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Phylogenetic Analyses of a Truffle-like Genus, *Boninogaster*, from Hahajima Island, the Bonin Islands, Japan

Kentaro Hosaka

Department of Botany, National Museum of Nature and Science,
Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan
E-mail: khosaka@kahaku.go.jp

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Abstract The DNA sequence data of the mysterious truffle-like basidiomycetes, *Boninogaster*, collected from Hahajima Island, the Bonin Islands were obtained and analyzed to infer its phylogenetic position. Parsimony analyses demonstrated that *Boninogaster* belongs to the family Sclerogastraceae in the order Geastrales. A brief description of habitat and macro- and microscopic characters were also provided.

Key words: *atp6*, 28S, Geastrales, *Pandanus*, *Sclerogaster*.

Introduction

The genus *Boninogaster* was described by Kobayasi (1937) based on the specimens collected from Mt. Asahiyama, Chichijima Island, the Bonin Islands, Japan. The original materials were found "growing solitarily on the decayed stumps of *Pandanus boninensis* Warburg" (Kobayasi, 1937). However, the type specimen is apparently missing, and there are no additional collections ever since. Therefore, detailed observation of morphological characters and molecular analyses were not possible. The only additional collections were made in 1995 from the Bonin Islands (Environment Agency of Japan, 2000), but no DNA data were obtained.

Boninogaster is a truffle-like basidiomycete with unknown phylogenetic affinity. It is a monotypic genus, and a specific epithet of a sole species, *Boninogaster phalloides*, refers to its similarity to immature stages of stinkhorns (Phallales) (Kobayasi, 1937). However, no close affinity of *Boninogaster* to Phallales was mentioned by Kobayasi (1937). Instead, some genera considered as closely related include *Alpova*, *Octaviania* (Boletales), and *Martellia* (Russulales) (Kobayasi, 1937). Although no detailed discussion was provided, Kobayasi (1937) also pointed out a similarity of *Boninogaster* to Nidulariaceae (Agaricales) and Sphaerobolaceae (Geastrales). The most recent classification by Kirk *et al.* (2008) included *Boninogaster* in the family Hysterangiaceae (Hysterangiales, Phallomycetidae).

Recent fieldwork at the Bonin Islands resulted in discoveries of several truffle-like fungi. One of such collections was made from Hahajima Island, the Bonin Islands, growing directly on rotten fruits and stems of *Pandanus boninensis*. Because their morphological and ecological characters were in well agreement with *Boninogaster*, the specimens were tentatively identified as *Boninogaster phalloides* and its phylogenetic position was inferred based on the DNA sequences from two loci.

Materials and Methods

Collecting Sites, Collecting Scheme, and Curation of Specimens

Fieldwork was conducted by the author in

June, 2013. The main collecting sites were located in Hahajima Island, the Bonin Islands, Japan. A general collection scheme (Castellano *et al.*, 1989) for hypogeous, truffle-like fungi was followed. At each collecting site, woody substrates, mostly Pandanaceae, were identified to species. If alternative plants were present near fruit bodies, those were also recorded.

Each specimen was photographed and macroscopic observation was conducted. All specimens were cut into half and dried with low heat and good air circulation. In addition to dried materials, small fragments of glebal tissue from freshly collected samples 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) under 4°C, following the procedures of Hosaka (2009) and Hosaka and Castellano (2008).

More detailed identification and description were conducted after returning to the mycology lab at the National Museum of Nature and Science, Japan. Specimens collected during the fieldwork were deposited at the fungal herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS).

Light Microscopy

For light microscopic observations, a small portion from the gleba was mounted in water, 3% or 5% (w/v) KOH and 30% ethanol solution on glass slides. Those samples were examined with Olympus BX53 microscope under Nomarski interference contrast. More than forty randomly selected basidiospores were measured under a light microscope at 1000 × magnification.

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 the large subunit (LSU) of the nuclear ribosomal DNA and mitochondrial ATPase subunit 6 (*atp6*). For amplifying the LSU, the combination of LR0R and LR5 (Vilgalys and Hester, 1990) was used. In addition, DNA sequence from the ITS region was obtained using the primers ITS5 and ITS4 (White *et al.*, 1990) for DNA barcode. For amplifying the atp6, the combination of atp6-3 and atp6-2 (Kretzer and Bruns, 1999) was used. 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 $MgCl_2$ (25 mM), 2 μl Bovine Serum Albumin (BSA). 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 (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions.

