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Macro- and microevolutionary perspectives on *Seynesiella juniperi*, a fungus in the Venturiales (Dothideomycetes, Ascomycota)

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Abstract. The present work represents the first comprehensive phylogenetic study of the genus *Seynesiella* (Dothideomycetes). The genus belongs into the family Cylindrosympodiaceae within the order Venturiales, based on a phylogeny reconstructed with five loci. The high genetic diversity found within the type species, *S. juniperi*, points towards cryptic speciation, with up to five distinct species that might be associated to different *Juniperus* hosts. Combining phylogenetics and multi-locus delimitation analyses with more detailed measurements of ascospores will be fundamental for a better understanding of species boundaries and the biogeographic history of the delimited species, as well as for revealing more specific fungal-plant association patterns.
Keywords. Fungi, Iberian Peninsula, phylogeny

[es] Perspectivas macroevolutivas y microevolutivas en *Seynesiella juniperi*, un hongo perteneciente a los Venturiales (Dothideomycetes, Ascomycota)

Resumen. Este trabajo representa el primer estudio filogenético detallado del género de hongos *Seynesiella* (Dothideomycetes). En base a una filogenia reconstruida con cinco marcadores se concluye que, dentro del orden Venturiales, este género se ubica en la familia Cylindrosympodiaceae. La alta diversidad genética hallada dentro de la especie tipo, *S. juniperi*, sugiere la existencia de especiación críptica, con hasta cinco especies distintas que podrían estar asociadas a diferentes huéspedes del género *Juniperus*. La combinación de análisis filogenéticos y de delimitación de especies basados en múltiples loci, junto con mediciones más detalladas de las ascosporas, será fundamental para una mejor comprensión de los límites entre las posibles especies distintas y su historia biogeográfica, así como para revelar pautas más específicas de asociación hongo-planta.
Palabras clave. Hongos, Península Ibérica, filogenia.

Introduction

The largest class of Kingdom Fungi is Dothideomycetes (Ascomycota), with ca. 19000 species that are distributed in two subclasses (Pleosporomycetidae and Dothideomycetidae), 1261 genera, 110 families and 23 orders (Wijayawardene et al. 2017). Members of this class are characterized by producing bitunicate asci with fissitunicate dehiscence and are extremely diverse ecologically, as they comprise endophytic, epiphytic, saprobic, phytopathogenic, zoopathogenic (including human pathogenic), lichenized, lichenicolous, nematode trapping, and rock-inhabiting taxa (Schoch et al. 2009; Hyde et al. 2013; Ametrano et al. 2019; Hongsanan et al. 2020). The number of known taxa is increasing at a steady pace as more geographic areas and habitats are investigated (e.g. Valenzuela-López et al. 2018; Pem et al. 2019; Hongsanan et al. 2020a). For example, a recent study on Venturiales Y. Zhang, C.L. Schoch & K.D. Hyde, which encompasses saprobes and pathogens of plants and animals (including humans) analyzed 115 taxa representing 30 genera and introduced a new family, eight new genera, and 12 new species (Shen et al. 2020). The use of molecular data within a multi-locus phylogenetic context has been crucial to revealing most of the known diversity. Recently, whole-genome sequence data generated for more than one hundred dothideomycete species have been used to refining phylogenetic relationships and understanding other evolutionary aspects, such as adaptation to stress, host specificity and the origin of pathogenicity (Haridas et al. 2020). Although these advances have allowed the resolution of several long-standing questions on Dothideomycetes diversity and evolution, many questions remain open about the evolutionary history of particular taxa, especially genera and species for which molecular data are not yet available.

One such genus is *Seynesiella* G. Arnaud, currently comprising five species worldwide (Sivanesan & Shivas 2002) and whose type species is *S. juniperi* (Desm.) G. Arnaud (Arnaud 1918). Ascomata of this genus are superficial thyriothecia, more or less conical to hemispheric, tapered with a rounded ostiole, and with a peridium formed by isodiametric cells towards the center and more rectangular cells towards the periphery; microscopically, asci produce eight spores, spores are bicellular, smooth, hyaline first

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and turning brownish at maturity. Although Müller & von Arx (1962) placed the genus in Venturiaceae E. Müll. & Arx ex M.E. Barr, other authors suggested its placement in Microthyriaceae Sacc. based on ascoma morphology and peridium structure (Luttrell 1965; Barr 1968; Lumbsch & Huhndorf 2010). A close relationship with the genera *Tothia* Bat. and *Arnaudiella* Petr. was also hypothesized (Wu et al. 2011). So far, the absence of molecular data for any of the known species in the genus has prevented us from answering these questions.

The species *Seynesiella juniperi* seems to be widespread across the Northern Hemisphere (GBIF 2019), from Ontario, British Columbia (Canada) and Wisconsin (USA) growing on *Juniperus communis* L., *J. communis* var. *depressa* Pursh and *J. scopulorum* Sarg. (Greene 1967–1968; Wu et al. 2011; Lee & Golinski 2020) to the United Kingdom, France and Scandinavia, growing on several *Juniperus* spp., and even Iran (Petrak 1949). In the Iberian Peninsula, there are a considerable number of occurrence reports from Catalunya and Huesca (Sierra López 2006) and Segovia (Olariaga 2018), growing on *J. communis*, *J. phoenicea* L. and *J. thurifera* L. The question arises whether this fungal species occurs in other areas of the Iberian Peninsula and on additional *Juniperus* L. hosts, such as *J. oxycedrus* L. and *J. sabina* L., and also whether there is any signal of genetic differentiation between specimens growing on different hosts.

The present study first proposes a macroevolutionary framework to test the placement of the genus *Seynesiella* in known high level taxa within Dothideomycetes using molecular data from several loci, and second, a microevolutionary framework that uses morphological and ecological traits, as well as phylogenetics and species delimitation analyses, to examine genetic differentiation in *S. juniperi* and determine whether there are signs of concordant evolutionary history between the fungi and their plant hosts.

Material and methods

Morphological studies

Leaves of *Juniperus* spp. were examined under a Leica S8APO dissecting microscope, and macroscopic photographs of ascomata were taken with a Leica EC3 image capture system. Preparations of entire ascomata were mounted in water, and microscopic details were observed using a Zeiss Axioplan 2 microscope fitted with Nomarski differential interference contrast (DIC). Photographs were taken with a Zeiss AxioCam digital camera. Microscopic measurements were made by means of the Zeiss Axiovision 4.8 image analyzer system and are given as the average and its standard deviation. Specimens are deposited in the Real Jardín Botánico of Madrid (MA herbarium). Author citations follow MycoBank (http://www.mycobank.org/).

