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Isolation and Identification of Mushroom Pathogens from *Agrocybe aegerita*

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Agrocybe aegerita* is an important mushroom cultivated in Korea, with good feel and a peculiar fragrance. *A. aegerita* can be cultivated throughout the year using culture bottles but is more susceptible to contamination than other mushrooms. Twenty-two pathogens were isolated from the fruiting bodies and compost of *A. aegerita*, and seven isolates were isolated from *Pleurotus ostreatus* to compare with the *A. aegerita* isolates, collected from Gimje, Iksan, Gunsan of Chonbuk, and Chilgok of Gyeongbuk Province in 2009. These isolates were identified based on morphological and molecular characteristics. Of the 29 isolates, 26 were identified as *Trichoderma* spp. and the remaining three were *Aspergillus* spp., *Mucor* spp., and *Penicillium* spp. A phylogenetic analysis revealed that the 26 isolates of *Trichoderma* were divided into four taxa, namely *T. harzianum, T. pleuroticola, T. longibrachiatum*, and *T. atroviride.* Among the *Trichoderma* spp., 16 isolates (55.2%) were identified as *T. harzianum*, six as *T. pleuroticola* (20.7%), two as *T. longibrachiatum*, and the remaining two were *T. atroviride.

KEYWORDS : ***Agrocybe aegerita,* Phylogenetic analysis, rDNA internal transcribed spacer sequence, *Trichoderma* spp.**

Higher fungi have been used by humans for millennia. First, they are used as part of a regular diet for their nutritional value, as they contain minerals, vitamins, and nutritive compounds such as proteins and polysaccharides and have a low fat content [1, 2]. Secondly, mushroom fruiting bodies are also appreciated as a delicacy. Indeed, their palatability is exploited as taste and flavor enhancers in food preparation and cooking [3]. Thirdly, higher fungi are used for medicinal purposes, and some species clearly demonstrate higher antioxidant properties than others [4, 5]. *Agrocybe aegerita* has a high antioxidant effect and free-radical scavenging ability, which is correlated with total phenolic content [6]. *A. aegerita* is a kind of saprophyte fungi and a basidiomycetes. It belongs to the family *Bolitiaece*, most of which are distributed in Korea, Japan, Europe, and Africa, *A. aegerita* is very fibrous, has a good feel and peculiar fragrance compared to others, and can be cultivated year round in bottle cultivation facilities. However, it requires long duration culturing in culture bottles and is more susceptible to contamination than others.

During this rapid expansion period, the mushroom industry suffered several disease epidemics. During severe outbreaks, no mushrooms are produced from a contaminated bed. Various pathogens such as *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., and *Trichoderma* spp. damage mushroom by inhibiting growth. *Penicillium* competes for preoccupancy with green spores and inhibits the formation of fruiting bodies, resulting in the spores spreading out in the middle and top portion of the mushrooms bottles. *Aspergillus* spp. and *Mucor* spp. form black colored mycelia on the top of mushroom culture bottles and inhibit the formation of fruiting bodies.

Green mold caused by *Trichoderma* species was once recognized as an indicator of poor compost quality but was of minor significance in the cultivation of the commercial mushroom, *Agricus bisporus*. Typical green mold symptoms are the appearance of green fungal sporulation on oyster mushroom substrates. Sinden and Houser [7] were the first to recognize *Trichoderma* spp. as a potentially important pathogen and/or competitor that affects white button mushroom production.

Trichoderma spp. grow rapidly under various conditions and utilize various kinds of substrates. *T. harzianum*, *T. viride*, *T. virens*, *T. longibrachiatum*, *T. koningii*, and *T. polysporum* are found frequently associated with mushrooms. Among these, *T. harzianum* has been recognized as the most important species and cause of potential losses [8]. The devastating nature of green mold was undocumented in the mushroom industry until 1985 when it was first observed in Ireland [9]. Since then, growers in England [10], Canada [11], and the United States [12] have experienced outbreaks of *Trichoderma* green mold resulting in millions of dollars in crop losses. However, in the past decade, green mold has become a destructive disease of cultivated mushrooms. Crop losses have been estimated at ≤ 3~4 million in the UK and Ireland [13] and at more than $20 million in Pennsylvania, USA [14].

With mushroom production located primarily in rural areas, the industry makes a significant contribution to the rural economy and provides a major alternative to traditional farming enterprises. However, the occurrence and diversity of *Trichoderma* spp. associated *A. aegerita* have

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not been well studied. The objectives of this study were to identify and characterize *Trichoderma* spp. and other pathogens present in fruiting bodies of commercial *A. aegerita* and substrates based on morphological and molecular characteristics.

