Molecular and Morphological Characterization of Endophytic Heterobasidion araucariae from Roots of Capsicum annuum L. in Korea

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A species of Heterobasidion was encountered during a diversity study of endophytic fungi from healthy root tissues of chili pepper (Capsicum annuum L.) in Korea. The fungal species (CNU081069) was identified as Heterobasidion araucariae based on phylogenetic analyses of the internal transcribed spacer and translation elongation factor gene sequences. Morphological descriptions of the endophytic isolate matched well with the previous references and supported the molecular identification. The fungus Heterobasidion araucariae CNU081069 is new to Korea.

KEYWORDS: Capsicum annuum, Endophytic fungi, Heterobasidion araucariae, Molecular taxonomy, Morphology

Introduction

Endophytes are microorganisms that form symptomless infections within healthy plant tissues [1, 2]. Endophytic microorganisms have been found in all plant families [3] and Dreyfuss and Chapela [4] estimated that there are at least 1 million species of endophytic fungi alone. Together, these findings indicate that endophytes are a rich and reliable source of genetic diversity and novel, non described species. The presence of endophytes within plant tissues may confer certain advantages to the host plant [5] by acting as a source of secondary metabolites [6, 7] and biological control agents [8, 9].

Chili peppers (Capsicum annuum L.) have been part of the human diet in the Americas since at least 7500 BC. Chili peppers are now probably the most widely consumed spice in the world [10]. Interestingly, we have isolated endophytic Heterobasidion, one of the most rarely isolated endophytic fungi in the world, from chili pepper.

Root and butt rot diseases of forest trees causes severe economic losses in some parts of the world, especially northern temperate zones [11]. In some areas, the rot or frequency of damage caused by it is 50% or more [12, 13]. Several closely related species of the genus Heterobasidion, primarily in living conifers, are responsible for these diseases [14]. Heterobasidion annosum (Fr.) Bref. (Aphylloohorales, Polyporaceae) is the most important pathogen among the fungi involved in the causal agent of necrosis of various conifers and angiosperms [15, 16]. In Europe, these fungi are primarily found in pine forests [17]. In general, Heterobasidion species affects the Norway spruce (H. parviporum), Picea abies, firs, Abies spp., etc. (H. abietinum) [14]. H. annosum and H. insulare (Murrill) Ryvarden have been widely recognized in China and Japan [15]. Phylogenetic studies have revealed that Japanese H. annosum s. lat. is closely related to European and Chinese populations of H. parviporum [18]. However, H. araucariae, which was first reported in Australasia (New Zealand) and later in Sweden, Scotland and the USA, causes dead conifer wood and butt rot of living Araucariaceae plants [14].

In this study, we characterized Heterobasidion species isolated as endophytic fungi from healthy symptomless root tissues of chili pepper in Korea by molecular and morphological analysis.

Materials and Methods

Isolation of endophytic fungi. Chili pepper plant (Capsicum annuum L.) tissues were collected from a Daejeon farmer’s field in Chungnam Province in the central portion of the Republic of Korea in 2009. Leaf, stem, and root samples of plants were randomly excised and brought to the laboratory in separate sterile polyethylene bags, where they were processed for isolation within 5 hr of collection. Briefly, samples were washed in running tap water to remove dust and debris, dried in air and then cut into 1 cm segments. For surface sterilization, the segments...
were cut from the margin of developing cultures, inoculated in V8A media [2]. Small discs (0.5 cm diameter) were examined on CMA, MEA, OMA, PDA and vegetable agar (Difco, Detroit, MI, USA) and potato dextrose agar (PDA; Difco) supplemented with the antibiotic streptomycin sulfate to inhibit bacterial growth. Developing hyphal tips of emerged colonies were collected after incubation at 25°C for 5, 10, and 25 days and sub-cultured on PDA for 8–10 days. Pure cultures of isolates were maintained in PDA slant tubes and 20% glycerol stock solution and deposited in the Culture Collection of the Chungnam National University Fungal Herbarium. In this study, molecular and morphological characteristics of the isolate CNU081069 were examined.