DNA extraction, PCR amplification and DNA sequencing

Up to five ascomata per leaf showing no contamination by hyphae from other fungi were isolated from each specimen and deposited into an Eppendorf tube. After grinding the ascomata with the help of micropestles, genomic DNA was extracted using the Speedtools Tissue DNA kit (Biotools® B&M Labs., S.A) following the manufacturer's recommendations. To reconstruct a phylogeny of orders in Dothideomycetes, the following five markers were selected and amplified by PCR: the nuclear small (nuSSU) and large (nuLSU) subunits of the ribosomal RNA, the RNA polymerase II largest (*RPB1*) and second largest (*RPB2*) subunits, and the elongation factor 1-alpha (*tef1-α*) gene. The nuclear ribosomal Internal Transcribed Spacer (nrITS), which includes the subregions nrITS1, 5.8S, and nrITS2, was additionally amplified to examine intraspecific variation in the focal species. This marker has been generally used for barcoding fungi due to its high nucleotide variability (Schoch et al. 2012); for the same reason, the nrITS is also useful for studying variation below the species level. The primers used as well as PCR conditions were the same as in Pérez-Ortega et al. (2016). PCR reactions were carried out using the Illustra Ready-To-Go GenomiPhi V3 DNA amplification kit (GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA) following the manufacturer's instructions. Amplicons were purified and cleaned using the QIAGEN quick spin columns (Qiagen®), and both complementary DNA strands were sequenced at MACROGEN (Madrid). Raw electropherograms were manually checked, trimmed and assembled using SeqmanII v.5.07© (Dnastar Inc.). GenBank accession numbers are provided in section Results.

Phylogenetic analyses at ordinal level

Following previous considerations of the genus *Seynesiella* being a member of the ascomycete class Dothideomycetes (Lumbsch & Huhndorf 2010; Wu et al. 2011; Hyde et al. 2013; Wijayawardene et al. 2017; Hongsanan et al. 2020b), a five-locus dataset was compiled with publicly-available DNA sequence data for 86 species representing most known orders in Dothideomycetes (Zhang et al. 2011). The arthoniomycete species *Schismatomma decolorans* (Turner & Borrer ex Sm.) Clauzade & Vězda and *Opegrapha dolomitica* (Arnold) Clauzade & Cl. Roux ex Torrente & Egea were included as the outgroup to root the phylogenetic tree. Alignments of the newly obtained and GenBank-downloaded nuSSU, nuLSU, *tef1-α*, *RPB1* and *RPB2* sequences were carried out with the software MAFFT v.7.308 (Katoh et al. 2002; Katoh & Standley 2013). The resulting alignments

were visualized in Geneious® v.9.0.2 to identify and manually remove in all five markers intronic regions, which were the ones that encompassed most ambiguously-aligned positions. The first, second and third codon positions of the protein-coding *tef1-α*, *RPB1* and *RPB2* markers were also delimited. Before producing a concatenated dataset, Maximum Likelihood phylogenies were constructed for each marker independently with the CIPRES' online RAxML-HPC2 v.8.2 version (Miller et al. 2010; Stamatakis 2006; Stamatakis et al. 2008), using 1000 bootstrap pseudoreplicates and the GTRGAMMA model, to test for topological incongruence among them, assuming bootstrap values ≥70 % as significant for conflicting relationships among the same set of taxa (Mason-Gamer & Kellogg 1996). Subsequently, RAxML-HPC2 was again used to build a multi-locus phylogeny, with the same parameters. Additionally, the software BEAST 1.8.1 (Drummond et al. 2012) was chosen to infer a phylogeny under a Bayesian framework, using an uncorrelated lognormal relaxed molecular clock and a Birth-Death tree prior. The *ucld.mean* parameter of each marker was given a uniform prior (lower= 0.0; upper= 5.0; initial= 1). A run using a chain length of 2×10^8 steps was implemented and parameters were logged every 2×10^4 steps. Resulting log files were checked in Tracer 1.7 to ensure that all parameters had ESS above 200 after removing the first 20% of saved trees as burn-in. Then, the median heights of the 1×10^4 post-burn-in tree samples were annotated with TreeAnnotator 1.8.1., and the tree was drawn with FigTree 1.4. The latter three programs are available at http://tree.bio.ed.ac.uk/. Optimal substitution models used in the above analysis for the five markers and their inner partitions were inferred with PartitionFinder v.1.1.1 (Lanfear et al. 2012) considering a model with linked branch lengths and the Bayesian Information Criterion (BIC). However, to avoid overparameterization and chain convergence issues, less complex substitution models, such as HKY, were used instead of GTR.

Phylogenetic analyses at the family level

Once the most likely placement of *Seynesiella* at ordinal level within Dothideomycetes was known, the work of Shen et al. (2020) on Venturiales was followed to test the placement of our focal genus in any known family. To do this, we used a different five-locus dataset comprising data of 5.8S, nuLSU, *tef1-α*, *RPB2* and the protein-coding β-tubulin (β-tub) gene. The same procedure and programs were chosen to construct a concatenated alignment and to infer Maximum Likelihood and Bayesian phylogenies (see previous section). To root the tree, the following species were chosen as outgroup: *Schismatomma decolorans* (Arthoniomycetes) and the dothideomycetes *Trichodelitschia munkii* N. Lundq., *T. bisporula* (P. Crouan & H. Crouan) E. Müll. & Arx, *Phaeotrichum benjaminii* Malloch & Cain and *Microthyrium microscopicum* Desm.

Intraspecific diversity and species boundaries

The alignment of nine nrITS sequences of *Seynesiella juniperi* was carried out with MAFFT v.7.308, and was manually optimized in Geneious v. 9.0.2 to trim alignment ends of longer sequences that included a section of the 18S–28S ribosomal subunits. The software MEGA 5.2 (Tamura et al. 2011) was employed to calculate several indices of nucleotide diversity and to infer a Maximum Likelihood phylogeny, using the T92 nucleotide substitution model and 500 bootstrap replicates. Then, the Automatic Barcode Gap Discovery method (ABGD, Puillandre et al. 2012), a model-based approach which automatically infers a one-sided confidence limit for intra- and interspecific divergence based on the distribution of all pairwise distances, was chosen to examine species boundaries in the dataset. The program was remotely run at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html, using the Kimura two parameters (K2P) model to calculate genetic distances between individuals, a TS/TV value of 2.01 obtained from MEGA 5.2, the Pmax value set to 0.01 (Puillandre et al. 2012), the relative gap width (*X*) to 1 and the remaining model parameters to default.

Results

Taxonomy and description

Seynesiella juniperi (Desm.) G. Arnaud, Annales de l'École Nationale d'Agriculture de Montpellier 16 (1-4): 203 (1918) [MB#157925] Fig. 1.

