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Multigene phylogeny coupled with morphological characterization reveal two new species of *Holmiella* and taxonomic insights within Patellariaceae

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Abstract – During our investigation of saprobic fungi on *Juniper* (Cupressaceae) of Uzbekistan, two novel species of *Holmiella* were collected from the host *Juniperus*. These new species are introduced based on morphological and molecular evidence, the latter generated to investigate their phylogenetic relationships. Taxonomic notes are also provided for the two previously described species of *Holmiella.* Phylogenetic reconstruction based on analyses of ribosomal DNA (ITS, LSU and SSU regions) of *Holmiella* species, strongly supports the three currently recognized taxa (*H. junipericola*, *H. juniperi-semiglobosae* and *H. sabina*) as a monophyletic group. The two novel species are morphologically distinct from *H. sabina*, the type species, with respect to size of ascomata, asci and ascospores, ascospore globules and peridium structure. Descriptions and illustrations, as well as notes on the

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taxonomy, and phylogenetic characterization of two new species are provided. An identification key to the four accepted species of *Holmiella* is also provided.

Ascomycetous microfungi / Central Asia / juniper trees / phylogeny / taxonomy / Patellariaceae / Uzbekistan

INTRODUCTION

The family Patellariaceae classified in the order Patellariales (Dothideomycetes) has been subjected to taxonomic research in recent years. Patellariaceae was introduced by Corda (1838) with three genera, *Cryptodiscus*, *Mellitiosporium*, and *Patellaria* (generic type) based on the structure of the apothecial fruiting bodies. The order Patellariales was established by Hawksworth & Eriksson (1986) which comprised only Patellariaceae. Kutorga and Hawksworth (1997) accepted 12 genera in Patellariaceae. Zhang and Hyde (2009) transferred the genus *Pseudoparodia* to Patellariaceae based on morphology. In the latest outline of Ascomycetes, the family Patellariaceae comprising 21 genera are accommodated in the order Patellariales (Wijayawardene *et al.* 2018). Species in Patellariaceae are saprotrophs or weak parasites on wood, and bark of a range of trees, and shrubs. They are characterized by superficial apothecioid ascomata, cupulate or discoid, exciple pseudoparenchymatous, pseudoparenchymatous or prosenchymatous hypothecium, with clavate to cylindric asci and usually 8-spored, bitunicate, fissitunicate, and ascospores varying in shape (Kurtoga & Hawksworth 1997, Yacharoen *et al.* 2015). The genus *Holmiella* was introduced by Petrini *et al.* (1979) with *H. sabina* (De Not.) Petrini, Samuels & E. Müll as type species and is characterized by apothecioid ascomata, a pseudoparenchymatous exciple and hypothecium, clavate asci and brown 1-septate ascospores. Currently, another species *H. macrospora* (Bonar & E.K. Cash) Kutorga & D. Hawksw is accommodated in this genus (Hyde *et al.* 2013). The asexual morph of *Holmiella* is unknown (Wijayawardene *et al.* 2017a). There have been very few molecular studies on members of Patellariaceae (Schoch *et al.* 2009, Hyde *et al.* 2013, 2014, Yacharoen *et al.* 2015).

In this study, several specimens were found on dead trunks and branches of *Juniperus semiglobosa* and *J. zerawschanica* from the mountains of Uzbekistan. After laboratory examination they were identified as species of *Holmiella*. In addition, phylogenetic analyses based on the nuclear large subunit (nLSU), nuclear small subunit (nSSU) and internal transcribed spacer (ITS) regions ribosomal RNA gene regions confirmed the identify of the two specimens as well as their placement within Patellariaceae. The two new species are described, illustrated and compared with similar taxa.

MATERIALS AND METHODS

Isolates and morphology

Samples were collected from dead trunks and branches of juniper trees in the mountain forests of Uzbekistan. Specimens were pressed and dried individually

between blotting papers and labelled. Samples were examined under a Motic SMZ 168 series microscope. Hand sections of the fruiting structures were made (Gupta *et al.* 2013) for morphological study and photomicrographs made with a Nikon Eclipse 80i compound microscope and Canon 600D digital camera using DIC microscopy. Measurements were made by the Tarosoft (R) Image Frame Work program v.0.9.7 and photoplates were made by Adobe Photoshop CS6 extended version 10.0 software (Adobe Systems, USA). Pure cultures were obtained from single spore isolation following the method designated in Chomnunti *et al.* (2014). Germinating single spores were transferred aseptically to malt extract agar (MEA) plates and incubated at 16°C. Cultures were grown for 2-4 weeks and morphological characters, such as colour, texture and colony characters were recorded. Holotype specimens of the two new species are deposited in Tashkent Mycological Herbarium (TASM) of the Institute of Botany, Academy of Sciences of Uzbekistan, Tashkent, Uzbekistan and isotype specimens are deposited in the Mae Fah Luang University Herbarium (MFLU), Chiang Rai, Thailand. Ex-type living cultures are deposited in the Culture Collection at Mae Fah Luang University (MFLUCC). Faces of Fungi and Index Fungorum numbers are provided as in Jayasiri *et al.* (2015) and Index Fungorum (2017). New taxa are established based on recommendations outlined by Jeewon and Hyde (2016).

