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Two *Erysiphe* species associate with recent outbreak of soybean powdery mildew: consequence from molecular phylogenetic analysis based on nuclear rDNA sequences

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Abstract  Serious outbreak of powdery mildew by a fungus belonging to the mitosporic genus *Oidium* subgenus *Pseudoidium* has been reported on soybean (*Glycine max*) in a wide area of eastern Asia since 1998. The taxonomic and phylogenetic placement of the causal fungus has not been determined yet due to lack of perfect stage. We found ascomata having mycelioid appendages on a single leaf of soybean infested by powdery mildew. Molecular phylogenetic analysis was conducted based on a total of 14 sequences of the rDNA internal transcribed spacer (ITS) region from 13 soybean and wild soybean (*Glycine soja*) materials collected in Japan, Korea, Vietnam, and the United States, combined with 47 sequence data obtained from the DNA databases. It was revealed that two *Erysiphe* species associate with the outbreak of soybean powdery mildew. There was 16% difference between the two species in genetic divergence of ITS sequence. One species with perfect stage has ITS sequence identical to *Erysiphe glycines* on *Amphicarpaea*, and is identified as *Erysiphe glycines* based on the ITS sequence and morphology of ascomata. The second species without perfect stage is likely to be *E. diffusa* (= *Microsphaera diffusa*) known as the causal fungus of soybean powdery mildew in the United States, because the ITS sequences are identical to those from materials collected in the United States. However, we need materials having ascomata of *E. diffusa* to confirm the species name.

Key Words  Erysiphaceae, *Glycine max*, ITS, *Microsphaera*, *Oidium* subgenus *Pseudoidium*
Introduction

Sawada et al. (1982) recorded first occurrence of powdery mildew of soybean (*Glycine max* (L.) Merr.) in Japan in 1980, and identified the causal fungus as *Erysiphe pisi* DC. based on anamorphic and teleomorphic characters. After the occurrence, there has been no report of the disease for 17 years in this country until second outbreak in Oita Prefecture, Kyushu Island, in 1998 (Hasama and Kato 2000). The disease has then expanded its distribution to a wide area of Kyushu and Honshu Islands within the last four years. Moreover, outbreak of the disease was first recorded in Korea in 1998 (Shin 2000), and in Vietnam in 2000 (N. Xuan Hong, personal communication). Morphological observation of anamorphic characters revealed that the causal fungus belongs to the mitosporic genus *Oidium* subgenus *Pseudoidium* (Cook et al. 1997). Detailed taxonomic and phylogenetic position of the fungus is, however, still unknown due to lack of perfect stage.

Soybean powdery mildew caused by the fungus having *Pseudoidium* anamorph, which is characterized by conidiophores producing conidia in solitary and lobed appressoria, was first reported in 1931 in the United States (Lehman 1931). The disease has since been reported in many countries of the North and South America (Brasil, Canada, Peru, Puerto Rico, Venezuela, and the United States; Amano 1986). Serious outbreaks of the disease were reported in southeastern and mid-western United States in 1970th (Arny et al. 1975; Dunleavy 1976; Leath and Carrooll 1982). The causal fungus is known as *Erysiphe diffusa* (Cooke & Peck) U. Braun & S. Takamatsu (syn. *Microsphaera diffusa* Cooke & Peck; Paxton and Rogers 1974; McLaughlin 1977). *Erysiphe diffusa* has characteristic dichotomously branched appendages on the ascomata, and conspicuously differs from the ascomata of *E. pisi*, which has mycelioid appendages, whereas the two *Erysiphe* species share *Pseudoidium* anamorph.

Our main questions in this study are, 1) does a single causal fungus associate with the recent outbreak of this disease in a wide area of eastern Asia?, 2) if so, is the fungus *E. diffusa* or *E. pisi*?, and 3) how and why did the fungus cause outbreak on soybean recent years? To address the questions, we collected powdery mildew specimens on soybean and wild soybean (*G. soja* Sieb. & Zucc.) in Japan, Korea, Vietnam, and the United States, and conducted
morphological and phylogenetic analyses of these specimens. During the study, we found formation of ascomata having mycelioid appendages on an infested soybean leaf. Analysis of the specimen revealed simultaneous occurrence of two *Erysiphe* species on a single individual of soybean.

**Materials and Methods**

**Sample sources**

Ten powdery mildew isolates from soybean collected in Japan, Korea, Vietnam, and the United States, and two isolates from wild soybean collected in Japan and Korea were used in this study. Their designation, host plants, locations of collection, and accession numbers of the nucleotide sequence databases (DDBJ, EMBL, and GenBank) are given in Table 1.