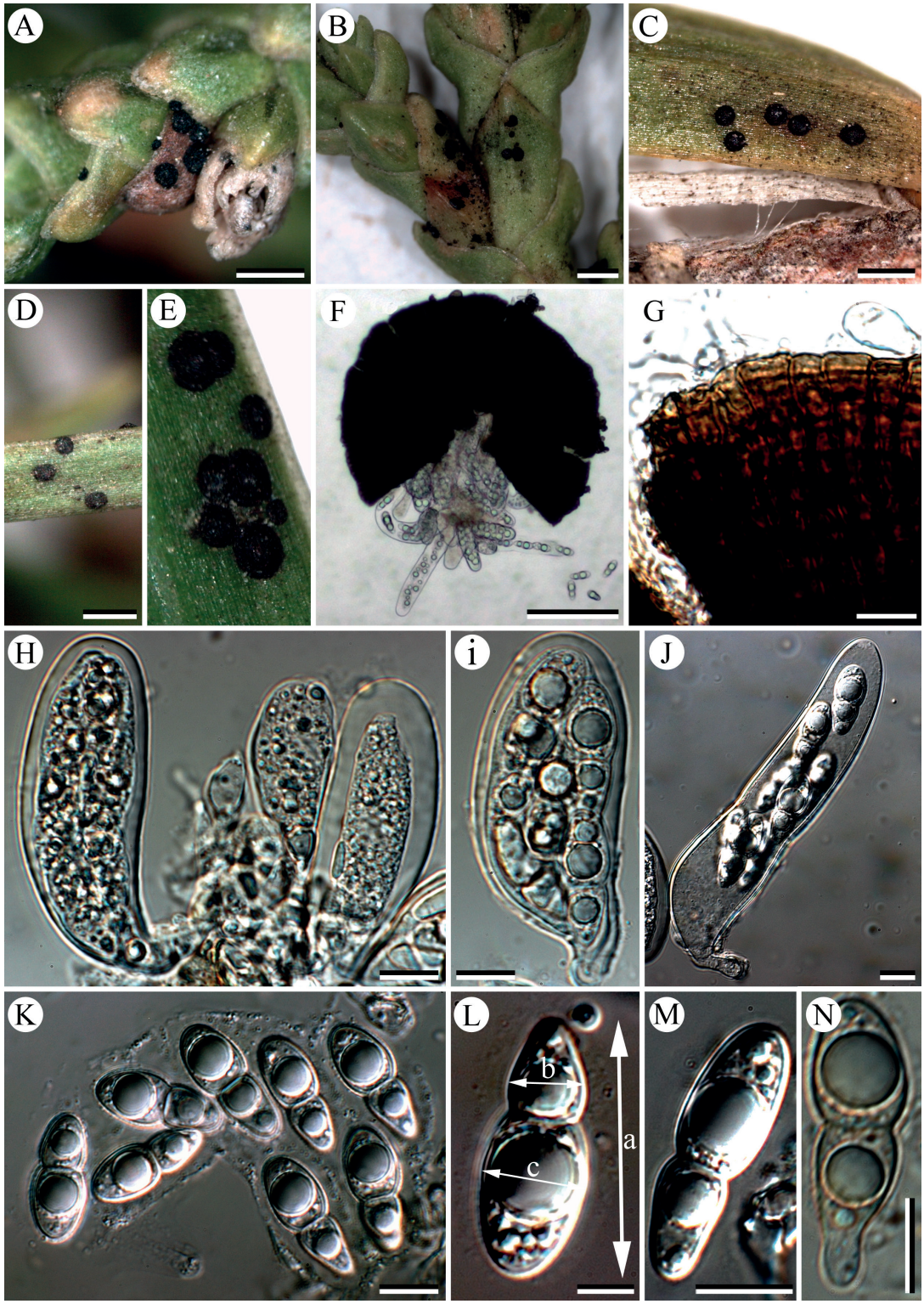

Figure 1. *Seynesiella juniperi* growing on *Juniperus phoenicea* (**A**, IGB748), *J. thurifera* (**B**, IGB749), *J. communis* (**C**, IGB879), *J. oxycedrus* (**D**, IGB778-I1200; **E**, IGB869, I1217). **F** Thyriothecium. **G** Peridium cells towards the ascoma margin. **H**–**J** Asci. **K** Ascospores released from an ascus. **L**–**N** Ascospores. Measured spore characters are indicated with lower case letters (**L**). **N** Germinating spore. Scale bars: **A**–**D**: 0'5 mm; **F**: 100 µm; **G**–**K**, **M**–**N**: 10 µm; **L**: 5µm.

Typus: FRANCE, without location, on lower part of living leaves of *Juniperus* sp., Desmazières Cryp. France exs. 1094 (K (M)164151, syntype)

Basionym: *Dothidea juniperi* Desm., Annales des Sciences Naturelles Botanique 15: 141 (1841) [MB#166229]

Obligate synonyms: *Gibbera juniperi* (Desm.) Auersw. ex Rabenh., Fungi Europaei Exsiccati Century 11: no. 1030 (1866); *Stigmatea juniperi* (Desm.) Winter, Rabenhorst's Kryptogamen-Flora Band 1, Abtheilung 2 (Lieferung 19): 340 (1885) [MB#581441]; *Microthyrium juniperi* (Desm.) Sacc., Mycotheca Veneta Century 13: no. 1269 (1878)

[MB#203335]; *Asterina juniperi* (Desm.) Ge. Boyer & Jacz., Bulletin de la Société Botanique de France 40 (Session Extraordinaire a Montpellier en Mai 1893): CCLXXXIV (1893) [MB#453800]; *Seynesia juniperi* (Desm.) Höhn., Annales Mycologici 15 (5): 373 (1917) [MB#268490]; *Hysterostoma juniperi* (Desm.) Bat. & Peres, Saccardoa 1: 94 (1960) [MB#332502].

Morphological description. Ascomata thyriothecia, shiny black to black, solitary, scattered or grouped in clusters, on the upper side of leaves (*Juniperus* spp. with squamiform leaves) or either in the upper or lower surface (*Juniperus* spp. with needle-like leaves), 238.5 ± 52.3 µm (n= 28) in diameter, first flattened and later conical to hemisphaerical, ending in an ostiole ca. 16–25 µm wide; peridium consisting of radial hyphae with deep brown, isodiametric cells towards the ostiole, and more rectangular cells towards the periphery, the latter with a size of 7–10 × 4–9 µm (n= 5). Hamathecium embedded in mucilage. Pseudoparaphyses present and abundant, branched, cylindrical, with a diameter up to 4.5 µm (n= 4). Asci 67.70 ± 15.79 µm long and 23.65 ± 3.18 µm wide (n= 40), claviform to subglobose, pedicellate, with eight spores arranged in an overlapping biseriate manner. Ascospores ellipsoidal to long ellipsoidal, septate, formed by two cells with different sizes (heteropolar), each with one guttule, with ends more or less rounded, wall smooth, surrounded by a mucilaginous layer, hyaline or rarely yellowish brown when very mature or injured, 23 ± 1.88 µm in length, and width of small and large cells 8.18 ± 0.82 µm and 9.91 ± 0.91 µm, respectively (n= 66).