DNA extraction, Amplification and Sequencing

Fungal isolates were grown on MEA media at 16 ± 2°C for 4 weeks. Total genomic DNA was extracted using the Biospin Fungus Genomic DNA Extraction Kit-BSC14S1 (BioFlux®, P.R. China). DNA amplifications were performed by Polymerase Chain Reaction (PCR) using known primer pairs, LROR/LR5 for nuclear large subunit rDNA (LSU) (Vilgalys & Hester 1990), NS1/ NS4 for SSU and ITS5/ ITS4 (White *et al.* 1990) for nuclear ITS regions. The amplification process was carried in a 50 µl reaction volume containing 2 µl DNA, 25 µl PCR mix, 19 µl distilled water 2 µl of each primer. The PCR reactions for amplification of ITS, LSU and SSU were carried out under standard conditions as outlined by Jeewon *et al* (2002, 2003). Purification and sequencing of PCR products with primers mentioned above were carried out at Shanghai Sangon Biological Engineering Technology and Services Co. (China).

Sequence alignment and phylogenetic analyses

Phylogenetic analyses were conducted on a combined dataset of LSU, SSU and ITS. Sequence data were downloaded from recent published data (Boehm *et al.* 2015) and some were obtained from GenBank to supplement the dataset (Table 1). The newly generated sequences were compared with other sequences in GenBank by nucleotide megablast searches. The multiple alignments were automatically performed by MAFFT v. 7. (http://mafft.cbrc.jp/alignment/server; 2016), using iterative refinements as E-INS-i method for ITS and G-INS-i method for LSU & SSU (Katoh & Standley 2013). The alignment was improved manually where necessary using Bioedit (Hall 1999). Maximum likelihood analysis was performed by using raxmlGUIv.0.9b2 (Silvestro & Michalak 2012) in the CIPRES Science Gateway platform (Miller *et al.* 2010). The search strategy was set to rapid bootstrapping and the analysis was carried out using the GTRGAMMAI model of nucleotide substitution. The number of replicates was inferred using the stopping

Table 1 Culture collection code and GenBank accession numbers of fungal strains used in this study.

Taxon	*Culture Accession No*[a]	*GenBank Accession No*		
		ITS	*LSU*	*SSU*
Anisomeridium ubianum	MPN94	KY486750	GU327709	JN887379
Cryomyces antarcticus	CCFEE 536		GU250365	GU250321
Cryomyces minteri	CCFEE 5187		KC315869	KC315858
Glyphium elatum	EB 0388	KM220946	KM220940	
Glyphium elatum	EB 0342	KM220945	KM220938	KM220935
Glyphium elatum	EB 0329		KM220937	KM220934
Glyphium elatum	EB 0365		KM220939	KM220936
Glyphium grisonense	EB 0376		KM220942	
Holmiella junipericola	**MFLUCC 18-0503**	**MH188902**	**MH188900**	**MH188901**
Holmiella juniperi semiglobosae	**MFLUCC 17-1955**	**MH188905**	**MH188903**	**MH188904**
Holmiella sabina	G.M. 2015-04-29.2	KY486750		
Hysteropatella clavispora	CBS 247.34		AY541493	AY541483
Hysteropatella elliptica	G.M. 2013-05-06 #01		KM220948	KM220948
Hysteropatella elliptica	CBS 935.97		DQ767657	EF495114
Hysteropatella prostii	H.B. 9934b	KT876980	KT876980	
Hysteropatella prostii	G.M. 2014-05-20 #01		KM220949	
Lichenothelia calcarea	L1324		KC015062	KR045803
Lichenothelia convexa	L1609		KC015071	KR045805
Patellaria atrata	BCC 28877	KM220950	GU371829	
Patellaria atrata	BCC 28876		KM220950	
Patellaria atrata	CBS 958.97		GU301855	
Yuccamyces citri	CBS 143161	MG386043	MG386096	
Yuccamyces pilosus	CBS 579.92		MG386097	

[a]**BCC**: BIOTEC Culture Collection, Bangkok, Thailand, **CBS**: Centraalbureau voor Schimmelcultures, The Netherlands, **CCFEE**: Culture Collection of Fungi from Extreme Environments, **EB**: E.W.A. Boehm, **G.M.**: Guy Marson, **H.B.**: H.O. Baral, **L:** Lucia Muggia, **MFLUCC**: Mae Fah Luang University Culture Collection, Chiang Rai, Thailand, **MPN**: Matthew P. Nelsen
Note: Sequences generated in this study are in bold.

criterion (Pattengale *et al.* 2009). Maximum-parsimony (MP) analysis was processed using PAUP v. 4.0b1027 to obtain the most parsimonious tree (Swofford 2003). Clade stability was assessed in a bootstrap analysis with 1000 replicates, random sequence additions with max-trees set to 1000 and other default parameters as implemented in PAUP. Parsimony bootstrap analyses were executed using the full heuristic search option, random stepwise addition, and 1000 replicates, with maxtrees set at 1000. Descriptive tree statistics for parsimony; Tree Length (TL), Consistency Index (CI), Retention Index (RI), Relative Consistency Index (RC) and Homoplasy Index (HI) were calculated for trees generated under different optimality criteria. Other details are outlined in Jeewon *et al.* (2003). The Bayesian inference

was conducted under different models for each partition of the matrix as evaluated by MrModeltest 2.2 (Nylander 2004). Six simultaneous Markov chains were run for 100 million generations and every 1000th generation a tree was sampled. MCMC heated chain was set with a "temperature" value of 0.15. All sampled topologies beneath the asymptote (25%) were discarded as part of a burn-in procedure and the remaining trees were used for computing posterior probabilities in the majority rule consensus tree. Bayesian Posterior Probabilities (BPP) equal or greater than 0.90 is given near to each node (Fig. 1). Phylogenetic data were viewed in MEGA v. 6 (Tamura *et al.* 2013), FigTree v. 1.4 (Rambaut & Drummond 2008). Facesoffungi numbers are as reported in Jayasiri *et al.* (2015). The new sequences were submitted in GenBank (Table 1). The alignment was deposited in TreeBASE (2018) under the accession ID 22550 (http://purl.org/phylo/treebase/phylows/study/TB2:S22550).