Phylogenetic Analyses

The dataset of Hosaka and Castellano (2008) was used to infer the phylogenetic position of the samples. DNA sequences of the LSU and *atp6* were aligned manually using the data editor of BioEdit ver. 7.0.1 (Hall, 1999). Ambiguously aligned regions were excluded from the analyses. The dataset was then analyzed 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). Support for the individual nodes was tested with bootstrap (BS) analysis under the equally-weighted parsimony criterion. BS analysis was based on 10,000 BS replicates using the heuristic search option (TBR and Multrees options off), with ten random addition sequences.

Fig. 1. Collecting site of *Boninogaster* at Nishiura, Hahajima Island, the Bonin Islands.

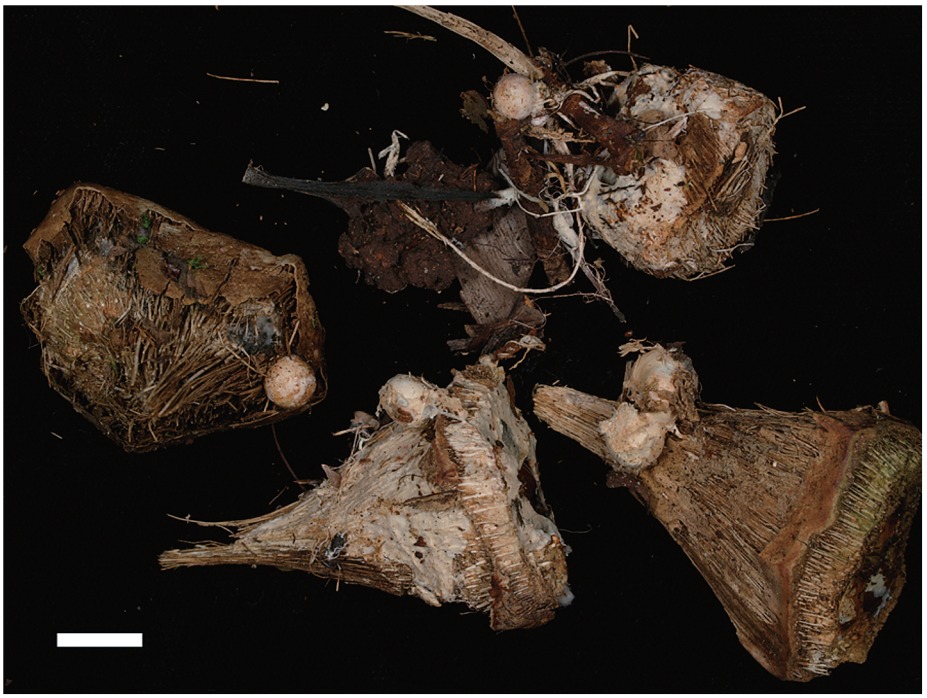

Fig. 2. Fruit bodies of *Boninogaster* (TNS-F-59692) growing on rotten fruits of *Pandanus.* Note globose fruit bodies with white mycelial mat on the surface of fruits. Bars = 1 cm.

Results

Collecting sites and habitat

Fruit bodies were collected from Nishiura, Hahajima Island, the Bonin Islands on June 26, 2013. The site was dominated by *Pandanus boninensis* with a few other tree species (Fig. 1). However, with a few exceptions, fruit bodies were always associated with *Pandanus*. The majority of fruit bodies were collected from rotten fruits of *Pandanus* (Fig. 2), but they were sometimes found from rotten branches of *Pandanus*, or other plant substrates. The specimen was deposited at the mycology herbarium of the National Museum of Nature and Science, Japan, under the specimen number TNS-F-59692.

Morphological observations

Fruit bodies were globose, 0.5–1.2 cm in diameter, with white peridium. They were often attached with white rhizomorphs at the base (Fig. 2, 3A, B). Peridium was composed of three layers: exoperidium and mesoperidium composed of hyaline, thin-walled, interwoven hyphae, and endoperidium composed of pseudoparenchymatous cells. Gleba was composed of minute peridioles, ochraceous with a slight olive tint, soft and slightly viscid when immature (Fig. 3C). Basidiospores were hyaline, smooth, ovoid to citriform, 5–5.5 × 3 μm (Fig. 3D).

