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Biological Species and Morphological Characteristics of Armillaria mellea Complex in Hokkaido: A. ostoyae and A. bulbosa

By

Joo Young Cha*, Jae Mo Sung** and Tsuneo Igarashi*

Abstract

The different morphological forms of Armillaria mellea (Vahl; Fr.) Kummer complex collected in Hokkaido were found to be composed of four intersterility groups or biological species by using all pairwise combinations with three single spore isolates of each fruit-body. Two of them, intersterility groups I and II, were identified by mating test with European and North American biological species as tester strains corresponding to two species, Armillaria ostoyae and Armillaria bulbosa, respectively. They are described and their occurrence and ecology documented.

Key words: Armillaria mellea, Armillaria ostoyae, Armillaria bulbosa, biological species, morphology

I. Introduction

Armillaria mellea (Vahl; Fr.) Kummer in the sense is a complex of plant pathogenic hymenomycetes well-known for its extremely wide host range and variability in fruit-body. Recently, it has been reported that this fungus consists of several reproductively isolated groups as biological species in North America (Anderson and Ullrich 1979, Anderson et al. 1980), Europe (Korhonen 1978), Australia (Kile and Watling 1983), Africa (Mwangi 1989) and Asia (Nagasawa et al. 1990, Sung et al. 1990). European and Australian biological species have been described in detail regarding their morphological and taxonomical characteristics (Berube and Dessureault 1989, Kile and Watling 1983, Roll-Hanson 1985). In nine North American biological...
cal species, seven have been determined regarding the morphological characteristics of each fruit-body (BERUBE and DESSUREAULT 1988, BERUBE and DESSUREAULT 1989, MOTTA and KORHONEN 1986).

Few studies of Japanese biological species of *A. mellea* complex have been described in detail (CHA and IGARASHI 1992, NAGASAWA et al. 1990). Moreover, morphological studies have not been undertaken in Hokkaido biological species. In the Experiment Forests of Hokkaido University, there have been occurrences of different morphological forms of *A. mellea* sensu lato. The objectives of this study were to determine the biological species occurring in Hokkaido, to describe the morphological forms and ecology, and to correlate morphological forms with biological species.

II. Materials and Methods

Seven fruit-bodies of *Armillaria* were collected in the fall of 1991 at various locations throughout the Experiment Forests of Hokkaido University, where they occur mainly in mixed and broad-leaved forests, in association with a variety of hosts. Table 1 lists hosts, geographical origins, and habitats. Dried voucher specimens were preserved and stored. To obtain single spore isolates, 2 cm × 2 cm plugs were cut from the pileus of each fruit-body with a knife. The upper surface of the pileus was attached with double-faced tape to the inside of the cover of a petri dish. Each cover was placed on a petri dish containing 1.5% water agar medium. Basidiospores were allowed to discharge for 3–5 hours or until several spores could be observed scattered on the surface of the medium with a stereomicroscope. After 12–24 hours incubation at 22°C, individual germinated spores were selected and transferred with a fine metal nee-

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Date</th>
<th>Intersterility groups</th>
<th>Locality &amp; regions</th>
<th>Hosts*</th>
<th>Habitats</th>
</tr>
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<tr>
<td>HUA 9112</td>
<td>9/25</td>
<td>I</td>
<td>Moshiri, Uryu</td>
<td>Be</td>
<td>Caespitose, living underground roots, mixed forest, e** 400</td>
</tr>
<tr>
<td>HUA 9113</td>
<td>9/25</td>
<td>I</td>
<td>Moshiri, Uryu</td>
<td>Be</td>
<td>Caespitose, solitary, dead underground roots, mixed forest, e. 410</td>
</tr>
<tr>
<td>HUA 9114</td>
<td>9/25</td>
<td>I</td>
<td>Moshiri, Uryu</td>
<td>Un</td>
<td>Solitary, dead underground roots, mixed forest, e. 400</td>
</tr>
<tr>
<td>HUA 9128</td>
<td>10/17</td>
<td>I</td>
<td>Tomakomai</td>
<td>Ud</td>
<td>Caespitose, living stem up to 0.5m, broad leaved forest, e. 50</td>
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<tr>
<td>HUA 9102</td>
<td>9/12</td>
<td>II</td>
<td>Otoineppu, Nakagawa</td>
<td>Fm</td>
<td>Caespitose, solitary on soils, decaying wood debris, broad-leaved forest, e. 150</td>
</tr>
<tr>
<td>HUA 9107</td>
<td>9/13</td>
<td>II</td>
<td>Otoineppu, Nakagawa</td>
<td>Be</td>
<td>Caespitose, solitary, decaying stump, mixed forest, e. 400</td>
</tr>
<tr>
<td>HUA 9125</td>
<td>10/8</td>
<td>II</td>
<td>Tomakomai</td>
<td>Ps</td>
<td>Caespitose, solitary, decaying trunk, mixed forest, e. 20</td>
</tr>
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</table>

