Identification of *Armillaria nabsnana* in *Gastrodia* Tubers

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The symbiosis between *Armillaria* species and an achlorophyllous orchid *Gastrodia elata* BLUME has been reported. The main species described as a symbiont is *Armillaria mellea* (VAHL: FR.) KUMMER, known widely as a primary root rot pathogen. Samples collected from the rhizomorphs attached to the tuber of *G. elata* were separated and analyzed. Molecular analysis based on sequencing of the intergenic spacer 1 (IGS-1) and internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) was performed, coupled with restriction fragment length polymorphism (RFLP) of the IGS-1 region. Cultural morphology and features of basidiomata were also used to characterize the isolates. Phylogenetic analysis and morphological data strongly suggested that the fungus present in the tubers of *G. elata* is *Armillaria nabsnana*. This is the first report of occurrence of this *Armillaria* species in association with *G. elata*.

Key words *Armillaria nabsnana*; *Gastrodia elata*; symbiosis; molecular analysis; intergenic spacer one; internal transcribed spacer

Armillaria species (Basidiomycota, Agaricales, Tricholomataceae) are well known to be the cause of root rot and death to woody plants in boreal, temperate and tropical regions worldwide.¹⁾ Generally this species has a broad variety of hosts, presenting pathogenic or symbiotic properties depending on the available substrate.^{1,2)}

Armillaria has been identified as an associate in certain achlorophyllous taxa of Orchidaceae including *Gastrodia elata* BL.^{3,4)} and *Galeola septentrionalis* REICHB.⁵⁾ Although many reports refer to *Armillaria mellea* as the main fungus symbiont of *G. elata*,^{3,6)} in recent years, other species of *Armillaria* have been found growing from mycorrhizal roots of this orchid. Five biological species, *A. ostoyae* (ROMAGNESI) HERINK, *A. gallica* MARXMULLER & ROMAGNESI (as *A. bulbosa* (BARLA) KILE & WATLING), *A. jezoensis* CHA & IGARASHI, *A. sinapina* BERUBE & DESSUREAULT and *A. singula* CHA & IGARASHI, associated with *G. elata* were reported in Japan.⁴⁾

In eastern traditional medicine, the tubers of this orchid have been used for the treatment of many kinds of diseases including headache, rheumatism, seizure, dizziness and circulation problems.^{7,8)} *G. elata* is considered as a difficult species to cultivate due to the need for different kinds of fungi colonization to achieve its full development.⁶⁾ To increase the knowledge of the cultivation process and understand its relationship with the *Armillaria*, it is important to first identify which *Armillaria* species is involved in the association.

In past decades, the identification of *Armillaria* spp. was based on mating tests and morphological characteristics.^{2,9,10)} However, those methods are very limited due to the great number of biological species and similar morphological characteristics among them.^{11,12)} Furthermore, mating pairings require fresh haploid cultures that sometimes are not available and a long period is required to obtain results.^{13,14)}

Methods based on molecular techniques are nowadays routinely used and provided a new perspective for phylogenetic and taxonomic studies.^{12,15–17)} PCR-RFLP analysis profiles of intergenic spacer 1 (IGS-1) have been successfully applied

to the *Armillaria* species identification.^{12,13,18,19)}

Sequencing of the internal transcribed spacer (ITS) and intergenic spacer 1 (IGS-1) regions^{14,20,21)} has also been used for identification. The ITS and IGS sequences available in the GenBank and the development of efficient software have provided a valuable tool for differentiation as well as for the detection of polymorphism and heterogeneity among species. The ITS and IGS-1 of the ribosomal DNA are known to be highly polymorphic enabling the accurate species differentiation of the *Armillaria*.¹¹⁾ In some cases, however, ITS1 region sequences were proven to be nearly uniform among the *Armillaria* of northern hemisphere species.¹¹⁾

The aim of this study was to apply molecular biological tools to identify which *Armillaria* species are involved in the association with *G. elata*. The ITS and IGS-1 regions of rDNA were amplified and sequenced for further comparison with other *Armillaria* species. PCR-RFLP of the IGS-1 region was performed with three different endonucleases. Morphological characteristics and compatibility tests were also used in the analysis.