RESULTS

Phylogenetic inferences

The phylogeny derived from analyses of the combined LSU, SSU and ITS gene dataset from 17 taxa in Patellariaceae is shown in Fig. 1. Topologies of trees generated from analyses of single gene datasets were also compared and the overall tree topologies were congruent to those obtained from the combined dataset. The RAxML analysis of the combined dataset yielded a best scoring tree (Fig. 1) with a final ML optimization likelihood value of -7204.268166. The dataset consisted of 2269 characters of which 1753 were constant, 273 variables were parsimony uninformative characters and 243 were parsimony informative characters. *Anisomeridium ubianum* MPN94 was the outgroup taxon. The most parsimonious tree had scores as follows: TL = 833, CI = 0.783, RI = 0.709, RC = 0.555 and HI = 0.217. All *Holmiella* species are in a strongly monophyletic clade. *Holmiella junipericola* clustered with *H. sabina* with a strong support of 96% ML/ 97% MP/ 1.00 PP while *H. juniperi-semiglobosae* is basal to *Holmiella sabina* with 98% ML/ 99% MP/1.00 PP supports. The new sequence data is deposited in GenBank (Table 1).

Taxonomy

Holmiella juniperi-semiglobosae D. Pem, Gafforov, R. Jeewon & K.D. Hyde***, sp. nov.***

Mycobank Number – MB 825001*, Facesoffungi Number:* FoF 04586

Fig. 2

Etymology – Name reflects the host from which the fungus is isolated.

Holotype – TASM 6135

Saprobic on dead trunks and branches of *Juniperus semiglobosa*. **Sexual morph**: *Ascomata* 100-150 µm high × 200-500 µm diam. ($\bar{x}$ = 225.8 × 110.6 µm, n = 10), apothecial, solitary or in groups, cracking irregularly into 3-6 or more lobes, receptacle carbonaceous, fragile, gelatinous when rehydrated, fleshy, leathery in texture, black. *Exciple* 45-75 µm wide, ($\bar{x}$ = 62.6 µm, n = 10), pseudoparenchymatous

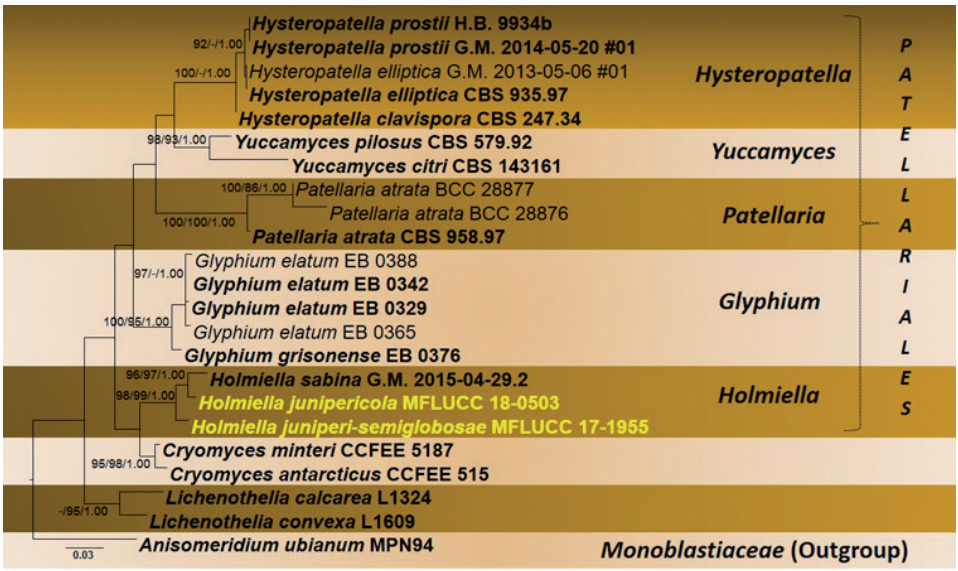

Fig 1. RAxML tree based on analyses of a combined dataset of LSU, SSU and ITS partial sequences. Bootstrap support values for ML and MP equal to or greater than 90%, Bayesian posterior probabilities (PP) equal to or greater than 0.95 are defined as ML/MP/PP above or below.

on the surface, merging into a thick, subhyaline plectenchyma up to 50 µm and thick near the base, narrower towards the margin, outer layer, to the sides composed of dark brown 11-30 µm thick, inner layer 25-50 µm, composed of thin walled hyaline to light brown cells of *textura globosa* merging with the host tissues. *Subhymenium* inverted conically, of marginally elongated and more or less horizontally oriented hyaline to brown cells. *Hypothecium* cannot be distinguished from the exciple. *Hamathecium* composed of 1-2 µm wide, filiform, branched, hyphal filaments, hyaline in the middle to light brown at the apex and agglutinated into a thick epithecium. *Asci* 91-196 × 29-48 µm ($\bar{x}$ = 134.6 × 35.8 µm, n = 10), 8-spored, clavate, bitunicate, fissitunicate short pedicellate, thick-walled, apex thickened, with an indistinct ocular chamber. *Ascospores* 20-35 × 12-18 µm ($\bar{x}$ = 26.5 × 15.3 µm, n = 10), 2-seriate overlapping, clavate to ellipsoidal, 1-septate at the middle, slightly constricted at the septum, ranging from hyaline when immature to light brown and dark brown when mature, slightly granulated, with a rounded apex. **Asexual morph**: Unidentified asexual structures of chlamydospores observed.

