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简 报

Punctularia atropurpurascens (*Punctulariaceae*, *Basidiomycota*): a new record to China

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Abstract: [Background] A fluffy fungal fruitbody (No. HMNWAFU-CF-HS002) was collected from the rotten stumps of *Paulownia tomentosa* in Shaanxi province, China. **[Objective]** Morphological description and molecular identification were carried out to determine its taxonomic status. **[Methods]** Macroscopic features were imaged, while microstructures were measured, counted, and drawn. Living culture was isolated and purified with PDA, and phylogenetic analysis of rDNA internal transcribed spacer (ITS) was performed with maximum likelihood (ML), maximum parsimony (MP), and bayesian inference (BI) methods. **[Results]** The morphological characteristics of HMNWAFU-CF-HS002 were highly similar to those of *Punctularia atropurpurascens*. Phylogenetic analysis showed that HMNWAFU-CF-HS002 nested into the *P. atropurpurascens* clade. **[Conclusion]** Based on the morphological evidences and phylogenetic results, HMNWAFU-CF-HS002 was identified as *P. atropurpurascens*, a new record species in China. *P. tomentosa* is a new record host species for *P. atropurpurascens*. So far, all the three species of *Punctularia* have been recorded in China.

Keywords: *Punctularia atropurpurascens*, biological resource, morphology, rDNA ITS phylogeny, new record species

担子菌门总革菌科中国新记录种紫黑点壳菌

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摘 要：【背景】在陕西省的毛泡桐腐木桩上观察和采集到一株绒毛状真菌子实体，编号为

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HMNWAFU-CF-HS002。【目的】为了确定该菌的分类地位，对其进行形态观察及分子鉴定。【方法】获取宏观彩色图像并对显微结构进行测量、统计和绘图。此外，用 PDA 培养基分离纯化该菌的培养物，并结合最大似然法、最大简约法和贝叶斯法进行 rDNA ITS 分子系统学研究。【结果】HMNWAFU-CF-HS002 的形态特征与 *Punctularia atropurpurascens* 高度相似。系统发育分析将 HMNWAFU-CF-HS002 聚在 *P. atropurpurascens* 的单系分支中。【结论】结合形态学特征与系统发育结果，HMNWAFU-CF-HS002 被鉴定紫黑点壳菌(*P. atropurpurascens*)，为中国的新记录种。此外，毛泡桐为其新记录寄主。至此，*Punctularia* 属下的 3 个种，均在中国有分布记录。

关键词：紫黑点壳菌，生物资源，形态学，rDNA ITS 分子系统学，新记录种

1 Introduction

The genus *Punctularia* consists of merely three accepted species, *P. atropurpurascens* (Berk. & Broome) Petch, *P. bambusicola* C.L. Zhao, and *P. strigosozonata* (Schwein.) P.H.B. Talbot[1-3]. In China, *Punctularia bambusicola* is reported from Yunnan province[2], *P. strigosozonata* is found in Shanxi province and Jilin province[4-5], but *P. atropurpurascens* has not been reported[6-7] or listed in the data-base Checklist of Fungi in China (http://fungalinfo.im.ac.cn/) till now. Its known distribution involved few countries in Africa, Asia, Europe, North America and South America[8-11] (https://www.gbif.org), and India[12] is the only Asia country documenting this species.

Studies on *P. atropurpurascens* involved morphological features[9], growth cycles[10], degradation function[13-14], antifungal and medicinal components, which have garnered huge attention in recent years due to its ecological, economical and medical values. For example, Anke et al.[15] extracted phlebiakauranol aldehyde from cultures of *P. atropurpurascens*, which exhibited strong antifungal, antibacterial and cytotoxic activities against several phytopathogens; Alborés et al.[16] detected and purified a novel lectin with specific compatibility towards *N*-acetyl-glucosamine from mycelia; latterly, Alborés et al.[17] continued extracting a laccase from the extracellular extract of *P. atropurpurascens*, which was able to degrade Remazol Brilliant Blue R and Acid Blue 25 dyes. In this study, we firstly reported this high-valuable *P. atropurpurascens* in China, and described its eye-catching morphologies, rDNA ITS phylogenetic relationship with the homogenesis species, and its growth cycles and hosts in details.