MATERIALS AND METHODS

Fungal Isolation and Cultivation Fungal samples were harvested in July 2005, found in different root parts of *G. elata*, which grows naturally in the forest of the Health Sciences University of Hokkaido, in Japan. Several fungi were isolated from the rhizomorphs attached to the tuber surfaces (Fig. 1).

The samples were rinsed overnight with distilled water and sterilized with 5% sodium hypochlorite solution for 60 s. Samples from the fruit body, collected in September 2005, were cut to a size of approximately 5 mm, rinsed with distilled water and sterilized with 70% ethanol. After sterilization, all the samples were carefully dried and plated onto malt extract agar medium (MEA) consisting of 2% glucose, 0.1% peptone, 2% malt dextrose, 2% agar, amended with 100 ppm streptomycin to avoid bacteria proliferation. Fungal colonies grown after 4 weeks cultivation under 25 °C temper-

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ature were transferred to new MEA media.

For single spore isolation, a 5 mm size fruit body was attached right side up to the cultivation petri dish lids using vaseline. Lids were placed onto the dish bottom, already filled with MEA media. The dish was incubated at room temperature for 60 min and gently rotated to scatter the spores in the culture media. The spores were grown in the same MEA and then transferred to fresh MEA, after substantial appearance of the colonies.

A total of 24 samples of the fungi were isolated from the rhizomorphs, the fruit body and sporefalls. Eight samples (Table 1) were chosen and subjected to DNA extractions according to slight differences in the culture characteristics. Tester strains of *A. mellea* purchased from NITE Biological Resource Center (Chiba, Japan) and *A. nabsnona* (Japanese tester strain 30987-3) kindly supplied by Dr. E. Nagasawa of the Tottori Mycological Institute (Tottori, Japan) were also cultivated and subjected to DNA extractions. Voucher specimens were preserved and stored.

DNA Extractions The fungal isolates were grown in 50 ml malt extract broth, consisting of 2% malt extract, 2% glucose, 0.1% peptone, in complete dark at 25 °C. Tester strains were also obtained and also subjected to DNA extractions. After 4 weeks, mats of the mycelium were collected, rinsed with sterile distilled water, freeze dried at -70 °C for 24 h and lyophilized. The freeze-dried mycelium was ground into fine powder. Genomic DNA was extracted by mixing the pulverized mycelium with NTE buffer (100 mM NaCl, 30 mM Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol) (pH 8.0) containing 0.5% (v/v) Nonidet P-40 and centrifuged at

10000 rpm for 1 min. The pellet was resuspended in NSE buffer (100 mM NaCl, 200 mM sucrose, 10 mM EDTA) (pH 4.0) before adding TES buffer (10 mM Tris-HCl, 250 mM EDTA (pH 9.2), 2.5% SDS). The homogenate was incubated for 30 min at 55 °C. The solution was extracted with an equal volume of phenol:chloroform:isoamylalcohol (50:48:2) and centrifuged at 10000 rpm for 20 min. The upper layer was mixed with 0.1 volume of 3 M sodium acetate, and then 0.54 volume of isopropanol. The DNA pellet was collected, rinsed with 70% ethanol and then dried under a vacuum.

PCR-RFLP of IGS Region The IGS-1 region was amplified with primers LR12R (5' CTGAACGCCTCTAAGT-CAGAA 3')²²⁾ and O-1 (5' AGTCCTATGGCCGTGGAT 3').²³⁾ The PCR reactions were performed in a 50 µl mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 200 µM of each of the four deoxynucleoside triphosphates, 5 pmol of each primer, 1.0 µl of DNA template, and 2.5 units of *Taq* polymerase. Amplification was carried out with a Thermal Cycler MP (Takara, Ltd., Shiga, Japan.). Reaction parameters consisted of initial denaturing at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. IGS-1 PCR products were then purified using QIAquick PCR Purification kit (Qiagen Inc., Valencia, CA, U.S.A.).

