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First Record of a Species in the Genus *Sphaerobolus* (Geastrales) from Myanmar

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Abstract The mushrooms with very small fruit bodies were collected in 2017 from Lampi Island, Myanmar, and they were demonstrated to be species of *Sphaerobolus* (Geastrales), which have never been reported from this country. Lack of some critical characters in the specimens made detailed morphological comparison difficult, but DNA sequence data clearly separated the Myanmar specimens from the three currently recognized species in the genus.

Key words: Agaricomycota, biogeography, distribution, fungi, inventory, mushrooms, Phallomycetidae, Southeast Asia.

Introduction

To clarify fauna and flora (including fungi) of Myanmar and the surrounding areas, a joint research team of National Museum of Nature and Science, Japan, and Forest Research Institute, Myanmar has been conducting biological inventories in Myanmar since 2016. During one of our fieldwork activities in 2017, we have collected numerous small-sized fruit bodies on woody substrate with earthstar-like morphology, which were strongly suspected as a species of the genus *Sphaerobolus* Tode (Geastrales, Phallomycetidae, Agaricomycota).

The genus *Sphaerobolus* is commonly known as “cannonball fungus” or “artillery fungus” due to their unique morphology and mechanism for basidiospore discharge (Ingold, 1972). Because they produce abundant fruit bodies on artificial media, they have been used for many experimental studies (Ingold and Peach, 1970; Flegler, 1984). Their phylogenetic relationship to earthstar fungus, the genus *Geastrum*, has long been speculated, but it has only recently been demonstrated to be true based on molecular analyses using multiple genes (Hibbett *et al.*, 1997; Hosaka *et al.*, 2006).

The online repositories of fungal names, such as Index Fungorum, currently list a total of 35 taxa for the genus *Sphaerobolus*. Some of them, however, need to be synonymized or transferred to other genera, and the remaining 15 species could tentatively be considered valid taxa in the genus. Some authors recognize only three species in the genus, i.e., *S. stellatus* (type species), *S. iowensis* and *S. ingoldii* (Geml *et al.*, 2005). Those three taxa all have similar macro-morphological characters, with slight differences in glebal size (Geml *et al.*, 2005). Microscopically, they all possess ellipsoid basidiospores but their sizes and length/width ratios are slightly different (Geml *et al.*, 2005). Geml *et al.* (2005) further demonstrated differences in hyphal growth rate and colony morphology using several artificial media.

Because some characters, especially growth

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rate on agar media, are sometimes difficult to obtain, DNA sequences are arguably the easiest and most straightforward data to compare species of *Sphaerobolus*. The study by Geml *et al.* (2005) is by far the only one including multiple taxa of *Sphaerobolus* in phylogenetic context. According to their study, each species of the genus was strongly supported as monophyletic and they could clearly be separated each other by long branches based on commonly used loci for fungal phylogenetic studies, such as ITS. Therefore, by obtaining DNA sequences from new samples and by analyzing them phylogenetically, it is easy to recognize whether they belong to the described species or potentially new species in science.

Colored photographs and brief description of basidiomata of *Sphaerobolus* from Myanmar are provided. In addition, DNA sequence data from the large subunit (nuc-LSU) of nuclear ribosomal RNA, elongation factor 1-alpha gene (EF-1α), and mitochondrial small subunit of ribosomal RNA (mt-SSU) were obtained to clarify the phylogenetic position of *Sphaerobolus* from Myanmar.

Materials and Methods

Collecting Sites, Collecting Scheme, and Curation of Specimens

Multiple basidiomata of *Sphaerobolus* were collected by the first author at the coast of Lampi Island, Myanmar (N10°41′26.9″, E98°14′38.8″; elevation 0 m) on May 23, 2017. The samples were photographed under natural light in the field and then wrapped with aluminum foil for transport to the laboratory facility in Bo Cho Island, Myanmar. In the laboratory, the samples were photographed again using artificial lighting and more in-depth macroscopic observation was conducted. The samples were dried with low heat and good air circulation using a food dehydrator for 24 hours. In addition to dried materials, small fragments (ca. 1 cubic millimeters each) of clean, sterile tissue from freshly collected materials were cut using a clean razor blade. Contamination of visible soil particles and other materials was carefully avoided. The tissue fragments were soaked in DMSO buffer (Seutin *et al.*, 1991) with an addition of 100 mM Tris-HCl (pH 8.0) and 0.1 M sodium sulfite (Na_2SO_3), and they were stored at room temperature until further molecular experiment became possible, following the procedures of Hosaka (2009) and Hosaka and Castellano (2008).