Culture characteristics: *Ascospores* germinating on MEA within 48 h, reaching 3 cm diam. in 1 week at 16°C. *Mycelium* superficial, medium dense, whitish to dark brown from above, from reverse dark brown in the middle to whitish at the edge, circular, flat, smooth edge, hairy, with a well-defined margin. On MEA (Figs 4b, 4c), colony comprising long, hyaline to brown, septate hyphae 2-2.6 µm wide. *Conidia* not observed. *Chlamydospores* 5.8-8.5 × 4.1-9 µm ($\bar{x}$ = 5.26 × 6.41 µm, n = 10), unicellular, globose to subglobose or ellipsoid, hyaline to pale brown, thick-walled, outer cell wall often rupturing before the formation of a new cell, arising directly on terminal hyphae or laterally, often remaining attached in terminal hyphae.

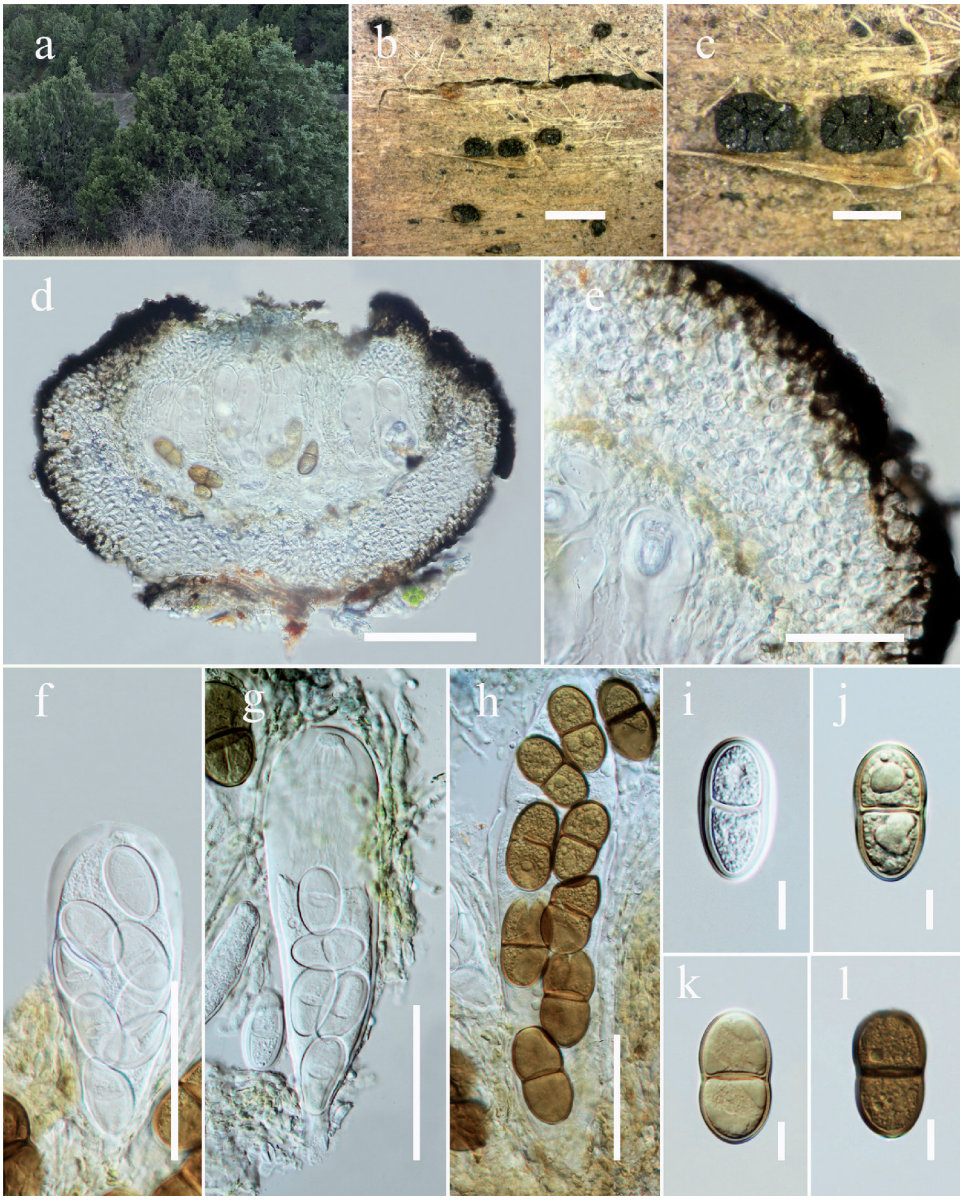

Fig 2. *Holmiella juniperi-semiglobosae* (TASM 6135**, holotype**). **a** Collection site. **b, c** Habit and appearance of ascomata on host surface. **d** Section through an ascoma. **e** Peridium. **f-h** Asci. **i, j** Immature ascospores **k, l** Mature ascospores. Scale bars: b = 1000 µm, c = 500 µm, d = 100 µm, e-h = 50 µm, i-l = 10 µm.

Distribution: Known only from the type locality.

Material examined: UZBEKISTAN, Tashkent Province, Bostanliq District, Beldorsoy, Katta Chimyon, Chatkal Range, Western Tien Shan Mountains, on dead trunks and branches of *Juniperus semiglobosa* (Cupressaceae), 7 May 2016, Y. Gafforov, YG-B39-1 (TASM 6135, **holotype**; MFLU 17-0090, **isotype**), ex-type living culture, MFLUCC 17-1955.