2 Materials and Methods

2.1 Materials

Wugong county, located in western Guanzhong plain in Shaanxi province, belongs to the semi-humid monsoon belt of warm temperate zone. Its average annual precipitation and sunshine duration are 552.6–663.3 mm and 2 094.9 h, respectively. From south to north, with an elevation from 415 m to 600 m, there are four parallel landforms, Weihe beach, terraced plain, loess tableland, and front and depression of piedmont pluvial fan. Weishui River, commonly known as back river in Wugong county, is a tributary of Weihe river, which traced back to the Yellow river[18].

The studied specimens were collected beside Weishui river. Voucher specimens were deposited in the Mycological Herbarium of Forestry College, Northwest A&F University (HMNWAFU-CF). Tiny sections were cut from the fruitbody and incubated on the potato-dextrose agar (PDA) medium at 25 °C in the dark for fungal isolation. About 5 days later, emerging fungal mycelia were transferred into fresh PDA plates to develop purified living cultures[19].

2.2 Morphological observation

The colorful photographs were captured by LUMIX G8M during each stage in the growth cycles, and fungal histological sections were observed at magnification up to 1 000× using an Olympus CX41 microscope. The microscopic observation followed Guan et al.[2] and Cui et al.[20]. Hand drawings were implemented with the aid of a digital pen tablet (GAOMON WH850) and the software Adobe Photoshop 2020. Slides of basidiocarps were stained by Cotton Blue and Melzer's reagent for microscopic observation, measurement and drawing. Conidia from the culture and basidiospores from basidiocarps were

observed and measured randomly. To determine the size variation of spores, 5% of the data were excluded from each end of the measurement range and noted in parentheses. In the text, the following abbreviations are used: IKI=Melzer's reagent, IKI–=inamyloid and indextrinoid, KOH=5% potassium hydroxide, CB=Cotton Blue, CB–=acyanophilous reaction of CB, *L*=mean spore length (arithmetic average of all spores), *W*=mean spore width (arithmetic average of all spores), *Q*=variation in the *L*/*W* ratios, *n*=number of spores from the given numbers of specimens. The width of a basidium was measured at its thickest part, and the length was measured from the apex (sterigmata excluded) to the basal septum. Terminological description of color followed Petersen[21].

2.3 DNA extraction and PCR amplification

A total of 0.02 g thalli from the specimen and a pure colony were separately ground to extract the genomic DNA by CTAB method according to Doyle et al.[22] and Qi et al.[23]. The concentrations of DNA extract from specimen and colony are 535.960 ng/μL ($OD_{260/280}$=1.95) and 462.237 ng/μL ($OD_{260/280}$=1.90), respectively. Internal transcribed spacer (ITS) regions were amplified using the universal primers ITS1/ITS4[24]. The PCRs were carried out in a final volume of 30 μL, containing 15 μL 2×*Taq* PCR MasterMix (CoWin Biosciences, China), 1.5 μL of 10 μmol/L each primer (Shanghai Sangon Biotech Company Limited, China), 3 μL template DNA, and 9 μL ddH_2O. Thermal cycling was implemented in a GeneAmp PCR TC-96 instrument (Bioer Technology Company Limited, Hangzhou, China) for pre-denaturation at 94 °C for 3 min, a subsequent PCR cycle lasting for 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C. Such 35 cycles and a final extension at 72 °C for 10 min were carried out until the procedure was terminated at 4 °C. The PCR products were then sequenced by Shanghai Sangon Biotech Company Limited, China, with primers ITS1 and ITS4 same as used in PCR reaction.

2.4 Sequence alignment and phylogenetic analysis

Sequences were assembled by BioEdit 7.2.5 (Bioedit Limited, UK), searched by BLAST, compared in the NCBI database and deposited in GenBank (Accession No. MW556264, MW556465). Other homologous sequences used for phylogenetic analysis were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), UNITE (https://unite.ut.ee) and Mushroom Observer (https://mushroomobserver.org/)[9-10]. All sequences were aligned using the MUSCLE algorithm in MEGA-X (https://www.megasoftware.net/home), and were manually adjusted to allow maximum alignment and minimum gaps, with all positions containing gaps or missing data eliminated completely. Two sequences of *Vuilleminia alni* Boidin, Lanq. & Gilles downloaded from GenBank were used as the outgroup. Aligned sequence matrix was exported as FASTA and MEGA format. Phylogenetic trees were constructed with maximum-likelihood (ML), maximum parsimony (MP) and bayesian inference (BI) methods.