Specimens collected during the fieldwork were deposited at Forest Research Institute, Myanmar (RAF), and the duplicate at the fungal herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS). All tissue samples were stored in freezers (−80°C) at the Center for Molecular Biodiversity Research, National Museum of Nature and Science.

DNA Preparation, PCR, and Sequencing

DNA was extracted from the tissue fragments stored in DMSO buffer. Tissues were ground under liquid nitrogen using a mortar and pestle. DNA extractions used a modified CTAB extraction followed by glass milk purification methods as summarized by Hosaka (2009) and Hosaka and Castellano (2008).

DNA sequence data were obtained from nuc-LSU, EF-1α and mt-SSU. The primer combinations used for PCR amplifications include: LR0R and LR5 (Vilgalys and Hester, 1990) for nuc-LSU, EF1-983F and EF1-1567R (Geml *et al.*, 2005) for EF-1α, and MS1 and MS2 (White *et al.*, 1990) for mt-SSU. PCR reactions were carried out using 20 µl reaction volumes each containing: 1 µl genomic DNA, 1 µl dNTPs (4 mM), 1 µl of each primer (8 µM), 0.5 units of Taq polymerase (TaKaRa, Tokyo, Japan), 2 µl MgCl2 (25 mM), 2 µl Bovine Serum Albumin (BSA). PCR reactions were performed using the same program for all genes: 95°C for 3 min; 35 cycles of 95°C for 1 min, 51°C for 45 sec, 72°C for 1 min; and 72°C for 15 min. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification bands were confirmed, PCR products were then purified using the ExoSap-IT

(Millipore, Molsheim, France) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit on ABI3500 (Applied Biosystems Inc., Foster City, CA, USA), following the manufacturer's instructions. Although the amplification of the ITS region was also performed and was successful, we could not obtain sequence data due to heterogeneity in chromatograms. Therefore, ITS sequence data of Myanmar *Sphaerobolus* are not reported in this study.

Molecular Analyses

The obtained raw sequences were edited using ATGC version 7.1.0 (GENETYX Corporation, Tokyo, Japan). Edited sequences were analyzed using the GenBank BLAST search (blastn) to confirm their phylogenetic affinities with *Sphaerobolus*. Default settings of blastn option were used. DNA sequences generated in this study (GenBank accession nos. MT514523 for nuc-LSU, MT531415 for EF-1α, and MT514522 for mt-SSU) were aligned manually with the data generated by Geml *et al.* (2005) using the data editor of BioEdit ver. 7.0.1 (Hall, 1999). The datasets of EF-1α and mt-SSU were then analyzed individually by maximum parsimony (MP) analysis. MP analyses were conducted under the equally weighted parsimony criterion using PAUP* version 4.0b10 (Swofford, 2002), with heuristic search option (with TBR and Multrees on, 100 random-addition-sequence replicates). Support for the individual nodes was tested with bootstrap (BS) analysis under the equally-weighted parsimony criterion. BS analysis was based on 100 BS replicates using the heuristic search option (TBR and Multrees options off), with ten random addition sequences.

Results and Discussion

Habitat and Curation

The collecting site was just along the shoreline of Lampi Island (Fig. 1A). The site was composed of some typical coastal plants such as *Pandanus*, but there were diverse broadleaved trees, including Dipterocarpaceae. Multiple fruit bodies (of up to 20) were found growing on a single fallen wood, which was identified as the family Pandanaceae (Fig. 1B). At the time of collecting, many fruit bodies still contained glebal mass in them as shown in Fig. 1B and 1C. However, all glebae were apparently discharged and lost during the transport to the laboratory. After careful examination of dried specimens, we have concluded that no data on mature basidiospores were obtainable from the current materials. Only mature fruit bodies without glebae or immature fruit bodies (Fig. 1D) without mature basidiospores were available. We therefore provide only a brief description of the materials which are summarized below.

Morphology

Basidiomata when immature (Fig. 1D) globose to slightly ellipsoid, white to slightly brownish, partially embedded in white hyphal mat, 1.8–2.3 mm in diameter. **Basidiomata when mature** (Fig. 1B and 1C) splitting apically into 8-10 rays, exposing inner layer of orange-yellow peridium and solitary peridiole (gleba), 2.6–3.0 mm in diameter (including the tips of rays). Mature, discharged glebae and basidiospores not observed.