Ecology and distribution. In the Iberian Peninsula, *Seynesiella juniperi* occurs on five *Juniperus* species: *J. communis*, *J. oxycedrus*, *J. phoenicea*, *J. sabina* and *J. thurifera*. Symptoms of injury due to parasitism were seldom observed in host leaves; in a few cases, however, a yellowish brown areole surrounding groups of thyriothecia was noticed. Based on the collections reported in the present study, this fungus displays a wide bioclimatic tolerance, being present in Mesomediterranean zones in the eastern Iberian Peninsula (e.g. low mountains near the coast in València and Alacant), as well as in more continental (Supramediterranean) areas of the inner Iberian Peninsula (Albacete, Soria) and mountains up to 1300–1600 m high (Castelló, Teruel, Madrid).

Specimens studied and GenBank accession numbers. **SPAIN. ARAGÓN**: TERUEL, La Puebla de Valverde, Corral de la Ceja, road to Javalambre summit, paramera, 40º 12’ 41.65” N, 0º 57’ 36.61” W, 1276 m, on *Juniperus phoenicea*, 17 Mar 2014, *I. Garrido Benavent* (IGB883); Nogueruelas, El Paso, 40º 15’ 5.58” N, 0º 37’ 24.80” W, 1370 m, on *J. phoenicea*, 21 Aug 2014, *I. Garrido Benavent* (IGB888). **CASTILLA Y LEÓN:** SORIA, Abejar, Sierra de Cabrejas, Las Cabezuelas, road to Calatañazor, 41º 46’ 37.50” N, 2º 46’ 41.20” W, 1161 m, on *J. thurifera*, 17 Mar 2014, *I. Garrido-Benavent* (IGB885). **CASTILLA-LA MANCHA:** ALBACETE, Chinchilla, pedanía Pinilla, 38º 43’ 15.64” N, 1º 35’ 58.68” W, 900 m, on *J. oxycedrus*, 27 Dec 2019, *I. Garrido Benavent* (IGB816, IGB817, IGB818); Férez, Mirador, 38º 24’ 08.90” N, 1º 56’ 06.73” W, 524 m, on *J. phoenicea*, 27 Dec 2019, *I. Garrido Benavent* (IGB827); Férez, Montes de Aguas Calientes, 38º 25› 24.82» N, 1º 52› 40.46» W, 501 m, on *J. phoenicea*, 27 Dec 2019, *I. Garrido Benavent* (IGB837); Hellín, Loma de Pinar Verde, road to Embalse del Cenajo, 38º 25’ 30.38” N, 1º 48’ 07.86” W, 569 m, on *J. oxycedrus*, 27 Dec 2019, *I. Garrido Benavent* (IGB825, DNA extraction code I1208; nrITS: MW405226); Hellín, Puente de Isso, Rio Mundo, 38º 27’ 53.98” N, 1º 47’ 35.00” W, 439 m, on *J. phoenicea*, 27 Dec 2019, *I. Garrido Benavent* (IGB822); Socovos, El Caunial, 38º 20’ 11.22” N, 1º 59’ 45.77” W, 769 m, on *J. oxycedrus*, 27 Dec 2019, *I. Garrido Benavent* (IGB829); ibídem, on *J. phoenicea*, *I. Garrido Benavent* (IGB831); CUENCA, Landete, Manzaneruela, 39º 55’ 38.52” N, 1º 18’ 07.63” W, 1051 m, on *J. phoenicea*, 19 Aug 2019, *I. Garrido Benavent* (IGB753); **COMUNITAT VALENCIANA:** CASTELLÓ, Vistabella del Maestrat, between Alt de l’Asevar and El Chaparral, 40º 15’ 55.94” N, 0º 22’ 57.52” W, 1624 m, on *J. sabina*, 21 Aug 2014, *I. Garrido Benavent* (IGB894); ALACANT, Beniarrés, Solloca, near the Beniarrés dam, 38º 49’ 01.96” N, 0º 21’ 16.97” W, 306 m, on *J. oxycedrus*, 3 Nov 2019, *I. Garrido Benavent* (IGB797); l’Orxa, La Pedrera, 38º 50’ 11.18” N, 0º 18’ 00.38” W, 473 m, on *J. phoenicea*, 28 Aug 2019, *I. Garrido Benavent* (IGB766); Vall de Gallinera, road to La Vall d’Ebo, Corral dels Morells, 38º 48’ 22.74” N, 0º 13’ 01.79” W, 579 m, on *J. phoenicea*, 29 Dec 2019, *I. Garrido Benavent* (IGB860; DNA extraction code I1209; nrITS: MW405224); Lloma de Penya Roja, 38º 50’ 49.96” N, 0º 15’ 33.41” W, 671 m, on *J. oxycedrus*, 14 Nov 2020, *I. Garrido Benavent* (IGB899); La Vall d’Ebo, Corrals de Pego, entrance to Barranc de l’Infern, 38º 48’ 23.59” N, 0º 08’ 23.41” W, 377 m, on *J. phoenicea*, 29 Dec 2019, *I. Garrido Benavent* (IGB858); VALÈNCIA, Alpuente, Atalaya Alta, 39º 53’ 51.42” N, 0º 59’ 00.68” W, 1035 m, on *J. phoenicea*, 19 Aug 2019, *I. Garrido Benavent* (IGB748); Chelva, Sierra de los Azagadores, Collado de Aguavientos, 39º 48’ 12.27” N, 0º 52’ 56.69” W, 1056 m, on *J. phoenicea*, 19 Aug 2019, *I. Garrido Benavent* (IGB745); Utiel, La Chula, 39º 37’ 37.62” N, 1º 08’ 50.63” W, 1034 m, on *J. phoenicea*, 22 Dec 2020, *S. Chiva & P. Moya* (IGB908); Siete Aguas, Venta Mina, 39º 27’ 23.59” N, 0º 52’ 46.89” W, 607 m, on *J. phoenicea*, 19 Aug 2019, *I. Garrido Benavent* (IGB751); La Font de la Figuera, road to Navalón, La Fontjordana, 38º 51’ 22.49” N, 0º 53’ 43.81” W, 734 m, on *J. oxycedrus*, 1 Nov 2019, *I. Garrido Benavent* (IGB778, DNA extraction code I1200; nrITS: MW405225; nuSSU: MW405219; nuLSU: MW405231; *RPB2*: MW619847; *tef1-α*: MW619849); Moixent, La Bastida, Les Alcusses,