Genomic DNA extraction and PCR amplification. Genomic DNA was extracted from mycelium using the method described by Deng et al. [19]. Amplification of the internal transcribed spacer (ITS) region was performed using primers ITS5 and ITS4. Additionally, the translation elongation factor 1-α (TEF) gene was amplified with primers EF-728F and 968R, after which the PCR product was purified using a Wizard PCR prep kit (Promega, Madison, WI, USA). Purified double stranded PCR fragments were then directly sequenced with BigDye terminator cycle sequencing kits (Applied Biosystems, Madison, WI, USA) according to the manufacturer’s instructions. Gel electrophoresis and data collection were performed using an ABI prism 310 Genetic Analyzer (Applied Biosystems).

Sequence analysis. The sequences were compared with those available in the GenBank database by BLAST search analysis. Sequences generated from materials in this study and retrieved from GenBank were initially aligned using CLUSTAL X program [20], after which the alignment was refined manually using the PHYDIT program ver. 3.2 [21]. Two neighbor-joining trees were reconstructed for ITS and TEF gene sequences with Kimura’s 2-parameter distance model [22] using the PHYLIP 3.57c package [23]. Bootstrap analysis using 1,000 replications was performed to assess the relative stability of the branches.

Sequence data were deposited in GenBank and assigned accession Nos. JQ691621 and JQ691622 for ITS and TEF sequences, respectively.

Morphology. Morphological characteristics of the isolate were examined on corn meal agar (CMA), malt extract agar (MEA), oat meal agar (OMA), PDA and vegetable juice agar (V8A) media [2]. Small discs (0.5 cm diameter) were cut from the margin of developing cultures, inoculated on the edge of agar plates and incubated at 20–30°C in the dark to determine the favorable growth conditions. The mycelia and conidiophores were observed using a BX50 microscope (Olympus, Tokyo, Japan). The conidia and conidiophores were measured using an Artcam 300MI digital camera (Artray, Tokyo, Japan). Colors were named using ‘A Mycological Colour Chart’ [24]. Morphological characteristics of the isolate were then compared with previous descriptions [15, 18].

Results and Discussion

Molecular analysis. To determine the phylogenetic relationship among the endophytic CNU081069 Heterobasidion isolate from the roots of chili pepper and its related species, the ITS region and TEF gene were compared. The results revealed 97~100% sequence similarity between the endophytic fungal isolate (CNU081069) and its relevant sequences in GenBank. The CNU081069 isolate, three isolates of H. araucariae (isolates B1083, 88-9-6 and 651100.1.3), and two isolates of H. insulare (isolates ATCC 26719, B1144) clustered together in a group based on their ITS sequences in which the three reference H. araucariae and the present endophytic isolate formed a sub-group with a high bootstrap value (99%). Based on the translation elongation factor gene sequence analysis, the isolate CNU081069 and two isolates of H. araucariae (isolates ICMP 9533 and ICMP 9529), as well as isolates of the two species, H. insulare and H. annosum, formed a group. The isolate CNU081069 and the two isolates of H. araucariae formed a sub-group with a bootstrap value of 100%, while H. annosum and H. insulare formed another sub-group. The endophytic isolate CNU081069 was identified as H. araucariae (Fig. 1).

Morphological characterization. Taxonomic descriptions, microphotographs of morphological structures of the species are shown in Table 2, Fig. 2.

Heterobasidion araucariae P. K. Buchanon 1988 (Table 1, Fig. 2).

Colony on CMA: Growth moderately fast, transparent white, grown well on 24~26°C, plates usually covered in 3 to 4 wk. Mycelia appressed, simple, sometime branched hyphae and conidiophores, colony reverse transparent to whitish. Generative hyphae from advancing zone thin walled, usually without clamp-connections, rarely clamped (Fig. 2A).

Colony on MEA: Moderate fast growth, plates usually covered in 2 to 3 wk. Optimum temperature for the colony growth is 24~26°C. Aerial mycelia erect or branched, sparse, appressed, whitish. Mat yellowish to pale yellow, cottony, color unchanged after 6 wk. Reverse grey yellow to pale yellow. Generative hyphae from advancing zone...
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thin walled, usually and commonly clamp connected (Fig. 2B).