Specimen examined. Myanmar: Lampi Island, Lampi Marine National Park (N10°41′26.9″, E98°14′38.8″), alt. 0 m, 23 May 2017, K. Hosaka KH-MYA17-115 (TNS, RAF).

Molecular Identification of Sphaerobolus

The BLAST search using the blastn option with default settings all resulted in *Sphaerobolus* as the top hit. However, the results from each gene showed less than 95% identity with the registered data, indicating the current materials from Myanmar represent different species form any other taxa registered in GenBank. The top hit of nuc-LSU blastn search was *S. ingoldii* (type material) with 100% query coverage and 94.7% identity. The nuc-LSU is usually considered slowly evolving. Therefore, their differences in 5% or more can be considered significant, indicating they are different species. Likewise, blastn

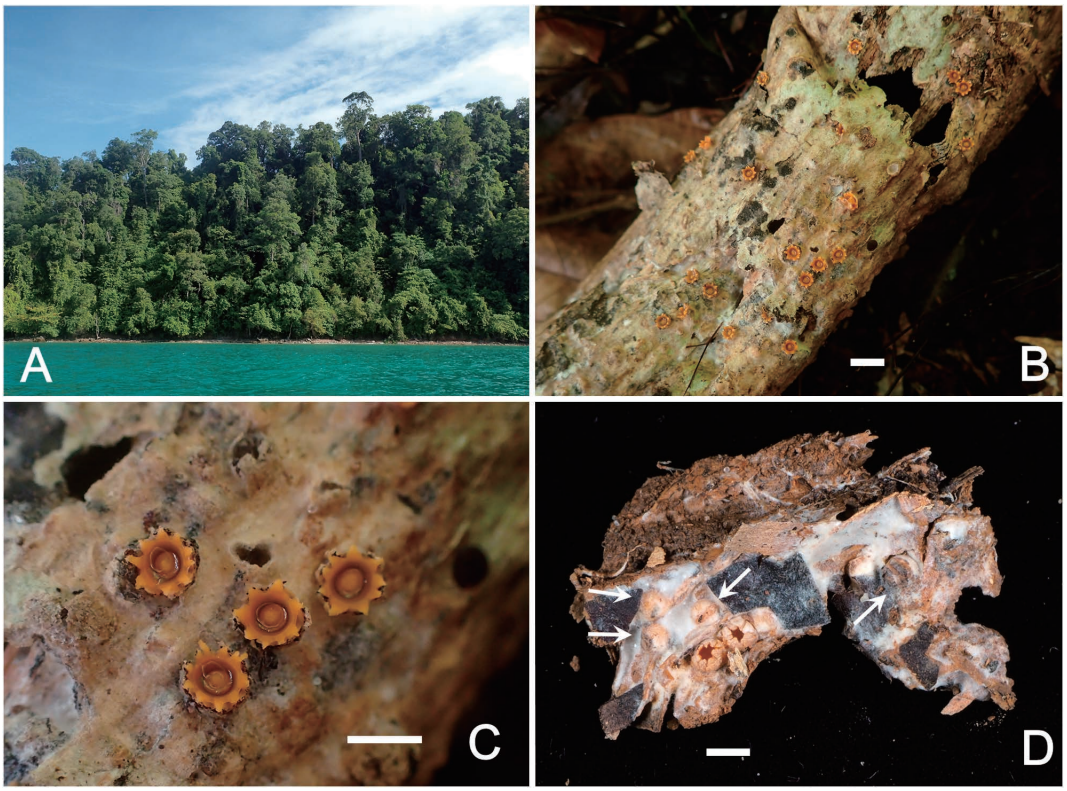

Fig. 1. *Sphaerobolus* sp. (KH-MYA17-115) from Lampi Island, Myanmar. A. Vegetation of collecting site (Lampi Island, Myanmar). B. Gregarious basidiomata on decaying wood. Bar = 5 mm. C. A magnifying view of basidiomata. Bar = 3 mm. D. Semi-dried, mature basidiomata without glebae and immature basidiomata partially embedded in hyphal mat (arrows). Bar = 3 mm.

searches of mt-SSU (moderate speed of evolution) and EF-1α (fast evolving) both showed very low identity with the Myanmar materials. The top hit of mt-SSU blastn search was *S. ingoldii* with 100% coverage and 89.7% identity. Finally, the top hit of EF-1α blastn search was *S. ingoldii* with 99% coverage and 84.4% identity. These results strongly indicate that the materials from Myanmar are different from the three currently recognized species in the genus, i.e., *S. stellatus*, *S. iowensis*, and *S. ingoldii*.