Materials and Methods

Collection of fungal isolates. Twenty-two pathogenic fungi were isolated from the fruiting bodies and compost of *A. aegerita.* Seven isolates from *Pleurotus ostreatus* were used to compare with the *A. aegerita* isolates. The isolates were collected from Gimje, Iksan, Gunsan of Chonbuk, and Chilgok of Gyeongbuk Province from January to April 2009.

Morphological characterization. Pure cultures of the collected isolates were grown in pre-sterilized plates on potato dextrose agar (PDA) using standard laboratory techniques. The cultures were incubated at 25°C in a biological oxygen demand incubator for 1 wk. Morphological characters such as colony color, size of hypha, and color and shape of conidia were recorded. Conidia and conidiophores were observed under a microscope and by scanning electron microscopy (JSM-5410LV; JEOL, Tokyo, Japan). These morphological data were compared with previous descriptions of related pathogens.

DNA extraction, PCR amplification of the rDNA internal transcribed spacer (ITS) region, and sequence analysis. For DNA extraction, mycelia cultures were raised individually on PDA at 25°C for 7 days. These pure mycelia were used for sequence analysis of the rDNA ITS region (ITS-1 region, 5.8S gene, and ITS-2 region). The genomic DNA was extracted using a DNeasy plant mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Amplification and sequencing of the isolates were performed using a pair of universal primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [15] for the region containing ITS1, ITS2, and the 5.8S rDNA. These primers were also used as a positive control in the subsequent diagnostic PCR. The amplification was conducted in a 20 μL reaction mixture containing 50 nM genomic DNA, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.2 mM dNTP, 200 ng of each primer, and 1 unit *Taq* DNA polymerase (Promega, Madison, WI, USA). The reaction mixtures were denatured at 94°C for 10 min and subjected to 35 cycles of 1 min at 94°C, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension step of 7 min at 72°C. The amplified PCR product was separated on a 1.5% agarose gel, followed by purification with a DNA purification kit (Core-one™; Core-Bio, Seoul, Korea), according to the manufacturer's instructions. Both amplicon strands were sequenced using the same primers, reactions were monitored with BigDye Terminator Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA), and run on an ABIPRISM 3130 automated DNA sequencer (Applied Biosystems), as described previously [16]. The rDNA ITS sequence data were analyzed using the DNASTAR program (DNASTAR Inc, Madison, WI, USA) and aligned by the CLUSTAL W method [17]. MEGA ver. 4.0 was used for the phylogenetic analysis [18, 19].

Results and Discussion

Of 29 isolates, 26 were *Trichoderma* spp. and one isolate each was *Mucor* spp., *Penicillium* spp., and *Aspergillus* spp. (Table 1). Contamination in mushrooms that spreads mainly due to contaminated air or by a worker can be easily identified from the outside, as the contamination is green to dark green, black, yellowish green, or red in color due to the spores (Fig. 1).

Symptoms of mushroom pathogens. *Mucor* spp., *Penicillium* spp., and *Aspergillus* spp. caused damage in a

Table 1. Pathogens isolated from *Agrocybe aegerita* and *Pleurotus ostreatus*

No.	Species	Host	Region
JB1	*Trichoderma harzianum*	*A. aegerita*	Chilgok
JB2	*T. longibrachiatum*	*A. aegerita*	Chilgok
JB3	*T. harzianum*	*A. aegerita*	Chilgok
JB4	*T. longibrachiatum*	*A. aegerita*	Iksan
JB5	*T. harzianum*	*A. aegerita*	Iksan
JB6	*T. harzianum*	*A. aegerita*	Iksan
JB7	*T. pleuroticola*	*A. aegerita*	Iksan
JB8	*T. harzianum*	*A. aegerita*	Iksan
JB9	*T. harzianum*	*A. aegerita*	Iksan
JB10	*T. pleuroticola*	*A. aegerita*	Iksan
JB11	*T. harzianum*	*A. aegerita*	Iksan
JB12	*T. harzianum*	*A. aegerita*	Iksan
JB13	*T. pleuroticola*	*A. aegerita*	Iksan
JB14	*T. harzianum*	*A. aegerita*	Iksan
JB15	*T. harzianum*	*A. aegerita*	Iksan
JB16	*T. pleuroticola*	*A. aegerita*	Iksan
JB17	*T. harzianum*	*A. aegerita*	Iksan
JB18	*T. harzianum*	*A. aegerita*	Iksan
JB19	*T. harzianum*	*A. aegerita*	Iksan
JB20	*Mucor racemosus*	*A. aegerita*	Chilgok
JB21	*Penicillin crustosum*	*A. aegerita*	Chilgok
JB22	*Aspergillus tubingensis*	*A. aegerita*	Iksan
JB23	*T. harzianum*	*P. ostreatus*	Gunsan
JB24	*T. pleuroticola*	*P. ostreatus*	Gunsan
JB25	*T. pleuroticola*	*P. ostreatus*	Gunsan
JB26	*T. atroviride*	*P. ostreatus*	Gimje
JB27	*T. harzianum*	*P. ostreatus*	Gimje
JB28	*T. harzianum*	*P. ostreatus*	Gimje
JB29	*T. atroviride*	*P. ostreatus*	Gimje