ancient Iberian town, 38º 48' 49.71" N, 0º 48' 09.80" W, 734 m, on *J. phoenicea*, 1 Nov 2019, *I. Garrido Benavent* (IGB787); Moixent, Cumbres de Valencia residential complex, 38º 53' 48.27" N, 0º 47' 29.01" W, 702 m, on *J. phoenicea*, 1 Nov 2019, *I. Garrido Benavent* (IGB791, DNA extraction code I1201; nrITS: MW405223; nuSSU: MW405220; nuLSU: MW405232; *tef1-α*: MW619850); La Puebla de San Miguel, Corral de Las Losas Blancas, 40º 2' 32.33" N, 1º 6' 31.20" W, 1587 m, on *J. thurifera*, 19 Aug 2019, *I. Garrido Benavent* (IGB749, DNA extraction code I1186; nrITS: MW405222; nuSSU: MW405218; nuLSU: MW405230; *RPB1*: MW619845; *RPB2*: MW619846; *tef1-α*: MW619848); Vallanca, Rio Bohílgues, Fuente Podrida, 40º 3' 50.08" N, 1º 20' 50.88" W, 958 m, on *J. thurifera*, 19 Aug 2019, *I. Garrido Benavent* (IGB755); Quatretonda, Pla dels Arenals, 38º 57' 31.85" N, 0º 22' 29.06" W, 364 m, on *J. oxycedrus*, 7 Dec 2019, *I. Garrido Benavent* (IGB798); Salem, road to Beniarrés, 38º 51' 01.93" N, 0º 22' 20.44" W, 433 m, on *J. phoenicea*, 3 Nov 2019, *I. Garrido Benavent* (IGB786); Sumacàrcer, road to Navarrés, 39º 05' 03.81" N, 0º 38' 03.87" W, 187 m, on *J. oxycedrus*, 5 Mar 2021, *I. Garrido Benavent* (IGB935); **COMUNIDAD DE MADRID:** Bustarviejo, track to El Pendón, near Fuente de la Víbora fountain, 40º 50' 41.89" N, 3º 43' 03.30" W, 1404 m, on *J. communis*, 25 Jul 2020, *I. Garrido Benavent* (IGB879); ibídem, on *J. thurifera*, *I. Garrido Benavent* (IGB880); Lozoyuela, Majada de las Ovejas, 40º 53' 15.60" N, 3º 39' 25.08" W, 1301 m, on *J. communis*, 12 Jul 2020, *I. Garrido Benavent* (IGB877, DNA extraction code I1223; nrITS: MW405229); Valdemanco, track under Cancho del Pastor, 40º 53' 17.74" N, 3º 39' 46.23" W, 1368 m, on *J. oxycedrus*, 18 Jul 2020, *I. Garrido Benavent* (IGB878); Venturada, close to Cotos de Monterrey residential complex, Canteras de Redueña, 40º 47' 41.81" N, 3º 35' 29.01" W, 930 m, on *J. oxycedrus*, 11 Mar 2020, *I. Garrido Benavent* (IGB868, DNA extraction code I1216; nrITS: MW405227); ibídem, on *J. oxycedrus*, *I. Garrido Benavent* (IGB869, DNA extraction code I1217; nrITS: MW405228); **REGIÓN DE MURCIA:** Moratalla, road from Benízar to Casicas del Portal, 38º 14' 49.05" N, 1º 59' 59.85" W, 1170 m, on *J. phoenicea*, 27 Dec 2019, *I. Garrido-Benavent* (IGB847); El Sabinar, close to Cortijo de La Leona, road to Letur, 38º 13' 01.38" N, 2º 08' 07.61" W, 1194 m, on *J. thurifera*, 27 Dec 2019, *I. Garrido-Benavent* (IGB841). **PORTUGAL. LISBON:** Monsanto Forest Park, 38º 44' 18.39" N, 9º 11' 06.33" W, 144 m, on *J. phoenicea*, 25 Jul 2019, *I. Garrido-Benavent* (IGB724, DNA extraction code I1183; nrITS: MW405221; nuSSU: MW405217).

Molecular datasets

The first five-locus dataset (nuSSU, nuLSU, *tef1-α*, *RPB1* and *RPB2*), which was used for elucidating the placement of *Seynesiella* at ordinal level in Dothideomycetes, consisted of 92 taxa and 6610 bp of which 3146 were variable, 2486 parsimony informative and 660 sites had singletons. The second compiled five-locus dataset (5.8S, LSU, *tef1-α*, *RPB2* and β-*tub*) that was used for establishing a closer phylogenetic placement of the focal genus within the order Venturiales was composed of 56 taxa and 1991 nucleotides; 904 of them were variable, 738 parsimony informative, and 166 sites with singletons. Finally, the nine nrITS sequences of *Seynesiella juniperi* generated a 506 bp-long alignment, with 23 and 8 variable and parsimony informative sites, respectively, together with 15 positions with singleton substitutions. The alignments are available in FigShare (doi: 10.6084/m9.figshare.14206964).

Phylogenetic relationships at the order and family levels

The ML phylogeny of Dothideomycetes orders calculated with RAxML had a lnL = -70080.4361. All parameters estimated in the BEAST analysis had ESS well above 200. Because these two phylogenies showed no supported topological conflicts, the ultrametric tree estimated in BEAST is shown in Fig. 2. The genus *Seynesiella* was placed at the base of the order Venturiales and this relationship was supported by both phylogenetic analyses (PP= 0.98; BS= 81%). Venturiales was composed of three well supported clades but relationships among them remained elusive. The two most closely related lineages to Venturiales were members of the family Phaeotrichaceae Cain and *Microthyrium microscopicum* (Microthyriales G. Arnaud). These lineages together with Venturiales formed a statistically supported monophyletic group within Dothideomycetes (PP= 1; BS= 78%). At the family level, the ML phylogeny of Venturiales calculated with RAxML had a lnL = -21754.7185 and all parameters estimated in BEAST reached ESS above 200. As before, the topology inferred with the Bayesian program is shown in Fig. 3. The genus *Seynesiella* was placed in the family Cylindrosympodiaceae P. Crous, M. Shen and Y. Zhang, and formed a well-supported clade (PP= 1; BS= 96%) sister to a representative of *Sympodiella acicola* W.B. Kendr; however, this relationship only received support from the BEAST analysis (PP= 0.96). The family Cylindrosimpodiaceae formed a monophyletic group supported only by the Bayesian analysis (PP= 1), and its phylogenetic relationships with the other two families within Venturiales, Venturiaceae and Sympoventuriaceae Y. Zhang, C.L. Schoch & K.D. Hyde, were not resolved with certainty in our reconstructions. The order Venturiales was again supported only by results of the Bayesian analysis (PP= 1).

