Phylogenetic analyses, morphological studies, and muscarine detection reveal two new toxic *Pseudosperma* (*Inocybaceae, Agaricales*) species from tropical China

Li-Na Zhao¹ · Wen-Jie Yu¹ · Lun-Sha Deng¹ · Jian-Hua Hu¹ · Yu-Peng Ge² · Nian-Kai Zeng¹ · Yu-Guang Fan¹

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Abstract
As a result of molecular phylogenetic analyses and morphological studies, two new species of the genus *Pseudosperma*, namely *P. fulvidiscum* and *P. singulare*, were discovered in Wuzhishan Nature Reserve of Hainan Province of China. The two new species are distinct from other known *Pseudosperma* species in the phylogram inferred from a combined nuclear ribosomal internal transcribed spacer region (ITS), the nuclear ribosomal large subunit (nrLSU), and the nuclear second-largest subunit of RNA polymerase II (RPB2) sequence data. Muscarine contents of the two new species were detected using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) approach. In addition, a new combination, *P. rubrobrunneum*, was proposed based on phylogenetic evidence.

Keywords *Inocybaceae* · Molecular phylogeny · New taxa · Toxin detection · Muscarine

Introduction

*Inocybaceae* is a family of ectomycorrhizal mushroom-forming fungi (Matheny and Bougher 2006), and many species in this family are poisonous (Kosentka et al. 2013). Multigene molecular phylogenetic analyses have now culminated in the recognition of at least seven major clades in the family (Matheny et al. 2020). *Pseudosperma* is a newly established genus in *Inocybaceae* and is supposed to contain muscarine, a neurotoxin that would cause a series of muscarinic symptoms in humans and animals (Deng et al. 2021b). Mushroom poisoning incidents caused by *Pseudosperma* species were reported in various regions around China (Li et al. 2022). The recognition of its species diversity, distribution, and toxin type and contents will be helpful to the prevention of poisoning incidents.

*Pseudosperma* is characterized by the rimulose to rimose pileus, the furfuraceous to appressed furfuraceous stipe with a pruinose apex, spermatic odor, elliptic to subphaseoliform basidiospores, the absence of pleurocystidia, the presence of thin-walled cheilocystidia, and symbiosis ecology with a large number of vascular plants (Matheny et al. 2020). At present, 93 taxa of *Pseudosperma* were recorded in the IndexFungorum database (www.indexfungorum.org, retrieved 26 Mar. 2022), and 40 of those were reported or originally described from Europe (Bandini and Oertel 2020). The species diversity in other continents was poorly addressed, especially in tropical areas. After the erection of the
genus, 16 new taxa were described in the past two years (Yu et al. 2020; Cervini et al. 2020; Saba et al. 2020; Jabeen and Khalid 2020; Jabeen et al. 2021; Bandini and Oertel 2020; Bandini et al. 2021). Currently, seven species of *Pseudosperma* were reported in China (Bau and Fan 2018; Yu et al. 2020), including four recently described species, viz. *P. neoumbrinellum*, *P. yunnanense* (Bau and Fan 2018), *P. citrinostipes* (Yu et al. 2020), and *P. arenarium* (Yan et al. 2022, in press).

Hainan is the southernmost provincial administrative region of China and is located on the north edge of the Chinese tropics. Central Hainan harbors the largest contiguous area of tropical rainforest and is a hotspot area of biodiversity in China (Zong 2020). Many new fungal species have been discovered in Hainan province every year (Wang et al. 2022, in press). In this study, we aim to describe two new *Pseudosperma* species using combined analyses of molecular, morphological, and ecological data. In addition, a comprehensive method of determination of these two new species using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) approach has been performed to detect their muscarine contents.

**Materials and methods**

**Field sampling and morphological studies**

Fresh materials were collected in the Wuzhishan and Yinggeling substations of Hainan Tropical Rainforest National Park and Fujian Province in China. Firstly, basidiomata were photographed using a digital camera in the field. Secondly, fresh specimens were recorded and described macroscopically later. Then, the specimens were dried overnight at 45 °C with an electronic drier, and the dried specimens were packed in sealed plastic bags with silica gel (Yu et al. 2020; Deng et al. 2021a). Color codes follow Kornerup and Wanscher (1978).

Microscopical characteristics were observed using an optical microscope (Olympus CX23) in the laboratory. Mushroom tissues from the pileus, the lamellae, and the stipes were cut into thin sections by freehand with the aid of a stereoscope (AV100-240V). Dried materials were rehydrated in KOH (5%) and stained with Congo Red (1%) when necessary. Microscopic structures, including basidiospores, basidia, cheilocystidia, hymenophoral trama, pileipellis, and stipitipellis, were examined and measured. Methods of measurements follow Fan and Bau (2013) and Yu et al. (2020). At least 100 basidiospores for each species were randomly measured from different specimens. The basidiospore measurements are expressed with the \([n/m/p]\), which indicates \(n\) basidiospores measured from \(m\) basidiomata of \(p\) specimens. In addition, the basidiospore size is given with the \((a) b–e–c\) (d) formula, where \(b–c\) contains a minimum of 90% of the measured values of the spores, \(e\) represents the average values of the spores, and \(a\) and \(d\) represent the minimum and maximum values of the spores (Ge et al. 2021). \(Q\) is the ratio length/width of individual spores \((Q\) value); \(Q_m \pm SD\) is the average \(Q\) of all basidiospores ± sample standard deviation (Na et al. 2022; Jean et al. 2022). Examined specimens were deposited in the Herbarium of Changbai Mountain Natural Reserve (ANTU) with FCAS numbers and the Fungal Herbarium of Hainan Medical University (FHMU).