Notes: *Holmiella juniperi-semiglobosae* (MFLUCC 17-1955) was collected from *Juniperus semiglobosa* in Uzbekistan. Morphological characters such as apothecial, black ascomata, a pseudoparenchymatous exciple, bitunicate clavate asci and 1-septate dark brown ascospores, fit well within the concept of *Holmiella. Holmiella juniperi-semiglobosae* differs from *H. sabina* in having shorter asci (91-196 × 29-48 µm v/s 115-135 × 30-37 µm), smaller ascomata (200-500 × 100-150 µm v/s 365-500 × 300-600 µm), in the shape of ascospores (ovate to ellipsoid v/s clavate to ellipsoidal) and number of globules in each ascospore cell (2 to 3 globules in each cells v/s 1 globule in each cell). A comparison of 475 ITS (+5.8S) nucleotides between *H. juniperi-semiglobosae* and *H. sabina* reveals a 5.8% base pair difference, which justifies the establishment of the new taxon (Jeewon & Hyde 2016) in *Holmiella*.

Holmiella junipericola D. Pem, Gafforov, R. Jeewon & K.D. Hyde***, sp. nov.***

Mycobank Number – MB 825002*, Facesoffungi Number:* FoF 04585

Fig. 3

Etymology – Name reflects the host from which the fungus is isolated.

Holotype – TASM 6136

Saprobic on dead trunks and branches of *Juniperus zerawschanica*. **Sexual morph**: *Ascomata* 500-2000 µm high × 200-1000 µm diam. ($\bar{x}$ = 1168.5 × 958.6 µm, n = 10), apothecial, solitary, sessile, superficial to erumpent, exposing a velvety hymenium when mature, round to elliptical, opening either by a longitudinal slit or cracking irregularly into 3-6 or more lobes to expose a flat dark brown to black disc, sub-gelatinous when rehydrated, with smooth outer surface of the receptacle, black. *Exciple* consisting of a dark-brown, leathery layer, 25-45 µm thick ($\bar{x}$ = 37.9 µm, n = 10), pseudoparenchymatous, two-layered, outer layer thick, comprised of dark brown to black, slightly cracked cells of *textura angularis*, inner layer, thin, composed of light brown cells of *textura epidermoidea*. *Subhymenium* inverted conically, of marginally elongated and more or less horizontally oriented brown cells. *Hypothecium* prosenchymatous consisting of *textura intricata*, light brown, 10-45 µm tall below the subhymenium base to 0.5 mm diam. *Hamathecium* of 1-2 µm wide, filiform, branched, interwoven hyphal filaments, forming a dark brown epithecium above the asci. *Asci* 110-145 × 38-41µm, ($\bar{x}$ = 128 × 39.8 µm, n = 10), 8-spored, clavate to broadly clavate, bitunicate, fissitunicate, with an ocular chamber in the apical dome. *Ascospores* 29-40 × 10-19 µm ($\bar{x}$ = 34.3 × 14.4 µm, n = 10), irregularly 2-3 seriate, overlapping, ellipsoidal, 1-septate in the middle and slightly constricted at the septum, a single globule in each cell, ranging from hyaline when immature to light brown and dark brown when mature, with a hyaline papilla at the ends, upper cell slightly wider than lower cell, thick and smooth-walled. **Asexual morph**: Unidentified asexual structures of chlamydospores observed.

Culture characteristics – *Ascospores* germinated on MEA within 48 h, reaching 2 cm diam. in 1 week at 16°C. *Mycelium* superficial, from above grey to dark brown, reverse dark brown at the margin to black in the middle, medium sparse, irregular, raised, smooth surface, edge crenate. On MEA (Figs. 5b, 5c), colony comprising long hyaline to pale brown, septate hyphae 1.5-4 µm wide. *Conidia* not