*Abbreviation; Be: Betula ermanii, Fm: Fraxinus mandshurica var. japonica, Ps: Prunus ssiori, Ud: Ulmus davidiana var. japonica, Un: Unidentified broad-leaved.
**Elevation (in meters above sea level).
**Table 2.** Origin of tester strains of the biological species of *Armillaria*

<table>
<thead>
<tr>
<th>Biological species</th>
<th>Original strain no.</th>
<th>Collector*</th>
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<tr>
<td><em>A. ostoyae</em></td>
<td>E12</td>
<td>KORHONEN</td>
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<tr>
<td><em>A. ostoyae</em></td>
<td>M1</td>
<td>MORRISON</td>
</tr>
<tr>
<td><em>A. bulbosa</em></td>
<td>M19</td>
<td>MORRISON</td>
</tr>
</tbody>
</table>

*Kindly supplied by K. KORHONEN, The Finnish Forest Research Institute, Unioninkatu 40A, SF-00170 Helsinki 17, Finland and by D. J. MORRISON, Forest Research Lab, 506 West Burnside Road, Victoria, B.C., V8Z 1M5, Canada.*

dle to each tube slants containing MA medium (1.25% malt extract (Difco) and 1.5% agar).

To determine intersterility groups, three single spore isolates from each fruit-body were mated in all possible pairwise combinations. Pairings were made by placing inocula 1 mm apart on a 1.25% MA petri dish and incubating at 22°C for 3 weeks. Single spore isolates and their incompatible pairings produce fluffy aerial mycelium, while flat crustose mycelium is produced by vegetative isolates and compatible pairings (HINTIKKA, 1973; KORHONEN, 1978; ULLRICH and ANDERSON 1978, ANDERSON and ULLRICH 1979). Two populations are judged fully interfertile when compatibility is determined solely by the bifactorial incompatibility system and intersterile if no compatible pairings are detected.

In order to obtain biological species status, three single spore isolates from each of the fruit-body were mated with all tester strains of each North American and European biological species. Tester strains were kindly supplied by D. J. MORRISON and K. KORHONEN. Table 2 lists the origins of tester strains used in this study. Vegetative isolates were made directly from the flesh of the fruit-bodies and placed in test tube slants containing 1.25% MA. Their cultural characteristics were observed on 3% MA medium at 22°C 3 weeks later.

The nuclear condition of subhymenial hyphae was determined by hand section hydrating dried hymenial tissue in McIlvaine buffer at pH 4.4 (0.1 M citric acid plus 0.2 M Na₂HPO₄), then macerating the tissue in a ground-glass tissue homogenizer. Drops of suspension were placed on microscope glass slides, air dried, and stained with the fluorochrome 4′-6-diamidino-2-phenylindole (DAPI) (COLEMAN et al. 1981). Stained material was observed with an Ortholux II microscope fitted with epifluorescence and utilizing the appropriate filter combinations. Dimensions of spores, basidia, and cells of the cuticle and veil were determined in material mounted in 4% KOH (w/v), using a calibrated eyepiece micrometer. Thick sections were made by hand with a knife. Sections were stained with 0.05% toluidine blue in 1% sodium borate before examination. Color names are indicated according to the manual of color names (JAPAN COLOR RESEARCH INST. 1973), and the color codes in parentheses are from MUNSELL (1965).