Phylogeny of Sphaerobolus

The EF-1α dataset was composed of a total of 10 taxa and 598 characters, of which 483 characters were excluded and the remaining 115 characters were parsimony informative. The MP analyses produced one parsimonious tree with a tree length 215 steps, CI of 0.7395, RI of 0.7838, and RC of 0.5796 (Fig. 2A).

The mt-SSU dataset was composed of a total of 10 taxa and 561 characters, of which 495 characters were excluded and the remaining 66 characters were parsimony informative. The MP analyses produced two equally parsimonious trees with a tree length of 86 steps, CI of 0.8837, RI of 0.9351, and RC of 0.8263 (Fig. 2B).

All currently recognized species (*S. stellatus*, *S. iowensis* and *S. ingoldii*) were recovered as monophyletic groups and the materials from Myanmar did not form a tight cluster with any one of them (Fig. 2). Based on these results alone, it is apparent that the Myanmar materials represent different species from the three currently recognized species in the genus.

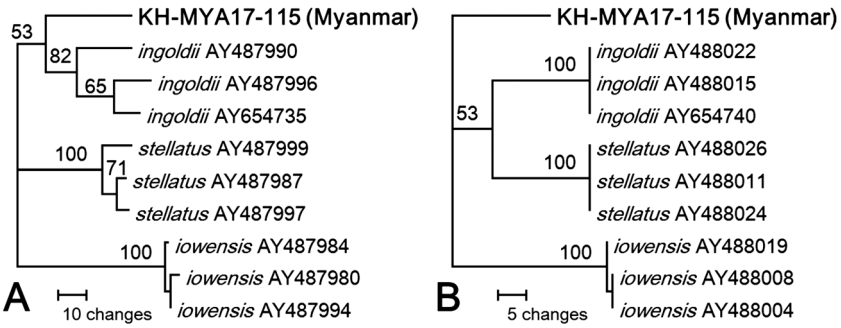

Fig. 2. Phylogenetic trees of *Sphaerobolus*. A. The most parsimonious tree based on EF-1α dataset. B. One of two equally parsimonious trees based on mt-SSU dataset. The trees are midpoint rooted. Taxon labels are shown as epithet, followed by GenBank accession numbers. The numbers on branches are bootstrap values.

Taxonomic Treatment and Biogeography

Although the genus has a worldwide distribution with records from all continents except Antarctica (Geml *et al.*, 2005), this is the first record of *Sphaerobolus* from Myanmar. Our molecular evidence strongly suggests the materials from Myanmar represent new species of *Sphaerobolus*. However, we decided not to formally describe a new species in this study because of several reasons.

Firstly, the voucher materials lack some important characters typically required to describe gasteroid mushrooms, e.g., glebae and basidiospores. Although it is not mandatory to include the characteristics of those structures for a formal description of species, we feel it worthwhile to make more effort to collect additional materials and/or to further scrutinize our current materials.

Secondly, the remaining twelve species in the genus have not been considered taxonomically although they were considered ambiguous or invalid taxa by some authors (Geml *et al.*, 2005). Most of them have been described in the 1800's to early 1900's and the type materials, if any, are probably in bad shape. In addition, their protologue tends to be overly simple, making morphological comparison very difficult. Based solely on the proximity of their type localities to Myanmar, however, *S. minimus* from the Philippines and *S. rubidus* from Sri Lanka are potentially the target taxa. In this case though, they probably represent very different taxa. According to the information obtained from the Index Fungorum (http://www.indexfungorum.org/), *S. minimus* possess brownish subiculum and globose spores, which indicate they do not belong to *Sphaerobolus*. *S. rubidus* grow on elephant dung and possess reddish fruit bodies, which imply they are probably ascomycetous fungi.

Thirdly, the study by Geml *et al.* (2005) has heavily focused on the materials from the Northern Hemisphere, but *S. stellatus* were also reported from Australasia, Africa and South America. By sampling globally, more intra-specific variations and/or additional new lineages in the genus might be revealed. This of course does not hinder us from formally describing a new species based on our current data, but we are hopeful to obtain additional samples from Myanmar in future fieldwork.

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