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研究报告

Isolation, identification and characterization of *Neonothopanus nambi* (Basidiomycota, Fungi), a new record from China

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Abstract: [Objective] A luminous Basidiomycete, designated MRC-lf3, was isolated from Fujian, China, and its taxonomic status was confirmed, and its life cycle was recorded. **[Methods]** The mycelial pure culture was obtained by tissue isolation method; morphological characteristics and the phylogenetic analysis of ITS rDNA sequence were used to analyze the taxonomic status of this fungus; the morphological and luminous characteristics were recorded by Digital Single Lens Reflex. **[Results]** Both morphological characteristics and ITS rDNA phylogenetic analysis results demonstrated that the strain MRC-lf3 was *Neonothopanus nambi*. Our study also finding that both ability to emit light and color of the fruiting body could be changed by different illumination intensity. **[Conclusion]** We isolated the luminous fungal strain from Fujian China, which was identified as *Neonothopanus nambi*, this is the first record of genus *Neonothopanus* in China. This new record of *Neonothopanus nambi* brings the total number of luminous fungi in the Chinese Mainland to 8 species.

Keywords: Luminescence, Basidiomycete, Morphology, ITS, Life cycle

中国野生发光真菌新记录种 *Neonothopanus nambi* 的分离、鉴定及其形态观察

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摘 要:【目的】在中国福建地区采集分离一株野生发光真菌，菌种编号为 MRC-lf3，对其进行形态观察及分子鉴定以确定其分类地位，并观察其整个生活史的形态特征。【方法】采用组织分离技术获得野生发光真菌纯培养物，结合形态学及 ITS 进化分析确定该菌的分类地位，应用单反相机记录子实体不同生长时期的形态特征及发光情况。【结果】该发光真菌的菌丝、子实体、孢子的形态均与 *Neonothopanus nambi* 最为相似，ITS 序列的进化分析结果也支持该发光真菌为 *Neonothopanus nambi*。其子实体颜色深浅和发光强度均受到光照强度的影响，光照可使子实体颜色变暗、发光强度减弱。【结论】从福建省福州市采集分离获得一株大型野生发光真菌，经鉴定该菌为 *Neonothopanus nambi*，是中国首次发现的 *Neonothopanus* 属真菌，该发现也将中国大陆的野生发光真菌总数增加至 8 种。

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关键词：萤光，担子菌，形态学，ITS，生活史

1 Introduction

Bioluminescence is a natural phenomenon that has fascinated humans throughout history. It represents one of the oldest fields of scientific study, dating from the first written records of the ancient Greeks[1]. Recently, bioluminescence is known to exist in certain animals, plants, and fungi. Aristotle (384–322 BC) described light emissions from rotten wood and distinguished this 'living light' from fire for the first time in written records[2]. In 2008, Desjardin et al. updated the number of luminous fungi species from 42[3] to 64, and classified them into three distinct lineages: *Omphalotus*, Mycenoid and *Armillaria*[4]. In 2010, seven new species of luminous fungi were discovered, increasing the number of known luminescent fungi species to 71[4-6], this data had been increased to 81 until 2014[7-9]. But there are only 7 species (including *Filoboletus yunnanensis*, *Filoboletus manipuolaris*, *Dictyopanus pusillus*, *Lampteromyces luminescens*, *Lampteromyces mangensis*, *Pleurotus prometheus* and *Omphalotus olearius*) have been reported from China mainland[7,10-13]; although there are 15 *Armillaria* species was reported in China, no one of them had been reported as luminescent fungi[14].

In this paper, we report a new record of a bioluminescent fungal species, namely, *Neonothopanus nambi* (Speg.) R.H. Petersen & Krisai, in China, based on analysis of morphological and molecular data.

2 Materials and Methods

2.1 Microorganisms isolation

Fungal fruiting bodies were collected from rotting wood at the Fujian Agriculture and Forestry University (119°22′−119°24′E, 26°08′−26°09′N), Fujian, China, in July 2007 and May 2012 (identified as the same species and designated as specimens LF1 and LF3). Mycelial colonies were isolated from stipes and cultured in potato dextrose agar (PDA) slants at 25±1 °C in darkness (The strain number was designated MRC-lf3). Both two specimens were deposited in Mycological Research Center of Fujian Agriculture and Forestry University and the living culture examined was deposited in both Mycological Research Center of Fujian Agriculture and Forestry University and China Center for Type Culture Collection (CCTCCM 2013668).

