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Research Article

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Sclerotia formation of *Phlebopus portentosus* in wild and artificial cultivation

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Abstract: *Phlebopus portentosus* is a favorite wild edible mushroom in the Xishuangbanna region, Yunnan, China and in northern Thailand. This bolete has a unique biotrophy. It can be saprobic but also form a tripartite association with root mealy bugs and plants. *P. portentosus* is the only edible fungus of Boletales in the world that can be cultivated artificially and anniversary produced at present. Sclerotium is often found at its natural environment and cultivated media, but the regularity and growth characteristics of the sclerotium are unknown. In this study the whole process of birth, growth, death and rebirth of the sclerotium of *P. portentosus* at the national and lab conditions was reported for the first time. The sclerotium formation in the nature is related to adversity, such as reduced rainfall and low temperature. The more rainfall, the less sclerotia. It seems that the lower temperature increased the sclerotium formation, however the relationship of the sclerotium formation to temperature was not obvious as the rainfall. Under artificial conditions the sclerotium formation of *P. portentosus* is related to the fungus physiological maturation, and the sclerotium occurrence always accompanied by appearance of the water drops on the colony. The result will set up a platform for research on importance

of the sclerotium in life circle of *P. portentosus*.

Sclerotium is a firm, frequently rounded resting body of fungal hyphae, which differentiated into a rind and a medulla¹. It can give rise to a fruiting body, a stroma or mycelia². The sclerotium can play a significant role in the fungal life cycle, such as overcoming adverse conditions and rapidly colonizing nearby substrates when favorable conditions return³⁻⁵. Normally the sclerotium is small, However, some edible or medicinal fungi, such as *Poria cocos*, *Cordyceps sinensis* and *Grifola umbellata* can develop big tuber-like sclerotia, which are harvested as food or medicine⁶⁻⁹. Recent success of morel cultivation showed that the morel sclerotia played a key role during production of its fruiting bodies. That is the sclerotia development is an essential stage for the producing morel fruit body¹⁰⁻¹⁴.

Phlebopus portentosus (Berk. and Broome) Boedijn is a delicacy in the tropical regions of China and Thailand. It is placed within the Boletinellaceae^{2,15}. This mushroom is extremely popular and sold at ¥60–100/kg (US\$9–14) in the Xishuangbanna region of Yunnan^{16,17}. Harvesting and trading the mushroom is an important means of livelihood for the local people. In recent years the production of the mushroom has declined due to uncontrolled commercial harvesting. Research on cultivation of *P. portentosus* has been carried out at the Yunnan Institute of Tropical Crops of Yunnan, China since 2003. Technologies for the cultivation of *P. portentosus* in mushroom houses and by field inoculation have been developed^{18,19}. During our research, the unique biotrophy of *P. portentosus* has been gradually unveiled. This mushroom can live a saprophytic lifestyle²⁰⁻²². However, it usually has a symbiotic association with soil mealy bugs, forming a special insect gall on plant roots²³⁻²⁶. In addition, sclerotium has often been discovered abundantly at its filed soils and artificial media. It is understandable that *P. portentosus* will produce the sclerotium under low-temperature or in dry seasons in the natural environment, which is a stress response to the harsh environmental conditions. When the temperature and moisture were well controlled within the suitable ranges in the process of artificial cultivation of the mushroom^{16,27,28}, however, a large number of sclerotia was still developed. This phenomena inspired us to study the interesting issue.

In this paper, the microscopic change process of *Phlebopus portentosus* sclerotia formation under field and artificial culture conditions was analyzed and established. The microscopic changes of *Phlebopus portentosus* from mycelium growth, mycelium kink, sclerotia formation, maturation, germination and senescence were clarified, which provides a platform to ascertain the importance of sclerotium formation in the cultivation of *P. portentosus*.

Results

Formation of sclerotium in the field. When the environmental conditions were getting non-conducive (lower temperature or less rainfall, or both) to the fungal growth, the fungal mycelia and rhizomorphs in the soil began to converge and tangle (Fig. 1a), and then a pale yellow, globose or irregular soft hyphal ball formed (Fig. 1b). As the hyphal ball gradually grew bigger its tissue became tighter and darker, and dense air hyphae developed on the surface (Fig. 1c). At this stage the ball case was soft, rough and covered by rhizomorphs (Fig. 1d), and its contained dark brown honey-like juicy inside (Fig. 1e). Finally, the hyphal ball solidified and separated from surrounding hyphae becoming a matured sclerotium.



Figure 1. Early stage of sclerotium formation of in the field. (a) mycelia and rhizomorphs began to converge and tangle. (b) a pale yellow, irregular soft hyphal ball formed. (c) the hyphal ball gradually grew bigger, tighter and darker with dense air hyphae on the surface. (d) the hyphal ball wall soft and its surface rough covered by rhizomorphs. (e) dark brown honey-like juicy inside the ball. Scale bars: c e=2 mm.

The matured sclerotium was solid, dark, glabrous, vein-like rhizomorphs scattered on the surface (Fig. 2a). Its peridium and internal tissues (pith) have differentiated. The peridium was made from dark hyphae, and the internal tissue was made from dense interwoven hyphae, wax-like, grayish to brownish with brown spots (Fig. 2b). The sclerotium normally produced singly (Fig. 2c), rarely beads-like (Fig. 2d), can be globose, subglobose or irregular, 1.42~14.98 mm in diam.

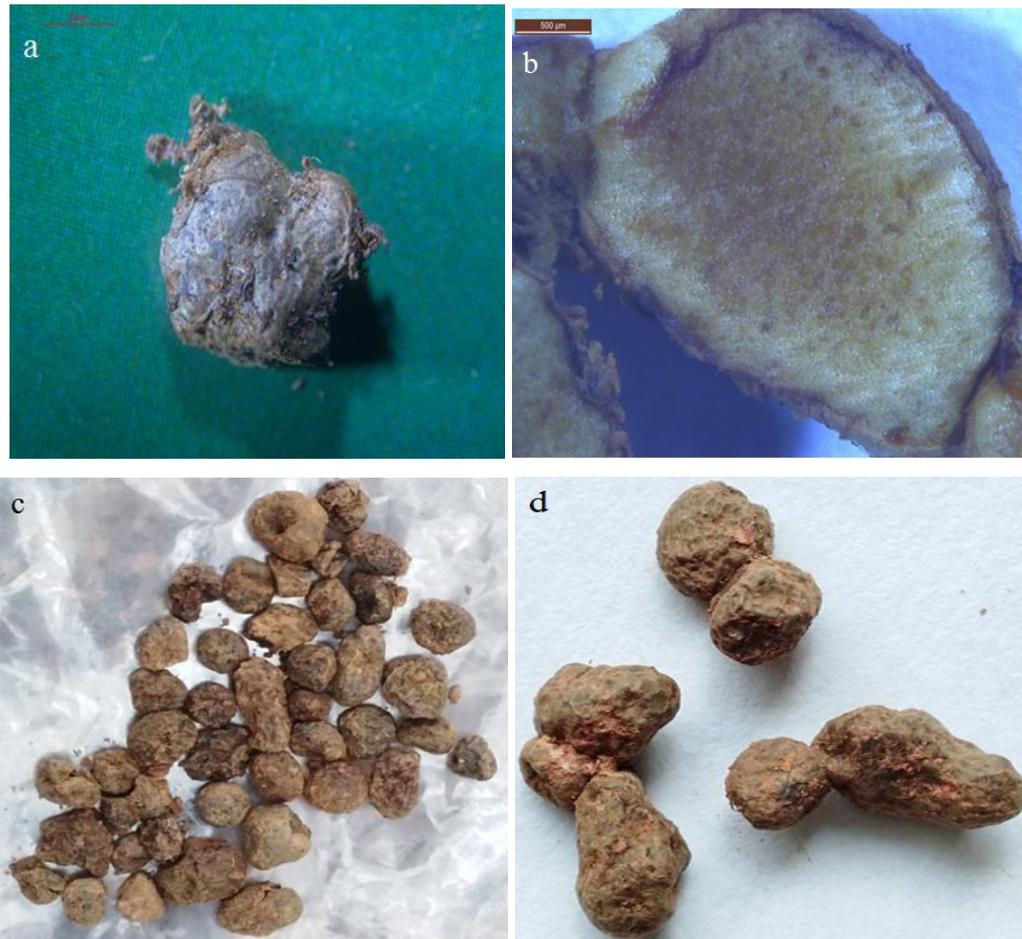


Figure 2. Matured sclerotium produced in the field. (a) appearance of a matured sclerotium. (b) a cross sectioned matured sclerotium showing the differentiated peridium and pith. (c) sclerotia in different shapes. (d) beads-like sclerotia. Scale bars: a, c=2 mm; b=3 mm.

