerous primer regions for the ribosomal operon, as well ITS sequences deposited on Genbank, and clustered them akin to metabarcoding studies. Our estimates reveal that species richness ranges from 50 to 60 species, depending on whether the ITS1 or ITS2 marker is used. Eastern Asia represents the biogeographic region with the highest species richness. We also assess the overlap of species across geographic regions and propose some hypotheses regarding

the drivers of variability in species diversity and richness between different biogeographic regions.

Keywords: DNA barcoding; basidiomycete fungi; diversification; meta-analysis; plant pathogenic fungi; ribosomal operon; symbiosis

INTRODUCTION

While the importance that fungi play as mutualists, decomposers, and pathogens is undisputed, researchers are just beginning to disentangle the processes that shape their global species richness and distribution (Tedersoo et al., 2014). One such globally important group is the armillarioid clade—a lineage composed of the mushroom-forming genera *Armillaria* and *Desarmillaria,* along with the sequestrate genus *Guyanagaster*. *Armillaria* species, representative of the annulate mushroom-forming species within the armillarioid clade, are globally distributed with an estimated 40 to 50 species (Volk & Burdsall, 1995; Baumgartner et al., 2011). In contrast, *Desarmillaria* – which was recently separated from *Armillaria* on the basis of morphological and molecular characters (Koch et al., 2017; Heinzelmann et al., 2019) – is representative of the exannulate mushroom-forming species within the clade. Within *Desarmillaria*, there are three described species, including *Desarmillaria ectypa*, which is restricted to northern European bogs (Zolciak et al., 1997), *D. tabescens,* which is likely found widely throughout Europe and Asia (Guillaumin et al., 1993), and *D. caespitosa,* which is found throughout North America (Antonín et al., 2021). While *Guyanagaster* – representative of the sequestrate species – is a member of the armillarioid clade (Henkel et al., 2010; Koch et al.,

46 2017), herein the discussion of the armillarioid clade will only refer to species of *Armillaria* and
47 *Desarmillaria*, and when necessary, *Guyanagaster* will be specifically referenced.
48
49 Armillarioid species are most often recognized as ecologically and economically important plant
50 pathogens, identified as the causal agents of Armillaria Root Rot. As facultative necrotrophs,
51 these species possess both parasitic and saprotrophic phases. During their parasitic phase, species
52 may attack a broad range of woody dicotyledonous hosts in both natural and agronomic systems
53 (reviewed in Baumgartner et al., 2011). However, during their saprotrophic phase, armillarioid
54 species play an important role in carbon cycling and nutrient exchange as they contain a suite of
55 genes that encode for enzymes that have the ability to degrade the entire spectrum of the major
56 components of plant cell walls (Kile et al., 1991; Sipos et al., 2017). Currently, all armillarioid
57 taxa are recognized as having the ability to be a pathogen during their life cycle – even the
58 putatively saprotrophic *A. cepistipes* has been recently reported to be pathogenic on *Populus*
59 *trichocarpa* (Alveshere et al., 2021). One largely diagnostic characteristic of many armillarioid
60 species is the production of rhizomorphs – discrete, long-lasting filamentous structures that
61 forage for new resources away from the primary resource base (Garraway et al., 1991).
62 Rhizomorphs can grow long distances to infect plants, resulting in genets with exceptionally
63 large sizes, some of which have been recognized as the largest and oldest organisms on earth
64 (Smith et al., 1992).
65
66 In addition to their importance as plant pathogens and biomass decomposers, armillarioid species
67 are ecologically important as they have the capacity to engage in several symbiotic associations
68 with various plants, other fungi, and insects. For example, the growth of sclerotia of the

polyporoid fungus, *Polyporus umbellatus*, is dependent on nutrition from symbiotic *Armillaria* species (Guo & Xu, 1992). *Armillaria* species in east Asia engage in a form of mycorrhizal relationship known as mycoheterotrophy with species of achlorophyllus orchids in the genera *Galeola* and *Gastrodia* (Kikuchi & Yamaji, 2010; Guo et al., 2016). Arguably, *Armillaria* sporocarps may be one the most documented mushroom target of other pathogenic fungi as they are specifically parasitized by the mushroom-forming species *Entoloma abortivum* (Lindner Czederpiltz et al., 2001; Koch & Herr, 2021) and putatively by species in the Ascomycete genus *Wynnea* (Xu et al., 2019). Lastly, the sequestrate genus *Guyanagaster* is the earliest diverging lineage within the armillarioid clade (Koch et al., 2017). The sporocarps of *G. necrorhizus* house nitrogen-fixing bacterial species, which likely facilitates a dispersal mutualism with wood-feeding termites (Koch et al., 2018; Koch et al., 2021).

Traditionally, species within the armillarioid clade have been delimited by morphological characteristics and, more recently, by mating compatibility studies (Bérubé & Dessureault, 1989; Cha et al., 1994; Volk & Burdsall, 1995; Qin et al., 2007). However, given the difficulty in identifying species based solely on these features, there has been significant emphasis on understanding species boundaries within the armillarioid clade using phylogenetic data (Guo et al., 2016; Klopfenstein et al., 2017; Koch et al., 2017; Coetzee et al., 2018). Initially, studies of the armillarioid clade, like most fungal diversity assessments, relied heavily on data from the nuclear ribosomal operon – which bridges the small subunit (18S), internal transcribed spacer region 1 (ITS1), 5.8S subunit, internal transcribed spacer region 2 (ITS2), the intergenic spacer region (IGS), and the large subunit (28S) (Chillali et al., 1998). However, the *translation elongation factor 1* (*tef1*) is the marker region that has emerged in more recent research on

92 armillarioid fungi, in that it provides adequate resolution to infer species boundaries and lineage
93 relationships within a global context (Maphosa et al., 2006; Guo et al., 2016; Klopfenstein et al.,
94 2017; Coetzee et al., 2018). While the research community interested in armillarioid fungi has
95 recently focused on the use of the *tef1* marker, this locus has not been utilized in any
96 environmental sequencing studies with which to measure global diversity and richness. In order
97 to evaluate both diversity and richness on a global scale, it is ideal to have a suite of characters –
98 including both morphological and molecular data – from many different sampling locations.
99 While the mycological research community is slowly moving toward the collection of molecular
100 data from multiple markers on a global scale, research has so far focused solely on characterizing
101 fungal species richness using the ITS1 and ITS2 regions that have consensus from the fungal
102 research community (Schoch et al., 2011; Herr et al., 2013; Hibbett et al., 2015).
103
104 Given the importance of armillarioid fungi, we wanted to understand the diversity and richness
105 of this group on a global scale. To do this, we assess – from the perspective of the widely
106 adopted in environmental barcoding markers, ITS1 and ITS2 – the overall global distribution
107 and species richness within this clade, much like has been done previously for soil fungi on a
108 large scale (Tedersoo et al., 2014). Here, we analyzed the totality of publicly available ITS
109 sequences submitted to NCBI-GenBank and fungal community sequencing data using
110 metabarcoding methodologies submitted to NCBI-SRA to gain a more complete understanding
111 of the species richness and the global distribution of the armillarioid clade. We recognize that it
112 is important to understand how this locus operates for this lineage given the immense number of
113 studies utilizing it, and we hypothesize that the ITS1 and ITS2 regions exhibit concordance. We
114 also discuss the utility of public data to inform species clusters, in this case operational