2.2 Culture medium

Sawdust medium: 78% sawdust, 20% bran, 1% anhydrite, 1% sugar. The ratio of material to water was 1:1.1.

2.3 Morphological analysis

Morphological observations were made of specimens cultured in PDA at 25±1 °C in darkness for 4 weeks. Macro-morphology was studied at this stage, while micro-morphological examinations were made during the second week of incubation, using an Olympus BX51 microscope. Nuclear staining of mycelia and basidiospores was performed using 4 mg/L DAPI (4′,6-diamidino-2-phenylindole) and 0.001% EB (Ethidium bromide). Micro chemical reactions of the fruiting bodies to 3% KOH and 3% NH_4OH were recorded from dried material revived in ethanol followed by water[15]. Basidiospores were monitored and characterized using a JSM-6380 LV scanning electron microscope (SEM), 15 kV. Spore statistics included: x, the arithmetic mean of spore length by spore width (± standard deviation); Q, the quotient of spore length by spore width in any one spore, indicated as a range of variation in n spores measured; and Q_m, the mean length/width quotient of all spores measured (±s). The specimens were photographed using a Nikon D90 camera, and morphology was described according to fungal classification books[16-17].

2.4 DNA extraction, amplification and sequencing

Genomic DNA was extracted, using an improved Hexadecyl trimethyl ammonium Bromide (CTAB) method[18], from mycelia cultured in liquid PDA and fruiting body, respectively. ITS sequences containing the 18S rRNA partial sequence, the internal transcribed spacer regions 1 and 2, the 5.8S rRNA complete sequence and the 28S rRNA partial sequence were amplified by polymerase chain reaction (PCR) using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′)[19-21]. The PCR reaction mixture contained 5 μL 10×Buffer (with

Mg^{2+}), 4 μL (10 μmol/L) dNTPs, 2 μL (5 μmol/L) each primer, 1 μL (5 U) *Taq* DNA polymerase, 34 μL H_2O, and 2 μL genomic DNA in a total volume of 50 μL. Samples were incubated in a thermal cycler at 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min; followed by a final extension at 72 °C for 10 min.

Amplifications that resulted in a single product were purified with Universal DNA Purification Kit (TIANGEN, Beijing, China). Purified fragments were then cloned into a pMD18-T vector (TaKaRa, Dalian, China), propagated in *E. coli* DH5α, and sequenced using an ABI-PRISM3730 sequencer at Sangon Biotech Co., Ltd (Shanghai, China).

2.5 Phylogenetic analyses

All 24 sequences used in this study were downloaded from GenBank, the sequences accession numbers and information on their origins were obtained from the NCBI database, and bioluminescence information was obtained from Desjardin et al[4-5]. Sequence information is listed in Table 1.

Table 1 Strains used for constructing the phylogenetic tree
图 1 用于构建系统进化树的菌株信息

Strains	Sequences' origin	GenBank number	Bioluminescence
Neonothopanus gardneri	Brazil	JF344713	+
Neonothopanus nambi	Vietnam	JN571729	+
Neonothopanus nambi	Vietnam	JN571726	+
Neonothopanus nambi	Vietnam	JN571728	+
Neonothopanus nambi	PuertoRico	AF525074	+
Neonothopanus nambi	Malaysia	DQ444306	+
Neonothopanus nambi	Malaysia	DQ444307	+
MRC-lf3	China	KC514805	+
Omphalotus iμudens	USA	AY313271	+
Omphalotus olivascens	USA	AY313281	+
Omphalotus nidiformis	*	EU424307	+
Omphalotus illudens	*	JN571727	+
Omphalotus illudens	USA	AY313273	+
Marasmieμus opacus	USA	AY256703	−
Marasmiellus opacus	USA	DQ450005	−
Marasmiellus ramealis	Sweden	DQ450030	−
Marasmiellus stenophyllus	USA	DQ450032	−
Marasmiellus aff. *pluvius*	USA	DQ450029	−
Marasmius brunneoaurantiacus	*	FJ917620	−
Marasmius occultatus	*	FJ917622	−
Marasmius sp. Z37	*	JN198388	−
Marasmius sp. RR-2010a	*	FJ917629	−
Pleurotus citrinopileatus	*	EU424285	−
Pleurotus salmoneostramineus	*	EU424302	−