Seasonal dynamics of sclerotium occurrence in the field. In the field, the sclerotium can be developed year around except July and August when they have good temperatures and adequate precipitations. From the December through the April of the following year when the temperature became lower and the rainfall was scarce, sclerotia were produced abundantly in the soil (Table 1, Fig 3). From the December through the February of the following year both young and matured sclerotia were discovered simultaneously. From the March through the June only matured and aging sclerotia were observed. After the April the sclerotia started to germinate as the temperature and rainfall gradually increased, and then amount of sclerotia discovered was gradually declined. In the meantime a few mushrooms of *P. Portentosus* emerged. From the May through the June a few matured and aging sclerotia were still existing in the soil.

Investigation time	Amount of sclerotium dicovered			
	Dongfeng farm	Hydropower Station	Mangajian	Total amount of sclerotium
2017.10	8	5	0	13
2017.11	7	3	4	14
2017.12	17	16	9	42
2018.1	15	20	14	49
2018.2	27	34	31	92
2018.3	19	43	36	98
2018.4	24	36	29	89
2018.5	7	25	11	43
2018.6	-	4	5	9
2018.7	-	-	-	0
2018.8	-	-	-	0
2018.9	4	-	-	4
2018.10	5	7	3	15

Table 1. Seasonal dynamics of sclerotium formation from 2017 to 2018.

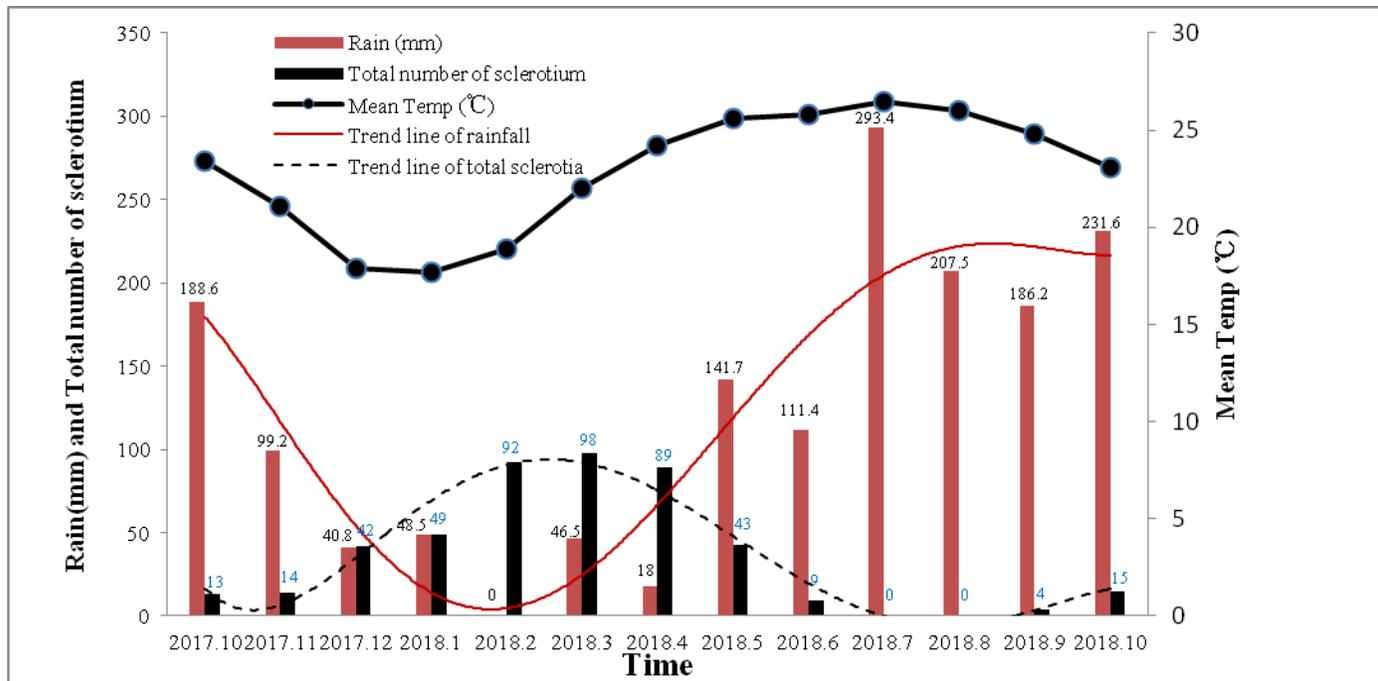


Figure 3. The relationship between the amount of sclerotia and monthly average temperature and rainfall. (the climate data provided by Xishuangbanna Meteorological Bureau).

Aging and died young of sclerotium in the field. Sclerotia dried out becoming shriveled when the climate was extremely dry and the soil was short of water (Fig. 4a). Their surfaces obviously wrinkled or became unevenness. At this moment the hyphal growth and accumulation of nutrients in sclerotia were interrupted due to water loss. The consequence is that the sclerotium became hollow and its peridium dried out becoming fragile, and finally ruptured to death (Fig. 4b, c). remained only the withered peridium left from the dead sclerotium (Fig. 4d). Amount of the dead sclerotia increased in the soil, as the dry weather continued, especially in the upper soil layer (0-10 cm). However, some of the matured sclerotia could survive during the difficult period even although they almost dried out. When the dried matured sclerotia were put in a petri dish lined with moistened double filter paper for 24 hours they could win rebirth.



Figure 4. Aging and died young sclerotia. (a) shriveled sclerotia. (b) the sclerotium becoming hollow. (c) a cross sectioned died sclerotium. (d) Peridium remains of died sclerotium. Scale bars: a, b=2 mm.

Germination of sclerotium in soil. When the temperature and moisture of soil reached the optimum conditions the survived sclerotium began to germinate. New mycelia developed from a single or multiple points of the sclerotium (Fig. 5a). The mycelium extended into the soil and the entire sclerotium was gradually surrounded by dense mycelium (Fig. 5b). As more and more mycelia continued to grow out from the sclerotium its accumulated nutrients were consumed and run out, and the sclerotia eventually disappeared from the soil. And then a new colony of *P. portentosus* formed, which fruiting bodies produced from the colony when the environmental conditions became suitable.