115 taxonomic units (OTUs), in armillarioid fungi and present caveats to its use. Additionally, we
116 speculate on factors, such as symbiotic interactions of armillarioid species, that affect the
117 diversity and geographic distributions of this lineage.
118
119 **MATERIALS AND METHODS**
120 **Sequences curated from public database vouchers.** To create a dataset of all publicly available
121 armillarioid ITS sequences, we queried NCBI-GenBank (https://www.ncbi.nlm.nih.gov) for
122 "*Armillaria*" with "ITS" (or "*Desarmillaria*" and "*Guyanagaster*" with "ITS") and downloaded
123 all of the nucleotide sequences matching these criteria. Taxonomy Browser (NCBI) (Federhen,
124 2012), which uses BLAST+ (Camacho et al., 2009), was then used to verify the correct
125 taxonomic names – those entered by the researchers who deposited the sequences to NCBI – for
126 each of the public sequences. We did this to remove sequences that were deposited to NCBI
127 under an incorrect genus-level designation (Bruns et al., 2008). The feature for geographic region
128 (such as "country") was also extracted for each sequence and any sequences without this
129 corresponding metadatum were excluded. Additionally, if sequences were associated with
130 another organism in a symbiotic relationship, this was also noted. Finally, all sequences with the
131 identifier "*Armillaria*" from the UNITE v8.3 database (Nilsson et al., 2018) were also included
132 in the dataset irrespective if they had corresponding location metadata. For these sequences, we
133 then extracted the ITS1 and ITS2 regions using ITSx (Bengtsson-Palme et al., 2013).
134
135 **Sequences curated from public amplicon studies.** We next mined the Sequence Read Archive
136 (SRA) database for public datasets of fungal amplicon sequencing data of the ITS1 and ITS2
137 region. We identified 454 pyrosequencing and various Illumina platform sequencing datasets

138 with the query (ITS2, ITS1 AND "biomol dna"[Properties] AND "platform ls454"[Properties]
139 AND “platform Illumina”[Properties]) to the SRA database (Leinonen et al., 2010) which
140 provided us with 183,838 sample files representing 1,253 public deposits to the SRA. We then
141 manually curated a list of datasets based on the availability of metadata from the NCBI SRA Run
142 Selector, such as geographic location, as we did previously for the NCBI sequences. Once
143 datasets were downloaded in the “.sra” format using the `prefetch` command, we converted the
144 datasets to “.fastq” format using the `fasterq-dump` command with the “--split_files” option.
145 Both tools are available from NCBI through the sra-tools scripts (https://github.com/ncbi/sra-
146 tools). Sequences from SRA deposited datasets were clustered against the NCBI list for
147 *Armillaria* (generated in the first stage) using VSEARCH version 2.17.0 (Rognes et al., 2016) at
148 90% sequence similarity using the `--usearch_global` command. To avoid off-target clustering
149 and to double check the VSEARCH clustering quality, we next identified the output from the
150 VSEARCH clustering using DECIPHER version 3.13 IDTAXA algorithm `Classify Organisms`
151 (Wright, 2016) and the UNITE2020 DECIPHER database
152 (http://www2.decipher.codes/Classification/TrainingSets/UNITE_v2020_February2020.RData)
153 selecting only those sequences being taxonomically identified as *Armillaria* and *Desarmillaria*.
154 Out of the 1,253 public studies deposited to the SRA that we queried, only 56 studies, restricted
155 to forest soil and plant matter, contained sequences with 90% homologous base pairs to
156 *Armillaria* ITS sequences. The remaining 1,197 studies, which mainly consisted of ecosystems
157 that did not contain woody plant hosts (i.e. built-environment, human, marine, etc.), did not
158 contain any sequences with homology to existing *Armillaria* sequences.
159

160 **Curation of total sequence datasets and geographic metadata.** Geographic location metadata
161 were also extracted from studies containing *Armillaria* sequences, which represented *Armillaria*
162 voucher specimens deposited in NCBI Genbank and from the SRA database metadata files.
163 Sequences of the ITS1 region from the Genbank nucleotide database and the SRA were
164 combined, and the same was done as a separate dataset for the ITS2 region. Any sequences less
165 than 150 base pairs in length were removed. To estimate species from these sequences, we then
166 clustered the ITS1 and ITS2 regions (combining the voucher specimen data and SRA sequences)
167 at 98% using USEARCH 11 (Edgar, 2010). We chose 98% nucleotide identity threshold because
168 this was the identity threshold utilized by Tedersoo et al. (2014). The biogeographic regions we
169 refer to largely follow those utilized by Tedersoo et al. (2014). Because some of the armillarioid
170 sequences associated with *Wynnea* species did not pass our quality filters and we were still
171 interested in utilizing them, we independently placed each of them into our dataset using
172 BLAST+, as above, to place them in the appropriate species cluster.
173
174 **Phylogenetic analysis.** To visualize how the armillarioid species clusters were related, we
175 utilized a phylogenetic approach. One exemplar sequence from each species cluster was chosen
176 to include in the multiple sequence alignment. We utilized the full-length sequence, when
177 possible, and not just the hypervariable regions. Species clusters for the ITS1 region and ITS2
178 region were analyzed separately. This was because in no study where we identified armillarioid
179 taxa did researchers employ both ITS1 and ITS2 markers from a single sampling location such
180 that we could absolutely ensure that both markers came from the same genetic individual or
181 genet. As ITS1 and ITS2 sequences could not be ascribed to a single genet or individual, we did
182 not attempt to statistically assess the phylogenetic tree correlation of the ITS1 and ITS2

183 sequences – this would be better suited for pangenomic studies of the armillarioid fungi using
184 multiple genes. The public sequences we identified as having homology to our armillarioid nexus
185 file were aligned using MAFFT version 7 (Katoh & Standley 2013) with refinements to the
186 alignment performed manually. RAxML-NG (Kozlov et al., 2019) was used to reconstruct this
187 phylogeny. The two known species of *Guyanagaster*, sister to *Armillaria* and *Desarmillaria*
188 species (Koch et al., 2017), were used to root this phylogeny. We defined well supported
189 branches as having 70% or greater bootstrap support. The location metadata associated with each
190 species cluster was mapped onto each phylogenetic tree using the 'ggtree' package (Yu et al.,
191 2018) within the R 4.1.0 environment (R Core Team 2013).
192
193 **RESULTS**
194 A total of 1,153 *Armillaria* ITS1 region sequences (1,119 from the nucleotide database and 34
195 from the SRA database) and 1,567 *Armillaria* ITS2 region sequences (1,246 from the nucleotide
196 database and 321 from the SRA database) were sorted into species clusters. Public data sets
197 which contributed to the high-throughput ITS1 and ITS2 region sequences in this study consisted
198 of six different primer pair combinations: fITS7/ITS4, gITS7/ITS4, ITS1F/ITS2, ITS1F/ITS4,
199 ITS3/ITS4, and ITS5/ITS2. Sequences for both ITS marker regions were identified from samples
200 collected from all continents except Antarctica. At a 98% nucleotide identity, 51 armillaroid
201 species clusters were recovered for the ITS1 region and 61 for ITS2 region. The geographic
202 distribution of these species clusters are shown in Fig. 1 and the symbiotic associations of these
203 species clusters is displayed in Table 1. Of the ITS1 region species clusters, sequences from the
204 SRA database clustered into five species clusters (species clusters 5, 6, 9, 15 and 23), all of
205 which also contained sequences queried from NCBI-Genbank. Of the ITS2 species clusters,