Note: +: Species of fungi reported as bioluminescent; −: Species of fungi not reported as bioluminescent; *: Information not available in the NCBI database.

The obtained sequences and other sequences downloaded from the GenBank database were aligned with MEGA 5.10 using ClustalW, followed by manual adjustment[22]. Phylogenetic analysis was performed with MEGA 5.10 using Maximum Composite Likelihood with a transition to transversion ratio. Phylogenetic trees were constructed using the Maximum Likelihood method. Bootstrap tests were performed using 1 000 replicates[22].

3 Results

3.1 Morphological observations

Growth habit: Fungal specimens were discovered growing at the base of rotting stumps and roots of hardwood trees (Figure 1A).

Fruiting bodies were solitary, clumped, and comprised overlaid layers. They could be divided into four different developmental stages: primordia, first developmental stage, middle developmental stage, and late development stage. The primordial stage was characterized by the presence of white or brown dots (Figure 1B). The first developmental stage presented the following: a semicircular pileus with a spatulate, rotund and approximately circular shape; stipe was central and eccentrically to centrally lateral; both the pileus and stipe were white or light tan in color on shady slopes and brown on sunny slopes; lamellae were decurrent, white, and somewhat dense (Figure 1B, 1C). The middle development stage was characterized by the following: pileus exhibited rotundity and an irregular round or reniform shape with involute edges; 10–50 mm in width, 0.3−0.7 mm thick; presence of radial stripes and a taupe coloration on sunny slopes, and white with some tan and light yellow patches on shady slopes; lamellae were decurrent, white, divergent and 0.1−0.5 mm broad (Figure 1D, 1G, 1I). The pileus was smooth, glabrous, lacked a veil or chap, and presented a hyaline or pale yellowish appearance in 3% KOH but greenish in 3% NH_4OH; stipes were clavate and solid with tough flesh, and were fairly short and thin (3−7 mm wide and 1−10 mm long). No annulus or volva was present. The context was fleshy, thin, white, odorless; the white part of the fruiting body was strongly luminescent (bright yellowish green; Figure 1F, 1H, 1J); in all development stages.

Basidiospores presented the following characteristics: spore prints were white; spores were hyaline, inamyloid, ellipsoid, and ruffled at the surface. Spore dimensions were, (3.4−5.5) μm×(2.1−3.4) μm, [x=(4.8±1.0) μm×(2.7±0.5) μm; Q=1.4−2.1; Q_m=1.75±0.18; n=20]. Four basidiospores were observed in each Basidia and the Pleurocystidia was absent; DAPI staining revealed that the spores were uninucleate (Figure 1O, 1P, 1Q).

The mycelium exhibited the following characteristics: white in color, vigorous growth, and bright yellowish green luminescence. A dark brown liquid was produced after one month when grown in a PDA culture medium (Figure 1K, 1L). Microscopic observations revealed that the mycelium had branch, septal and clamp connection, 2.5−6.2 μm in diameter. EB staining showed that cells were binucleate (Figure 1M, 1N).

N. nambi is distinguished by the ability to form luminescent and pleurotoid-like basidiomes with decurrent and distant lamellae, eccentric and solid stipes, inamyloid and ellipsoid basidiospores (4.0−6.5) μm×(2.8−4.0) μm, non-gelatinized, inamyloid hyphae with clamp connections, and white to grayish-tan fruiting bodies accommodate this isolate in *N. nambi* (Petersen & Krisai-Greilhuber 1999)[23].