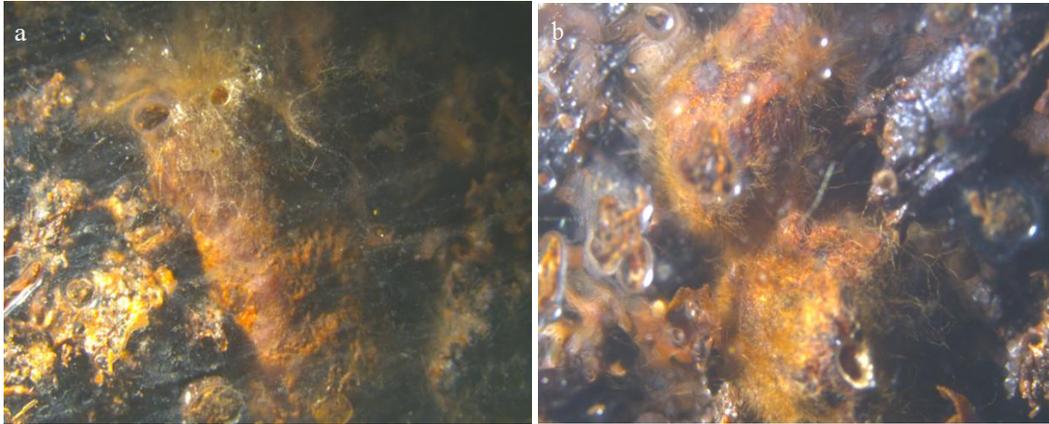
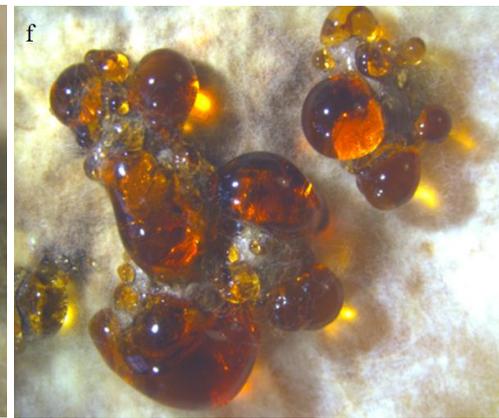
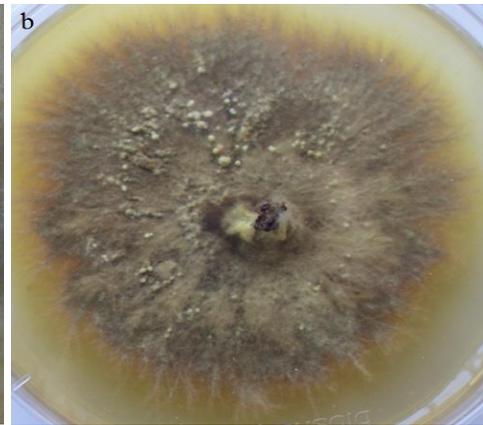
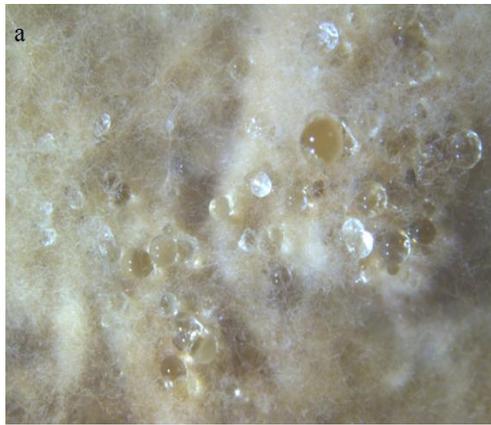


Figure 5. germination of the sclerotium. (a) a germinated sclerotium. (b) two sclerotia surrounded by new mycelia. Scale bars: a=2 mm; b=1 mm.

Culture of sclerotium in the lab. The 3 isolates of *P. portentosus* grew well on the agar medium and produced round colonies. When the colonies started developing cotton wool-like tangled interwoven hyphae a few small transparent liquid drops would appear on their surfaces (Fig. 6a). More liquid drops developed as the fungal colonies grew further and they became bigger and darker. And then a mass of curly, fluffy tangled mycelia knots raised up from the surface of colony (Fig. 6b). They soon developed into dense mycelia balls and had a lot of liquid drops on their surfaces. They were baby sclerotia (Fig. 6c). At this stage the peridium differentiated from its juicy internal tissue (Fig. 6d). As the sclerotium grew up, the interior tissue solidified due to nutrient accumulation and hyphal intensive growth (Fig. 6e). And then the sclerotium became harder and harder and the color of the liquid drops turned dark red brown (Fig. 6f). At this stage fluffy hyphae disappeared from the hardening peridium surface and a few dark pits appeared (Fig. 6g). The interior tissue was composed of fresh hyphae with more nutrients accumulated, juicy, brown to dark brown (Fig. 6h). Most of the sclerotia were globose to subglobose when young and became irregular cluster shapes due to adjacent sclerotia fused together, which could grow up to 20 mm long or more (Fig. 6g).



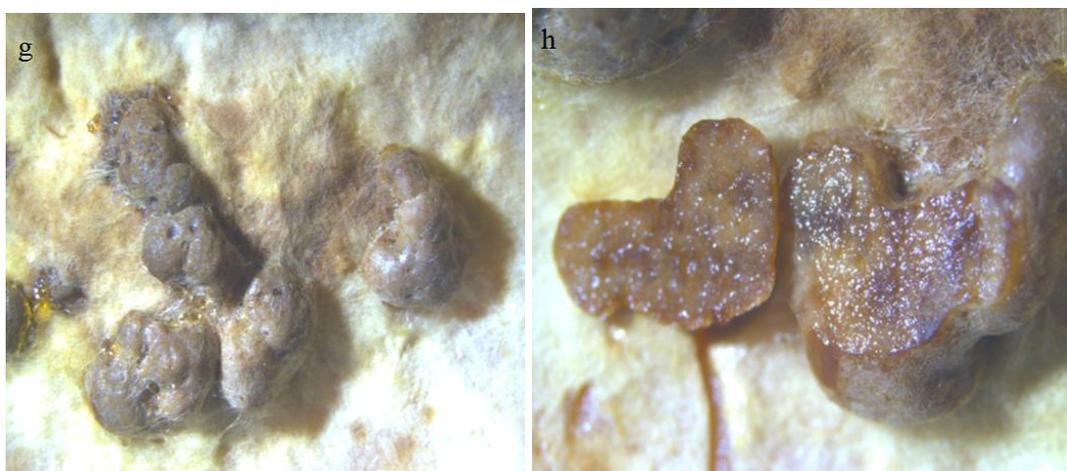


Figure 6. Sclerotium formation on agar medium. (a) liquid drops appeared on the colony. (b) curly, fluffy tangled mycelia knots raised up. (c) a baby sclerotium. (d) Inside the young sclerotium juicy and soft. (e) the internal tissue solidified with nutrients and hyphae accumulated. (f) liquid drops became dark brown. (g) brown and fluffy hyphae disappeared and pits appeared. (h) Cross sections showed the interior was brown to dark brown with fresh hyphae and juicy stored nutrients. Scale bars: a, c, d, e=1 mm; f, g, h=2 mm.

The time and positions of the sclerotium occurrence on the agar medium were different among the three isolates (Fig. 7, table 2). The isolate 17076 started producing the liquid drops as soon as it colonized the agar plate. The sclerotia occurred nearby the place where the isolate lump was put and scaled out. The isolate 18004 started producing sclerotia at the 9th day after the inoculation, the sclerotia formed a ring around the isolate lump 1 cm away. However, the isolate 18106 would not produce sclerotia until it colonized the whole agar plate and the most of sclerotia scattered around the edge of the colony. The sclerotia are smaller but with huge amount. The sclerotia produced from the agar medium could not germinate until incubating them at 4°C for 24 hours.