**DNA extraction, PCR, and sequencing**

In this study, the ITS, nrLSU, and *RPB2* gene fragments were analyzed. Genomic DNA was extracted using the NuClean Plant Genomic DNA kit (ComWin Biotech, Beijing), and the extracted DNA products were generally stored at −20 °C. Primers used for PCR amplification and sequencing include ITS1-F and ITS4 for ITS (White et al. 1990), LR0R and LR7 for nrLSU (Vilgalys and Hester 1990), and *RPB2*-6F and *RPB2*-7.1R for *RPB2* (Matheny 2005). The amplification reaction mixture (final volume 25 μL) contained the following: 9.5 μL ddH2O, 12.5 μL 2×Taq Plus MasterMix (Dye), 1 μL forward primer, 1 μL reverse primer, and 1 μL DNA template. Amplification reactions were performed in a TPProfessional Standard thermocycler (Biometra, Göttingen, Germany) under the following program: 5 min at 95 °C; 1 min at 95 °C; 30 s at 65 °C (add −1 °C per cycle); 1 min at 72 °C; cycle 15 times; 1 min at 95 °C; 30 s at 50 °C; 1 min at 72 °C; cycle 20 times; 10 min at 72 °C (Wang et al. 2021b). Sequencing work was commissioned to the Beijing Genomics Institute. Sequencing results were read with BioEdit version 7.0.9.0 software and assembled with SeqMan software. The newly generated sequences of new species were submitted to GenBank.

**Alignment assembly and phylogenetic analyses**

Sequences obtained from previous studies (Pradeep et al. 2016; Bau and Fan 2018; Yu et al. 2020; Cervini et al. 2020; Saba et al. 2020; Jabeen and Khalid 2020; Jabeen et al. 2021; Bandini and Oertel 2020; Bandini et al. 2021) and the Blastn results of close matches from GenBank were selected for phylogenetic analyses. *Nothocybe distincta* and *Mallowcybe terrigena* were used as outgroups. The dataset for each locus was aligned by MAFFT online service (https://mafft.cbrc.jp/alignment/server/) (Katoh et al. 2019) and manually adjusted by BioEdit version 7.0.9.0 (Hall 1999). The three datasets were concatenated by MEGA 6.0 (Tamura et al. 2013). Maximum likelihood (ML) analyses were conducted in W-IQ-TREE Web Service (http://iqtree.cibiv.univie.ac.at/) with 1000 replicates (Trifinopoulos et al. 2016). The best evolutionary model of each gene partition
was selected using MrModeltest v2.3 with the Akaike information criterion for Bayesian analyses (Nylander 2004). Bayesian inference (BI) analyses were performed using MrBayes v3.2.7a (Ronquist et al. 2012). Four Markov Chain Monte Carlo (MCMC) chains were sampled over 5,000,000 generations, sampling every 1000 generations, the standard deviation below 0.01, and BI posterior probabilities (BI-PP) were determined after removing the first 25% of trees. The results were edited by using the FigTree v1.4.3 software. The ML bootstrap proportions (ML-BP ≥ 70) and BI-PP (≥ 95%) were shown on each branch.

**Toxin detection**

Dried mushroom samples from holotypes of the two new species (FYG6311 and FYG6363) were ground into a fine powder; 0.02 g was accurately weighed and put into a 5-mL centrifugation tube with 2 mL of methanol-water (5:95, v/v), respectively. The mixture was vortexed for 30 min at first and then was extracted ultrasonically for another 30 min. After centrifugation at 1000 rpm for 5 min, the total supernatant was collected using a 0.22-μm organic filter membrane to filtrate before UPLC-MS/MS analysis and diluted with acetonitrile-water (7:3, v/v) when necessary. *Lentinula edodes* was used as the blank sample. The optimal UPLC and MS parameters and other settings followed Xu et al. (2020). The muscarine content was estimated in the mushroom extraction by using standard muscarine (Sigma-Aldrich, Chemical purity ≥ 98%). The analytical results are reported as mean ± SD g/kg, where the mean is the average content of muscarine, and SD represents the standard deviation.

**Results**

**Phylogenetic analyses**

We newly generated 16 ITS, 7 nrLSU, and 7 *RPB2* sequences and submitted them to GenBank. The best-fit models selected by MrModeltest for each gene are GTR+I+G equally. The concatenated dataset (Supplementary information) comprises 144 taxa (Table 1) and 3180 nucleotide sites with 958 bp ITS, 1445 bp nrLSU, and 777 bp *RPB2*, of which 1814 are constant and 884 are parsimony informative. The phylograms resulted from Bayesian inference (BI) and maximum likelihood (ML) analyses are similar with a few statistical differences, and thus, only the ML tree is shown in Fig. 1. All the *Pseudosperma* taxa were clustered in a full support clade (BP = 100%, PP = 1). Two major subclades were retrieved in *Pseudosperma*, and one of them was only supported by ML analysis. Thirteen specimens form an independent lineage with full support (BP = 100%, PP = 1), representing *P. fulvidiscum*, and three specimens cluster together and form an independent lineage representing *P. singulare*. The two new species are grouped into a full support subclade (BP = 100%, PP = 1) that is sister to all the remainders of the genus. The subclade unifying *P. araneosum* (Matheny and Bougher) Matheny and Esteve-Rav., *P. aff. araneosum* (PL58410), *P. brunneosquamulosum* K.P.D. Latha and Manim., *P. rubrobrunneum* (K.P.D. Latha and Manim) Y.G. Fan, and *P. sp.* (MCA562) as well as the two new species. In this subclade, *P. aff. araneosum* (PL58410) is sister to *P. fulvidiscum*, and *P. araneosum* is sister to *P. singulare*. *Pseudosperma brunneosquamulosum* and *P. sp.* (MCA562) clustered in a full support lineage that is sister to the lineage unifying *P. fulvidiscum* and *P. aff. araneosum* (PL58410); *P. rubrobrunneum* is sister to the lineage unifying *P. singulare* and *P. araneosum*.

**Taxonomy**

*Pseudosperma fulvidiscum* Y.G. Fan, L.N. Zhao, and W.J. Yu, sp. nov., Figs. 2 and 3

**MycoBank:** MB843662

**Etymology:** “fulvidiscum” refers to its brown to smoky brown pileus.

**Diagnosis:** *Pseudosperma fulvidiscum* differs from *P. brunneosquamulosum* by its relatively larger habit, pallid stipes with brownish tiers of squamules in the lower part, broader lamellae, longer and usually subcapitate cheilocystidia, and its association with *Carpinus* trees.

**Holotype:** China. Hainan Province: Wuzhishan Nature Reserve, 109°40′E, 18°51′53″N, 688 m asl., 29 July 2021, leg. Y.-G. Fan, L.-N. Zhao, L.-S. Deng, and J.-H. Hu, FYG6311 (FCAS3513).