3.2 ITS sequence and phylogenetic analysis

The ITS sequences of fruiting body (LF3) and mycelia culture (MRC-lf3) were exactly the same, and the length was 726 bp (GenBank number: KC514805). The final ITS dataset comprised 24 sequences and 706 characters including gaps, after excluding regions deemed too ambiguous for alignment. Phylogenetic analysis of the aligned sequences was performed using the Maximum Likelihood method (Figure 2). MRC-lf3 and six *N. nambi* ITS sequences were identified in the same clade (Bootstrap value=99%), and clustered with *Neonothopanus gardneri* in the *Neonothopanus* genus (Bootstrap value=89%). The *Neonothopanus* clade is strongly supported (Bootstrap value=88%) as the sister clade to *Omphalotus*. Both clades were identified to be Omphalotus lineages, belonging to Omphalotaceae. Four Marasmiaceae species and two Pleurotaceae species were used as out-groups; they were in the different clades to Omphalotaceae.

The ITS sequences of 24 strains built by the Maximum Likelihood tree showed our isolate is *N. nambi*, a species not previously recorded from China; this result is supported by previous studies[24].

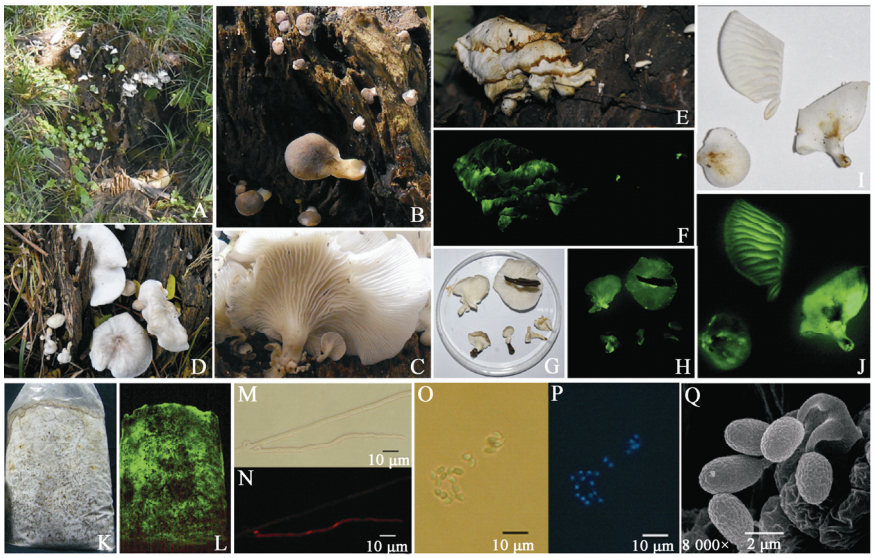

Figure 1 The luminous fungus *Neonothopanus nambi* (LF3)

图 1 发光真菌 *Neonothopanus nambi* (LF3)

Note: A: The habitat of LF3; B and C: Primordial and first developmental stages of the fruiting bodies on sunny and shady slopes, respectively; D: Middle developmental stage of the fruiting bodies (sunny slope); E and F: Late developmental stage of fruiting with daylight exposure and 10 min dark exposure, respectively; G and H: Mature fruiting bodies grown on sunny slopes with daylight exposure or 10 min dark exposure, respectively; I and J: Mature fruiting bodies grown on shady slopes with daylight exposure or 10 min dark exposure, respectively; K and L: Colonies grown on sawdust media with daylight exposure or 10 min dark exposure, respectively; M and N: Microstructure of mycelia, unstained or stained by EB, respectively; O−Q: Microstructure of basidiospores, unstained, stained by DAPI, or observed by SEM, respectively.