206 sequences derived from the SRA database were clustered into 14 species at 98% similarity
207 (species clusters 3, 9, 10, 13, 17, 18, 19, 23, 24, 26, 30, 35, 43 and 51); one of these clusters,
208 species cluster 17, was solely composed of sequences from the SRA database and did not cluster
209 with any sequences from the NCBI nucleotide database.
210
211 The most species rich biogeographic region was East Asia, with 23 and 19 species clusters at
212 ITS1 and ITS2, respectively (Fig. 1 and 2). With the ITS1 region data, East Asia also contained
213 the most species clusters that are found in no other biogeographic region (Fig. 2). The other
214 species rich biogeographic regions included western and eastern North America and Europe,
215 with five, six, and nine armillarioid species clusters at the ITS1 region and 13, 12, and 12
216 armillarioid species clusters at the ITS2 region, respectively. These geographic regions appear to
217 be the best sampled as a majority of the total barcoded armillarioid sporocarps, rhizomorphs, and
218 mycelial tissue are from these regions (Fig. 3). In the southern hemisphere, species richness is
219 lower when compared to the northern hemisphere, with temperate South America, Africa,
220 Australia, and New Zealand having five, six, three, and four species clusters at the ITS1 region
221 and eight, five, six, and nine species clusters at the ITS2 region. Largely tropical geographic
222 areas, including Hawaii, India, tropical South America, and Southeast Asia have the lowest
223 species richness with one to two mushroom-forming species in each location. Tropical South
224 America also has two species of the sequestrate genus, *Guyanagaster*.
225
226 Shared species clusters between biogeographic regions were much more pervasive in the
227 northern hemisphere compared to the southern hemisphere. Between locations, the most shared
228 species clusters include a link between East Asia and Europe, as well as a link between Australia

229 and New Zealand. Evidence for shared species clusters throughout the southern hemisphere is
230 limited to only Australia and New Zealand (Figure 2), but according to our analysis, these
231 regions do not share any species clusters with other geographic location (Fig. 1). At the ITS1
232 region, four species clusters (species clusters 1, 4, 13, and 22), and at the ITS2 region, three
233 species (species clusters 1, 7, and 12), were shared between locations in the northern and
234 southern hemisphere. Two of the species clusters derived from the ITS1 region and all of the
235 species clusters from the ITS2 region included sequences from specimens or environmental
236 samples in the northern hemisphere and Subsaharan Africa.
237
238 Species clusters (or species complexes) for the armillarioid lineage were determined from the
239 combined dataset of sequences derived from community sequencing efforts and accessions
240 deposited into NCBI Genbank. We identified two known sequestrate armillarioid species—
241 species of *Guyanagaster*—from the nucleotide database. Of the *Desarmillaria* species, there was
242 one species cluster detected at both the ITS1 (species cluster 14) and ITS2 (species cluster 22)
243 regions, from Europe and East Asia, which likely corresponds to *D. ectypa*. Four and three
244 species clusters that correspond to other *Desarmillaria* species at the ITS1 (species clusters 5, 6,
245 11 20) and ITS2 (species clusters 9, 25 and 41) regions, respectively, were also detected. The
246 *Desarmillaria* species clusters form a well-supported monophyletic lineage in analyses of both
247 ITS1 and ITS2 marker regions. All of the other species clusters, 45 at the ITS1 region and 56 at
248 the ITS2 region, likely correspond to *Armillaria sensu stricto*. There were 12 species clusters at
249 the ITS1 (species clusters 4, 7, 9, 19, 23, 26, 28, 29, 33, 39 and 41) region and ten at the ITS2
250 (species clusters 7, 12, 16, 19, 27, 48, 49, 52, 54 and 55) region that included sequences
251 annotated as *A. mellea*. These *Armillaria mellea* species clusters, along with two *Wynnea*

symbionts (from both an ITS1 and ITS2 marker region perspective) that likely represent as-yet undescribed species, form a well-supported monophyletic lineage in both analyses (Fig. 3), indicating that this is a genuine species complex as illustrated in the phylogenetic trees in Fig. 3. Species clusters for both the ITS1 and ITS2 regions are available in Tables S1 and S2.

DISCUSSION

Previous studies within the armillarioid clade have used morphology (Bérubé & Dessureault, 1988; Watling et al., 1991; Volk & Burdsall, 1995), hyphal compatibility tests (Anderson & Ullrich, 1979; Banik & Burdsall, 1998) and phylogenetic assessment (Maphosa et al., 2006; Guo et al., 2016; Coetzee et al., 2018) to draw conclusions of diversity and global richness. In this study, we utilized a complementary methodology — data derived from both the ITS1 and ITS2 marker regions taken from barcoding and community sequencing analysis studies — to investigate global species richness and distribution of species. As the universal barcode for fungi (Schoch et al., 2013), the ITS regions are the most widely available in terms of number of species that are represented in public databases (Nilsson et al., 2018), and the region is the predominant marker for fungal molecular ecology studies (Herr et al., 2013, Hibbett et al., 2016). Additionally, there has been an increasing awareness of the importance of barcoding macroscopic fungal collections, most notably those stored in fungal herbaria, using ITS primers to aid in identification and create more complete databases (Truong et al., 2017; Haelewaters et al., 2018; Vu et al., 2019). Therefore, the strength of this approach is that it allowed us to utilize the increasing numbers of publicly available datasets (notably using the ITS1 and ITS2 marker regions) from fungal ribosomal operon barcoding and metabarcoding efforts that have previously gone unused in *Armillaria* phylogenetic studies.

The data we analyzed for the ITS1 region suggests there are 51 species clusters in the armillarioid clade, while the ITS2 region data indicates there are 61 species clusters. These numbers are largely consistent with what has been previously determined through multigene phylogenetic efforts focused largely on fungal sporocarp tissue samples (Guo et al., 2016; Coetzee et al., 2018). Given our filtering scheme for short and spuriously aligned sequences, and the fact that we did not include any singletons – recognized here as species clusters here that only contained a single sequence – our results have likely underestimated the species richness of this clade. Additionally, as the number of environmental sequencing studies increase, particularly in under-sampled regions across the globe, we will likely uncover an even greater species richness. In additional to increased geographic sampling, future studies may also find – when using a greater number or a more phylogenetically informative marker region – that overall species richness of the armillarioid clade is greater than we report here.

There are known biases with ITS as a marker within the armillarioid clade, including that it may be too conserved to discriminate some species (Chillali et al., 1998; Coetzee et al., 2001). Our findings corroborate this, particularly when it comes to discriminating between *A. gallica*, *A. sinapina*, *A. cepistipes*, *A. calvescens* and *A. ostoyae*. (Figure 3). Both the ITS1 and ITS2 marker regions failed to resolve these taxa with any certainty. However, the other possibility for this observation is not because of poor resolution from the markers, but because of a misapplication of names deposited into GenBank (Bruns et al., 2008). In other instances, the ITS regions are also too variable and may lead to an overestimation of individuals in other species complexes (Dunne et al., 2002). This may certainly be the case with *A. mellea*, which could represent up to