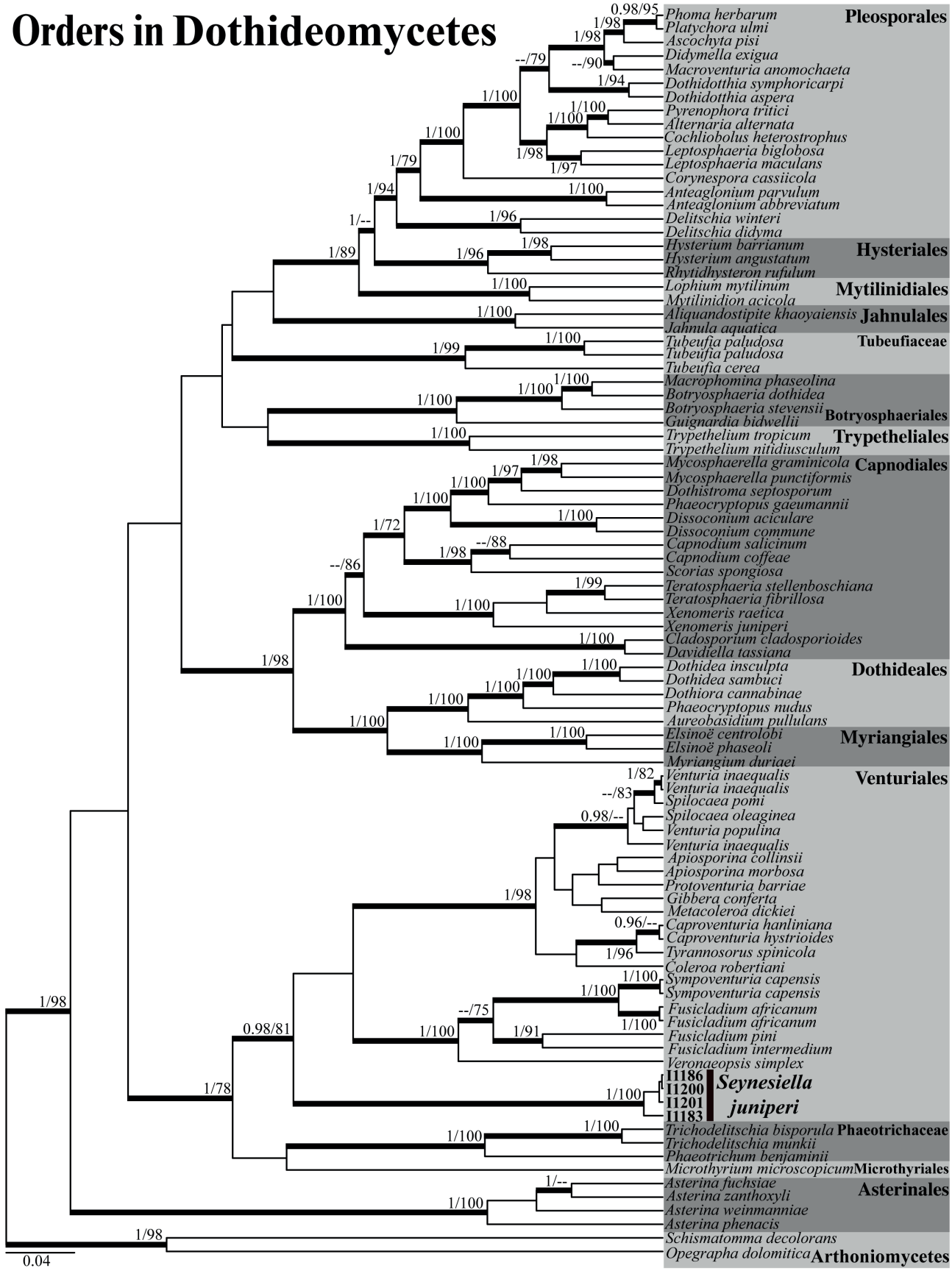

Figure 2. Phylogram showing the placement of the genus *Seynesiella* in the Dothideomycetes Tree of Life based on a five-locus dataset (nuSSU, nuLSU, *tef1-α*, *RPB1* and *RPB2*). The represented topology was obtained with BEAST 1.8.1. Posterior Probabilities (PP; BEAST analysis) and bootstrap support values (BS, RAxML analysis) are represented on branches leading to nodes on the left and right, respectively. Branches in bold had a significant statistical support (PP ≥ 0.95; BS ≥ 70 %).