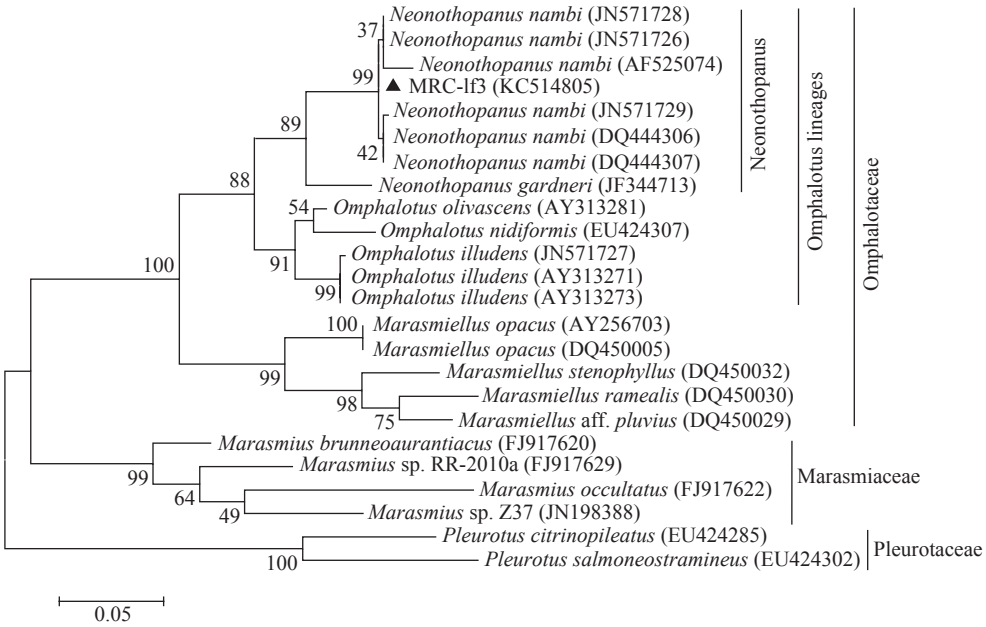

Figure 2 Maximum Likelihood tree obtained from heuristic research based on the ITS sequences

图 2 基于 ITS 序列构建的系统进化树

Note: All of sequences were downloaded from NCBI database (http://www.ncbi.nlm.nih.gov/pubmed/). Taxon name was composed of "Organism (NCBI ID)". Scale bar indicates evolutionary distance. Values above branches indicate the degree of bootstrap support from a 1 000 replicate analysis.

4 Discussion

Neonothopanus was established based on *N. nambi*, which was formally recognized as *Agaricus nambi*[23]. According to Species Fungorum 2014 (http://www.indexfungorum.org/Names/Names.asp), there are three species, namely, *N. nambi*, *N. gardneri* and *N. hygrophanus* belonging to the *Neonothopanus* genus. Both *N. gardneri* and *N. hygrophanus* were transferred to the *Neonothopanus* genus in 2011[25-26]. And several main differences could be found among these three species. For instance, *N. nambi* and *N. hygrophanus* forms white to pale grayish tan (brown) basidiomes and ellipsoid basidiospores, but *N. gardneri* forms yellow basidiomes and globose basidiospores; the fruiting body of *N. nambi* and *N. gardneri* could luminescent, but *N. hygrophanus* has not been explicitly reported as bioluminescent[9,25-26].

N. nambi has been reported in Vietnam, South America, Central America and the Caribbean region, Australasia including Papua New Guinea and New Caledonia, South Asia and Southeastern Asia, but it is a new species in China[4,9,24,27]. Compared with the 19 specimens which had been described by Audrey et al[9], the specimens we collected have some special and new characteristics, such as: both the pileus and stipe were brown on sunny slopes when young, and weakly, sometimes no luminescent; and the basidiospores were ruffled at the surface according to scanning electron microscope results.

As a species of *Neonothopanus*, *N. nambi* has been extensively studied. In 2009, a luminous fungus isolated from dead wood in rainforests in southern Vietnam was successfully cultivated on a large scale and then identified as *N. nambi* based on a combination of morphological and molecular data[24,28]. This made *N. nambi* an important species in research on bioluminescent systems, bioactive compounds, and related applications[29-31]. However, few photographs of *N. nambi* in the wild have been reported[9], especially with regards to the entire developmental process. Our research addressed this deficiency by photographing the entire process from sporulation and mycelial production to fruiting body development. A notable finding was that illumination intensity changed the color of the pileus. The pileus became light tan in color and exhibited a partial or complete inability to emit light when grown under high illumination conditions. This phenomenon can provide certain reference values in the application of *N. nambi* in bioluminescence research.

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