298 14 species based on analysis of the ITS1 region (Figure 3). While our results suggest that ITS1
299 and ITS2 may provide estimates of species richness that are consistent with morphological,
300 reproductive behavior, and multigene phylogenetic assessment, these markers are unlikely to
301 provide an accurate reflection of precise delimitation in the armillarioid fungi. Species
302 delimitation is best employed with large datasets with multiple different characters and suites of
303 genes – we have found that this is most certainly the case with the armillarioid fungi. This study,
304 which is based on two public marker regions using seven primer pairs, underscores the need for
305 additional ecological markers for fungi and for the development of sequence analysis from
306 fungal metagenomic and metatranscriptomic studies (Kuske et al., 2015; Hibbett et al., 2016).
307 However, because the aim of this work is to understand diversity and distribution patterns, and
308 not to resolve taxonomy issues within this clade, at present we are limited by species clusters
309 identified by our marker regions and taxonomic identification derived from previous
310 phylogenetic analyses and the expertise and reliability of data submitters on Genbank. We argue
311 that further taxonomic work is accomplished via a combination of morphological, ecological,
312 and phylogenomic data.
313
314 Reconciling species clusters – which we identify as OTUs – and separating species boundaries is
315 challenging problem in microbial ecology, taxonomy, and phylogenetics (Mysara et al., 2017).
316 The species clusters we generated here, analogous to the reference sequences in the UNITE
317 database for fungal ITS, were generated by clustering homologous sequences at 98.0% similarity
318 (Nilsson et al., 2018). Like the UNITE database, as well as other databases for the ribosomal
319 operon, our clustering is a close approximation to species but arbitrary in designation. We rely
320 here on manual curation to choose a species designation for a particular cluster or OTU. We have

321 roughly designated species in Figures 1 and 2, but these should only be considered accurate
322 when multiple factors, such as multi-locus or genomic sequence data, as well as morphological
323 and mating studies are incorporated (Hibbett et al., 2016).
324
325 ***Patterns of distribution and species richness***
326 From the perspective of the ITS1 marker region, all armillarioid sequences derived from our
327 curated SRA-derived sequences clustered with existing sequences from the nucleotide database
328 based on voucher collected material. The ITS2 marker region is utilized more frequently in
329 fungal community sequencing efforts due to its level of variability (Hibbett et al., 2016), so it is
330 not surprising that we detected more armillarioid species clusters when using this marker region.
331 The sequences from our curated amplicon sequencing dataset that are not represented by
332 voucher-derived sequence reads represent a single armillarioid species cluster from temperate
333 South America, a geographic area warranting increased surveys for sporocarp diversity. Because
334 several *Armillaria* species from that region do not have sequenced type or voucher specimens, it
335 remains unknown if our armillarioid species cluster represents an undescribed species or
336 represents a species that has been described in the literature but has not been previously
337 sequenced. Another interesting example, and one that elucidates the importance of barcoding
338 studies, is the species cluster 5 from the ITS1 region which is composed of one sequence from a
339 voucher specimen (Vu et al., 2019) and all the other sequences being from an environmental
340 study with no associated voucher material. This further illuminates the importance of continued
341 efforts to barcode, by using multiple marker regions or genomic sequencing, well documented
342 voucher material.
343

As an important pathogen in forest and agronomic systems there has been an emphasis on providing data to advance species delimitation of *Armillaria*. Additionally, the relatively characteristic signs of *Armillaria* presence in the environment, including white mycelial fans under tree bark and black shoestring-like rhizomorphs (Baumgartner et al., 2011) are easy to identify, collect, culture, and sequence species of *Armillaria*, which certainly aids in our understanding of the distribution of these species even in the absence of sporocarps. Lastly, the entirety of the armillarioid clade has relatively few species when compared to other genera found in the Agaricales (Liimatainen et al., 2014; Oliveira et al., 2020). Of course, the available community sequencing studies from which we mined do not comprehensively represent all natural communities in which armillarioid species occur, so there are undoubtedly more species that are left to be found as sampling increases. However, these results also suggest that *Armillaria* species are not contributing substantially to the pool of species known based purely on sequence data (Hawksworth and Lücking, 2017).

Our analyses here largely agree with previously phylogenetic assessments on diversity of this clade and which geographic regions harbor the greatest species richness in the armillarioid clade. This study shows that armillarioid species richness is concentrated in the northern hemisphere, specifically in East Asia. While this finding can certainly be attributed in part to an outsized collecting effort of this group throughout this geographic area (Fig. 3), it may also be reflective of the life history of this lineage. Eurasia is hypothesized to be the ancestral range of *Armillaria* (Koch et al., 2017) and these results are consistent with this region also being the center of species diversity. East Asia, and China in particular, was previously known to have a high species richness of *Armillaria* species (Qin et al., 2007; Zhao et al., 2008; Guo et al., 2016).

367 Furthermore, phylogenetic analyses have provided significant evidence of genetic divergence
368 between *Armillaria* specimens collected from China that were thought to be conspecific
369 throughout the northern hemisphere, suggesting that endemicity may be prevalent in the northern
370 hemisphere in this clade (Guo et al., 2016) and which our analyses also agree with (Fig. 2).
371
372 Another feature of northern hemisphere diversity of the armillarioid clade is the high degree of
373 connectedness between the three primary geographic regions (Fig. 1). Certain *Armillaria* species
374 clusters in the northern hemisphere appear to be broadly distributed throughout the entire region.
375 There are three and five *Armillaria* species clusters, respectively, that are present in North
376 America, Europe and East Asia when considering the ITS1 and ITS2 markers. These species
377 clusters are largely in circumscription with existing designations of the *A. gallica*, *A. calvescens*,
378 *A. sinapina*, and *A. cepstipes* morphological and molecular species complexes. This finding
379 supports the notion that some armillarioid species are distributed throughout the entire northern
380 hemisphere (Coetzee et al., 2015; Coetzee et al., 2018). However, within the northern
381 hemisphere, there are also many species that appear to be restricted to just a certain
382 biogeographic region, suggesting that a narrower interpretation of specific species is more
383 appropriate (Figure 2). For example, the name *A. mellea* have been applied to specimens
384 collected throughout the northern hemisphere. However, our results suggest that what is typically
385 identified as *A. mellea* is in fact a broad species complex (Figure 3). This confirms a previous
386 study which detected significant phylogenetic structure in specimens matching the description of
387 *A. mellea* (Coetzee et al., 2000). While the same has been suspected for *D. tabescens*,
388 inconclusive results from mating studies (Darmono et al., 1992; Guillaumin et al., 1993; Ota et
389 al., 1998; Qin et al., 2007) and phylogenetic analyses (Tsykun et al., 2013; Coetzee et al., 2015),

390 have clouded efforts to disentangle this complex. However, a recent study has separated the *D.*
391 *tabescens* species complex into two separate taxa based on a five gene phylogeny of voucher
392 collections previously identified as *D. tabescens* (Antonín et al 2021). Our results suggest that
393 there may be even more species in this complex.
394
395 Our geographic assessment of diversity is in agreement with numerous other studies that found a
396 distinct suite of *Armillaria* species throughout the southern hemisphere (Coetzee et al., 2010;
397 Koch et al., 2017; Coetzee et al., 2018). Previous studies have suggested that some species,
398 including *A. novae-zelandiae*, are shared between the two regions (Coetzee et al., 2018).
399 However, results from our community analysis of these same data suggest that no armillarioid
400 species are shared between temperate South America and Australasia (Figure 1) but that
401 morphologically similar species may be present in both geographic regions. Our observation is
402 supported by previous phylogenetic analyses of *Armillaria* species from these regions which
403 shows considerable intraclade substructure from sporocarps in the different biogeographic
404 regions (Pildain et al., 2009) and further suggests that dispersal between the two regions may not
405 be common. This could be due to the closure of overland dispersal routes throughout Antarctica
406 several million years ago, and, as a result, the geographic barrier allowed for two lineages of
407 armillarioid fungi to begin diversification. Our analysis also suggests that Australia and New
408 Zealand are relatively restricted from other geographic regions (Figure 1), following the pattern
409 that dispersal between southern hemisphere biogeographic regions is a much rarer occurrence
410 when compared to the northern hemisphere most likely due to the spatial distance of land masses
411 that have been separated for millions of years. Lastly, we should again point out that our analysis
412 here relies only on the ITS1 and ITS2 markers and to fully assess the level of species distribution