### III. Results

Two intersterility groups were determined by the all pairwise combinations mating
test with three single spore isolates from each of the seven fruit-bodies (Table 1). These groups were mated with tester strains of European and North American species and identified with group I and II according to A. ostoyae and A. bulbosa.

**Descriptions**

*Armillaria ostoyae* (ROMAGN.) HERINK in Hasek

Misapplied Name: *Armillaria obscura* (SECRETAN) HERINK, according to TERMORSHUZEN and ARNOLDS (1987) (Fig. 1A).

Pileus 4–14 cm diam., when young, hemispherical-companulate or obtusely conic, then plano-convex and finally plane, sometimes irregularly undulating in age. Surface dry, when young light brown (5YR-6/6) at the center and pinkish beige (5YR-8/2) or pale beige (10YR-8/2) toward the margin, but usually reddish brown (2.5YR-4/6), brownish gold (5YR-6.5/6), dark reddish brown (10R-3/6) and light grayish brown (10YR-8/4) toward the margin, and deep brown (2.5YR-4/6), usually covered with distinct long fine hairs in shades of light yellowish brown (10YR-4/6) and whitish partial veils at the margin when young, later distinct scales in shades of dark grayish brown (2.5YR-3/2), dark reddish brown (10R-3/6), distributed over the surface but more densely concentrated toward the center, on dull reddish yellow (2.5Y-8/6) or pale reddish yellow (2.5Y-8/4) background toward the center. Margin inrolled at first then plane, striated, usually concolorous to cap but sometimes paler, pale reddish yellow (2.5Y-8/4). Flesh firm, very thick at the apex of the stipe, context white.

Lamellae white when young, to light reddish brown later, close, sinuate, subdecurrent, 1 cm thick at the point of attachment to the stipe but thinner toward the margin.

Stipe central, 4.5–12 cm long, 0.7–2.2 cm thick at the apex, cylindric, clavate when young becoming more or less equal, fibrous, solid when young, stuffed when old, concolorous with the lamellae or pinkish beige (10YR-8/2) but becoming darker toward the base, reddish brown (2.5YR-4/8), dark reddish brown (10YR-8/2), dark yellowish brown (5YR-4/4), sometimes with strong yellow (5Y-7/10) mycelial fibers at base, covered with longitudinally fibrillose when young, striate at the apex, the striations continuous with the lamellae.

Annulus thick, membranous, usually white to concolorous with lamellae, underside with dark yellowish brown (7.5YR-4/4) or dark brown (2.5YR-3/2) floci.

Spores ivory in mass, broadly elliptical to ovate, 5.2–7.5×7.5–12.5 μm, smooth, hyaline, with a distinct apiculus, nonamyloid. Basidia clavate, 6.3–8.8×35–52.5 μm four-spored, usually with a clamp connection (Fig. 2H). Pleurocystidia absent. Pileipellis composed of three layers: a suprapellis (Fig. 2A) consisting of an array of parallel, thick-walled hyphae, regular, 7.5–20×25–70 (170) μm, uppermost layer with brownish pigmentation; a mediopellis (Fig. 2B) consisting of a tight mosaic of parallel thick-walled hyphae and irregular elliptical cells, 7.5–15×10–56 μm; a subpellis (Fig. 2C) composed of a loose network of filamentous thin-walled hyphae, 8.7–12.5×42.5–125 (200) μm, staining blue in toluidine blue. Subhymenial hyphae filamentous, sometimes with a clamp connection, binucleate (Fig. 1C, 2G). Lamella trama bilateral.

**INTERSTERILITY GROUP:** I.

**SPECIMENS EXAMINED:** HUA9112, HUA9113, HUA9114, HUA9128.
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Fruit-bodies found from late September to mid-October. Occurring most commonly caespitose but sometimes solitary on decaying stem of declining tree, dead roots, and healthy roots. Hosts consist of Betula ermanii, Ulmus davidiana var. japonica and unidentified broad-leaved. In pure culture, rhizomorphs are flat and belt shaped in shade of dark brown and white at first with dichotomous branched. Although not reported in Hokkaido, A. mellea s. str. can be distinguished from A. ostoyae by its no-clamp connection in the uninucleate subhymenium and at the base of the basidia. Moreover A. ostoyae has a brown pigments in the cell wall of cuticle. A. ostoyae is very widely distributed in the northern temperature region throughout the world (Anderson and Ullrich 1979, Berube and Dessureult 1989, Guillaumin et al. 1983, Korhonen 1978, Lung-Escarmant et al. 1985, Morrison et al. 1985, Nagasawa et al. 1990, Proffer et al. 1987, Rishbeth 1982, Roll-Hansen 1985, Romagnesi 1973, Sung et al. 1990, Ullrich and Anderson 1978).