**Description:** Basidiomata small. Pileus 5–20 mm diam., spherical to hemispherical when young, becoming convex to applanate with an umbo upon expansion; margin initially incurved, then decurved for a long time, straight or uplifted when overmatured; surface dry, wooly fibrillose when young, then radially fibrillose-rimulose to appressed scaly, rimose to strongly split in age; yellowish brown (5C8) to brownish (6E6) or dark brown (6F5) around the center, gradually shallowing to yellowish (5B6) to pale yellowish-brown (5B4) outwards, uniformly brown (6D5) to dark brown (6E5) when overmatured; background creamy yellowish (5B2). Lamellae adnexed to emarginate, moderately crowded to subdistant, 1.3–3.5 mm wide, unequal in length, alternately distributed with 3–4 tiers of lamellulae, white (1A1) to creamy white (1A2) when young, then turns pale yellow (5A2) to yellowish-brown (5B3), and reddish ochraceous-brown (6C5) when overmatured, edge pallid, fimbriate. Stipe 7–41 mm in length, 1.3–3 mm in width, terete, solid and becoming fistulose upon maturity, slightly swollen at the apex, somewhat tapered at the base, pruinose at the apex, fibrillose with sparse protruding whitish (1A1) fibrils downwards to the
| Taxa                                      | Collection number (Herbarium) | Locality          | GenBank accession number | ITS    | LSU    | RPB2   |
|-------------------------------------------|-------------------------------|-------------------|--------------------------|--------|--------|--------|
| Mallocybe terrigena                      | JV16431                       | Finland           |                          | AM88264 AM380401  | AM833309 |
| Nothocybe distincta                       | CAL-1310 (holotype)           | India             |                          | KG171343  EU604546  | KG171345 |
| ’Pseudosperma bruneicohurnata             | PBM1889 (TENN)                | USA               |                          | JQ408787  JQ319707  | JQ846493 |
| P. aestivum                               | BK18089706 (UTC, holotype)    | USA               |                          | EU600847  EU600846  | EU555465 |
| P. aff. araneosum                         | PL58410                       | Australia         |                          | KJ729880  KJ729906  | KJ729937 |
| P. aff. reisiporum                        | PBM12195 (PERTH-E7042)       | Western Australia |                          | JQ408771  EU555466  | EU555465 |
| P. aff. rimosum                           | TR183-05                      | PNG               |                          | JQ408773  JN975005  | JQ815425 |
| P. aff. rimosum                           | TR75-05 (M)                  | PNG               |                          | JQ408774  JQ815425  | JQ815425 |
| P. aff. sororium                          | ADW0057/TENN063512           | USA               |                          | JQ408778  JN975004  | JQ421076 |
| P. aff. sororium                          | REH8245 (NY)                 | Costa Rica        |                          | JQ408783  JN975004  | JQ421076 |
| P. albobrunneum                           | LAH135047 (holotype)         | Pakistan          |                          | MG495392  |        |        |
| P. alboflavellum                          | TGB11280 (isotype)           | India             |                          | KP636859  KP171058  | KM656097 |
| P. amabile                                | BAN3100                       | Austria           |                          | MW010036  |        |        |
| P. amabile                                | BAN3100                       | Germany           |                          | MW010031  |        |        |
| P. amoris                                 | BAN2931 (holotype)            | Germany           |                          | MW010038  |        |        |
| P. amoris                                 | BAN2913                      | Germany           |                          | MW010037  |        |        |
| P. araneosum                              | PBM3755 (isotype)             | Australia         |                          | KJ729878  KJ729904  | KJ729937 |
| P. arenarium                              | NXYC20201005-01 (holotype)   | China             |                          | OM304278  OM304287  | OM304287 |
| P. arenarium                              | NX20210922-57                | China             |                          | OM304279  OM304287  | OM304287 |
| P. arenicolans                            | SX20210930-65                | France            |                          | FJ904134  FJ904134  | FJ904134 |
| P. arenicola var. mediterrineum            | JV14920F (WTU)               | Italy             |                          | JQ408748  JQ408748  | JQ408748 |
| P. aureocitrinum                          | BAN2903                      | Spain             |                          | MW010047  |        |        |
| P. aurora                                 | AU10245 (WTU)                | Canada            |                          | HQ201337  HQ201338  | HQ201338 |
| P. aurora var. inodoratum                 | Sz5573                       | USA               |                          | MH024847  |        |        |
| P. breviterincarnatum                     | BK28080407                   | USA               |                          | EU555451  EU555451  | EU555451 |
| P. breviterincarnatum                     | PB1914                       | USA               |                          | JQ408750  JQ319677  | JQ846465 |
| P. bruneosquamulosum                      | CAL 1308 (holotype)          | India             |                          | KX073582  KX073586  | KX073586 |
| P. brunneoumbonatum                       | MSM0053 (holotype)           | Pakistan          |                          | MG742419  MG742420  | MG742422 |
| P. brunneoumbonatum                       | MSM00545                    | Pakistan          |                          | MG742421  MG742422  | MG742422 |
| P. bulbissimum                            | EL6605                       | Norway            |                          | AM882765  AM882765  | AM882765 |
| P. bulbissimum                            | EL75-07                      | Sweden            |                          | FJ904160  FJ904160  | FJ904160 |
| P. cercocarpell                           | BK20069806 (UTC)             | USA               |                          | EU600889  EU600889  | EU600889 |
| P. cf. flavellum                          | EL2010a/PAM05062502          | France            |                          | FJ904128  FJ904128  | FJ904128 |
| P. cf. microfastigiatum                    | EL113-06                     | Sweden            |                          | FJ904156  FJ904156  | FJ904156 |
| P. cf. rimosum                            | PBM2958                      | USA               |                          | JQ408777  JQ42071  | JQ42071  |
| P. cf. rimosum                            | PC080925                     | UK                |                          | FJ904153  FJ904153  | FJ904153 |
| P. cf. rimosum                            | PAM05061101                 | France            |                          | FJ904155  FJ904155  | FJ904155 |
| P. cf. rimosum                            | PBM2574 (TENN)               | USA               |                          | JQ408776  EF561633  | EU307858 |
| P. cf. rimosum                            | JV8125                       | Finland           |                          | FJ904152  FJ904152  | FJ904152 |
| P. cf. rimosum                            | JV22619                      | Estonia           |                          | FJ904157  FJ904157  | FJ904157 |
| P. cf. rimosum                            | EL-2010d/JV26578             | Estonia           |                          | FJ904154  FJ904154  | FJ904154 |
| P. citrinostipes                          | FGY2903                      | China             |                          | MT072897  MT071202  | MT071202 |
| P. citrinostipes                          | FGY2909 (holotype)           | China             |                          | MT072898  MT071203  | MT071203 |
| P. conviviale                             | AMB18243 (holotype)          | Italy             |                          | MT095091  MT095115  | MT095115 |
| P. dulcamaroides                          | EL29-08                      | USA               |                          | FJ904127  |        |        |
Table 1 (continued)