Topologies were constructed based on ML analysis with MEGA formatted file[25]. Twenty-four different nucleotide substitution models were considered, and the model with the lowest bayesian information criterion (BIC) score was chosen as the best-fit evolutionary model. Initial trees for the heuristic search were obtained automatically by applying neighbor-joinning and BioNJ algorithms to calculate a matrix of pairwise distances, which was further estimated using the maximum composite likelihood (MCL) approach; and the topology with superior log-likelihood value was selected. The MP tree was obtained by using the subtree-pruning-regrafting (SPR) algorithm[26] with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates). Both MP and ML bootstrap analysis were conducted 1 000 replicates by software MEGA-X[27].

The FASTA formatted file was firstly transformed into NEXUS format using SequenceMatrix 1.8 and then was applied for calculating BI using MrBayes V3.1.2[28-29]. Four Markov chains were run from random starting trees for 1 000 000 generations till the average standard deviation of split frequencies was below 0.01, and trees were sampled every 100 generations. The first 25% of the sampled trees were discarded as burn-in and the remaining ones were used to reconstruct a majority-rule consensus and to calculate BI posterior probabilities (Bpps) of the clades. Tree reconstruction,

visualization and editing were implemented with FIGTREE V1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and MEGA-X. The Bpps were followed by bootstrap values of ML and MP at the nodes in the topology. Branches that received bootstrap support for ML (≥75%), MP (≥75%) and Bpp (≥0.95) were considered as significantly supported[20].

3 Results

3.1 Phylogenetic analysis

Twenty-three sequences of rDNA ITS were employed to construct the phylogenetic tree, eight of which were downloaded from UNITE (prefix with UDB), twelve from GenBank, one from Mushroom Observer (without prefix) and two newly generated from our collections. Tamura 3-parameter model was estimated as the best-fit evolutionary model for the aligned dataset. ML analysis generated a tree topology that was almost identical to the MP and Bayesian tree. Phylogenetic analysis revealed that two sequences from the specimen (Accession No. MW556264) and the colony (Accession No. MW556465) of the HMNWAFU-CF-HS002 were finely fitted in the *Punctularia atropurpurascens* clade, which included ITS sequences from Italy, India, USA, South Africa and Costa Rica, and formed an independent sub-clade (Figure 1).