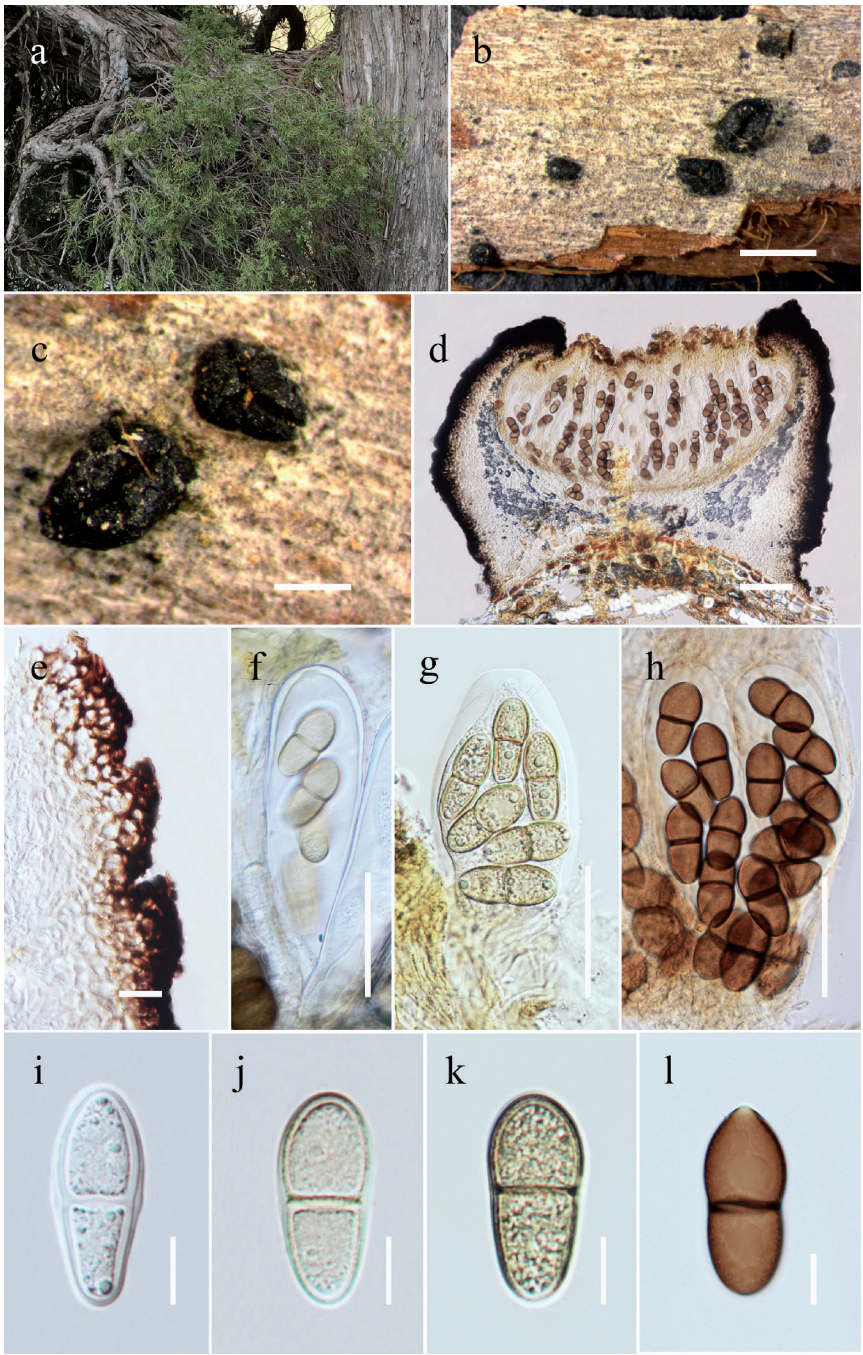

Fig 3. *Holmiella junipericola* (TASM 6136, **holotype**). **a** Collection site. **b, c** Habit and appearance of ascomata on host surface. **d** Section through an ascoma. **e** Peridium. **f-h** Asci. **i-l** Ascospores. Scale bars: b = 1000 µm, c = 500 µm, d = 50 µm, e = 20 µm, f-h = 50 µm, i-l = 10 µm.

observed. *Chlamydospores* 4-6.5 µm × 5.5-7 µm ($\bar{x}$ = 5.26 × 6.41 µm, n = 10), unicellular, globose to subglobose or ellipsoid, hyaline to pale brown, thick-walled, with outer cell wall often rupturing before the formation of a new cell, arising directly on terminal hyphae or laterally or produced at the apex of another chlamydospore, often remaining attached to form a short chain of 2 or in terminal hyphae.

Material examined. UZBEKISTAN, Surxondaryo Province, Boysun District, Qizilnaur Village, South-Western Hissar Mountains, on dead trunks and branches of *Juniperus zerawschanica* (Cupressaceae), 14 May 2016, Y. Gafforov, YG-S096 (TASM 6136, **holotype**; MFLU 17-0054, **isotype**), ex-type living culture, MFLUCC 18-0503.

Distribution. Known only from the type locality.

Notes: *Holmiella junipericola* was found on dead trunks and branches of *Juniperus zerawschanica*. It morphologically resembles *H. sabina* (type species), in having apothecioid black ascomata cracking irregularly to expose a flat dark brown to black disc, a hypothecium of *textura intricta*, bitunicate asci and dark brown ascospores with a hyaline papilla at the ends. However, *H. junipericola* differs from *H. sabina* in having larger ascomata (500-2000 × 200-1000 µm v/s 365-500 × 300-600 µm), the number of globules in each ascospore cell (2-3 globules v/s 1 globule) and ascospore apex shape (slightly pointed at the apex v/s rounded apex). On multi-gene sequence analyses, our new isolate MFLUCC 18-0503 clusters with *H. sabina* with strong bootstrap support (98%ML, 99%MP, 1.00PP). However, a comparison of 475 nucleotides across the ITS (+5.8S) gene regions reveals 21 base pair differences. Hence, *H. junipericola* is introduced as a new species in the genus *Holmiella.*

DISCUSSION

The genus *Holmiella* was established by De Not. (1979) for the single species *Triblidium sabinum* De Not. (1867) and is currently known as *Holmiella sabina* and can be considered as a discomycete (apothecial ascomycete) *sensu* Ekanayake *et al.* (2017). Species of *Holmiella* show morphological similarities to those of *Rhytidhysteron* Speg. (1881) and were traditionally grouped together with the genera *Tryblidiella* and *Eutryblidiella* (Kutorga & Hawksworth 1997). However, it has now been shown that these taxa are unrelated and *Holmiella* and belong to Patellariaceae in the genus *Patellaria* (Ekanayake *et al.* 2017, Wijayawardene *et al.* 2018). *Holmiella* is characterized by apothecioid black ascomata, a flat disc with a dentate margin, hypothecium of *textura intricata*, thick epithecium, bitunicate asci, brown 1-septate ascospores with germ pores and appears to be specific to *Juniper* trees (Kutorga & Hawksworth 1997). Currently, only two species are accommodated in *Holmiella* (Wijayawardene *et al.* 2017b) and DNA sequence data is available only for *H. sabina*, hence we could compare our new species phylogenetically only to *H. sabina*.