| Taxa                        | Collection number (Herbarium) | Locality       | GenBank accession number |
|-----------------------------|-------------------------------|----------------|--------------------------|
|                             |                               |                | ITS | LSU | RPB2 |
| *P. emberizanum*            | STU:SMNS-STU-F-0901461        | Germany        | MW647630 |
| *P. fissuratum*             | PBM2206 PERTH (E7054) (holotype) | Western Australia | JQ408770 | AY732213 | JQ421069 |
| *P. flavellum*              | EL13705                       | Sweden         | AM882776 |
| *P. flavorinumosum*         | LAH35042 (holotype)           | Pakistan       | MG495391 |
| *P. friabile*               | PBM3914/TEEN068384 (holotype) | USA            | MH216095 | NG_067823 |
| *P. fulvidiscum*            | FYG6331                       | China          | OM1135589 |
| *P. fulvidiscum*            | FYG6288                       | China          | OM1135590 |
| *P. fulvidiscum*            | FYG6310                       | China          | OM1135591 |
| *P. fulvidiscum*            | FYG6311 (holotype)            | China          | OM1135592 | OM349998 | OM747849 |
| *P. fulvidiscum*            | FYG6058                       | China          | OM1135593 | OM349999 | OM780121 |
| *P. fulvidiscum*            | FYG6067                       | China          | OM1135594 | OM350000 | OM780119 |
| *P. fulvidiscum*            | FYG6096                       | China          | OM1135595 |
| *P. fulvidiscum*            | FYG6099                       | China          | OM1135596 |
| *P. fulvidiscum*            | FYG6321                       | China          | OM1135597 | OM350001 | OM780120 |
| *P. fulvidiscum*            | FYG6327                       | China          | OM1135598 |
| *P. fulvidiscum*            | FYG6328                       | China          | OM1135599 |
| *P. fulvidiscum*            | FYG6308                       | China          | OM1135600 |
| *P. fulvidiscum*            | FYG6379                       | China          | OM1135601 |
| *P. godfrinioides*          | 371                            | Italy          | JF908099 |
| *P. gracilissimum*          | PBM3735                       | Australia      | KJ729919 | JF904137 |
| *P. gracilissimum*          | NLB937                        | Australia      | KP171122 | KJ801178 | KJ729946 |
| *P. griseorubidum*          | CAL1253                       | India          | KT180326 | KT180327 | KT180328 |
| *P. himalayensis*           | K7 (holotype)                 | Pakistan       | MH745138 |
| *P. holoxantha*             | ACAD:11683                    | Canada         | MH024853 | MH024884 |
| *P. huginii*                | STU:SMNS-STU-F-0901564 (holotype) | Austria   | NR_173974 | MW647628 |
| *P. hygrophorus*            | EL97-06                       | Sweden         | FJ904137 | FJ904137 |
| *P. illudens*               | II06 (TENN:065726)           | Australia      | JQ408769 | JQ319699 | JQ421068 |
| *P. keralense*              | K(M):191712 (holotype)        | India          | KM924523 | KM924518 | KY53234 |
| *P. lepidotellum*           | MCA1881 (BRG, holotype)       | Guyana         | JN642233 | JN642235 |
| *P. luteobrunneum*          | CAL1260 (holotype)            | India          | KX073580 | KX073584 | KX073588 |
| *P. melleum*                | MCVE30145 (holotype)          | Italy          | MT095090 | MT095114 |
| *P. melliolens*             | EL224-06                      | France         | FJ904149 | FJ904149 |
| *P. melliolens*             | PAM05052303                   | France         | FJ904148 | FJ904148 |
| *P. melliolens*             | G00110921 (holotype)          | France         | MN901255 | MN901255 |
| *P. melliolens*             | MCVE30344                     | Italy          | MT095095 |
| *P. mimicum*                | EB961997                      | Sweden         | FJ904124 | FJ904124 |
| *P. napaeanum*              | BAN2947 (holotype)            | Germany        | MW1001040 |
| *P. napaeanum*              | BAN2948                       | Germany        | MW1001044 |
| *P. neglectum*              | ZT13022/DED8063 (SFSU)        | Thailand       | EU600829 | EU600829 |
| *P. neoambrinellum*         | HMJAU25742 (holotype)         | China          | MH047249 | MG844977 |
| *P. niveivelatum*            | PB2337 (WTU)                  | USA            | JQ313566 | JQ313566 | AY333776 |
| *P. niveivelatum*            | BK21089714                    | USA            | JQ319695 | JQ319695 |
| *P. notodryinum*            | CO4463                        | USA            | MH578028 | MK421970 | MH577509 |
| *P. obsoletum*              | PB2332 (WTU)                  | USA            | JQ408766 | JQ408766 |
| *P. occidentalis*            | BK27089703 (UTC, holotype)    | USA            | EU600893 | EU600893 | EU600892 |
| *P. occidentalis*            | PB2552                        | USA            | AY038321 | AY038321 | AY333775 |
| Taxa            | Collection number (Herbarium) | Locality   | GenBank accession number |
|-----------------|-------------------------------|------------|--------------------------|
|                 |                               |            | **ITS**                  | **LSU** | **RPB2** |
| *P. pakistanense* | LAH35283                      | Pakistan   | MF575849                 |
| *P. perlatum*    | EL7404 (holotype)             | Sweden     | AM882771                 | AM882771 |
| *P. pinophilum*  | JV10247 (WTU)                | Finland    | JQ408767                 | JQ319698 |
| *P. pinophilum*  | MSM0046 (holotype)           | Pakistan   | MG742414                 | MG742418 |
| *P. pluviorum*   | PL23408                       | Australia  | KP170980                 |
| *P. ponderosum*  | MCVE: 30144 (holotype)        | Italy      | MT095092                 | MT095116 |
| *P. pinophilum*  | MSM0047 (holotype)           | Pakistan   | MG742417                 | MG742415 |
| *P. ponderosum*  |                             |            |                          |          |
| *P. pinophilum*  |                             |            |                          |          |
| *P. ponderosum*  |                             |            |                          |          |
| *P. rimosum*     | AO2008-0250                   | UK         | FJ904147                 | FJ904147 |
| *P. rimosum*     | EL118-08                      | Sweden     | FJ904146                 | FJ904146 |
| *P. rimosum*     | EL211-06                      | France     | FJ904145                 | FJ904145 |
| *P. rimosum*     | PAM06112703                  | France     | FJ904143                 | FJ904143 |
| *P. rimosum*     | EL75-05                       | Sweden     | AM882762                 | AM882762 |
|                 |                               |            |                          |          |
| *P. rimosum*     | PAM03110904                   | France     | FJ904144                 | FJ904144 |
| *P. rimosum*     | DJL-SJ14 (TENN)               | USA (Virgin Islands) | JQ408784 | EU600851 |
| *P. rimosum*     | BK28080513 (UTC)              | USA        | EU600850                 | EU600848 | EU600849 |
| *P. rimosum*     | PBM2601 (TENN)               | USA        | EU600852                 | EU600852 |
| *P. rubrobrunneum* | CAL1307 (holotype)         | India      | KX073583                 | KX073587 | KX073590 |
| *P. salentinum*  | MCVE 30342                    | Italy      | MT095093                 | MT095117 |
| *P. singulare*   | FYG6339                       | China      | OM135605                 | OM149380 | OM780122 |
| *P. singulare*   | FYG6363 (holotype)            | China      | OM135606                 | OM149381 | OM780123 |
| *P. singulare*   | FYG6365                       | China      | OM135607                 | OM149382 | OM780124 |
| *P. solare*      | BAN3078 (holotype)            | Germany    | MW647627                 | MW647627 |
| *P. sororium*    | MCA859/PBM3901 (TENN) (holotype) | USA   | JQ408772                 | JQ319700 | MH249810 |
| *P. sororium*    | PBM2654 (TENN)               | USA        | EU600853                 | EU600853 |
| *P. sororium*    | PBM3055/TENN063504            | USA        | JQ408781                 |          |
| *P. sororium*    | J152000                       | Sweden     | FJ904151                 | FJ904151 |
| Pseudosperma sp. | MCA562                        | Japan      | JQ408785                 | JQ421077 | JN975016 |
| Pseudosperma sp. | TR194-02                      | PNG        | JQ408793                 | JN975032 | JQ421080 |
| Pseudosperma sp. | TR194-02                      | PNG        | JQ408793                 | JN975032 | JQ421080 |
| Pseudosperma sp. | DVO4132011                    | USA        | KP636835                 | KP170140 |
| Pseudosperma sp. | PBM3766                       | Australia  | KP636852                 | KP170154 | KM555146 |
| Pseudosperma sp. | TR104-05                      | PNG        | JN975011                 | JN975011 |
| Pseudosperma sp. | TR133-05 (M)                  | PNG        | JQ408791                 | JQ319709 |
| Pseudosperma sp. | TR138-05 (M)                  | PNG        | JQ408792                 | JN975009 |
| Pseudosperma sp. | TR49-05                       | PNG        | JQ408790                 | JN975014 | JQ421079 |
| Pseudosperma sp. | MTS2494A (UC)                 | USA        | JQ408786                 | JN975008 |
| Pseudosperma sp. | MCA704 (TENN)                 | Japan      | JQ408765                 | JN975007 |
| Pseudosperma sp. | TM02-130                      | Canada     | EU22733                   |
| *P. spurium*     | SJ92017 (holotype)            | Sweden     | AM882784                 |
| *P. squamatum*   | PAM05052301                   | France     | FJ904132                 | FJ904132 |
| *P. squamatum*   | SJ08003                       | Sweden     | FJ904136                 | FJ904136 |
| *P. triaciculare* | MSM0039 (holotype)           | Pakistan   | MG742423                 | MG742424 |
| *P. triaciculare* | MSM0041                       | Pakistan   | MG742429                 | MG742430 |
| *P. umbrinellum* | JV13699                       | Finland    | FJ904165                 | FJ904165 |
middle, lower part with appressed to protruding tiers of brownish (5B4) fibrils. Context fleshy in pileus, white (1A1), yellowish white (1A2) near the cuticle, with a slightly darker tinge under the umbo, becoming brownish (5C4) with age, 1–3 mm thick under the umbo, 0.5–1.2 mm thick at mid-radius; fibrillose and shiny in the stipe, whitish (1A1) to beige (1A1–1A2). Odor fungoid or mild.