413 and diversity, most notably for the *A. novae-zelandiae* species complex, a thorough multi-gene
414 phylogeny (perhaps including a mating compatibility component) would be needed to assess the
415 level of speciation due to allopatry in the Southern Hemisphere.
416
417 Notably, armillarioid species are also depauperate in the lower latitudes—an area characterized
418 by largely tropical climate conditions. For example, *A. puiggarii* is the only known mushroom-
419 forming armillarioid species that appears in tropical South America. Similarly, two to three
420 species were broadly found throughout Africa. Perhaps most interesting are the specimens
421 collected in Southeast Asia, which correspond to one species cluster and show no biogeographic
422 connectedness to other regions (Fig. 1), suggesting that further collecting effort here may
423 uncover a novel suite of species that appear to remain unconnected from other biogeographic
424 regions. A temperate bias in collecting efforts undoubtedly plays a role in this finding as this
425 could certainly be a direct reflection of the disproportionate collecting efforts in temperate
426 regions compared to the tropics. Another explanation could be that species in this lineage are
427 adapted to the cooler and harsher climate in temperate and boreal environments (Koch et al.,
428 2017), or have adapted to temperate woody host species over tropical hosts. A final explanation
429 could be that armillarioid sporocarps are adapted to fruit in cooler environments (Ford et al.
430 2015), which could explain why they are not observed as frequently in warmer climates.
431 However, it is the tropical rainforests of the Guiana Shield that are the exclusive habitat of the
432 two known sequestrate species of *Guyanagaster* (Henkel et al., 2010; Koch et al., 2017). Given
433 the subhypogeous habit of these sporocarps, it is not implausible that other sequestrate species
434 have yet to be collected and described.
435

436 The northern and southern hemispheres have been typically thought to not overlap species
437 distributions (Volk & Burdsall, 1995). While our results are largely consistent with this, there are
438 some exceptions. One of these includes a single *Armillaria* collection from Argentina (species
439 cluster 7 for ITS1 and species cluster 12 for ITS2), which supports a connection from temperate
440 South America with northern hemisphere species. However, whether this species is a relic from a
441 time when certain *Armillaria* species had a much broader distribution, or a recent migrant to the
442 region on imported plant material, remains unknown. When assessed from a 99% clustering
443 threshold, the specimen from Argentina no longer clusters with the northern hemisphere species,
444 suggesting that it does not represent a recent introduction to the region. We also detected several
445 species dominant throughout the northern hemisphere that were also present in South Africa.
446 Previous phylogenetic analyses showed support for at least two introductions of northern
447 hemisphere *Armillaria* species to South Africa which were believed to be introduced centuries
448 ago from potted plants from Europe (Coetzee et al., 2001; Coetzee et al., 2003b; Wingfield et al.,
449 2010). At least one other human-mediated introduction of *Armillaria* from Asia to East Africa
450 has been identified, perhaps introduced to the region on tea plants (Mwenje et al., 2006; Guo et
451 al., 2016). Of the six and five *Armillaria* species clusters at the ITS1 and ITS2 region,
452 respectively, identified from sequences from African specimens, three of them were also
453 represented in samples from the northern hemisphere. This observation supports previous work
454 that there may be three, or more, independent *Armillaria* introductions to Africa. The other two
455 to three *Armillaria* species clusters from Africa include species only present on that continent.
456 This is consistent with our current understanding of the African diversity of *Armillaria*, and if
457 this remains true and no more putatively native *Armillaria* species are discovered, it represents
458 one of the biogeographic zones with the most species-poor representations of *Armillaria*.

459

460 ***Symbiosis as a driver for distribution and species richness***

461 Another possible contributing factor related to the high species richness in East Asia could be the
462 fact that *Armillaria* species engage in mycorrhizal relationships with mycoheterotrophic plants,
463 most notably the species of achlorophyllus orchids in the genera *Galeola* and *Gastrodia* (Kikuchi
464 & Yamaji, 2010; Guo et al., 2016). Using the dataset of Guo et al. (2016), four of our *Armillaria*
465 species clusters associate with orchids from an ITS1 perspective, and seven species clusters
466 associate with orchids from an ITS2 perspective (Table 1). None of the species clusters are
467 specific to only orchid associates. However, an interesting hypothesis emerges from this
468 association, as to whether this symbiosis is a driver of species diversification, as some
469 mycorrhizal relationships—particularly ectomycorrhizal—are known to enhance diversification
470 of the symbionts (Koide et al., 2008; Wilson et al., 2017; Sato & Toju, 2019). However, to
471 address this question, we need a more thorough understanding of the geographic extent of this
472 interaction across the global mycoheterotrophic orchid distribution. Such research, particularly in
473 tropical regions where these mycoheterotrophic orchids are found, may also lead to the discovery
474 of new *Armillaria* species.

475

476 Another symbiosis that may play a role in shaping the distribution of the *Armillaria* species is
477 that with species of the ascomycete genus *Wynnea*. Sclerotia produced by *Wynnea* species,
478 regardless of collecting locality, always contain *Armillaria* hyphae suggesting a dependency of
479 these two lineages on one another (Xu et al., 2019), but it is unclear what is exactly happening in
480 this interaction. Because most of the *Armillaria* sequences from *Wynnea* scloerotia did not pass
481 our filtering thresholds, we independently compared those sequences to our armillarioid species

482 clusters. Armillarioid symbionts of *Wynnea* fell into four species clusters: the clusters 1, 7, 50
483 and 51 for ITS1 and the clusters 8, 31, 60 and 61 for ITS2. Two of the *Armillaria* symbionts of
484 *Wynnea*, one from West Virginia, USA (species cluster 50 for ITS1 and 60 for ITS2) and the
485 other from Brazil (species cluster 51 for ITS1 and 61 for ITS2), are not conspecific with any of
486 the *Armillaria* species clusters detected in any study and may represent new species. The other
487 two species clusters where *Wynnea* symbionts were detected were those that have large
488 geographic distributions. For species cluster 1, which includes one sporocarp from Central
489 America, there are two additional sequences from *Wynnea* symbionts, collected from Guatemala
490 and Costa Rica. In species cluster 7, the *Wynnea* symbiont from Brazil extends the geographic
491 distribution of this largely East Asian cluster to tropical South America. One possibility for why
492 some *Wynnea* symbionts were collected in locations that are largely disjunct from where the
493 majority of the sporocarps were collected may have to do with the specialization of the
494 sclerotium environment. It is possible that certain armillarioid species were once more
495 widespread or abundant than they currently are but were outcompeted by better adapted
496 armillarioid species. *Wynnea* symbionts may have been buffered from local extinction,
497 particularly in Central and South America, due to the extreme specialization of *Armillaria*
498 species for the *Wynnea* environment. However, an increase in the number of voucher collections
499 of *Wynnea* sclerotia and armillarioid fruiting bodies will be needed to fully test this hypothesis. If
500 this hypothesis is supported, *Wynnea* sclerotia could offer a glimpse into the previous
501 distribution of armillarioid species.
502
503 In the *Polyporus umbellatus* interaction, all the *Armillaria* isolates collected by Xing et al. (2017)
504 fell into five and six species clusters when using both ITS1 and ITS2 marker regions,

respectively (Table 1). With the ITS1 marker, there is one armillarioid species cluster composed of sequences only known from the association with *P. umbellatus*, suggesting that this may be a source for new *Armillaria* species. This is a similar situation with the putative mycoparasitic interaction between *E. abortivum* and *Armillaria* (Lindner Czederpiltz et al., 2001; Koch & Herr, 2021). In this interaction, the role of symbiosis in driving the distribution and species richness is unclear, often because methods other than the use of ITS region sequencing were used to identify the *Armillaria* associate. Therefore, we do not have a precise understanding of the species that are involved in this interaction. Instead, the distribution of this interaction seems to be driven more so by the distribution of *E. abortivum*, which is restricted to eastern North America and eastern Asia.