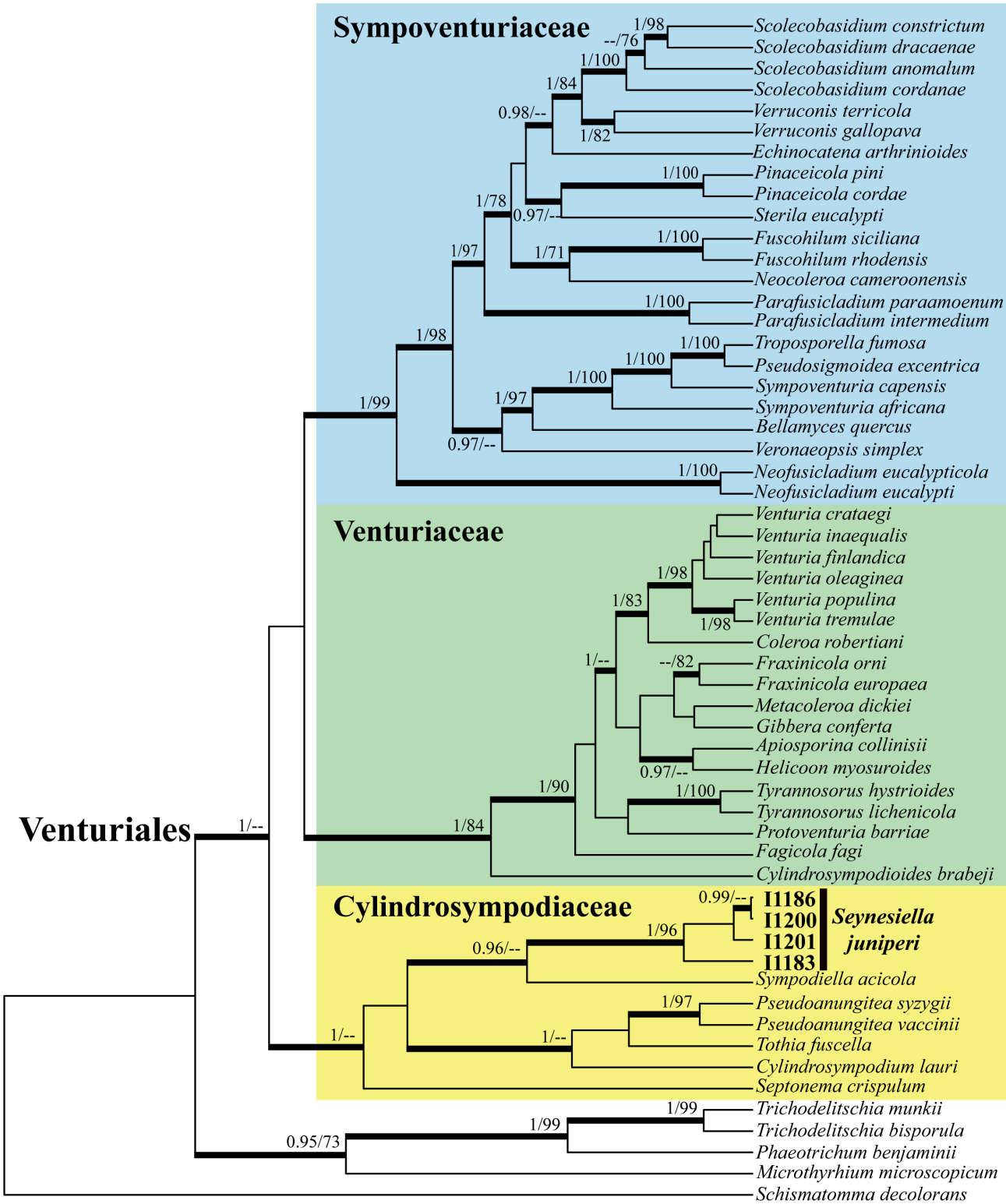

Figure 3. Ultrametric tree obtained with BEAST 1.8.1 showing the placement of the genus *Seynesiella* in the family Cylindrosympodiaceae within the order Venturiales, based on a five-locus dataset (5.8S, nuLSU, *tef1-α*, *RPB2* and β-tub). Numerals on branches represent Posterior Probabilities (PP, value on the left) and bootstrap support values (BS, RAxML analysis; value on the right) and denote significant statistical support (bold branches).

Diversity within the focal species Seynesiella juniperi

The ML phylogeny of *Seynesiella juniperi* nrITS sequences revealed three statistically-supported monophyletic clades Fig. 4B. One encompassed four specimens collected on *Juniperus oxycedrus* in the eastern and central Iberian Peninsula (BS= 71%). A sample collected on *J. thurifera* leaves in El Rincón de Ademuz (València, I1186) and another from Lisbon on *J. phoenicea* (I1183) were placed close to those four specimens, with a BS value of 93%. Two

other specimens collected on *J. phoenicea* in València and Alacant at approximately the same latitude than Lisbon were genetically more divergent (see samples I1201 and I1209 in the alignment Fig. 4D). Finally, the most genetically distinct specimen was collected on *J. communis* in Madrid (central Iberian Peninsula; see sample I1223 in Fig. 4D). Although the ABGD analysis did not reveal a clear barcode gap in the distribution of genetic distances (data not shown), the number of partitions (or putative species) inferred ranged between five (at values of P= 0.001292 to 0.001668), three (P= 0.002154 to 0.007743) and two (P= 0.010000). In Fig. 4B only the two- and three-putative species solutions are represented. If two species were assumed, specimens growing on *J. communis* would correspond to a distinct species to those occurring on *J. oxycedrus*, *J. thurifera* and *J. phoenicea*. Accepting the existence of three different species would mean separating the specimens growing on València and Alacant *J. phoenicea* into a third species. Considering the least conservative hypothesis of five species would imply that specimens occurring on different *Juniperus* species are also distinct; in that case, specimens occurring on western and eastern *J. phoenicea* would be also different taxa.