Armillaria bulbosa (BARLA) KILE & WATLING, Trans. Brit. Mycol. Soc. 81 (1): 135. 1983 (Fig. 1B).

Pileus 1.8—5.5 cm diam., convex then plano-convex and finally plane to slightly umbilicate, center almost always with a mammilate, irregularly undulating. Surface sometimes hygrophanous but usually dry, sometimes with shades of grayish brown (2.5YR-3/4) but always light yellowish brown (5YR-6/6), light reddish yellow (10YR-8/6), bright yellowish orange (7.5YR-7/10), gold (2.5Y-7/10) covered with distinct hairs and fibrills in shades of grayish brown (7.5YR-7/10) distributed over the surface but more densely concentrated toward the center when young, usually dull reddish yellow (7.5Y-7/10), grayish brown (7.5YR-4/4) small scales on a pale reddish yellow (2.5Y-8/4) background toward the center.

Margin inrolled at first then plane, strongly translucent-striate, concolorous to cap or light yellow (2.5YR-8/6), deep yellowish brown (5YR-4/6). Flesh firm, thick at the apex of the stipe, context white.

Lamellae white when young, light brown later, close, subdecurrent, 0.3—0.5 cm thick at the point of attachment to the stipe but thinner toward the margin.

Stipe central, 1.5—6.8 cm long, 0.2—0.7 cm thick at the apex, bulbous when young, but usually clavate-bulbous, fibrous, solid when young but slightly hollow when old, concolorous with the lamellae or light orange (5YR-7/4) at the apex, but pale beige (10YR-8/2) to grayish yellow (2.5Y-8/2), gold (10YR-7/10) longitudinally fibrous toward the base, striate at the apex.

Annulus cortinate, sometimes fibrous submembranous, obscure in age and leaving the stipe only slightly fugacious annular zone consisting of white to cream fibrils, nonpigmented.

Spores ivory in mass, broadly ellipitical to ovate, 3.8—5×7—10.3 μm, smooth, hyaline, apiculate and nonamyloid. Basidia clavate, 5—7.5×32.7—50 μm, four-spored with a clamp connection (Fig. 2H). Pleurocystidia absent. Pileipellis composed of three layers: a suprapellis (Fig. 2D) consisting of an array of parallel, loose, regular, thick-walled hyphae, 5—12.5×25—50 μm, uppermost layer with brownish pigmentation;
a mediopellis (Fig. 2E) consisting of a tight mosaic of elliptical and parallel hyphae, thick-walled hyphae, 3.8-25 × 7.5-130 μm; a subpellis (Fig. 2F) composed of a loose network of filamentous thin-walled hyphae, 5-30 × 20-200 μm, staining blue in toluidine blue. Subhymenial hyphae filamentous, with a clamp connection, binucleate (Fig. 1C, 2G). Lamella trama bilateral.

INTERSTERILITY GROUP: II
SPECIMENS EXAMINED: HUA9102, HUA9107, HUA9125.

Fruit-bodies found from mid-September to early October. Occurring most commonly caespitose on decaying wood debris, stump, and trunk but sometimes solitary on soil. Hosts consist of Betula ermanii, Fraxinus mandshurica var. japonica and Prunus ssiori. In pure culture, rhizomorphs are cylindrical and sharp tips with monopodial branches in shades of dark brown to black. A. bulbosa may occur in the same locations as A. ostoyae and A. mellea s. str. but can be differentiated by its cortinate annulus and clavate-bulbous stipes. Moreover, rhizomorphs can be used to differentiate A. bulbosa from A. ostoyae, the former being cylindrical and the latter belt-shaped in medium. A. bulbosa is differentiated from A. mellea s. str. not only by its clamp connection on basidia but also by binucleate subhymenium cells. A. bulbosa occurs in very wide regions, similar to A. ostoyae (ANDERSON and ULLRICH 1979, MARXMULLAR 1980, MARXMULLAR 1982, MOTT and KORHONEN 1986, NAGASAWA et al. 1990, ROMAGNESI and MARXMULLAR 1983, SUNG et al. 1990).