Finally, as was previously mentioned, the South American tropics have only a single known mushroom-forming armillarioid species, *A. puiggarii*, but do have two known species of the sequestrate genus *Guyanagaster*. Phylogenetic and biogeographic analyses show that *Guyanagaster* represents the earliest diverging lineage of the armillarioid clade (Koch et al., 2017). This finding was particularly illuminating because sequestration is a unidirectional process that has never been reversed (Hibbett et al., 1997), suggesting that the ancestor of *Guyanagaster* was a gilled mushroom with widespread distribution (Koch et al., 2017). We hypothesized that, because *Guyanagaster* is the only extant lineage, that it is highly specialized for its environment and, as a result, was buffered from extinction unlike its mushroom progenitors (Koch et al., 2017). Further studies have suggested the symbiosis with wood-feeding termites may play a role in its dispersal and ecosystem specialization and, therefore, its persistence (Koch & Aime, 2018; Koch et al., 2021).

CONCLUSION

In the present study, we used publicly available data to address the global species richness of the genus *Armillaria sensu lato*, a group of fungi most notably recognized as mushroom-forming plant pathogens. By mining public data, we estimate that the overall richness of taxa ranges from 50 to 60 species. The highest species richness was observed in Eastern Asia. We also assessed the overlap of species across geographic regions. We proposed numerous hypotheses regarding the drivers of variability in species diversity and richness between different biogeographic regions and along with symbiotic associations with other fungi and host species. Lastly, we show a proof of concept for the use of publicly available sequence data, from individual voucher specimens and from community sequencing efforts, in understanding fungal species richness in a global context.

Data and code availability.

We used publicly available data as the basis for this study. The public data we used, along with all other associated data that we curated for this study, as well as code for data analysis, can be found at https://github.com/HerrLab/Koch_Global_Armillaria_2021.

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Conflict of Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author Contributions

RAK and JRH initiated the work, analyzed the data, designed the figures, and drafted the manuscript.

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850 **FIGURE LEGENDS**

851

852 **Fig. 1. Phylogenetic trees illustrating the number of sequences that correspond to each**

853 **species cluster.** Maximum likelihood phylogeny constructed from the full length ITS region

854 using one exemplar sequence per species cluster. Two species of *Guyanagaster*, sister to all other

855 armillarioid species, were used to root this phylogeny. Well-supported branches (greater than

856 70% bootstrap support) are thickened. The color of the corresponding circles corresponds to the

857 biogeographic region from which the voucher material producing the sequences was collected.

858 The size of the circles is scaled by the number of sequences that were grouped into a particular

859 species cluster from a particular region. (A) Phylogeny of species clusters as determined from

860 analysis of ITS1 and (B) phylogeny of species clusters as determined from analysis of ITS2.

861

Fig. 2. Species richness in each of the biogeographic regions. Bar graphs showing the total richness of our species clusters in each biogeographic region and the combination of biogeographic regions. (A) ITS1 and (B) ITS2. The species richness for each vertical bar corresponds to the number of species that are solely found in the biogeographic regions corresponding to the colored circle(s) below. The species richness for the horizontal insets correspond to the total species richness within each biogeographic region.

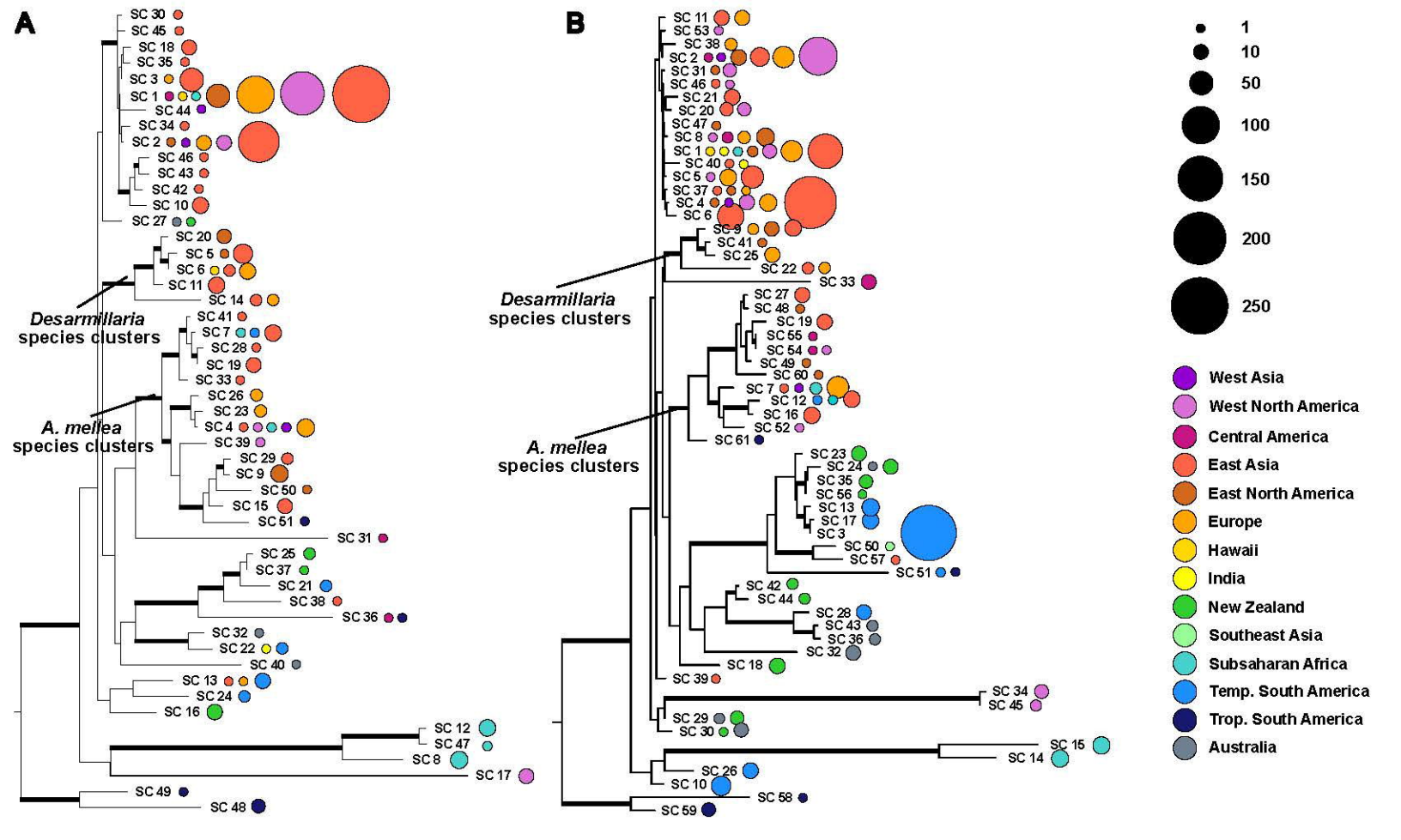
869 **Fig. 3. Connectedness of biogeographic regions by shared armillarioid species clusters.** The
870 width of the lines and diameter of circles are proportional to the number of connections and
871 number of species, respectively. The blue line corresponds to ITS1 and the magenta line to ITS2.
872 The numbers within the circles indicate the number of armillarioid species clusters detected in
873 each region, with the top number referring to the number of species clusters at ITS1 and the
874 bottom number at ITS2.
875

Table 1. Armillarioid sequences (and corresponding GenBank accessions and location metadata) from tissue engaging in symbiotic associations.