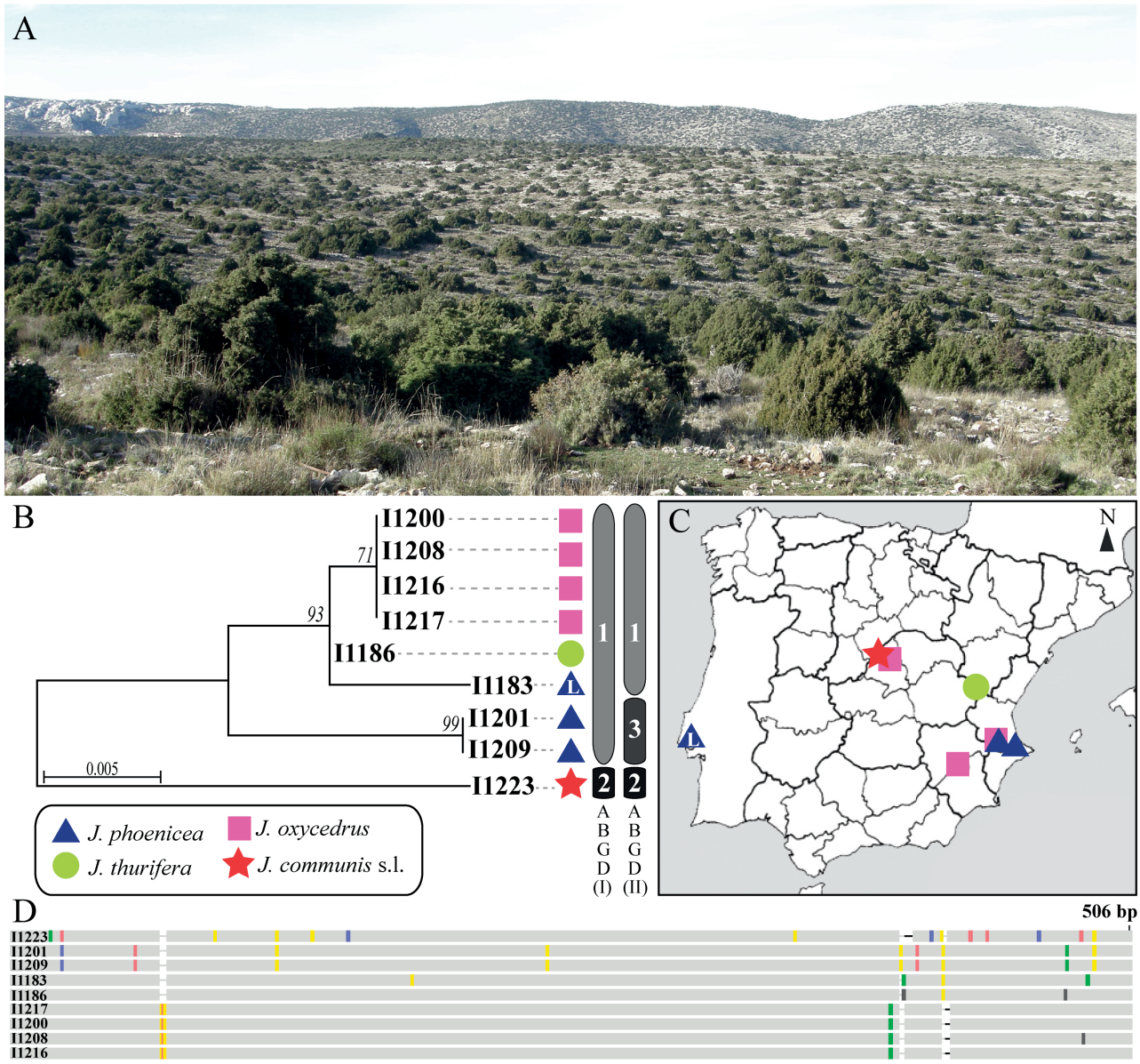

Figure 4. Habitat and genetic differentiation in *Seynesiella juniperi*. **A:** A mixed stand of *Juniperus oxycedrus* and *J. phoenicea* in El Sabinar (Moratalla, Región de Murcia). **B:** Phylogenetic relationships of selected *S. juniperi* specimens occurring on different *Juniperus* spp. based on nrITS data; each specimen is indicated by its DNA extraction code; columns on the right represent the results of the ABGD analysis, which segregated the focal species into two (option I) or three (II) putative species. **C:** Map of the Iberian Peninsula showing the geographic location of the samples included in the phylogeny. **D:** Screenshot of the nrITS alignment as represented in the software Geneious; boxes in different colors are mutations (each color represents a different nucleotide).

Discussion

The methodological approach conducted in the present work has allowed the phylogenetic placement of the genus *Seynesiella* in the Dothideomycetes Tree of Life. Based on data from five loci, the genus is placed in the family Cylindrosympodiaceae within the order Venturiales. This family was recently introduced in Shen et al. (2020) to include the genera *Cylindrosympodium* W.B. Kendr. & R.F. Castañeda, *Pseudoanungitea* Crous, *Sympodiella* W.B. Kendr. and *Tothia*, which contain saprophytic species usually forming hyphomycetous asexual structures on woody plant hosts (e.g., Pinaceae, Lauraceae, Myrtaceae and Ericaceae, Shen et al. 2020). The inclusion of the focal genus *Seynesiella* within this family spans the known morphological and reproductive variability of Cylindrosympodiaceae as this genus is recognized by reproducing sexually and forming typical thyriothecia. The genus' type species *S. juniperi* has been generally accepted as parasite on leaves of several species in Cupressaceae (Sierra López 2006; Hyde et al. 2013), although there are also reports of its growth on already dead leaves (Sierra López 2006; Wu et al. 2011). Therefore, the concept of Cylindrosympodiaceae may be expanded to include probable parasite species as well. The placement of *Seynesiella* in the order Venturiales contradicts earlier placements in the family Microthyriaceae (order Microthyriales) due to similarities in the type of ascomata and peridium structure (Luttrell 1965; Barr 1968; Lumbsch & Huhndorf 2010). Therefore, our results are more in line with the proposal of Müller & von Arx (1962) about a closer affinity with members of the family Venturiaceae, and also with Wu et al. (2011), who suggested a close similarity of *Seynesiella* with the thyriothecia-forming genus *Tothia*. However, our phylogenetic reconstruction suggests that *Seynesiella* and *Tothia* are not the closest genera within the family Cylindrosympodiaceae; instead, *Seynesiella* is sister to *Sympodiella*, whose type species, *S. acicola*, was described by Kendrick (1958) to introduce a hyphomycete genus inhabiting decaying needles of *Pinus sylvestris* L. Molecular data of additional species within the various genera in Cylindrosympodiaceae, including the five *Seynesiella* species described so far, are needed for deepening our understanding of the diversity and phylogenetic relationships within this family.

The analysis of intraspecific genetic diversity in *Seynesiella juniperi* based on Iberian Peninsula specimens points towards the existence of more than one species. Although the sampling only covered a reduced portion of the species' distribution, the considerably high number of variable positions in the nrITS alignment together with the observation of several well-supported clades in the ML phylogeny and the ABGD outcome supports the split of the species into two, three or even five distinct taxa. This result seems to indicate a case of cryptic speciation, i.e. two or more distinct species classified as a single species (Bickford et al. 2007), which is a common phenomenon in tiny fungi (e.g. Crous et al. 2004; Pringle et al. 2005; Alves et al. 2008). Although the studied specimens cannot be discriminated by macroscopical characters, microscopical and ecological traits (e.g. host identity) could be combined to provide a more straightforward species delimitation. Thus, central Iberian specimens on *Juniperus communis* are phylogenetically well separated from the remaining clades and produce larger asci and longer spores compared with the remaining specimens on other *Juniperus* spp. (Tab. 1). Similarly, phylogenetic isolation of *S. juniperi* specimens growing on *J. thuri-fera* may be supported by the production of smaller asci and spores. In contrast, the separation into distinct species of specimens growing on *J. oxycedrus* and *J. phoenicea* only seems feasible on the basis of phylogenetics, as the measured asci and spore size values are largely overlapping. However, the fact that genetic differences are relatively high among specimens occurring on *J. phoenicea* from Lisbon and eastern Iberian Peninsula suggests that genetic differentiation at the geographical scale may be playing an important role as well. The lack of a clear barcode gap found in the ABGD analyses might align with the latter observation, which would indicate that the overall genetic differences observed are just intraspecific and controlled by geography. In any case, to further evaluate whether morphological characters are informative enough for species separation, we re-commend performing more detailed measurements of spores and asci for specimens growing on different *Juniperus* hosts and test any differences statistically. In particular, measurements of the length and width of each of the two spore cells would be recommended (see Fig. 1L for reference).

The hypothesis of evolutionary events in *Seynesiella juniperi* recapitulating divergence events in the phylogeny of their *Juniperus* hosts has not been validated in the present work. Based on the reconstructed *Juniperus* spp. phylogenies of Mao et al. (2010) and Adams & Schwarzbach (2013), *J. communis* is close to *J. oxycedrus* and both belong in *Juniperus* section *Juniperus*, whereas *J. thurifera* and *J. phoenicea* are placed in *Juniperus* section *Sabina*. The phylogeny of the fungus, however, places specimens growing on *J. communis* away from those occurring on *J. oxycedrus*. This lack of concordance between fungal and host phylogenies could be an artifact of the molecular markers used, though. Moreover, our knowledge about the range of likely *Juniperus* hosts in the Northern Hemisphere to which *S. juniperi* associates is still limited, and therefore host switching may be a likely phenomenon occurring in this peculiar fungal-plant association.

Conclusion

The present work provides crucial information to understand the evolutionary history of *Seynesiella juniperi*, a tiny but fairly common ascomycete fungus growing on *Juniperus* spp. leaves. A denser sampling of specimens, localities and molecular markers is needed for further evaluating species boundaries in this fungus, as well as gaining a deeper understanding of the evolutionary events that shaped current geographic distributions and associations with different *Juniperus* hosts. In particular, it would be interesting to test whether the origin of the fungus occurred in a temporal window that coincides with the origin of its plant hosts.

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