RFLP analysis of the IGS-1 PCR products was performed according to methods described by Fukuda and Ono.²⁴⁾ PCR products were digested separately with three restriction endonucleases: *Hinf*I, *Msp*I and *Hae* III (Takara, Ltd., Shiga, Japan), following the supplier's instructions. Electrophoresis was carried out on 3.0% Nusieve 3:1 Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME, U.S.A.) and visualized with ethidium bromide, under UV light.

Sequencing of ITS and IGS Regions IGS-1 PCR products were submitted to a cloning method which was carried out using a Dyna Express TA PCR cloning kit (BioDynamics, Laboratory Inc., Tokyo, Japan). PCR cycle sequence reaction was performed using a Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Biosciences, U.K. limited), according to the manufacture's instructions. Reaction was carried out with a thermal cycler MP (Takara, Ltd., Shiga, Japan) and consisted of 20 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. After reaction, 6 µl of formamide loading dye was added to each sample. Sequencing was performed

Fig. 1. *Armillaria* Rhizomorphs Attached to *Gastrodia* Tuber

Table 1. Specimens of the Biological Species Submitted to DNA Extraction and Their Cultural Characteristics

Isolates	Origin	Culture characteristics	Accession number	
			ITS	IGS
C-3-3	Rhizomorphs	Flat, crustose, dark brown	AB368926	AB368921
C-4-3	Rhizomorphs	Rhizomorphic	AB368927	AB368922
C-7-3	Rhizomorphs	Flat, crustose, dark brown	AB368928	AB368923
S-40	Spore	White and fluffy		
78	Fruit body	Rhizomorphic	AB368925	AB368920
F-2	Fruit body	Flat, crustose, dark brown		
F-3	Fruit body	Rhizomorphic, dark brown		
F-4	Fruit body	Flat, crustose, dark brown		
31616	Japanese <i>Armillaria mellea</i> tester strain (IF031616)	Rhizomorphic		
31166	Japanese <i>Armillaria mellea</i> tester strain (IF031166)	Rhizomorphic		
30987-3	Japanese <i>Armillaria nabsnona</i> tester strain	White and fluffy	AB368929	AB368924

using a Hitachi SQ5500E Sequencer (Hitachi Co., Ltd., Tokyo).

The ITS regions (ITS-1, 5.8S and ITS-2) of the rRNA were amplified using primers ITS-5 (5' GGAAGTAAAA-GTCGTAACAAGG 3') and ITS-4 (5' TCCTCCGCTTATT-GATATGC 3').²⁵ PCR reaction was performed using the same conditions described for the IGS-1 region. PCR products were cloned and then sequenced equally as described previously.

DNA Sequences Similarity and Phylogenetic Analysis

DNA similarity of the IGS-1 and ITS regions was performed using a DNASIS software package (HITACHI). Comparison of other *Armillaria* species was carried out using previously characterized sequence data from GenBank. Phylogenetic relationships among the four isolates and the other *Armillaria* strains were also determined by cladistic analyses of IGS-1 region. The sequences were aligned using the DNASIS software package. The alignment was manually edited in MacClade 4.0.²⁶ Regions of ambiguous alignment were excluded from further phylogenetic analysis. Other regions containing single-nucleotide insertion or deletion were included in the analysis, with gaps treated as missing data. Phylogenetic analysis was performed using the maximum parsimony criterion in PAUP* 4.0.²⁷ Branch support was assessed by bootstrap analysis based on 500 replicate heuristic searches using the option in PAUP*.

Mating Test The sporefalls were collected and plated onto MEA using the procedure described previously. For haploid-haploid pairings, the isolate S-40 which demonstrated a white and fluffy appearance, were paired with the haploid Japanese *Armillaria nabsnana* tester strain. Pairings were made by placing inocula 0.5 mm apart on a MEA culture media and incubated at 25 °C for 3 weeks.

RESULTS

Basidiomata and Cultural Morphology Characteristics

Basidiomata of this *Armillaria* species generally grows in mid September, when the temperature drops to approximately 15 °C at night. High humidity and adequate day temperature is also required. Basidiomata usually occurs in nature in clusters of 5 to 10, fused at the attachment point in the base of a decaying tree root. The pileus varied from 4.5–7 cm in diameter with a honey-brown color and appearing smooth and slimy on the surface, slightly concave at the disk center and slightly incurved in the margins. The stipe was creamy-white in color, measured 4.2–5.5 cm high and 2–5 mm in diameter. The basidiospores measured 7–10 µm × 3–6 µm, smooth surface with an ovoid to sub-globose aspect.