**Colony on OMA:** Growth slower than on MEA media. Plates covered in 3 to 4 wk. Optimum temperature was

![Phylogenetic trees](image)

![Morphology of the isolate](image)

**Fig. 1.** Phylogenetic trees of the endophytic isolate *Heterobasidion araucariae* CNU081069 and related species generated from neighbor-joining analysis of the data sets of (A) internal transcribed spacer and (B) translation elongation factor gene sequences, respectively. Numbers above the nodes are bootstrap values (> 50%) from 1,000 replications. The bar indicates the number of substitutions per position. The present *Heterobasidion* isolate is marked in bold. The outgroup is *Echinodontium tsugicola* WD1215.

**Fig. 2.** Morphology of the isolate CNU081069 (*Heterobasidion araucariae*). Colonies on corn meal agar (A), malt extract agar (B), oat meal agar (C), potato dextrose agar (D) and V8 juice agar (E) after 4 wk at 25°C, generative hyphae with clamp-connections (F), conidiophores with and without vesicles (G~I), conidiogenous vesicle attaching with conidia bearing denticles (J), conidia of different sizes and shapes (K) (scale bars: F, G = 50 μm; H, I = 20 μm; J, K = 10 μm).
Table 1. Isolates of *Heterobasidion* used in this study for phylogenetic analyses

| Species          | Isolate | Host                     | Origin             | Accession No. |
|------------------|---------|--------------------------|--------------------|---------------|
| *H. araucariae*   | CNU081069 | *Capsicum annum* L. | Korea               | JQ691621      |
| *H. araucariae*   | B1083   | *Agathis* sp.            | New Zealand        | GQ162432      |
| *H. araucariae*   | 88-9-6  | *Araucaria* sp.          | Sweden              | FJ627527       |
| *H. araucariae*   | 651100.1.3 | Conifers             | Scotland           | X70028        |
| *H. araucariae*   | ICMP 9533 | Dead conifer wood      | New Zealand        | -             |
| *H. araucariae*   | ICMP 9529 | Dead conifer wood      | New Zealand        | -             |
| *H. abietinum*    | 92179/3 | Dead wood               | Sweden              | FJ627542      |
| *H. abietinum*    | FB18    | Dead wood               | Sweden              | FJ627571      |
| *H. annosum*      | B651    | *Pinus* sp.             | USA                 | QG162430      |
| *H. annosum*      | B1092   | *Abies* sp.             | Japan               | GQ162423      |
| *H. annosum*      | MSCL 532 | Unknown                | Latvia              | GU296436      |
| *H. annosum*      | wh391   | Unknown                 | Austria             | AF455452      |
| *H. annosum*      | 97015/3 | Unknown                 | Finland             | AF289931      |
| *H. annosum*      | 98307   | Unknown                 | Unknown             | -             |
| *H. annosum*      | TC111-3 | Unknown                 | USA                 | -             |
| *H. insulare*     | ATCC 26719 | Unknown            | USA                 | FJ411717      |
| *H. insulare*     | B1144   | *Abies* sp.             | Japan               | QG162431      |
| *H. insulare*     | WD651   | Decayed wood            | Japan               | -             |
| *H. parviporum*   | B1436   | *Abies concolor*        | USA                 | GQ162425      |
| *H. parviporum*   | B146    | *Abies* sp.             | USA                 | GQ162420      |
| *H. parviporum*   | HR32    | *Abies* sp.             | Sweden              | FJ627575      |
| *H. parviporum*   | B1314   | *Populus* sp.           | China               | -             |
| *H. parviporum*   | 87-210-7 | Decayed wood          | Sweden              | -             |
| *H. parviporum*   | B1142   | *Abies* sp.             | Mexico              | GQ162421      |
| *H. parviporum*   | B1317   | *Abies* sp.             | Russia              | GQ162424      |
| *H. parviporum*   | 91132/1 | Decayed wood            | Sweden              | FJ627376      |
| *H. parviporum*   | E1      | Decayed wood            | Sweden              | FJ903330      |
| *H. parviporum*   | HR20    | Decayed wood            | Sweden              | -             |
| *Coprinus cirneus*| KACC 49396 | Unknown            | Korea               | AF345816      |
| *Bondarzewia berkeleyi* | Li 1097 | Firs                    | China               | FJ644288      |
| *Echinodontium tsugicol* | WD1215 | *Abies* sp.          | Japan               | AF218398      |

ITS, internal transcribed spacer; TEF, translation elongation factor.