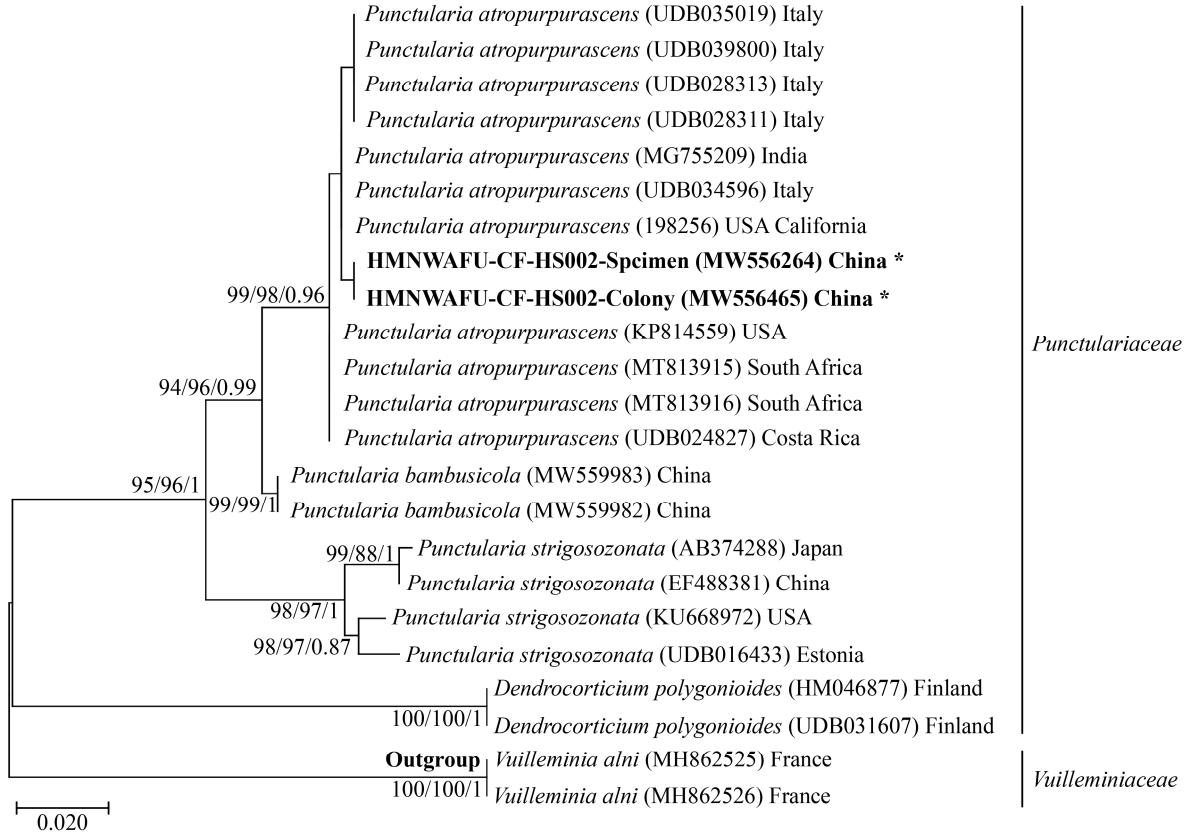

Figure 1 Phylogenetic tree constructed by the maximum-likelihood (ML) method based on sequences of the ITS region of nuclear ribosomal DNA

图 1 基于 rDNA ITS 区序列构建的最大似然法系统发育树

Note: The phylogenetic tree is drawn to scale, with branch lengths measured in the number of substitutions per site[27]. The Bayesian inference posterior probabilities (Bpps) are followed by bootstrap values of ML and maximum parsimony (MP) at the nodes in the topology. Sequence data are shown with species name, accession number in GenBank or UNITE (prefix with UDB) or Mushroom Observer (without prefix), and location of the voucher specimen

注：发育树按比例绘制，用每个位点的替换次数来衡量树枝的长度[27]。在拓扑结构的节点处标出了最大似然法(ML)、最大简约法(MP)的自展值，以及贝叶斯后验概率(Bpps)。序列信息有：物种名称、GenBank 或 UNITE (前缀为 UDB)或 Mushroom Observer (无前缀)登录号，以及凭证标本所在地

3.2 Taxonomy

Punctularia atropurpurascens (Berk. & Broome) Petch, Ann. R. bot. Gdns Peradeniya 6: 160, 1916. Figure 2

=*Thelephora subhepatica* Berk., London J. Bot. 5: 3, 1846.

≡*Thelephora atropurpurascens* Berk. & Broome, J. Linn. Soc., Bot. 14: 64, 1873.

=*Corticium tuberculosum* Pat., in Patouillard & Lagerheim, Bull. Soc. mycol. Fr. 8: 118, 1892.

=*Punctularia tuberculosa* (Pat.) Pat. & Lagerh., Bull. Herb. Boissier 3: 57, 1895.

=*Punctularia subhepatica* (Berk.) Hjortstam, Mycotaxon 54: 191, 1995.