IV. Discussion

Two species of the Armillaria mellea complex, as they occur in the Experiment Forests of Hokkaido University, are readily distinguishable. In A. ostoyae, the predominant colors are brownish gold to dark reddish brown, the pileus surface is covered with distinct long hairs and scales in shades of dark grayish brown to dark brown, the lamellae are close and thick and the annulus is prominent, membraneous, thick, pigmented and persistent (Fig. 1). In A. bulbosa, the predominant colors are light yellowish brown to bright yellowish orange. The surface of the pileus is small scales and fibrillae in shades of grayish brown, the lamellae are thick and close and the annulus is cortinate and evanescent with occasional submembraneous fibrils (Fig. 1). Morphologically, the two species have nearly identical spores and pileipellis tissue. A. ostoyae and A. bulbosa have clamp connection in the basidia and binucleate to the subhymenial hyphae (Fig. 1), while in A. mellea they are unclamped and uninucleate.

Hokkaido collections of A. ostoyae are very similar to their European and North American counterparts, as described by ROMAGNESI (1970), GUILLAUMIN et al. (1983) and BERUBE and DESSUREAULT (1988). However, our collections of A. ostoyae differed from their North American counterparts. The subpellis cells of our species were longer and subhymenial hypha were clamped (Fig.). A. bulbosa also resembles the European and North American species as described by MARXMULLER (1980), ROMAGNESI and MARXMULLER (1983) and MOTT and KORHONEN (1986). The European species of A. bulbosa have pigments on annulus, whereas in our collections, pigments are lacking. The main conspicuous difference between Hokkaido and North American or European
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species of *A. bulbosa* is their habitat, since the former is caespitose on decaying substrates and sometimes solitary on soil but the latter only solitary on soil. The shape of stipe *A. bulbosa* is clavate-bulbous but usually bulbous in European species. This difference may not be of significance since it is reported that bulbless form *A. bulbosa* occur frequently in Europe (MOTTA and KORHONEN 1986). Although microscopical features such as the hymenial trama structure, the pileipellis structure, and basidiospore shape and size have been of less value for distinguishing both *A. ostoyae* and *A. bulbosa*, morphology of cap, annulus, and stipe as macroscopical features are useful for differentiating the species. Moreover, branching type and shape of rhizomorph proved to be reliable characters for two species differentiation.

**Acknowledgements**

The authors would like to thank Dr. A. OGOSHI and Mr. S. AKINO for technical comments and assistance.

**Literature Cited**


摘　要

北海道産ナラタケの子実体から分離した单胞子菌株を相互に交配させ、生物学的な分類について検討した。分類された不和合性集団は、ヨーロッパと北アメリカのナラタケの生物学的種のテスト菌株と交配試験を行い、*Armillaria ostoyae*と*Armillaria bulbosa*が同定された。これらの2種について、子実体の形態的特徴と分布生態などについて記載を行った。
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Fig. 1. A. Fruit-body of Armillaria ostoyae (×0.3).
B. Fruit-body of Armillaria boulbosa (×0.6).
C. Phase contrast photomicrograph of subhymenial hyphae with a clamp connection (× 1000).
Fig. 2. A–F. Cross section of pileipellis of (A–C) Armillaria ostoyae and (D–F) Armillaria bulbosa: (A, D) suprapellis (A: ×250, D: ×380), (B, E) mediopellis (B: ×1000, E: ×380), (C, F) subpellis (C: ×250, F: ×100).

G. Fluorescence micrograph of DAPI-stained lamella trama showing predominantly binucleate subhymenium (×380).

H. Phase contrast photomicrograph of basidia, illustrating clamp connections (×1000).

I. Fluorescence micrograph of DAPI-stained spores showing predominantly uninucleate (×380).