similar manner by competing for space resulting in growth inhibition and hampered fruiting body development. The pathogens grew quicker than the mushrooms and occupied all of the surface area, which prevented growth of the mushrooms. The pathogens could be identified with the naked eye due to their colored mycelia. A contaminated bottle can spread infection to others so it must be discarded quickly. The *Mucor* spp. and *Aspergillus* spp. formed a black color on the top of mushroom bottles after sporulation (Fig. 1). Initially, *Penicillium* spp. appeared as a green-gray colored powder and turned black as timed passed. The spores of *Trichoderma* spp. and *Penicillium* spp. were round and globular in shape. The *Mucor* spp. and *Aspergillus* spp. had shapes similar to an onion flower stake, and their spores had spikes on the outer surface (Fig. 2). Similar morphological characters

Fig. 1. Fruiting bodies and symptoms of some pathogens occurring on *Agrocybe aegerita*. A, *A. aegerita* fruiting body; B~D, Symptoms occurring in *A. aegerita* as a result of pathogens.

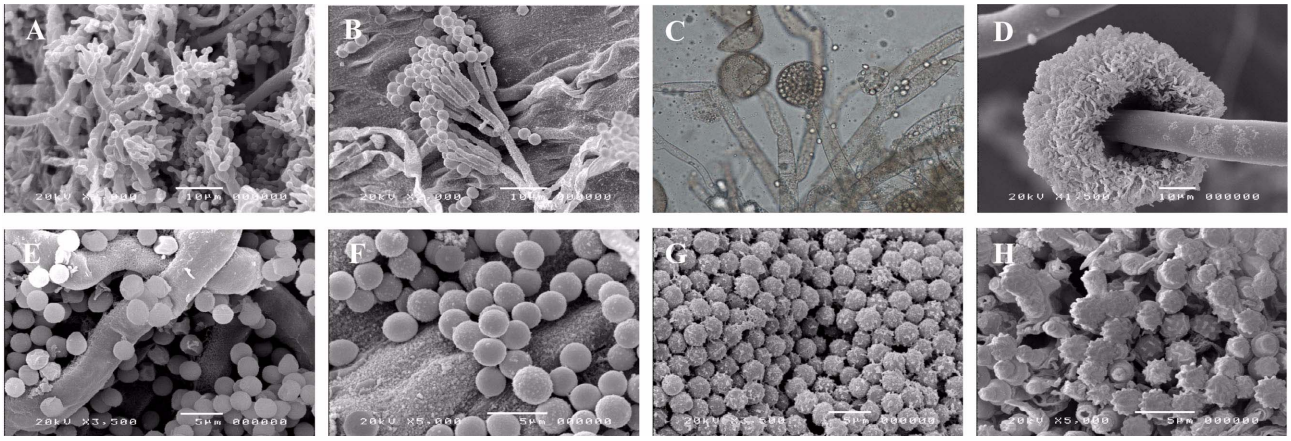

Fig. 2. Morphological characteristics of the pathogenic fungi from *Agrocybe aegerita*. A, E, *Trichoderma pleuroticola*; B, F, *Penicillium crustosum*; C, G, *Mucor racemosus* f. *racemosus*; D, H, *Aspergillus tubingensis*.