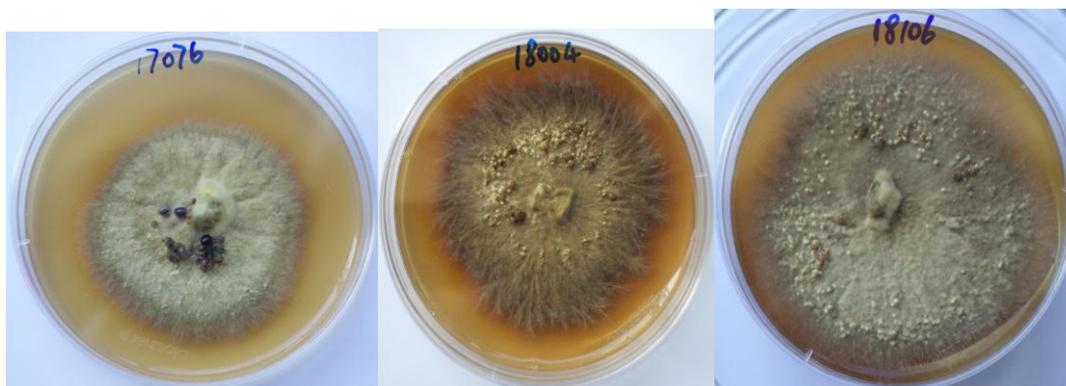


Figure 7. Sclerotium formation of three strains on the agar medium.

Isolate	Time of sclerotia formed	Number of sclerotia	Sclerotia size (mm)	Position of sclerotia on the medium
17076	4d	132	1.80~21.77	Near the inoculation site
18004	9d	194	1.31~12.34	1cm away from the inoculation site
18106	16d	217	1.61~9.76	Far away from the inoculation site

Table 2. Characteristics of Sclerotia formation of three isolates on the agar medium. The number of sclerotia refers to the total number of sclerotia in 10 petri dishes. Sclerotia size refers to the range of smallest and largest sclerotia in 10 petri dishes.

Molecular identification of the sclerotium. The ITS sequences of six sclerotium samples of *P. portentosus* were submitted to the GenBank and their accession numbers listed in the Table 3.

Six DNA fragments, 690 bp, were obtained from six sclerotium samples. A total of 14 ITS sequences were included in the phylogenetic analysis. *Neoboletus* sp.iNat31878612 (MN498124.1) was used as outgroup. The results indicated that the nucleotide sequences of the six sclerotium samples were almost the same and grouped with four sequences of *P. portentosus*. However, they were well separated from *Phlebopus marginatus* REH8883 (EU718109) and *Phaeogyroporus portentosus* group (Fig. 8).

Species	Voucher No	GenBank accession No	DNA size(bp)	Reference
<i>Phaeogyroporus portentosus</i>	Php1	DQ534569.1	702	29
<i>Phlebopus portentosus</i>	Php1	EU718110.1	707	Wilson et al .Unpublished
<i>Phlebopus portentosus</i>	WPPH2	FJ603112.1	813	21
<i>Phlebopus portentosus</i>	CMU320-2	JN639898.1	797	Cao et al.Unpublished
<i>Phlebopus portentosus</i>	CMU51-281-1	JQ695907.1	678	Kumla et al.Unpublished
<i>Phlebopus portentosus</i>	CMU52-320-2	KF768405.1	750	Kumla et al.Unpublished
<i>Phlebopus marginatus</i>	REH8883	EU718109.1	687	Wiison et al.Unpublished
<i>Neoboletus</i> sp.	iNat31878612	MN498124.1	726	Clements et al.Unpublished
<i>Phlebopus portentosus</i>	sclerotium 1	MT362458	690	This paper
<i>Phlebopus portentosus</i>	sclerotium 2	MT362459	690	This paper
<i>Phlebopus portentosus</i>	sclerotium 3	MT362460	690	This paper
<i>Phlebopus portentosus</i>	sclerotium 4	MT362461	690	This paper
<i>Phlebopus portentosus</i>	sclerotium 5	MT362462	690	This paper
<i>Phlebopus portentosus</i>	sclerotium 6	MT362463	690	This paper

Table 3. Sequences used in the analysis. Sclerotium 1 from strain of *P. portentosus* 17076; sclerotium 2 from strain of *P. portentosus* 18004; sclerotium 3 from strain of *P. portentosus* 18106; sclerotium 4 from Dongfeng farm; sclerotium 5 from Hydropower Station; sclerotium 6 from Mangajian.

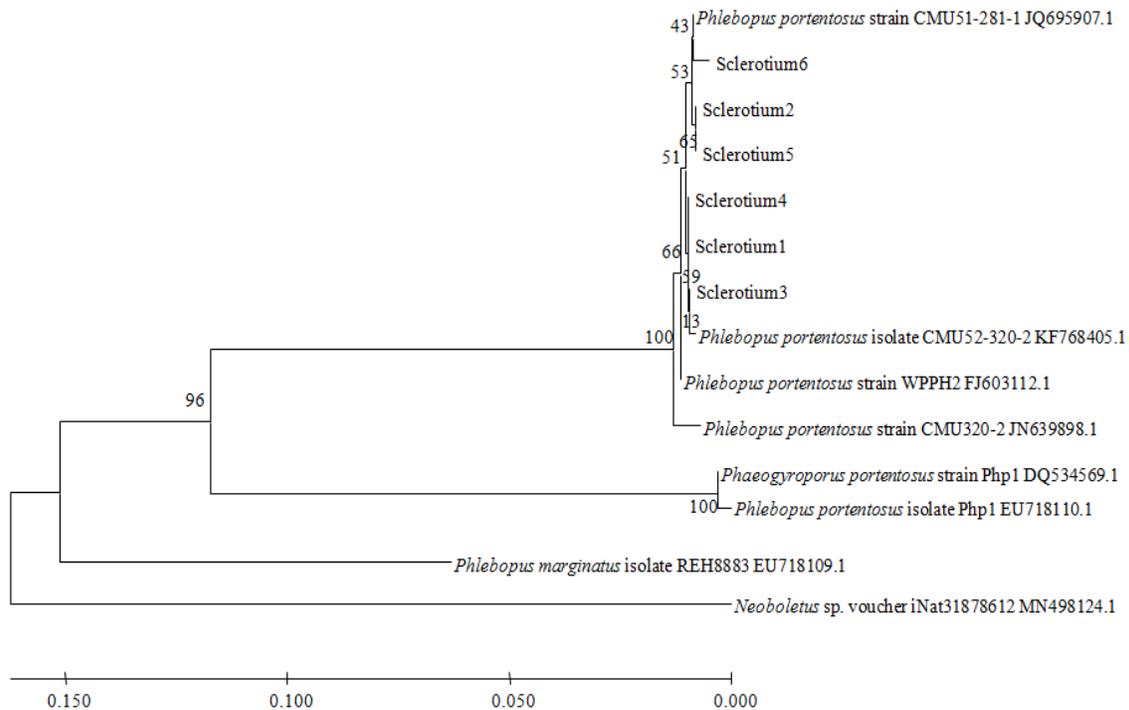


Figure 8. Neighbor-joining tree based on ITS region sequences of sclerotium1~6 and related GenBank sequences. Bootstrap tests were performed with 1000 repetitions.

Discussion

The whole process of the sclerotium formation of *P. portentosus* at both the national and lab conditions was reported for the first time. At the national conditions the sclerotium occurrence was closely related to climate seasonal dynamics, especially to the rainfall (Fig. 3). More sclerotia were discovered in the drier months, December through April of the following year, that is the dry season in the Xishuangbanna region. During the dry season the rainfall was lower than 50 mm/month. When the rainfall is over than 100 mm/month, the amount of sclerotia discovered was lower than 15/month. The more rainfall, the less sclerotia. It seems that the lower temperature increased the sclerotium formation. However the relationship of the sclerotium formation to temperature was not obvious as the rainfall. As can be seen, in the nature the sclerotium formation is an

important strategy of the fungus, *P. portentosus* for overcoming the unfavorable conditions to survive.

It is not easy to distinguish the different kinds (formation stages) of sclerotia, especially the died from shriveled but still alive ones. After the sclerotium germination test was done we recognized that the died sclerotium could not germinate but the shriveled germinated well under the suitable conditions. No any residues were left from the germinated shriveled sclerotia. However, when the sclerotium died out it left the dried out peridium in the soil (Fig. 4).