	Species cluster	
***Wynnea* symbionts**	**ITS1**	**ITS2**
MK271357: Pennsylvania, USA	1	8
MK281621: China	1	8
MK281620: Guatemala	1	8
MK271358: Costa Rica	1	8
MK271360: New York, USA	1	8
MK271359: New York, USA	1	8
MK271361: West Virginia, USA	1	8
MK599142: China	1	8
MK271362: Brazil	7	12
MK281622: Japan	7	12
MK281623: West Virginia, USA	50	60
MK281619: Brazil	51	61
***Polyporus* symbionts**	**ITS1**	**ITS2**
KY389148: China	1	1
KY389149: China	1	1
KY389150: China	1	1
KY389170: China	1	1
KY389172: China	1	1
KY389174: China	1	1
KY389176: China	1	1
KY389189: China	1	1
KY389154: China	1	4
KY389167: China	1	4
KY389168: China	1	4
KY389169: China	1	4
KY389171: China	1	4
KY389184: China	1	4
KY389185: China	1	4
KY389191: China	1	4
KY389192: China	1	4
KY389160: China	1	5
KY389161: China	1	5
KY389162: China	1	5
KY389163: China	1	5
KY389164: China	1	5
KY389165: China	1	5
KY389166: China	1	5
KY389159: China	1	6
KY389178: China	1	6
KY389182: China	1	6
KY389183: China	1	6
KY389155: China	2	2

KY389147: China	2	4
KY389153: China	2	4
KY389177: China	2	4
KY389180: China	2	4
KY389181: China	2	4
KY389186: China	2	4
KY389187: China	2	4
KY389179: China	2	6
KY389188: China	2	6
KY389193: China	2	6
KY389190: China	3	2
KY389152: China	3	4
KY389157: China	3	4
KY389158: China	3	4
KY389151: China	3	6
KY389173: China	3	6
KY389156: China	35	3
KY389175: China	47	1