Colonies that arose from basidiospores were characteristically white and fluffy with raised mycelium becoming dark brown, flat and crustose in appearance as they aged. In contrast, colonies resulting from the tissues of the fruit body and the rhizomorphs were consistently flat, crustose and sometimes rhizomorphic.

PCR-RFLP of IGS-1 The IGS-1 of the isolates was amplified by PCR and a single product was detected for all 8 isolates. The RFLP analysis performed using the three different restriction endonucleases resulted in the same restriction patterns among the 8 isolates. These restriction patterns

demonstrated the same band patterns with *A. nabsnana*, clearly differing from those of *A. mellea* (Fig. 2).

ITS and IGS-1 Sequences and Phylogenetic Relationship

ITS and IGS-1 sequence comparison between the isolated fungi with other *Armillaria* species deposited in the GenBank resulted in high homology with *A. nabsnana* (Table 2).

Four isolates (C-3-3, C-4-3, C-7-3, 78) and the Japanese *A. nabsnana* tester strain were submitted to sequencing. Similarity comparison of the ITS regions of C-4-3, C-7-3 and 78 demonstrated a 100% match among them. The isolate C-3-3 had two mutation points and compared with the Japanese *A. nabsnana* tester strain, the similarity was 99.6%. ITS comparison with other *Armillaria* deposited in the GenBank resulted in a high similarity with *A. nabsnana*, *A. gallica*, *A. calvescens*, *A. cepistipes* and *A. sinapina* (Table 2).

IGS-1 sequence comparison of the four isolates with the Japanese *A. nabsnana* tester strain resulted in a perfect similarity match. A deletion event consisted of 22 bp from the 430th to the 451st position counting from the 3' end of LR12R primer was present in all isolates including the Japanese tester strain. Comparison of the IGS-1 region of the four isolates resulted in the highest similarity (94–95%) with *A. nabsnana* deposited in the GenBank, while the four isolates shared a comparatively low value, ranging from 68 to 92%, with the other *Armillaria* species (Table 2). In the parsimony tree (Fig. 3), the isolates associated with *G. elata* and the Japanese tester strain (30987-3) were grouped within the well-supported clade that corresponds to *A. nabsnana* (99% bootstrap support). Phylogenetic analysis of IGS-1 sequences

Fig. 2. Band Patterns of *Msp*I, *Hae*III, *Hinf*I Digestion Products of the IGS-1 Region of Our Isolate (1), *A. nabsnana* (2) and *A. mellea* (3)

Size markers (100 bp ladder) in both sides.

Table 2. Similarity Comparison of the ITS and IGS-1 Regions of Our Isolates with Other *Armillaria* Species Deposited in the GenBank

Biological species	Similarity comparison			
	ITS-1, 5.8 S, ITS-2		IGS-1	
	C-3-3	78	C-3-3	78
<i>A. nabsnana</i>	98–99%	99%	94–95%	94–95%
<i>A. gallica</i>	98%	98–99%	91%	91–92%
<i>A. calvescens</i>	98%	99%	92%	92%
<i>A. sinapina</i>	98%	98%	91–92%	91–92%
<i>A. cepistipes</i>	98%	98%	90–91%	90–92%
<i>A. tabescens</i>	90–91%	91%	91%	91%
<i>A. mellea</i>	83–88%	83–89%	68–71%	68–71%
<i>A. borealis</i>	97%	97%	90%	91%
<i>A. ostoyae</i>	98%	98%	91%	90–91%

Fig. 3. One of the Most Parsimonious Trees Based on Heuristic Search Analysis of IGS-1 Sequences Showing the Placement of the Fungi Associated with *Gastrodia elata* among Different Species of *Armillaria*

Tree length is 317 steps with 245 phylogenetically informative characters. Consistency index (CI)=0.820, retention index (RI)=0.895, and rescaled consistency index (RC)=0.734. Percentage bootstrap support (500 replications) is shown above branches.

also indicated that the fungi isolated from *G. elata* in this study are apparently different from *A. gallica*, *A. singular*, *A. sinapina*, *A. jezoensis* and *A. ostoyae* previously isolated from mycorrhizal roots of this orchid.