Basidiospores [327/16/16] (7)8–9.3–11(13) × (4.2)5–5.6–6.5(8) μm, Q = (1.23)1.37–2.00(2.18), Qm ± SD = 1.67 ± 0.177, ellipsoid, broadly ellipsoid or ovoid, sometimes slightly oblong, less often subphaseoliform, smooth, thick-walled, golden yellow (2A6) to yellowish brown (4B5), with one large nearly rounded oily droplet and numerous yellowish (2B6) granular inclusions. Basidia 16–41 × 9.5–12.5 μm, clavate to broadly clavate, thin-walled, rounded or obtuse at apex, and tapering toward the base, occasionally with a wider constriction, with 4 sterigmata 3–6 μm in length, colorless to yellowish (1B7), with one or more colorless to bright yellow (2A7) oily inclusions when mature. Lamellae edge sterile. Cheilocystidia absent. Pleurocystidia absent. Cheilocystidia 20–58 × 9–20 μm, in clusters, clavate, broadly clavate to utriform, apices obtuse or rounded, tending to subcapitate or capitate, sometimes septate, thin-walled, colorless, occasionally with golden yellow (2A7) inclusions. Hymenophoral trama 50–187 μm thick, regular to sub-regular, transparently colorless or pale yellowish (1B7), composed of smooth, thin-walled, cylindrical to inflated hyphae 20–30 μm thick. Pileipellis a cutis, 137–250 μm thick, compact, regular to subregularly arranged, golden brown (4B6) to dark brown (4F5) in mass, composed of cylindrical to inflated, thin-walled and encrusted hyphae 3–14 μm wide. Stipitipellis a cutis frequently disrupted by loose hyphal projections, hyphae 3–17 μm wide, thin-walled, and colorless. Caulocystidia 22–39 × 7–15 μm, present at stipe apex, vesiculose, clavate, utriform or subcapitate, obtuse at the apex, thin-walled, smooth, colorless and hyaline. Stipe trama regularly and densely arranged, yellowish in mass, hyphae thin-walled, colorless, 4–23 μm wide. Oleiferous hyphae abundant, 2–14 μm wide, present in pileal, stipe, and hymenophoral trama, bright yellow (4B7), less often colorless, smooth, often bent, occasionally branched. Clamp connections present, common in all tissues.