Orchid symbionts	**ITS1**	**ITS2**
KT822259: China	1	1
KT822279: China	1	1
KT822297: China	1	1
KT822314: China	1	1
KT822334: China	1	1
KT822336: China	1	1
KT822286: China	1	4
KT822303: China	1	4
KT822329: China	1	4
KT822260: China	1	5
KT822301: China	1	6
KT822270: China	1	20
KT822322: China	2	46
KT822296: China	3	1
KT822264: China	3	6
KT822268: China	3	6
KT822273: China	3	6
KT822284: China	3	6
KT822285: China	3	6
KT822289: China	3	6
KT822275: China	3	21
KT822276: China	3	21
KT822302: China	30	1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Desarmillaria species clusters
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A. mellea species clusters
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West Asia
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New Zealand
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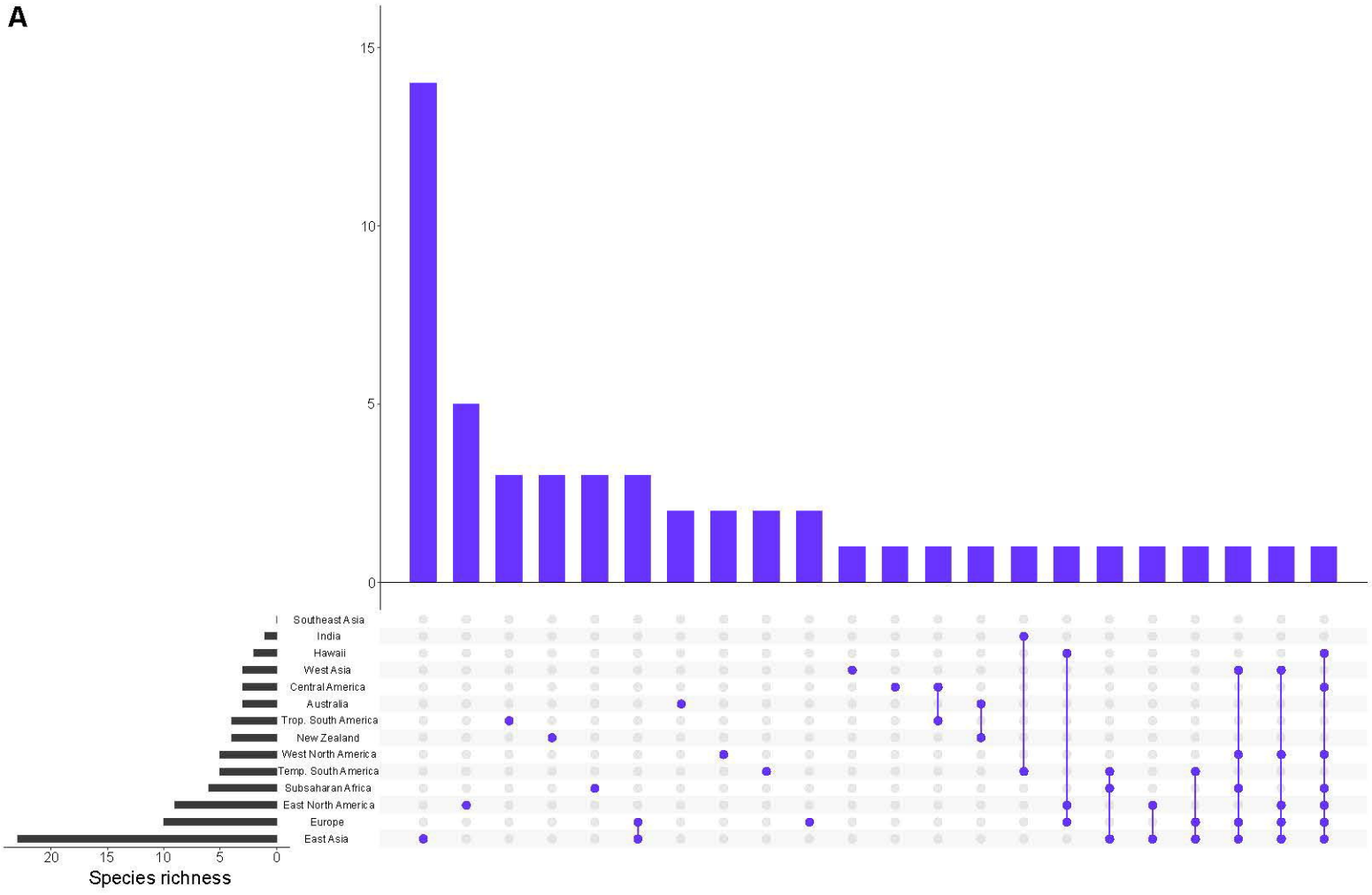
Temp. South America
Trop. South America
Australia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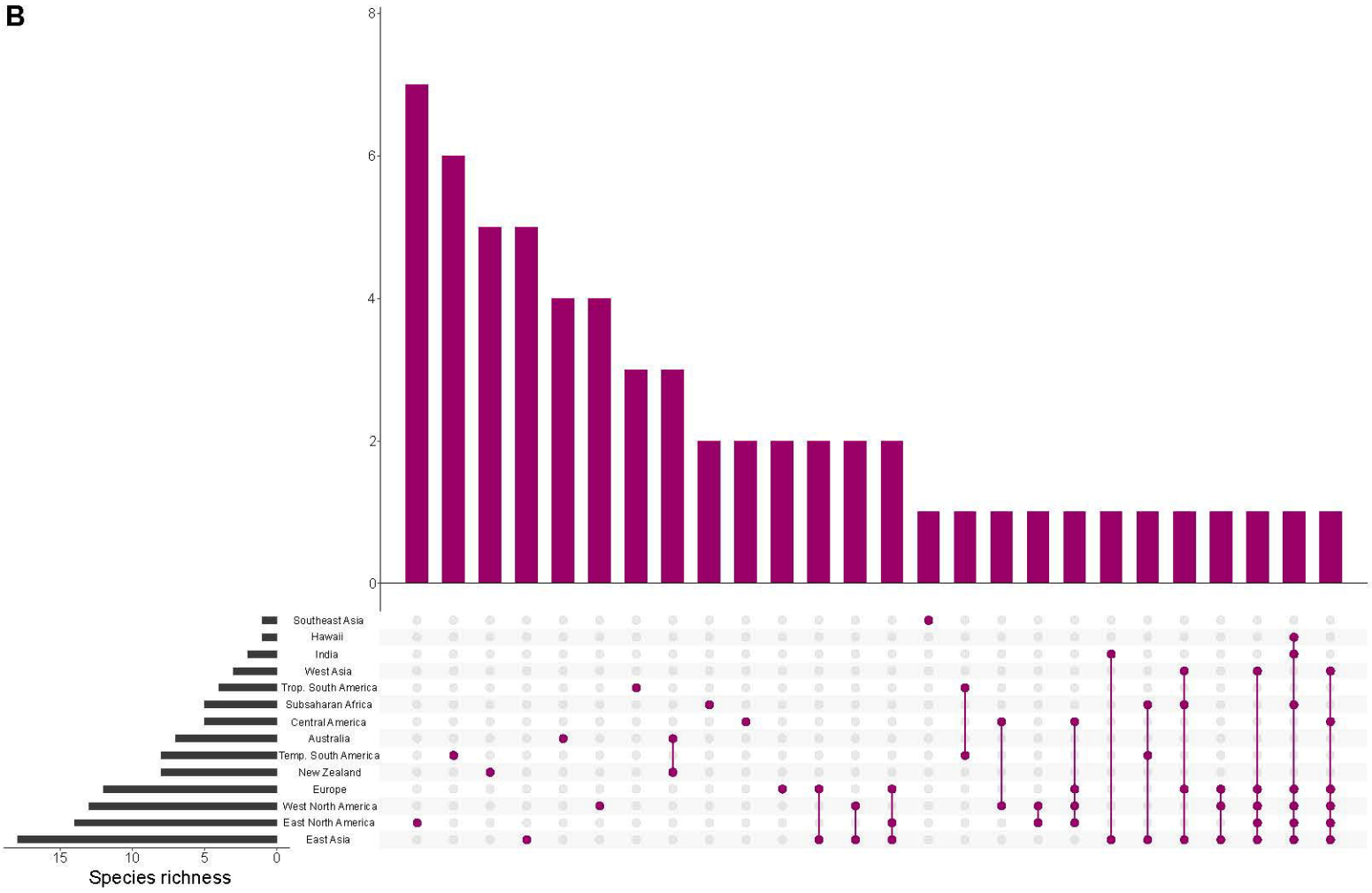
Species richness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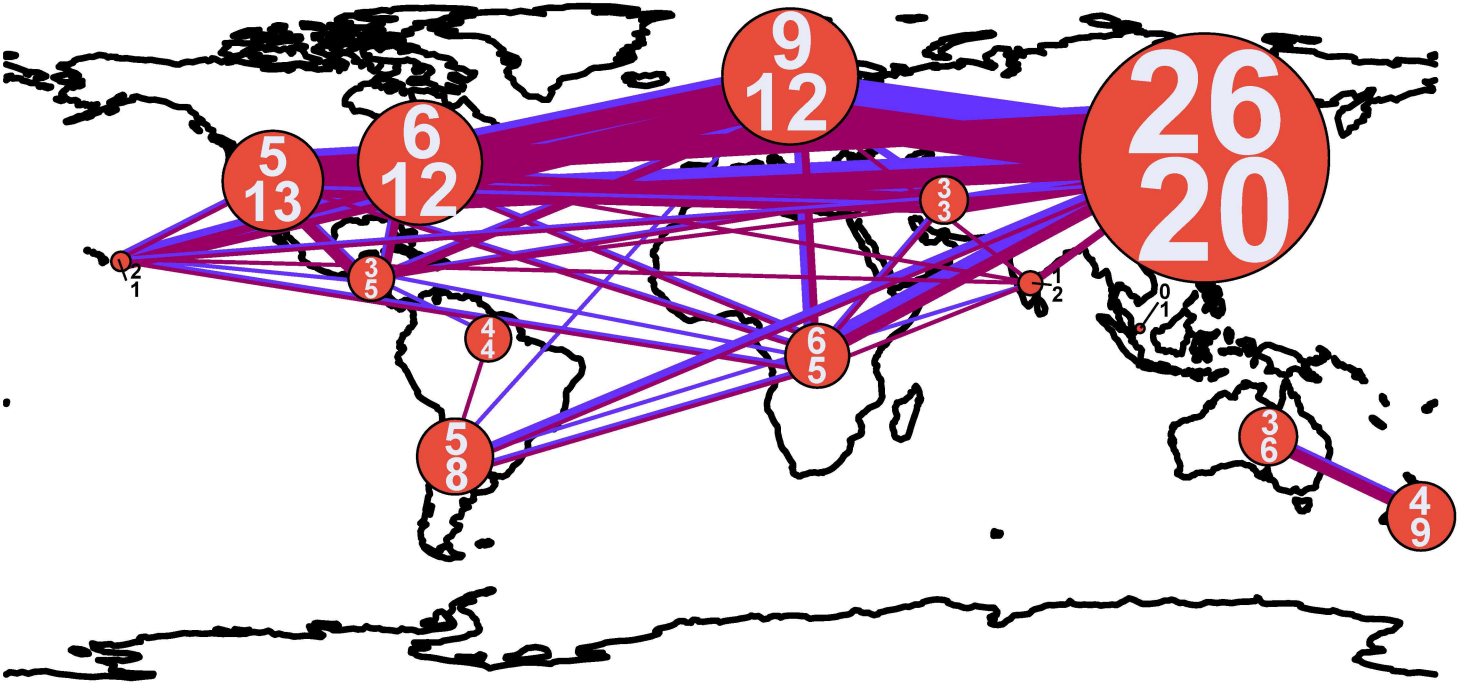
Species richness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