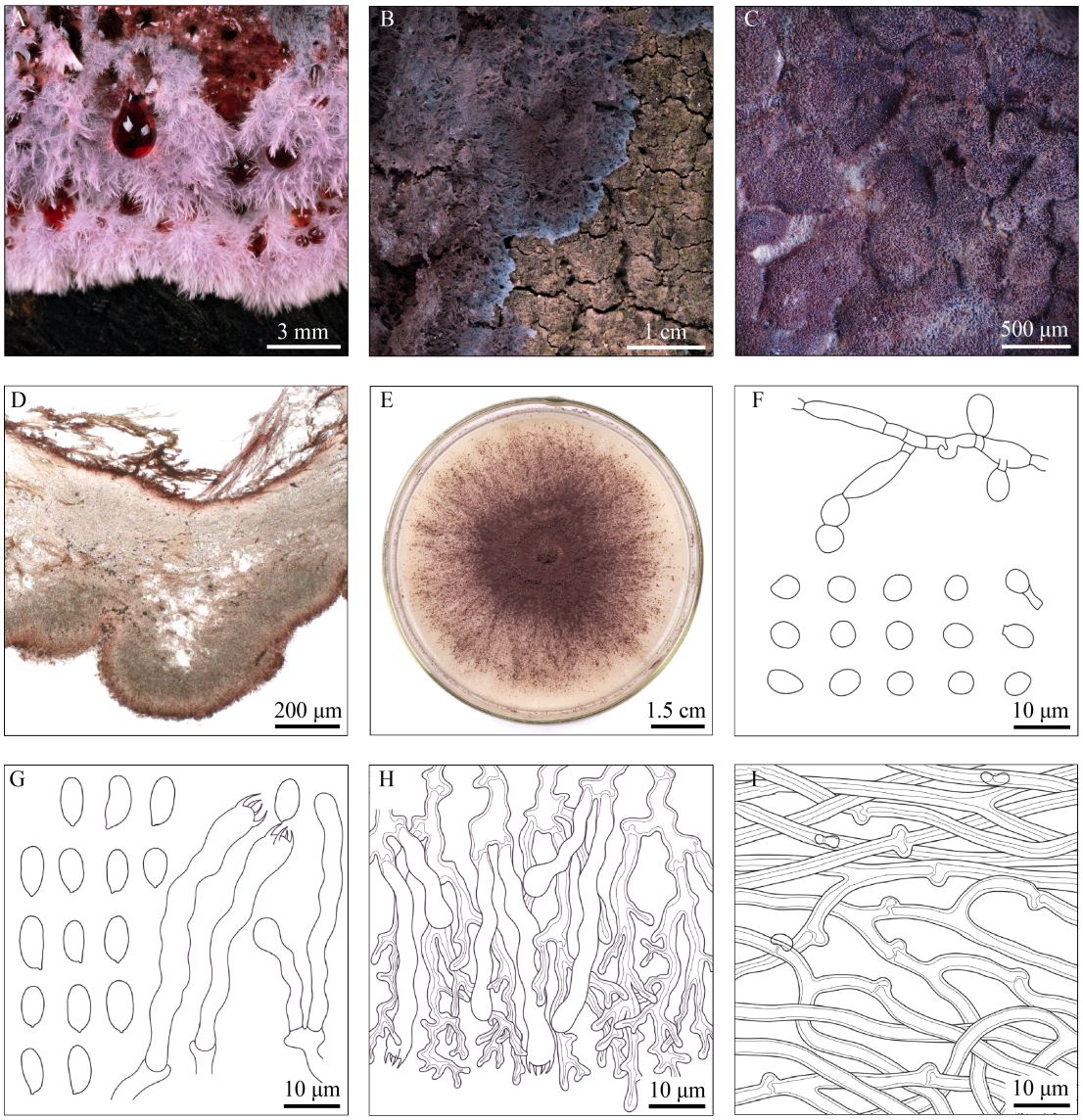

Figure 2 Features of *Punctularia atropurpurascens* (drawn from HMNWAFU-CF-HS002)

图 2 紫黑点壳菌形态特征(绘自 HMNWAFU-CF-HS002)

Note: A: Morphological characteristics in autumn with red droplets secreted; B: Morphological characteristics in winter; C: Hymenium; D: Vertical section through the basidiocarp; E: Colony; F: Conidia and segments of supporting hyphae; G: Basidiospores, basidia and basidioles; H: Subhymenial generative hyphae, dendrohyphidia, basidia and basidioles; I: Subicular generative hyphae and abhymenial generative hyphae

注：A：秋季形态特征，伴有红色液滴渗出；B：冬季形态特征；C：子实层；D：担子果切片；E：菌落；F：分生孢子及支撑菌丝片段；G：担孢子、担子及幼担子；H：子实基层生殖菌丝、树状子实层端菌丝、担子及幼担子；I：菌丝层生殖菌丝及远子实层生殖菌丝

Fruitbody—Basidiocarps effused to effuse-reflexed, adherent on the substrate, in patches about a few centimeters across, up to 1 mm thick. Subiculum thin layered, 17–84 (–105) μm thick, fibrose, coffee brown to dark brown. Hymenium surface papillate or shallowly tuberculate, becoming crustose to corneous when dry, reddish brown to dark purplish brown. Superior surface cottony, fluffy, membranous, light pink to rose, with reddish aqueous droplets secreted from pores in autumn; compact, woolly, partly powdered, pinkish purple to purplish brown in winter.

Hyphal structure—Hyphal system monomitic, generative hyphae with clamp connections. Subicular generative hyphae 2.3–3.9 μm wide, with thick solid walls, yellowish brown to coffee brown, sometimes with multiple clamp connections on the single septum. Abhymenial generative hyphae 1.8–3.7 μm wide, thick-walled, branched distantly from septa, hyaline. Subhymenial generative hyphae 1.9–4.3 μm wide, thin-walled to slightly thick-walled, subhyaline or very pale brown, frequently branched.

Hymenium—Dendrohyphidia numerous, luxuriant, dendritic or coralloid, up to 75 μm long, 3.0–6.6 μm wide, with thick walls and very narrow or absent lumens at tips, ochraceous to coffee brown, darker from bases to extremities. Basidia tubular or clavate, flexuose, infrequent, (39.1–66.3) μm×(5.1–6.9) μm; 4 sterigmata up to 5 μm long. Basidioles similar to basidia in shape, but smaller in size, without obvious sterigmata. Cystidia and cystidioles absent.