Phylogenetic Analyses

The newly generated sequences were deposited in GenBank under the accession numbers KJ629153, KJ629154 and KJ629155. The dataset was composed of a total of 52 taxa, including the outgroup. The initial alignment resulted in 1471 bp long (LSU = 781 bp long, atp6 = 690 bp

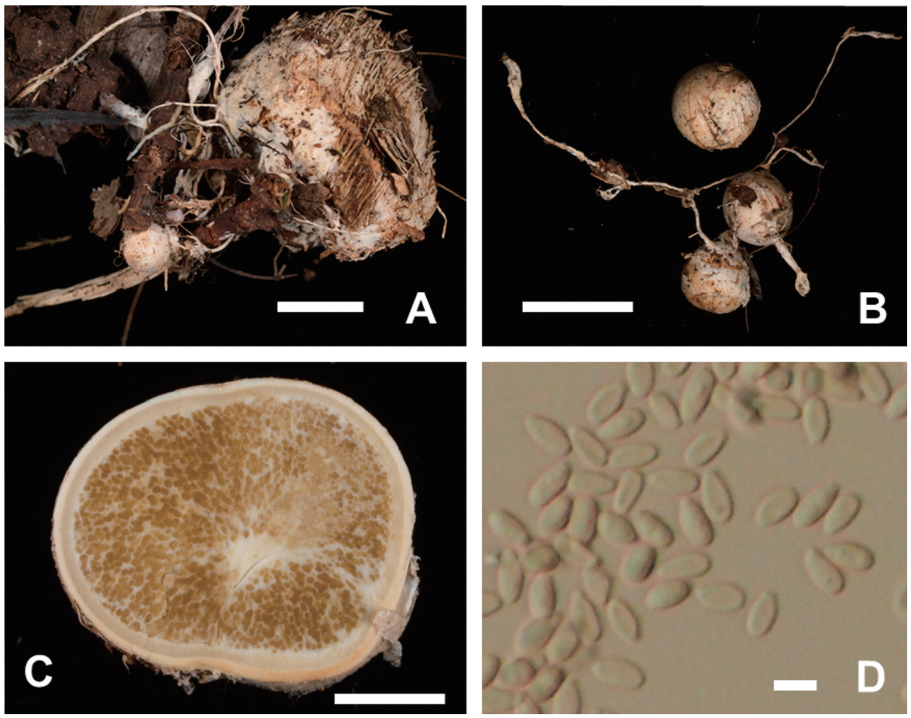

Fig. 3. Macro- and microscopic characters of *Boninogaster* (TNS-F-59692). A: A single fruit body with long white rhizomorphs and mycelial mat growing on rotten fruit of *Pandanus*. Bars = 1 cm. B: Fruit bodies with long rhizomorphs. Bars = 1 cm. C: Longitudinal section of fruit body. Bars = 0.5 cm. D: Light microscopy of basidiospores. Bars = 5 μm.

long). After excluding the ambiguously aligned region, a total of 1220 characters (LSU = 572, atp6 = 648) were kept for the analyses, of which 438 characters (LSU = 129, atp6 = 309) were parsimony uninformative. The MP analyses produced six equally parsimonious trees with a tree length of 1899 steps, CI of 0.3712, RI of 0.6745, and RC of 0.2504 (Fig. 4).

Discussion

The materials were found growing on *Pandanus* substrate (Figs. 1, 2), which is consistent with the finding by Kobayasi (1937). Although Kobayasi (1937) collected *Boninogaster* from the "decayed stumps of *Pandanus*", the collections of this study were mostly from rotten fruits of *Pandanus* (Fig. 2). Morphological characters