On the agar medium all the 3 isolates produced abundant sclerotia under the optimum conditions. It means that the sclerotium formation under artificial cultivation is not related to the unfavorable temperature or water conditions. The sclerotium occurrence always accompanied by appearance of the water drops on the colony (Fig. 6). The fungal colony on the agar medium produced water drops known as “spitting water”, which is a common phenomena when it reached the reproductive stage from vegetative stage^{4,30}. The results of recent research on *Morchella* cultivation indicated that occurrence of fruiting bodies followed the sclerotium formation, which accompanied by the water spitting^{31,32}. *Morchella* spitting water was due to the changes of cytoplasmic movement and osmotic pressure caused by the internal physiological balance or mycelial metabolism⁴, and the occurrence of sclerotium is the prerequisite of fruiting bodies formation of culturing *Morchella*^{4,11,33}. Under the artificial cultivation when *P. portentosus* fully colonized the substrate it produced abundant sclerotia on its surface. At this moment the fungus reached reproductive stage and ready to produce mushrooms. It can be said that under artificial conditions (on the agra medium) the sclerotium formation of *P. portentosus* is caused by its physiological changes in itself. However, in the nature during mushroom season (June through October) there was few sclerotia in the soils. The relationship of the sclerotium formation to the production of fruiting bodies of *P. portentosus* needs further study in the future.

Materials and methods

Preparation of fungal isolates. The fungal isolates of 17076、18004 and 18106 were used in this research. They were isolated from fruiting bodies of *P. portentosus*, which

were collected from Jinghong region of Xishuangbanna, Yunnan and kept at the Plant Protection and Microbial Utilization Research Center of Yunnan Institute of Tropical Crops, Yunnan, China.

Field investigation sites. Three sites were set up to investigate the sclerotium production. Two of them are the Grapefruit orchards, the Dongfeng Farm Grapefruit Orchard and Mangajian Village Grapefruit Orchard. They also are our experimental bases for the fungal field inoculation. The inoculation trail at the Dongfeng Farm Grapefruit Orchard started in 2015 and produced mushrooms in 2016. The inoculation trail at the Mangajian Village Grapefruit Orchard started in 2017 and produced mushrooms in the same year. The 3rd was the Jinghong Hydropower Station where wild *P. portentosus* was growing and fruiting. The three sites are separated over 30km away and all located at Jinghong City, Xishuangbanna, Yunnan(Fig. 9).



Figure 9. Investigation sites. (a) Dongfeng Farm Grapefruit Orchard. (b) Jinghong Hydropower Station. (c) Mangajian Village Grapefruit Orchard.

Field investigation methods. The investigation of the sclerotium formation was carried out in the second or last ten days of every month from October 2017 to October 2018. A small pit of 20 × 20 × 20 cm was dug out, number, size and shape of the sclerotia discovered from the pit were recorded and sampled. Attention also was paid to the changes of the sclerotium development at different collection time in order to construct the picture of the sclerotium formation in the field. Sampling the sclerotia was repeated twice at each sites. The sclerotia and soils from each sampled pits were taken to the Mycological Lab of the Yunnan Institute for Tropical Crop Research for further examination.

Morphological examination of the sampled sclerotia in the lab. Under a stereomicroscope (LEICA M125) and Electronic digital display Vernier caliper (GUANGLU 0~200mm), the morphological characteristics and dynamic changes of the sclerotia collected at different time were examined and recorded.

Sclerotium formation trail in the lab. The M1 agar medium²⁸ was used for culturing the isolates of 17076、18004 and 18106, which were incubated at 28-30°C. Each isolate had 10 plates. Mycelial growth and process of sclerotium formation were examined and recorded.

Molecular identification of Sclerotium, DNA extraction. Genomic DNA was extracted from a small (100 mg) piece of the sclerotium with cwbio's fungal genomic DNA isolation kit (BeiJing cwbio Biotech China). Sclerotium was identified based on the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene cluster. Two universal primers of fungus, namely, TIS1 (5'- CCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'), were used for DNA amplification. Amplification conditions include 94°C for 5 min, 30 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min; and then, 72°C for 10 min. The PCR product was gel purified and sequenced by TSINGKE (Kunming, China).

Phylogenetic analysis. For phylogenetic analysis, ITS sequences of representative taxa were obtained from GenBank by BLAST query (TABLE I). Comparative analysis of nucleotide sequences was obtained by conducting BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A phylogenetic tree was generated by the neighbor-joining (NJ) method on MEGA 7.0. Bootstrap analysis was conducted based on 1,000 reemblings.

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Author Contributions

Conception and design: T.W.Y., Y.W. and C.X.Z.; Investigation: T.W.Y., J.L., X.J.X., M.X.H., and C.X.Z.; Acquisition of data: X.J.X., M.X.H., F.G., Y.W.F. and W.B.W.; Analysis and interpretation of data: T.W.Y., J.L., L.M.D. and Y.W.; Writing original draft: T.W.Y., Y.W. and C.X.Z.

Competing interests

The authors declare that they have no competing interests.