In this study, we provide taxonomic details for two new species, *Holmiella junipericola* and *H. juniperi-semiglobosae*. The taxonomic affinities of species within Patellariaceae warrant further studies given the paucity of sequence data in GenBank. Phylogenetic investigations herein, despite limited taxon sampling, provide some insights into the taxonomy of Patellariaceae species (Fig 1). Our

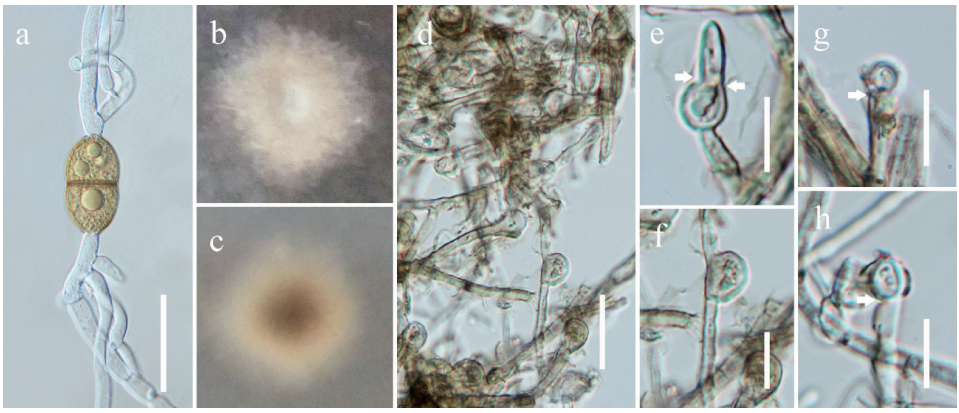

Fig. 4. Chlamydospores and culture of *H. juniperi-semiglobosae* (MEA, 16°C). **a** Germinated ascospore on MEA. **b, c** culture characters on MEA. (**b**: from above, **c**: reverse view). **d-h** Chlamydospores and hyphae. Arrows indicate ruptures of the thick outer cell wall of chlamydospores before development of a new cell. **Scale bars**: a = 40 µm, d = 20 µm, e-g = 10 µm.

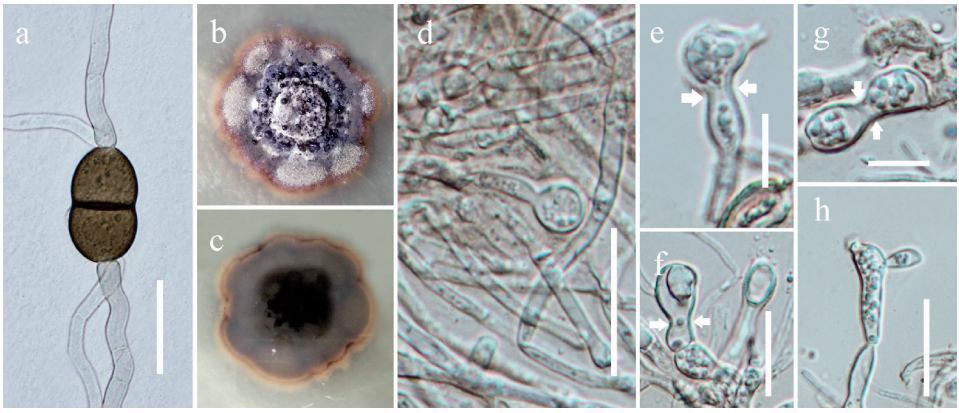

Fig. 5. Chlamydospores and culture of *H. junipericola* (MEA, 16°C). **a** Germinated ascospore on MEA. **b, c** Culture characters on MEA (**b**: from above, **c**: reverse view). **d-h** Chlamydospores and hyphae. Arrows indicate ruptures of the thick outer cell wall of chlamydospores before development of a new cell. **Scale bars**: a = 30 µm, d, h = 20 µm, e, g = 10 µm, f = 15 µm.

phylogenetic tree shows that *Holmiella* constitutes a strongly supported monophyletic clade, basal to *Glyphium* which is also a member of the Patellariaceae. Our new taxa fit well with the diagnosis of *Holmiella* as previously determined by other taxonomists (Kutorga & Hawksworth1997, Yacharoen *et al.* 2015). A close phylogenetic affiliation of *H. junipericola* to *H. sabina* was found. This would make sense taxonomically as both of them are similar in having a dark brown exciple composed of *textura globulosa-angularis* at the exterior and *textura epidermoidea* at the interior layer, a prosenchymatous hypothecium consisting of *textura intricata*, clavate, bitunicate asci and dark brown, 1-septate ascospores. However, *H. junipericola* differs remarkably from *H. sabina* based on size of ascomata, globule number in each ascospore and ascospore shape. Moreover, *H. junipericola* was isolated from *Juniper*

zerawschanica, while *H. sabina* was isolated from *Juniper macropoda* (Table 2). *Holmiella juniperi-semiglobosae* is also similar to *H. sabina* in having clavate, bitunicate asci, dark brown, 1 septate ascospores, but is different in having shorter asci, smaller ascomata, in the shape of its ascospores and number of globules in each ascospore cell. Also, the hypothecium in *H. juniperi-semiglobosae* cannot be distinguished from the exciple, while a brown prosenchymatous hypothecium of *textura intricata* can be seen in *H. sabina*. Our new taxa can also be compared to *H. macrospora* based on its clavate bitunicate asci, dark brown, 1-septate ascospores and germ pore at each end of the ascospores. *Holmiella macrospora* is also remarkably different from the two new species in the size of ascomata, asci and ascospores. A comparison of the morphological characters of *Holmiella* species is provided in Table 2. DNA sequence analysis of another larger dataset with the