**Habitat:** Single or scattered on clay soils under *Carpinus londioniana* var. *lanceolata* (Hand.-Mazz.) P.C.Li.

**Geographical distribution:** Known from Hainan and Fujian provinces, China.

**Additional specimens examined:** China. Hainan Province, Wuzhishan Nature Reserve, 109°40′43″E, 18°51′53″N, 688 m asl., 1 May 2022, leg. Y.-G. Fan, L.-N. Zhao, L.-S. Deng, and J.-H. Hu, FYG7044 (FCAS3574), FYG7045 (FCAS3575); same location, 27 July 2021, leg. Y.-G. Fan, L.-N. Zhao, L.-S. Deng, and J.-H. Hu, FYG6942 (FCAS3510), FYG6530 (FCAS3521), FYG6289 (FCAS3523); same location, 29 July 2021, leg. Y.-G. Fan, L.-N. Zhao, L.-S. Deng, and J.-H. Hu, FYG6331 (FCAS3510), FYG6321 (FCAS3518), FYG6327 (FCAS3519), FYG6328 (FCAS3520), FYG6379 (FCAS3522), FYG6568 (FCAS3524); 26 June 2020, leg. Y.-G. Fan and W.-J. Yu, FYG5029 (FCAS3525); same location, 12 Aug 2020, leg. N.-K. Zeng, Zeng4615 (FCAS3548); Ledong Li Autonomous County, Yinggeling substation of Hainan Tropical Rainforest National Park, 109°23′33″E, 19°1′20″N, 550 m asl., 2 April 2021, leg. Y.-G. Fan and L.-S. Deng., FYG6058 (FCAS3514), FYG6067 (FCAS3515), FYG6096 (FCAS3516), FYG6099 (FCAS3517); Fujian Province, Shunchang Country, Yangkou forestry farm, 24 Jun 2021, leg. Y.-P. Ge, Q. Na, B.-R. Ke, and L.-L. Qi, GN0942 (FCAS3547).

**Remarks:** *Pseudosperma fulvissicum* is a common species under *Carpinus* forests; it fruits from late April to late August. In the field, it is a typical little brown mushroom with a slender habit and brownish pileus. The pileus status could be fibriullose to rimose with appressed scales or fibrils in different specimens. The stipe surface is usually finely felt or appressed-fibrillose with brownish tomentose hyphae toward the lower part, however usually hard to observe after handling. Unlike most species in the genus, the new species has moderately crowded to subdistal lamellae in certain specimens. The differences between individuals or collections were mostly due
to the weather conditions or microclimates. In terms of microscopic intraspecific variation in *P. fulvidiscum*, the basidios- spores of FYG6311 are smaller and more spherical ($Q_m = 1.54$, $n = 20$); however, FYG6327 has larger and narrower basidiospores ($Q_m = 1.74$, $n = 20$). Phylogenetically, an undescribed Australian specimen (PLS8410) labeled *P. aff. araneosum*, is the closest taxa with moderate support. *Pseudosperma brunneosquamulosum*, originally described in tropical India, shares similarities in its small and slender habit, brownish pileus color and status, and the size and the shape of the basidiospores; however, it differs by brownish orange stipes, broader basidiospores ($Q_m = 1.5$), shorter and non-capitate cheilocystidia, and an association with trees of Dipterocarpaceae (Tibpromma et al. 2017).

**Pseudosperma singulare** Y.G. Fan, L.N. Zhao, and W.J. Yu, sp. nov., Figs. 4 and 5

**MycoBank:** MB843663

**Etymology:** “singulare” refers to its solitary habit.

**Diagnosis:** *Pseudosperma singulare* differs from *P. araneosum* by its small basidiomata, squamulose pileus with a brown tinge, shorter basidiospores, broader and longer cheilocystidia usually constrained at the middle, and association with different plants.

**Holotype:** China. Hainan Province, Wuzhishan Nature Reserve, 109°40′43″E, 18°51′53″N, 662 m asl., 29 July 2021, leg. Y.-G. Fan, L.-S. Deng, L.-N. Zhao and J.-H. Hu, FYG6363 (FCAS3526).

**Description:** Basidiomata small, slender. Pileus 6–10 mm diam., spherical to subshpherical at first, then campanulate to convex, margin incurved when young, then decurved for a long time; surface dry, woolly tomentose to subtomentose with finely squamules toward the disc, radially fibrillose to rimulose outwards, usually split at margin; brown (6B4) to dark brown (6B5) toward the center, brownish (6A3) to lightly brown (6A4) outwards, uniformly brown (6D5) to dark brown (6E5) when overmatured. Lamellae adnexed, moderately crowded to subdistant, 0.6–1.5 mm wide, unequal in length, alternately distributed with 2–3 tiers of lamellae, whitish to yellowish brown (5B6) at first, then brownish (5D6), ochraceous-brown (5D7) with age, completely brown upon drying; edge fimbricate, pallid. Stipe 18–22 mm in length, 1–1.5 mm in width, terete, equal, solid, somewhat enlarged at both the apex and the base, furfuraceous or scurfy at apex, with appressed or hairy yellowish (5A3) to brownish (6B5) fibrils downward, background white (1A1) to cream white (1A3), brownish (5C5) toward the base. Context fleshy in pileus, white (1A1) to creamy white (1A2), 0.8–2.5 mm thick under the umbo, 0.3–1.2 mm thick at mid-radius; fibrillose in stipe, whitish, pale yellowish near the cuticle. Odor grassy or mild.