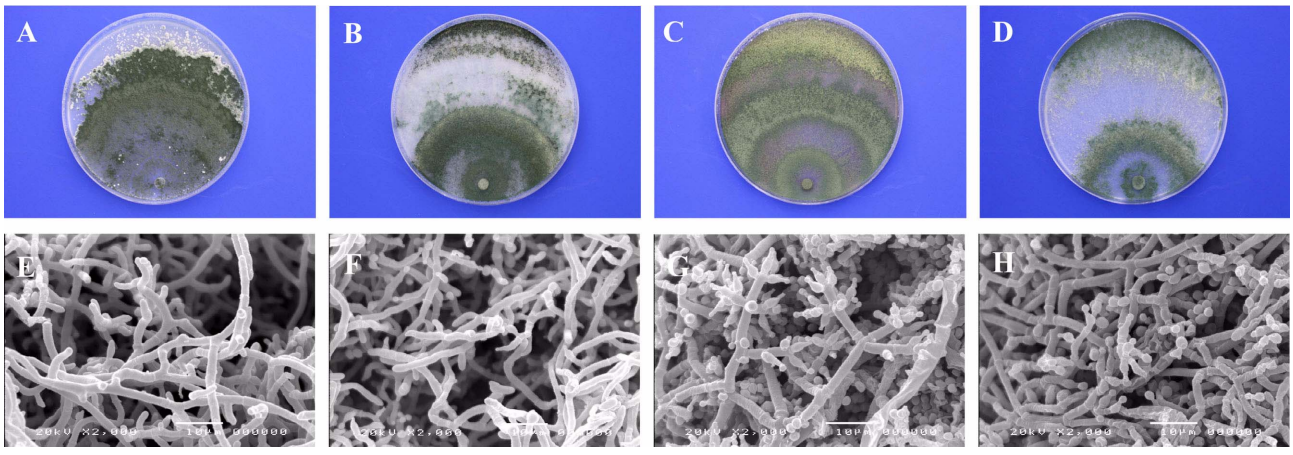

Fig. 3. Cultural and morphological characteristics of isolated *Trichoderma* spp. from *Agrocybe aegerita* and *Pleurotus ostreatus* on potato dextrose agar for 10 days. A, E, *Trichoderma longibrachiatum*; B, F, *T. harzianum*; C, G, *T. pleuroticola*; D, H, *T. atroviride*.

were reported in *Penicillium* spp. by Jo *et al*. [20], and in *Mucor* spp. [21], and *Aspergillus* spp. [22].

Trichoderma spp. initially produced a dense pure white mycelium, which was difficult to distinguish from the mushroom mycelium. However, the mycelial mat gradually turned green in color due to heavy sporulation, which is a characteristic symptom of green mold disease. *Trichoderma* spp. infected the newly developing primordia. This produced brownish lesions and spots on the developing fruiting bodies, which later joined and completely covered the mushroom fruiting bodies. The emerging fruiting bodies in the affected portion of the substrate were badly spotted, brownish in color, and showed reduced growth and yield [8]. The main *Trichoderma* spp. have a green, green-yellow, or white color on the mushroom bottles and cause parasitic damage, compete with other mushrooms for nutrients, and produce a mycotoxin [23].

Morphological characteristics of *Trichoderma* spp. Four species of *Trichoderma* were isolated from *A. aegerita* and *P. ostreatus.* Differences in the morphological characteristics of the four species are described in Table 2 and Fig. 3. All *Trichoderma* spp. were cultured on PDA for 10 days. They developed hyaline mycelia and produced conidiophores on exposed fertile branches after 1 wk. The conidia of *Trichoderma* are generally oblong, rarely globose, and mostly green or hyaline but rarely yellow. The sizes of the *T. longibrachiatum, T. harzianum*, *T. pleuroticola*, and *T. atroviride* conidia were 3.8~5.4 × 2.7~3.2 μm, 2.7~3.7 × 2.4~3.4 μm, 2.6~3.7 × 2.1~3.0 μm, and 2.7~4.0 × 2.4~3.4 μm, respectively. The conidia size and length/weight ratio of *T. longibrachiatum* was the largest followed by *T. atroviride*, whereas the size of the *T. pleuroticola* hyphae was longest followed by *T. longibrachiatum*, and the smallest was *T. atroviride*. The conidia of all four species were ovate and green in color, while the *T. pleuroticola* conidia were yellowish green. Growth of *T. atroviride* was slower than that of the other species, and conidia were restricted to concentric rings. *T. longibrachiatum* tended to form fasciculate conidiation initially, which turned coalescent, often forming greenish yellow conidial crusts with dense conidiation. *T. longibrachiatum* was distinguished by aggregated conidiophores with weakly developed pustules. As a representative isolate, *T. longibrachiatum* was usually cultivated until dark green spore bands were 3 cm from the colony center, as the pathogen was mixed together with white mushroom mycelia and light-green pathogen spores. *T. harzianum* was mass-produced at the end of mycelium with black spores, unlike *T. longibrachiatum* and *T. pleuroticola.* Differences in the anamorphs, growth characteristics, and DNA sequences were observed among the species. For the closely related species, significant differences were more likely visible in the *Trichoderma* anamorph morphology than in the anatomy or morphology of the telemorph. Seaby [24] reported that distinguishing *Trichoderma* spp. using classic microscopic features alone was difficult, as their characteristics differ widely on different media, and spore sizes vary significantly with incubation temperature. Choi *et al*. [25] reported that the conidia of *Trichoderma* spp. are ellipsoidal and ovoid and that the phialides were lageniform and bowling pin shaped. The phialides of *T.* cf. *virens* and *T. harzianum* tended to cluster but were solitary in *T. longibrachiatum. T.* cf. *virens* was characterized by predominantly effuse, penicillate type conidiation, which was sparingly branched and fertile to the apex. Samuels *et al*. [26] also reported similar dimensions for *Trichoderma* spp. isolated from *Agaricus bisporus.* The most critical and vulnerable stage of mushroom cultivation is the culture stage, so the utmost care has to taken at this time. If mushrooms become infected at the culture stage, none of them can be harvested.