Figures

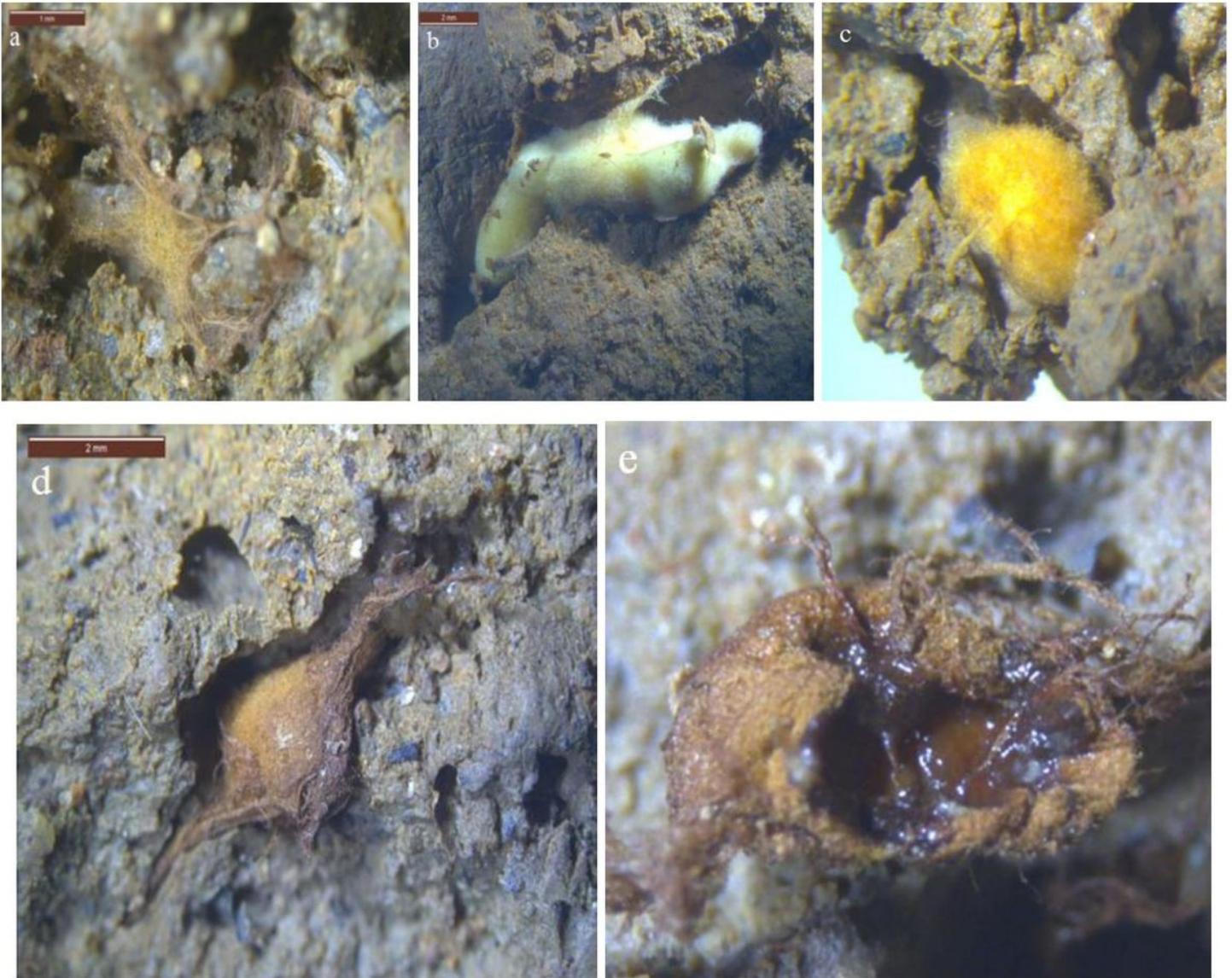


Figure 1

Early stage of sclerotium formation of in the field. (a) mycelia and rhizomorphs began to converge and tangle. (b) a pale yellow, irregular soft hyphal ball formed. (c) the hyphal ball gradually grew bigger, tighter and darker with dense air hyphae on the surface. (d) the hyphal ball wall soft and its surface rough covered by rhizomorphs. (e) dark brown honey-like juicy inside the ball. Scale bars: c e=2 mm.

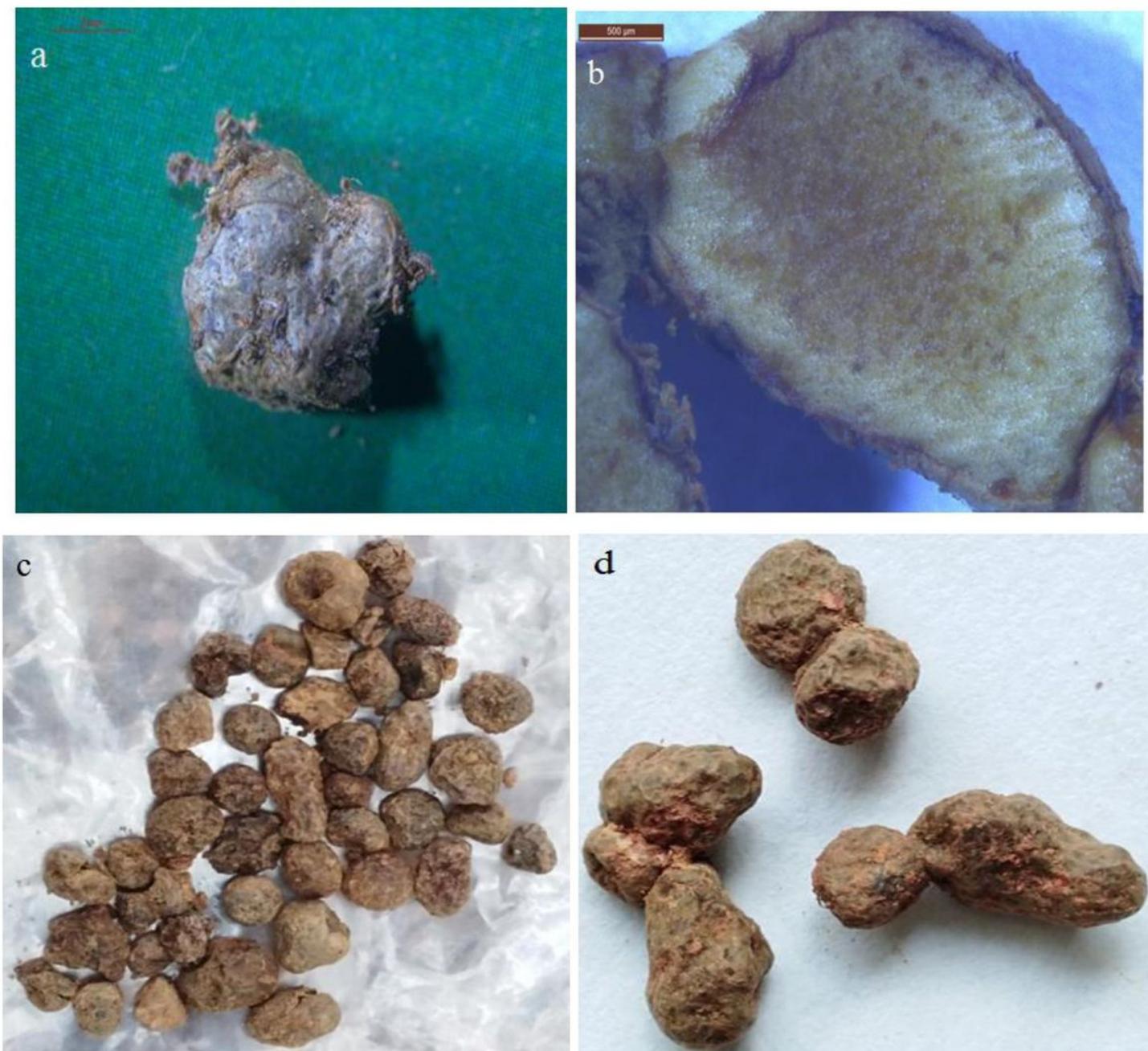


Figure 2

Matured sclerotium produced in the field. (a) appearance of a matured sclerotium. (b) a cross sectioned matured sclerotium showing the differentiated peridium and pith. (c) sclerotia in different shapes. (d) beads-like sclerotia. Scale bars: a, c=2 mm; b=3 mm.