Table 2. Morphological characteristics that differentiate the new species from recorded *Holmiella* species

Species	*Habitat/ Host*	*Ascospores color/ shape/septation*	*Size (μm)*			
			Ascospores	*Asci*	*Ascomata*	*Exciple (thick)*
H. sabina	*Juniperus macropoda* (Cupressaceae)	Light brown when immature, dark brown when mature, clavate to ellipsoidal, 1-septate, and constricted near median septum.	33-40 × 17-20	115-135× 30-37	365-500 × 300-600	22-58
H. macrospora	*Libocedrus decurrens* (Cupressaceae)	Dark brown, broadly ellipsoid, 1-septate in the middle and slightly constricted at the septum	50-70 × 25-33	150-230 x 55-65	700-1000 × 500-700	25-40
H. junipericola	*Juniperus zerawschanica* (Cupressaceae)	Hyaline when immature, dark brown when mature, ellipsoidal to elongated, 1-septate in the middle and slightly constricted at the septum	29-40 × 10-19	110-145 × 38-41	500-2000 × 200-1000	25-45
H. juniperi-semiglobosae	*Juniperus semiglobosa* (Cupressaceae)	Hyaline when immature, dark brown when mature, ovate to ellipsoid, 1-septate in the middle and very slightly constricted at the septum.	20-35 × 12-18	91-196 × 29-48	200-500 × 100-150	45-75

inclusion of several more sequences, revealed that *Fungal* sp. (ARIZ AZ0676B, HM123390 U'ren *et al.* 2010) and Botryosphaeriales sp. (AM262-P6T8T; KT264537, Torres Cruz *et al.* unpublished) also cluster within the *Holmiella* clade. Both *Fungal* sp. and Botryosphaeriales sp. are related to *H. juniperi-semiglobosae*. *Fungal* sp is an unidentified endophyte, hence the morphological details cannot be compared. Further comparison of the ITS nucleotide sequences of *Fungal* sp. and Botryosphaeriales sp to *H. juniperi-semiglobosae* reveals a difference of 4.6% and 5.0% base pair differences respectively. The affinities of these species to *Holmiella* is interesting, but cannot be explained herein. It could also be possible that *Homiella* is not a monophyletic genus or there is need for a taxonomic reassessment of the *Fungal* sp. (ARIZ AZ0676B, HM123390) and Botryosphaeriales sp. (AM262-P6T8T; KT264537). It is noteworthy that both of our new species produce

Ascospores germ pores/ globule	*Exciple (Pseudoparenchymatous*	*Hypothecium*	*Asexual morph*	*Source References*
Germ pores at both ends A single globule in each cell	- Outer layer of textura globulosa angularis - Inner layer textura epidermoidea.	Prosenchymatous composed of loose textura intrictata, colourless	Cornicuriella-like	Yacharoen *et al.* (2015) Kutorga and Hawksworth (1997)
A polar germ pore in each end A single globule in each cell	Unreported	Prosenchymatous of textura intricate, brown.	Unreported.	Kutorga and Hawksworth (1997)
Germ pore in each end A single globule in each cells	- Outer layer textura globulosa angularis - Inner layer textura epidermoidea.	Prosenchymatous consisting of textura intricate, light brown	Formation of chlamydospores.	This study
Not visible, 2−3 globules in each cells	Outer and Inner layer of textura globulosa	Hypothecium cannot be distinguished from the exciple.	Formation of chlamydospores.	This study

chlamydospores in culture and such observations have not been reported so far from other *Holmiella* species. Our molecular based phylogeny also highlights some peculiar taxonomic findings pertaining to the familial placement of *Yuccamyces*. This asexual genus was established by Gour *et al.* (1979) and is characterized by sporodochial conidiomata composed of walls of *textura intricata* and *textura oblita*, with holoblastic, hyaline, cylindrical conidia (Dyko and Sutton 1979). The two species analyzed herein are *Yuccamyces pilosus* and *Yuccamyces citri*. There has been speculation concerning the placement of *Yuccamyces* and recently Crous *et al.* (2017) suggested an affinity to the genus *Hysteropatella*, but it has been treated as Patellariales *incertae sedis* (Crous *et al.* 2017). As indicated in the phylogeny herein, our results are in agreement with Crous *et al.* (2017) and a close relationship to *Hysteropatella* is reported with strong support (Fig 1). However, given that *Yuccamyces* is nested between *Hysteropatella* and *Patellaria*, which are members of the Patellariaceae, we believe that the most probable family that *Yuccamyces* should be accommodated in is Patellariaceae. It is recommended to collect more samples and analyse more DNA sequence data to better resolve taxonomic relationships of Patellariaceae species. The discovery and detailed description of the two new species will help in the further delimitation of the genus in Patellariaceae.

KEY TO EXTANT SPECIES OF *HOLMIELLA*

1. Ascospores < 40 µm long, < 20 µm wide, on *Juniperus*..... ***2***
1. Ascospores > 50 µm long, > 20 µm wide, on *Libocedrus*..... ***H. macrospora***
2. Hypothecium in section colourless..... ***H. sabina***
2. Hypothecium in section brown..... ***3***
3. Ascomata 200-500 × 100-150 µm, ascospores without visible germ pares, containing 2-3 globules in each cell..... ***H. juniperi-semiglobosae***
3. Ascomata 500-2000 × 200-1000 µm, ascospores with germ pores at each end, with a single globule inside each cel ..... ***H. junipericola***

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