PCR amplification of the rDNA ITS region and a sequence analysis. The rDNA ITS sequence data of 38 isolates were compared and analyzed phylogenetically, including 29 isolates from Jeonbuk and Gyeongbuk Provinces, six isolates of *Trichoderma* spp., and one isolate each of *Mucor*, *Penicillium*, and *Aspergillus* spp. with the publicly available gene sequences by BLAST against the GenBank database (htpp://www.ncbi.nlm.nih.gov/).

The size of the entire rDNA ITS region was 500~600 bp, yielding 539 bp for *Trichoderma* spp., 515 bp for *Penicillium* spp. and *Aspergillus* spp., and 600 bp for *Mucor* spp. aligned nucleotide positions. The nucleotide sequences obtained from the GenBank BLAST search in the rDNA ITS region of the isolates was compared with that of other pathogens (Table 1). The phylogenetic analysis of the nucleotide sequence revealed that 26 isolates of *Trichoderma* were divided into four taxa, namely *T. harzianum*, *T. pleuroticola*, *T. longibrachiatum*, and *T. atroviride.* However, JB20, JB21, and JB22 were classified as *Mucor racemosus* f. *recemosus*, *Penicillium crustosum*, and *Aspergillus tubigenesis*, respectively. Among the *Trichoderma* spp., 16 isolates (55.2%) were identified as *T. harzianum*, six as *T. pleuroticola* (20.7%), two as *T. longibrachiatum*, and the remaining two as *T. atroviride*. All four groups of *Trichoderma* spp. isolates were different in their cultural and morphological characteristics, as described earlier (Table 2, Fig. 3). The introduction of DNA sequencing and cladistic analysis in the early 1990s opened a new era for fungal systematics by providing a new set of independently derived data that could be analyzed in tandem with more classically derived data [23]. The molecular and morphological characterization of green mold, *Trichoderma* spp., isolated from *P. ostreatus* and *P. eryngii* beds, were grouped into three species. The occurrence of different species of *Trichoderma* was *T.* cf. *virens* (70.8%), *T.*

Table 2. Morphological characteristics of *Trichoderma* spp. from *Agrocybe aegerita* and *Pleurotus ostreatus*

Isolates	*T. longibrachiatum*	*T. harzianum*	*T. pleuroticola*	*T. atroviride*
Conidia				
Size (μm)	3.8~5.4 × 2.7~3.2	2.7~3.7 × 2.4~3.4	2.6~3.7 × 2.1~3.0	2.7~4.0 × 2.4~3.4
L/W	1.3~1.8	0.9~1.2	1.0~1.3	0.9~1.5
Shape	Ovate	Ovate	Ovate	Ovate
Color	Green, dark green	Green	Green, yellowish green	Green
Formation	After 3 days	After 3 days	After 3 days	After 3 days
Hypha				
Size (μm)	4.4~7.9	3.5~6.3	5.2~13.4	2.9~5.4
Colony color	Dilute green~dark green	Green~dark green, black	Dilute green~yellowish green	Dilute green~green

L/W, length/width.