**Basidiospores** [107/2/2] 8–9.1–10 (10.5) × 5–6.1–7.8 (9) μm, $Q = (1.25) 1.31–1.73 (1.8)$, $Q_m = ± SD = 1.51 ± 0.13$, ellipsoid to broadly ellipsoid, sometimes ovoid, occasionally irregular or subglobose, smooth, thick-walled, golden yellow (4A6) or yellowish brown (5B6), with one large nearly round- ed oily droplet and numerous yellow (2A6) granular inclusions. Basidia 19–27 × 9–12 μm, clavate to broadly clavate, thin-walled, obtuse at the apex, and gradually tapering toward the base, colorless to yellowish (1B1), with one or more bubble-like, bright yellow (1B6) oily inclusions, with 4- or 2- sterigma, 2–6 μm in length. Lamellae edge sterile. Pleurocystidia absent. Cheilocystidia 26–70 × 8–25 μm, in clusters, versiform and usually median-constrained, clavate to broadly clavate, broadly fusiform, occasionally utriform, rounded or obtuse at apex, tending to capitate or subcapitate, base often tapered, colorless and hyaline, thin-walled, occasionally with amorphous inclusions. Hymenophoral trama 112–200 μm thick; regular to sub-regular, hyphae 16–32 μm wide, smooth, thin-walled, colorless, ellipsoid or fusiform. Pileipellis a cutis often with erected elements, 50–163 μm thick, bright yellow (4B5) to dark brown (4F4), hyphae of cuticular layer 6–15 μm wide, septate, cylindrical, slightly thick-walled, brownish (4B5), walls bright yellow, encrusted or smooth; Pileal trama colorless or yellowish, subregularly arranged, composed of compact, thin-walled, colorless, inflated or fusiform hyaline 2–12 μm wide. Stipitipellis a cutis, colorless to pale yellow (1A2) in mass, regularly and densely arranged, smooth, thin-walled hyphae 3–7 μm wide, often disrupted with protruding elements. Caulocystidia not observed. Stipe trama regular, yellowish in mass, hyphae 5–10 μm wide, smooth, thin-walled, colorless. Oleiferous hyphae present in pileus and stipe trama, not abundant, 2–8 μm wide, yellowish (2B5), smooth, bent, and occasionally branched. Clamp connections present and common in all tissues.

**Habitat:** Single on clay soils under Carpinus lanceolata under the umbo, 0.3–1.2 mm thick at mid-radius; fibrillose in stipe, whitish, pale yellowish near the cuticle. Odor grassy or mild.

**Geographical distribution:** currently known only from the type locality in Hainan Province, China.

**Additional specimens examined:** China, Hainan Province: Wuzhishan Nature Reserve, 109°40′43″E, 18°51′53″N, 688 m asl., 29 July 2021, leg. Y.-G. Fan, L.-N. Zhao, L.-S. Deng, and J.-H. Hu, FYG6339 (FCAS3652), FYG6365 (FCAS3528).

**Remarks:** *Pseudosperma singulare* is encountered under Carpinus forests; it fruits solitarily in late July in Hainan.
Province, China. Comparing to *P. fulvidiscum*, its pileus is uniformly brown without discoloring toward the margin, the squamules toward the pileus center are smaller and irregularly erected, the Q value of basidiospores is smaller, and the pileipellis is composed of smooth and encrusted hyphae. Phylogenetically, *P. araneosum*, originally described from

**Fig. 2** Basidiomata of *Pseudosperma fulvidiscum*. (a–e) Basidiomata. (d–e) Rimose to rimulose pileus. (f–g) Stipe. (h–i) Lamellae. (a, e) FYG6327 (holotype); (b, i) FYG6311; (c) FYG6880; (d) FYG6321; (f) FYG6308; (g) FYG6096; (h) FYG6310. Scale bars: a–c, e–f, h–i = 5 mm; d, g = 2 mm. Photos by Y.-G. Fan, L.-S. Deng, and L.-N. Zhao
Queensland, is a sister to the new species; however, it has medium-sized basidiomata, non-squamulose and yellowish tinged pileus, longer basidiospores measured (8.0–) 8.5–11.5 × 5.0–7.0 μm ($Q_m = 1.68$), slender cheilocystidia measured 27–53 × 10–17 μm, and is associated with different plants (Matheny and Bougher 2017). Pseudosperma

Fig. 3 Microscopic features of Pseudosperma fulvidiscum (FYG6327, holotype). (a, b) Basidiospores. (c, d) Basidia. (e–h) Cheilocystidia. (i) Lamella edge: shows cheilocystidia in clusters. (j) Hymenophoral trama. (k) Pileipellis. (l) Oleiferous hyphae. (m) Pileipellis hyphae. (n) Caulocystidia. (o) Stipitipellis. Scale bars: a–h, l, m = 20 μm; n = 10 μm; i–k, o = 50 μm. Photos by L.-N. Zhao.
rubrobrunneum, described in India, is similar in outwards appearance and outline and size to basidiospores; it differs by brownish orange stipe, non-constrained and shorter cheilocystidia measured $17–39 \times 11–18 \mu m$, and ecology with Dipterocarpaceae trees (Tibpromma et al. 2017).
Fig. 5 Microscopic features of *Pseudosperma singulare* (FYG6327, holotype). (a, b) Basidiospores. (c–f) Basidia. (g–k) Cheilocystidia. (l) Hymenophoral trama. (m–o) Pileipellis. (p) Oleiferous hyphae. Scale bars: a–k, n–p = 10 μm; l = 50 μm; m = 100 μm. Photos by L.-N. Zhao
New combination

*Pseudosperma rubrobrunneum* (K.P.D. Latha and Manim)

Y.G. Fan, comb. nov.

≡*Inocybe rubrobrunnea* K.P.D. Latha and Manim, 
Tibpromma et al., Fungal Diversity 83: 174 (2017)

*Mycobank*: MB843881

Notes: This new combination is made based on phylogeny (nrLSU and RPB2) of *Inocybe sensu lato*. *P. rubrobrunneum* was nested in the Pseudosperma clade (Latha and Manimohan 2017), which is now treated as *Pseudosperma* genus (Matheny et al. 2020). In our three-gene phylogeny of the genus (ITS, nrLSU, and RPB2, Fig. 1), *P. rubrobrunneum* is sister to the lineage unifying *P. singulare* and *P. araneosum*.