Spores—Basidiospores infrequent, ellipsoid to narrowly ellipsoid, (7.0–9.2 (–9.9)) μm×(3.5–4.6 (–4.9)) μm in size, *L*=8.0, *W*=4.0, *Q*=2 (*n*=30/1), smooth, thin-walled, hyaline. Conidia prevalent, abundant, in branched chains, subglobose to ellipsoid, sometimes irregular, (4.3–6.9 (–7.8)) μm×(3.6–4.6 (–4.8)) μm, *L*=5.5, *W*=4.1, *Q*=1.3 (*n*=30/1), with thick walls, pale brownish to pale purplish brown, with lipid bodies inside, breaking off from each one and showing the remains of the linking walls at both ends, sometimes shed with segments of supporting hyphae.

Colonies—Colonies tufted, pulverulent, violet at first, becoming dark reddish-brown later, with white margin, forming a thick mass of conidia.

Chemical reaction—CB–; IKI–; tissues darkening in KOH.

Specimen examined—China. Shaanxi province, Xianyang city, Wugong county, Weishui river, on rotten stumps of *Paulownia tomentosa* Steud., 4 October 2020 & 3 January 2021, Peng ZJ, Yu ZD & Luo ZY (HMNWAFU-CF-HS002).

4 Disscussion

For the first time, the species *P. atropurpurascens* was reported in China based on morphological and phylogenetic evidences in this study. Phylogenetically, *P. atropurpurascens* is more closely related to *P. bambusicola* than *P. strigosozonata* based on rDNA ITS sequence analysis (Figure 1), which is in accordance with that of Knijn et al.[9] and Guan et al.[2]. Morphologically, the genus *Punctularia* lacks cystidia and cystidioles, while usually has obvious sterile dendrohyphidia, however, three species in this genus displayed diverse morphologies in basidiocarps thickness, color of hymenial surface and margin, basidia size, and dendrohyphidia length. *P. atropurpurascens* appeared to be thicker basidiocarps (up to 1 mm), larger basidia ((39.1–66.3) μm×(5.1–6.9) μm) and longer dendrohyphidia (up to 75 μm) than *P. bambusicola*, of which is 100–300 μm thick, (17.5–21.5) μm×(4.0–5.5) μm size and up to 42 μm long[2], respectively. The hymenial surface in the former is reddish brown to dark purplish brown or bluish[10], but from pink to rose in the latter. And both of them have basidiocarps with more or less whitish fibrillose margin[2,9-10]. *Punctularia strigosozonata* differs from the above two species in its resupinate to effuse-reflexed basidiocarps, with orangish brown to maroon or dark violaceous hymenial surface and brown velutinous margin[2,30-31]. Among them, only *P. atropurpurascens* developed the distinct structure of subiculum. Interestingly, *P. atropurpurascens* displays morphological changes during the annual growth cycle[10]. Two cycles were found under the different habitat humidity, a long one in wet conditions and a short one under drought stress[10]. The superior surface of *P. atropurpurascens* showed pink feathery morphology and exuded reddish aqueous droplets from pores in autumn (Figure 2A), but compact, woolly, partly powdered, pinkish purple to purplish brown without exudates when given an enough humidity in winter (Figure 2B), which were as same as Knijn et al.[9] reported. In addition, Knijn et al.[10] reported that these droplets were composed of phlebiarubrone derivatives and lipid molecules

involved in various cellular signal transduction pathways in fungi and plants[32-35].

All three species of *Punctularia* displayed excellent abilities of degradation and were considered as white rot saprobes according to the previous studies[2,9-10,14]. *Punctularia strigosozonata* was found on branch of *Pistacia*, *Populus* and *Quercus*[3,31], *P. bambusicola* on a dead bamboo, *P. atropurpurascens* on *Acacia*[1-2], *Celtis australis* L.[10], *Juglans regia* L.[4], *Mangifera*[1-2], *P. radiata* D. Don[14], *Platanus acerifolia* (Aiton) Willd., *Quercus ilex* L., *Q. cerris* L., and *Q. suber* L.[10]. Additionally, we found *P. atropurpurascens* can colonize and gravely decompose the stumps of *P. tomentosa*, an indigenous tree in Guanzhong Plain. This is the first report of the novel host *P. tomentosa* in China.

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