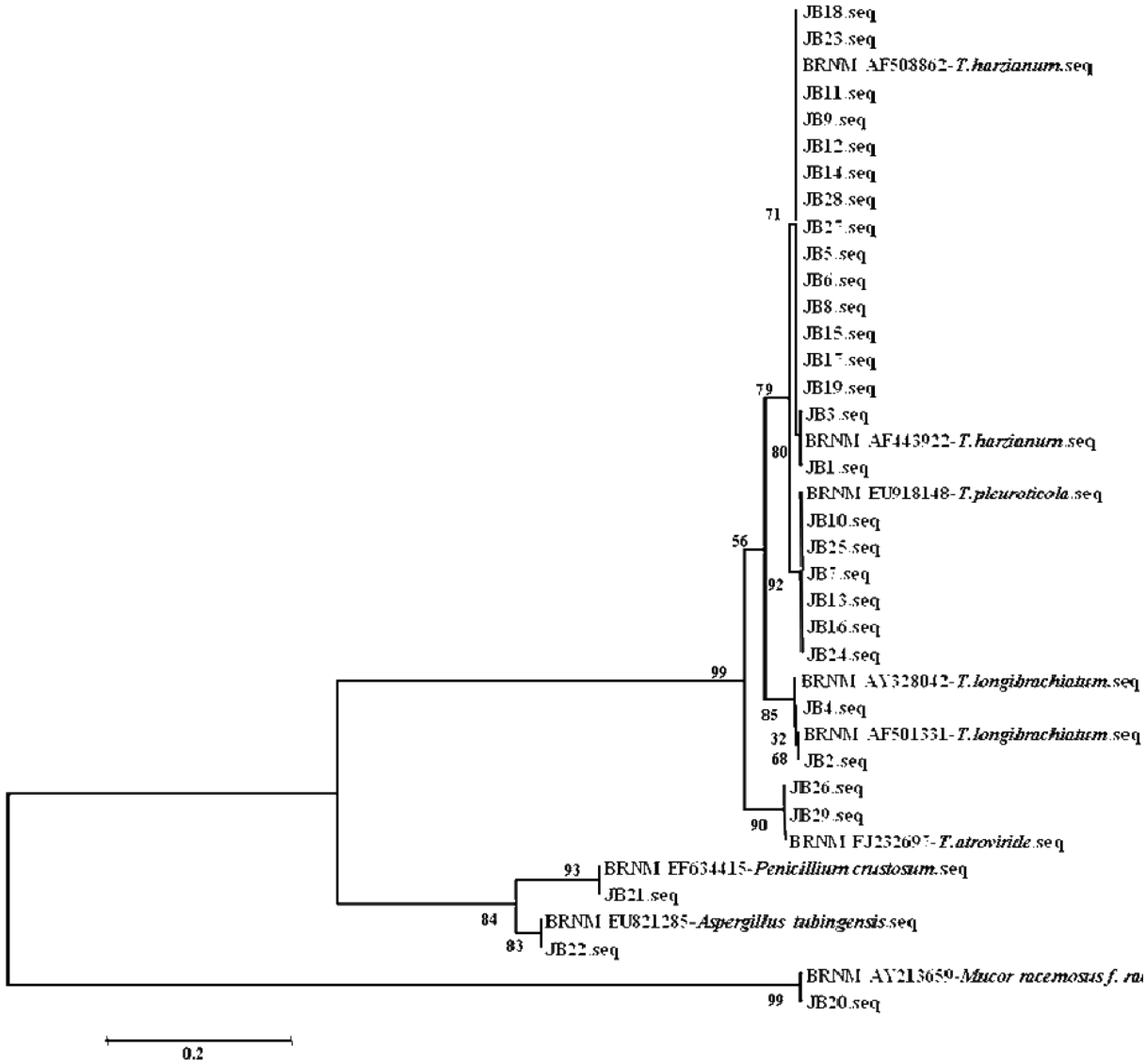

Fig. 4. Phylogenetic tree of the rDNA internal transcribed spacer sequences of *Agrocybe aegerita* isolates and other reference sequences obtained from GenBank (by Laser gene 7.0).

longibrachiatum (16.7%), and *T. harzianum* (12.5%) [25]. In addition to showing phylogenetic relationships among *Trichoderma* spp., studies in which phenotype and DNA analyses were combined have tended to confirm species monophyly, as distinguished by Bissett [27, 28] (Fig. 4). Many *Trichoderma* spp. have demonstrated antifungal or

plant-growth-stimulating activities, which has led to their exploitation as biological control agents, and some isolates are used in commercially available applications [26].

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