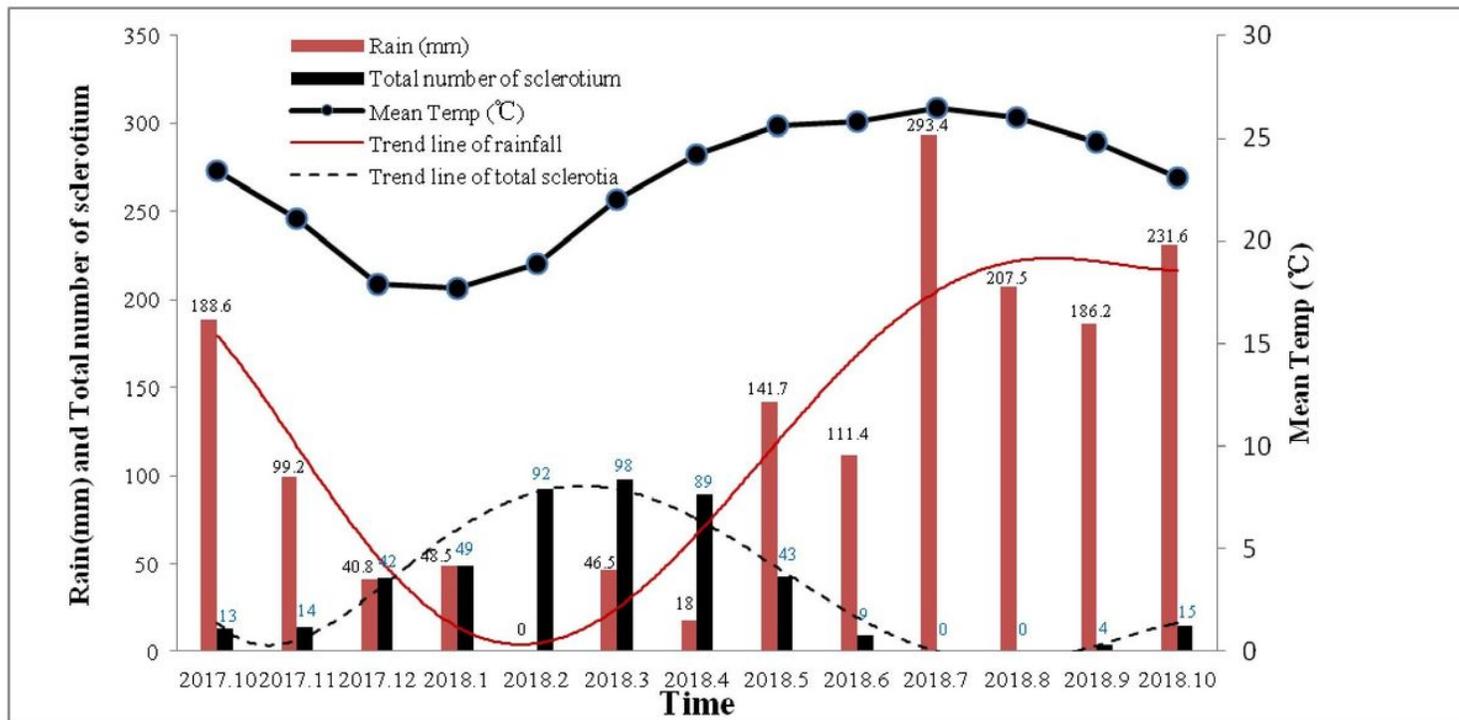


Figure 3

The relationship between the amount of sclerotia and monthly average temperature and rainfall. (the climate data provided by Xishuangbanna Meteorological Bureau).

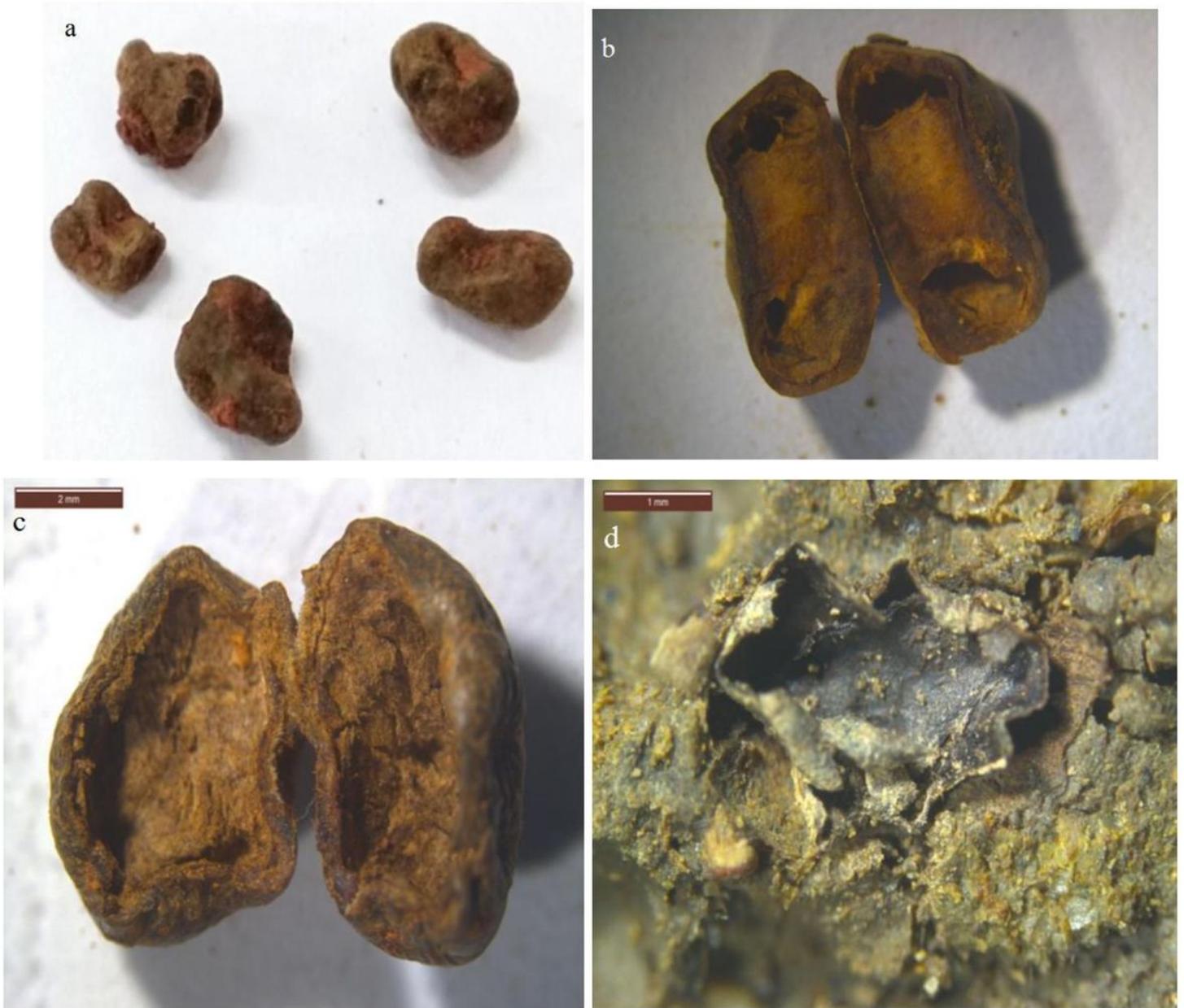


Figure 4

Aging and died young sclerotia. (a) shriveled sclerotia. (b) the sclerotium becoming hollow. (c) a cross sectioned died sclerotium. (d) Peridium remains of died sclerotium. Scale bars: a, b=2 mm.

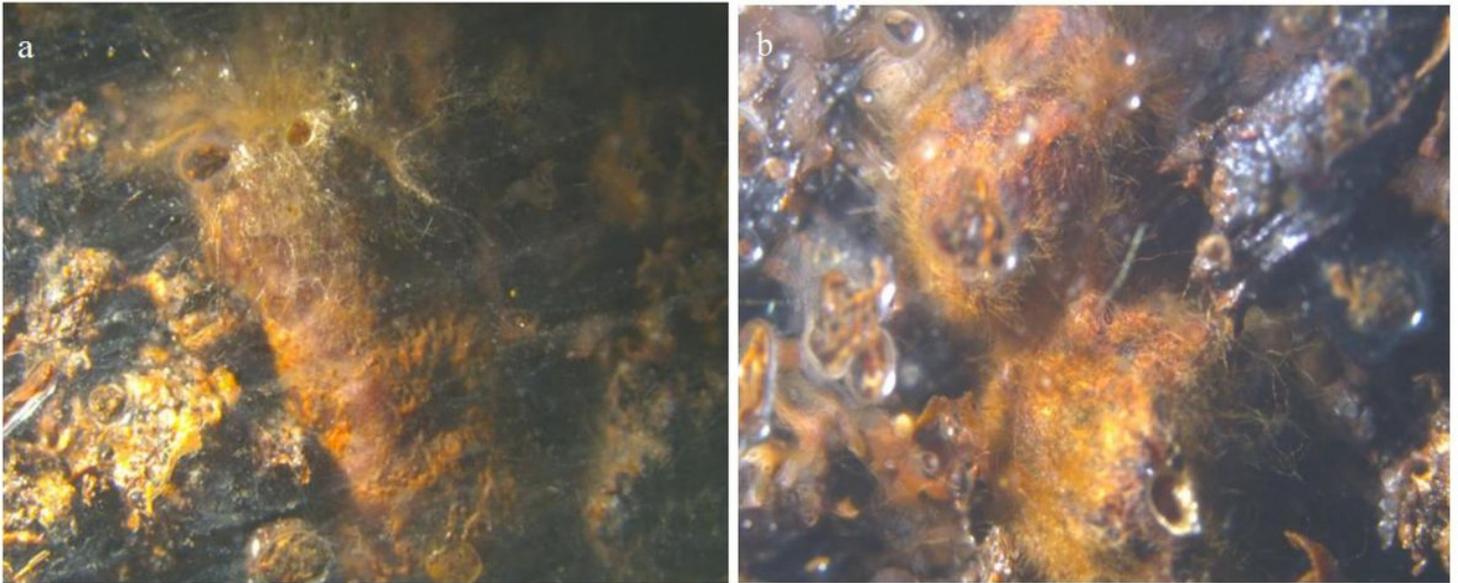


Figure 5

germination of the sclerotium. (a) a germinated sclerotium. (b) two sclerotia surrounded by new mycelia. Scale bars: a=2 mm; b=1 mm.