Table 2. Comparison of cultural and morphological characteristics of *Heterobasidion araucariae* and the present endophytic isolate, CNU081069

| Characteristics          | Present isolate CNU081069 | *H. araucariae* \(^a\) |
|--------------------------|---------------------------|------------------------|
| Colony: Color            | Mat yellow to pale yellow, buff; reverse pale yellow | White to pale yellow, translucent; Colony reverses white or pale yellow |
| Growth                   | Plates usually covered in 2 to 3 wk | Moderate fast grow |
| Optimum temperature      | 24–26°C                   | 23–25°C                |
| Hyphae: Clamp            | Generative hyphae produced clamp connections in the margin region | Hyphae clamped, clamps present on wide, straight hyphae, especially near the margin |
| Conidiophores: Characters| Granular materials formed at the base after 4 wk; aroused from prostate hyphae, often constricted at the junction; usually simple, conidiophores sometime branched | Arising from prostate hyphae, often constricted at the junction, granular materials at the base, simple or branched |
| Length (µm)              | Up to 300                 | Up to 400              |
| Width (µm)               | (5.5–) 8.1 (~10.5)        | 5.5–13.5               |
| Conidia: Shape           | Ovoid, subglobose, asperulate, sometimes pyriform, single celled and smooth-walled | Subglobose, ovoid or pyriform, unicellular, hyaline, smooth-walled |
| Size (µm)                | (4–) 7.8 (~11.5) x (2–) 4.2 (~7) | (5–) 7.6 (~16.0) x (3–) 5.3 (~13.5) |

Sources of description [15, 18].
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24–26°C. Aerial mycelia were common, transparent white to white. Appressed mycelia, cottony to woolly, reverse white to transparent white and unchanged after 6 wk. Generative hyphae from advancing zone thin walled, usually without clamp-connections, clamp-connections rarely found (Fig. 2C).

**Colony on PDA and V8A:** Growth moderately fast at 25°C. Colony was whitish to pale yellow or pale brown. Surface textures were plane velutinous. Aerial mycelium was observed, sparse and appressed, cottony to woolly. Reverse white to light yellowish brown (V8A) and yellowish brown and blackish spots surrounding the colony root (PDA). Generative hyphae from advancing zone thin walled, usually forming clamp-connections on PDA media and rarely on V8A media, mostly single and extremely rarely double. Clamp connections were common in both media (Fig. 2D and 2E).

**Conidiophores and conidia:** Conidiophores were usually straight to slightly flexuose, arising as erect branches from prostrate hyphae, often constricted at the junction of the hyphae, almost single, rarely branched, variable in length up to 300 μm long and (5.5~) 8.1 (~10.5) μm in diameter. Conidia produced from apical, inflated conidiogenous vesicles that bear denticles to produce conidia on the top. Vesicles were (5.5~) 11.6 (~17.0) μm in diameter and denticles were 1.0~2.0 μm in size (Table 2, Fig. 2J and 2K). These morphological characteristics matched well with the previous descriptions of *Heterobasidion araucariae* [15, 18].

**Isolate examined:** On roots of chili pepper; CNU081069.

**Hosts and distribution:** Recorded on Araucariaceae ([*Agathis australis* (D. Don) Lindley from, *A. vitiensis* (Seem.) Benth. & Hook. f. ex Drake, *Araucaria bidwillii* Hook., *A. cunninghamii* Aiton ex Don) and Pinaceae (*Pinus kesiya* D. Don) Lindley from, *P. patula* Schlecht. & Cham., *P. taeda* L.), on stumps or dead standing and fallen wood, rarely on living wood, as endophytic fungi in *Cham.* (*D. Don)* Lindley from, *P. taeda* (L.)

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