Mating Test Pairing between haploid isolate and haploid Japanese *A. nabsnona* tester strain resulted in changes of morphology of haploid mycelia into the crustose type and stained the media dark brown. No black demarcation line was visible between the inocula.

DISCUSSION

Many studies refer to *A. mellea* as the main symbiotic species of *G. elata*.^{2,6,28} However, recent studies have shown that other *Armillaria* spp. are involved.⁴ Our study, based on sequence similarity of the ITS and IGS-1 regions of the rDNA and PCR-RFLP and phylogenetic analysis of IGS-1 strongly suggested that the association of *G. elata* is with *A. nabsnona*.

Mating test was performed in order to analyze the compatibility of the isolates from the gastrodia tubers with the Japanese *A. nabsnona* tester strain. The tests resulted in a clearly compatible pairing of haploid isolates with this tester

strain.

Analysis of the ITS region resulted in a perfect match within our isolates and the Japanese *A. nabsnona* tester strain. Sequences comparisons with other *Armillaria* deposited in the GenBank revealed significantly high similarity with *A. nabsnona*, *A. gallica*, *A. calvescens*, *A. cepistipes* and *A. sinapina*.

These results indicate that ITS sequences are highly conserved among these species. However, enough variability in the IGS-1 region was present enabling a clear distinction with these *Armillaria* species.

PCR-RFLP analysis of the IGS-1 region using *Hinf*I, *Hae*III and *Msp*I of our isolates produced restriction fragment patterns consistent with those found in *A. nabsnona* tester strain. Those results matched with those found by Fukuda *et al.*²⁹

The sequencing of the IGS-1 region demonstrated a perfect match between them and with the Japanese *A. nabsnona* tester strain. Comparison of our isolates with other *A. nabsnona* deposited in the GenBank indicated a deletion event consisted of 22 bp, between the 430th and 451st position from the 3' end of the LR12R primer sequence. However, comparison of the IGS-1 region of our isolates with the data

of other *Armillaria* species deposited in the GenBank resulted in the highest similarity with *A. nabsnana*, whether or not the deletion event was considered. Furthermore, phylogenetic analysis of the IGS-1 data also revealed a well supported group that corresponds with previously recognized *A. nabsnana*.

A. nabsnana is reported as a species distributed on the west coast of North America and its range was described primarily in hardwoods of riparian areas.³⁰⁾ Ota *et al.*³¹⁾ have described the occurrence of *A. nabsnana* in the region of Honshu island, in central Japan. Based on mating tests, Mohammed *et al.*³²⁾ reported a compatible pairing of an isolate collected from Hokkaido Prefecture (Japan) with an *Armillaria* species described as NABS IX. Later, Volk *et al.*³³⁾ described NABS IX as *A. nabsnana*, a new species from western North America. The authors performed a compatibility test between NABS IX samples collected from North America and Japan, however, their result showed no compatible pairing between the Japanese and North America isolates. We believe that some peculiarities are present among the species that diverge from continents and in these cases mating tests might not be the best technique for the identification of *Armillaria*.

With respect to *Armillaria* species associated with *G. elata*, five biological species (*A. ostoyae*, *A. gallica*, *A. jezoensis*, *A. sinapina*, and *A. singula*) have been reported by Cha and Igarashi.⁴⁾ Furthermore, Mohammed *et al.*³²⁾ reported a clearly compatible pairing of the isolates collected from *G. elata* with European and North American testers of *A. gallica*.

This is the first report concerning the association of *A. nabsnana* with *G. elata* based on a molecular level. However, it should be pointed out that as only a limited number of isolates were used in these studies more fungi sampling from the roots of *G. elata*, especially from different areas, would be necessary. Furthermore, the accurate identification of the fungi associated with *G. elata* might unveil its complex relationship and increase the knowledge of the cultivation process.

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