Figure 6

Sclerotium formation on agar medium. (a) liquid drops appeared on the colony. (b) curly, fluffy tangled mycelia knots raised up. (c) a baby sclerotium. (d) Inside the young sclerotium juicy and soft. (e) the internal tissue solidified with nutrients and hyphae accumulated. (f) liquid drops became dark brown. (g) brown and fluffy hyphae disappeared and pits appeared. (h) Cross sections showed the interior was

brown to dark brown with fresh hyphae and juicy stored nutrients. Scale bars: a, c, d, e=1 mm; f, g, h=2 mm.

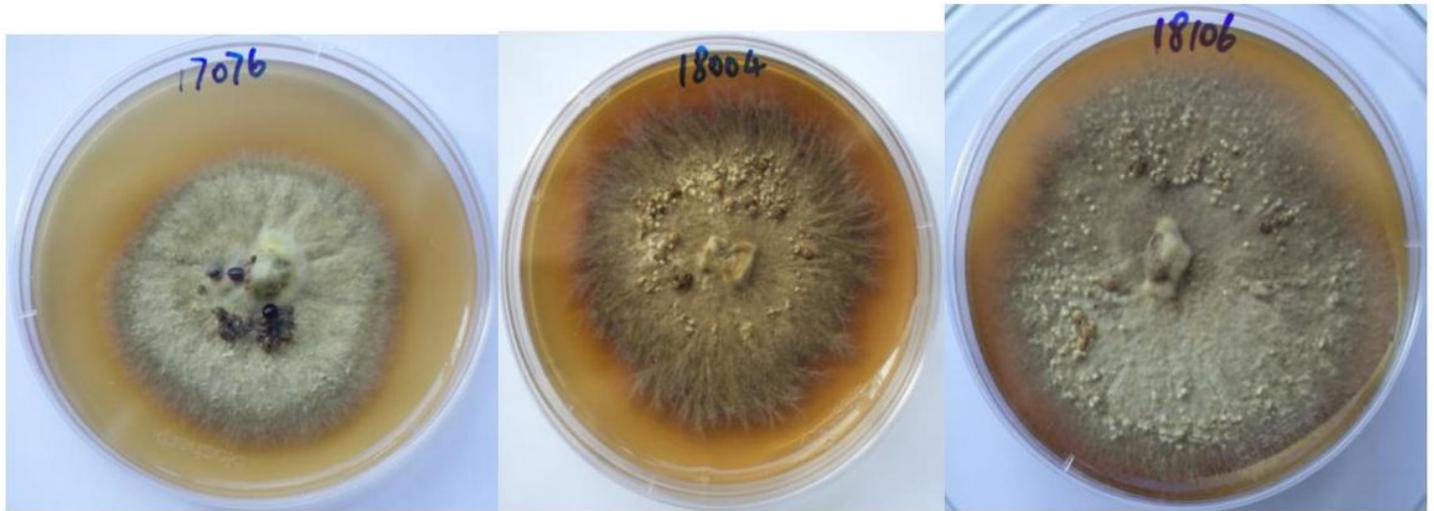


Figure 7

Sclerotium formation of three strains on the agar medium.

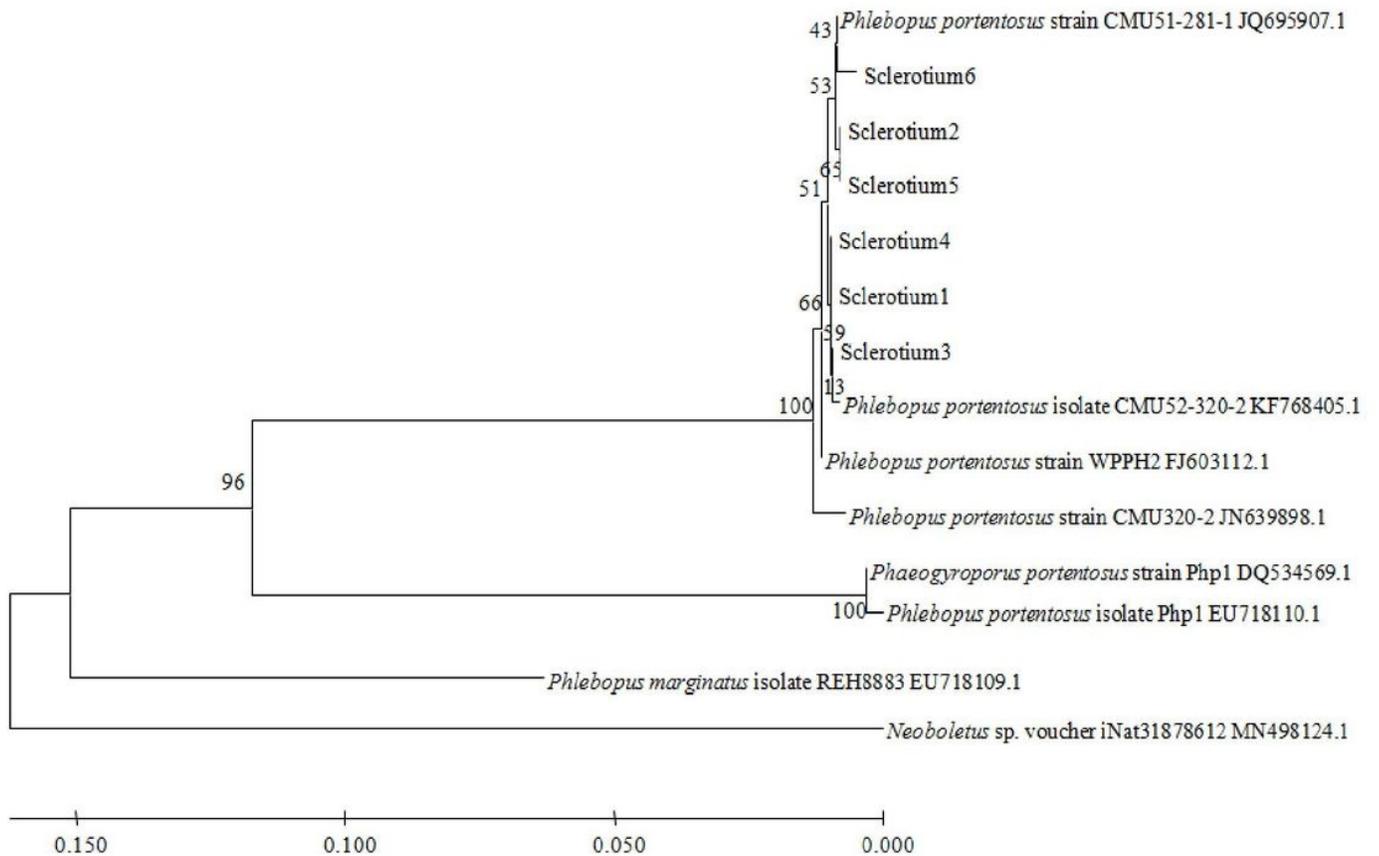


Figure 8

Neighbor-joining tree based on ITS region sequences of sclerotium1~6 and related GenBank sequences. Bootstrap tests were performed with 1000 repetitions.



Figure 9

Investigation sites. (a) Dongfeng Farm Grapefruit Orchard. (b) Jinghong Hydropower Station. (c) Mangajian Village Grapefruit Orchard. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research

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