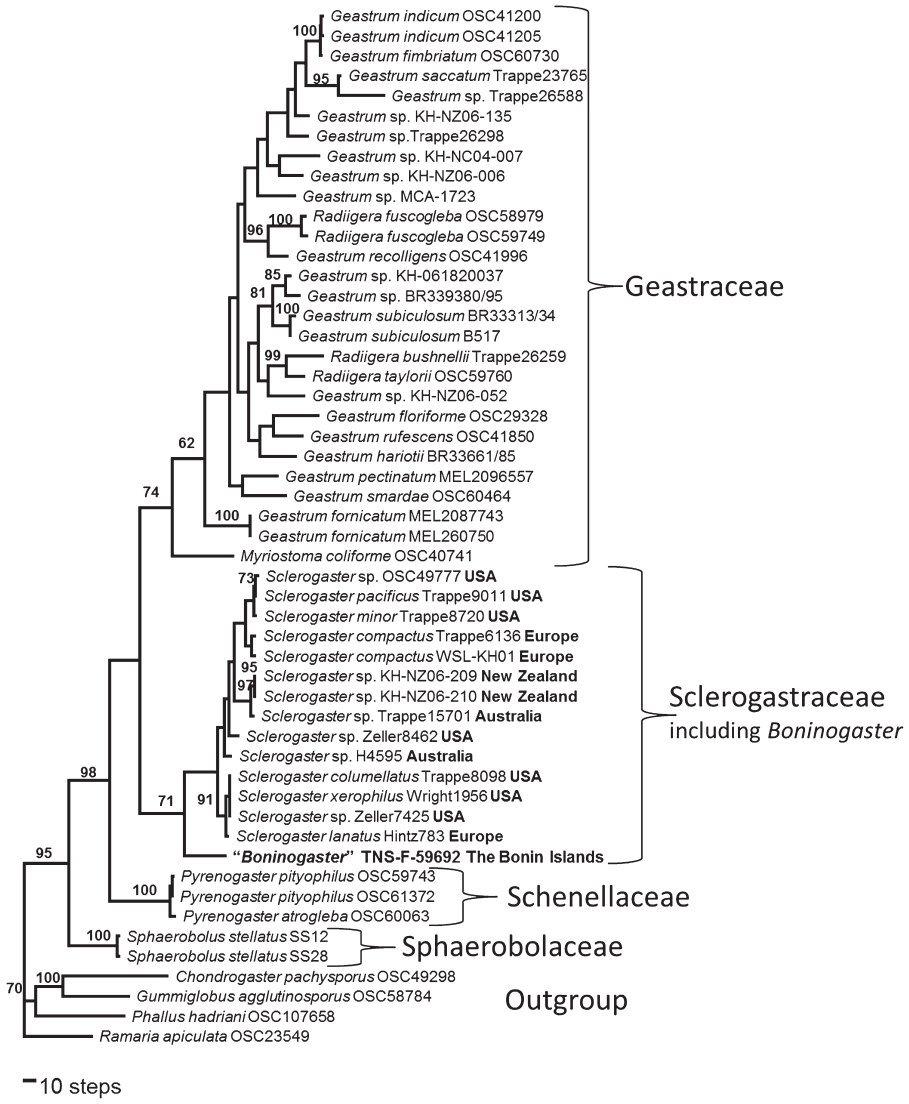

Fig. 4. One of the most parsimonious trees of Geastrales derived from a combined atp6 and LSU dataset for a supermatrix of 52 taxa. Taxon names followed by voucher numbers, and by area of distribution in bold face (only for the Sclerogastraceae clade). Numbers on branches are nodal supports (Bootstrap values).

were in well agreement with *Boninogaster*, having white, globose fruit bodies (Figs. 2, 3A, B) with three-layered peridium and ochraceous gleba (Fig. 3C), and smooth, ovoid to citriform basidiospores (Fig. 3D). The illustrations by Kobayasi (1937) seem to indicate that gleba is dark colored (almost black), but in the descriptions, glebal color is indicated as "Dark Olive Buff" (Kobayasi, 1937). It is unclear whether the materials from this study (TNS-F-59692, Fig. 3C) have gleba of dark olive buff defined by Kobayasi (1937), but no materials collected for this study had gleba darker than Fig. 3C. Furthermore, spores are described as ovoid to citriform (Kobayasi, 1937), which are in well agreement with the current materials (Fig. 3D), but the illustrations seem to indicate having more strongly lemon-shaped than Fig. 3D.

Because the type specimen could not be located, direct comparison of morphological characters was only possible with the literature information. Therefore the identification of recent materials to *Boninogaster phalloides* should be considered tentative. However, morphological characters from two additional specimens collected in 1995 from Hahajima Island (TNS-F-42909 & TNS-F-238174) were also investigated for comparison. The specimens were identified as *Boninogaster phalloides* by Shoichi Yoshimi, and morphological characters were in well agreement with the literature information and the material for this study (TNS-F-59692).

Phylogenetic analyses revealed a unique position of *Boninogaster* in the order Geastrales (Fig. 4). It is moderately supported as monophyletic in Sclerogastraceae, but *Boninogaster* represents the earliest branch of the clade. Because of its close relationship with *Sclerogaster*, it is possible to synonymize *Boninogaster* to *Sclerogaster* based on this result. However, *Boninogaster* is treated as an independent genus in this study with the following reasons. First, *Boninogaster* is separated from all other species of *Sclerogaster* by rather long branches (Fig. 4). Second, all described species of *Sclerogaster* possess basidiospores with warts or spines (Hosaka and Castellano, 2008) whereas *Boninogaster* possesses smooth spores (Fig. 3D). However, this treatment should also be considered tentative because not all species of *Sclerogaster* were included in phylogenetic analyses. In addition, recent fieldwork by the author also discovered some truffle-like fungi with smooth basidiospores, which can be identified as *Sclerogaster* (unpublished data).

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