Toxin detection

The weights of the tested samples for the two new species were 0.0115 g for FYG6311 and 0.0020 g for FYG6363, respectively. The representative chromatograms of muscarine are shown in Fig. 6. After comparing the retention time (0.95 min) and relative deviation (0.32%) with standard muscarine in the allowance of ±25% relative range, the toxin muscarine was identified. The calibration curve for muscarine generated during the validation was $y = 20223.15025x + 18054.61816 (r = 0.99837)$ for muscarine concentration in the range of 2–100 ng/mL ($y$ means the peak area, and $x$ is the muscarine concentration, $r$ represents correlation coefficient). The muscarine contents are $3.1340 \pm 0.4078$ g/kg and $0.5505 \pm 0.0026$ g/kg in *P. fulvidiscum* and *P. singulare*, respectively. Precision was performed, injecting six times the standard mixture, and the relative standard deviation (RSD) were 13.01% and 0.48%, respectively. Percentages of recovery was 98.47–100.18%, and the average recovery was 98.78%.

Discussion

In recent years, an increasing number of *Pseudosperma* species have been discovered, especially after *Inocybaceae* was re-divided into seven genera by Matheny et al. (2020) and the establishment of the genus. These include *P. albobrunneum*,...
P. brunneombonatum, P. flavorimosum, P. pinophilum, and P. triacicularis from Pakistan (Saba et al. 2020; Jabeen and Khalid 2020; Jabeen et al. 2021), P. amabile, P. amoris, P. napaeanum, P. emerizanum, P. huginii, and P. solare from Germany or Austria (Bandini and Oertel 2020; Bandini et al. 2021), P. conviviale, P. melleum, and P. ponderosum from Italy (Cervini et al. 2020), and P. citrinostipes from southwestern China (Yu et al. 2020). These works have greatly facilitated the species diversity of the genus. In the present study, P. fulvidiscum and P. singulare were identified as new species through morphological studies and phylogenetic analyses. They share high similarities in appearance. It would be very difficult to distinguish them by morphomorphology or even by most of the microfeatures. However, the appressed scaly pileus and brownish tiers of fibrils in the lower part of the stipe in P. fulvidiscum and the presence of non-encrusted pileipellis hyphae in P. singulare could help discriminate them. Detailed comparisons between the two new species were listed in Table 2.

Tropical Asian elements of Pseudosperma or Inocybaceae spp. are usually encountered in Fagaceae or Dipterocarpaceae forests (Horak 1980; Fan and Bau 2014; Latha and Manimohan 2017). However, the present two new species are both collected under the Carpinus forests. Carpinus is a genus of Betulaceae distributed mainly in temperate and extending to subtropical to northern tropics (Liu et al. 2021). Based on our field observations, the only putative host of the two new species, Carpinus londinianana var. lanceolata, was described from Wuzhishan of Hainan, where there is a tropical mountain climate. One specimen (GN0942) of P. fulvidiscum was collected from Fujian province, where there is a subtropical monsoon climate, but this collection lacks detailed host information.

In our multi-gene phylogeny, the two new species grouped into a full support clade that is sister to all the remainders of the genus. The subclade unifying P. brunneosquamulosum and P. rubrobrunneum from tropical India, P. arraneosum and P. aff. araneosum (PL58410) from tropical Australia, and Pseudosperma sp. (MCAS62) from subtropical Japan. Interestingly, the two Australian taxa, P. aff. araneosum (PL58410) and P. araneosum, are sisters to P. fulvidiscum and P. singulare, respectively. Pseudosperma brunneosquamulosum and P. sp. (MCAS62) clustered in a full support lineage that is sister to the lineage unifying P. fulvidiscum and P. aff. araneosum (PL58410); P. rubrobrunneum is sister to the lineage unifying P. singulare and P. araneosum. Matheny et al. (2009) suggested that northern and southern South America, Australia, and New Zealand are primarily the recipients of immigrant taxa during the Palaeogene or later. The present multi-gene phylogeny confirms close relationships between these Pseudosperma taxa from tropical Asia and tropical Australia. Similarly, Inocybe hainanensis T. Bau and Y.G. Fan, a formerly described species from tropical China, has the closest affinities to an Australian species I. violaceucaulis Matheny and Bougher based on nLSU phylogeny (Fan and Bau 2014). With more tropical taxa being discovered and described in the genus, we will reach a better understanding of their distribution pattern and evolutionary history.

The alkaloid muscarine, an ammonium quaternary compound that stimulates the parasympathetic nervous system of animals, is found in Inocybaceae and several mushroom genera (Lurie et al. 2009; White et al. 2019). Among the seven major clades of Inocybaceae, the presence of muscarine was suggested to be a derived trait for an inclusive clade containing Nothocybe, Pseudosperma, and Inocybe s. str. Pseudosperma is supposed to contain many muscarine-positive species, but only five species have been assayed in this genus (Kosenstka et al. 2013). Of these, P. rimosum, P. niveivelatum, P. sororium, and P. spurium contain muscarine, and only P. perlatum was reported to lack muscarine (Kosenstka et al. 2013). There are still numerous taxa in Pseudosperma waiting for toxin detection. In recent years, cases of poisoning caused by Pseudosperma mushrooms were
constantly reported in China (Li et al. 2020, 2021, 2022; Xu et al. 2020). The present two new species are both toxic mushrooms due to the fact that they contain high concentrations of muscarine. Even though these two new species are just “little brown mushrooms” and may be ignored in the field, the absence of aposematic coloration makes them more easily collected or consumed by mushroom hunters. Accordingly, publicity of the two new toxic mushrooms is undoubtedly needed, especially for the more frequently encountered species *P. fulviduscum*. Our present work provides scientific details for the identification and publicity of these two toxic species.

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**Author contribution** Conceptualization: Y-G.F. and W-J.Y. Methodology: L-N.Z. Performing the experiment: L-N.Z. Formal analysis: L-N.Z., W-J.Y., and L-S.D. Resources: Y-G.F., L-S.D., J-H.H., and Latha KPD. Data curation: L-N.Z., W-J.Y., and L-S.D. Writing—original draft preparation: L-N.Z. Writing—review and editing: Y-G.F. and W-J.Y. Supervision: Y-G.F. Project administration: Y-G.F. Funding acquisition: Y-G.F. and L-S.D. All authors have read and agreed to the published version of the manuscript.

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**Data availability** The sequence data generated in this study are deposited in NCBI GenBank.

**Declarations**

**Ethics approval and consent to participate** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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