**Light microscopy**

Hyphae, conidiophores and conidia of fresh materials were stripped off the leaf surfaces with clear adhesive tape, mounted on a microscope slide with the fungal materials uppermost and examined in water under a compound microscope. Herbarium materials were rehydrated before examination by boiling a small piece of infected leaf, with the fungal mycelium downwards, in a drop of lactic acid on a slide as described by Shin and La (1993) and Shin (2000). After boiling, the rehydrated mycelium was scrapped off the leaf and mounted either in lactic acid or in cotton blue in lactic acid for light microscopy. The following information was noted during the examination: size and shape of conidia; presence or absence of fibrosin bodies; nature of conidiogenesis; characteristics of the conidiophore, e.g. size and shape of foot cell, position of the basal septum; shape and position of hyphal appressoria; position of germ tubes of conidia, when found; and shape of appressoria on germ tubes of conidia.

**Scanning electron microscopy (SEM)**
Leaf specimens were prefixed in 2.5% glutaraldehyde and washed in 0.2M Milonig’s phosphate buffer. They were fixed in 1% osmium tetroxide (OsO4) for 90 min and washed in deionized distilled water (DDW) three times. After dehydration through a graded ethyl alcohol series, they were treated with amylacetate. Subsequently they were dried in a critical-point dryer, coated with gold, and finally observed by a scanning electron microscope (LEO 440).

DNA extraction, PCR, and sequencing

Whole-cell DNA was isolated from mycelia by the chelex method (Walsh et al. 1991; Hirata and Takamatsu 1996). The nuclear rDNA region, including the internal transcribed spacer (ITS) regions (ITS 1 and ITS 2), and the 5.8S rRNA gene were amplified by the polymerase chain reaction (PCR) using the primers ITS5 (White et al. 1990) and P3 (Kusaba and Tsuge 1995). PCR reactions were done in 50 µl volumes as previously described (Hirata and Takamatsu 1996). A negative control lacking template DNA was included for each set of reactions. One microliter of the first reaction mixture was used for the second amplification with the partial nested primer set ITS1 (White et al. 1990) and P3. The PCR product was subjected to preparative electrophoresis in 1.5% agarose gel in TAE buffer. The DNA product of each amplification was then excised from the ethidium-stained gel and purified using the JETSORB kit (GENOMED, Germany) following the manufacturer's protocol. Nucleotide sequences of the PCR products were obtained for both strands using direct sequencing in an Applied Biosystems 373A sequencer (Applied Biosystems). The sequence reactions were conducted using the PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) following the manufacturer's instructions.

Molecular phylogenetic analysis
The sequences were initially aligned using the Clustal V package (Higgins et al. 1992). The alignment was then visually refined with a word processing program, using color-coded nucleotides. The alignment was deposited in TreeBASE (http://www.herbaria.harvard.edu/treebase) as S746. Phylogenetic trees were obtained from the data using distance and parsimony methods. For distance analysis, the most appropriate evolution model was determined for a given data set using PAUP* 4.0b8 (Swofford 2001) and Modeltest 3.06 (Posada and Crandall 1998). A starting tree was obtained with neighbor-joining method. With this tree, likelihood scores were calculated for 56 alternative models of evolution by PAUP*. The output file was then imported to Modeltest to compare the models by likelihood ratio test. Once a model of evolution was chosen, it was used to construct phylogenetic trees with the minimum-evolution (ME) method by a heuristic search option of PAUP*.

For the parsimony analysis, we used the maximum-parsimony (MP) method with a heuristic search using PAUP*. This search was repeated 100 times with different random starting points, using the stepwise addition option to increase the likelihood of finding the most parsimonious tree. All sites were treated as unordered and unweighted, with gaps treated as missing data. The branch-swapping algorithm was TBR, the MULPARS option was in effect, and zero-length branches were collapsed. The strength of the internal branches from the resulting trees were tested by bootstrap analysis using 1000 replications (Felsenstein 1985) and by decay analysis (Bremer 1988; Donoghue et al. 1992).

Results

Morphology of anamorph

Irregular white patches are formed on upper and lower side of leaves, stems, and pods, which finally cover the whole leaf surface and young stems. Hyphae are substraight to flexuous, branching at right or narrow angle, with a septum near the branching point (Figs. 1, 2). Appressoria on hyphae are formed opposite in pairs or single, and multilobed to moderately
lobed (Fig. 2). Conidiophores are formed single or occasionally two on a hyphal cell, arising from the upper part of mother cells, 45-95 x 7-9 µm in size, straight or occasionally slightly flexuous at the base of foot-cells, producing conidia singly followed by 1-3 cells, with a basal septum at the branching point of the mycelium (Fig. 1). Conidia are oval, ellipsoid, or cylindrical without conspicuous fibrosin bodies, 25-38(-44) x 13-18(-21) µm in size, producing polygoni-type germ tubes (Braun 1987) on the shoulder (Fig. 3). These characters, all in good agreement among all specimens observed, indicate that this fungus belongs to the mitosporic genus *Oidium* subgenus *Pseudoidium*.

Discovery and morphology of perfect stage

In November of 2001, we found ascomata on a part of soybean leaf covered with whitish powdery mildew colonies at a soybean field of Mie University. Several matured dark-brown and many immatured yellowish ascomata were embedded in colonies forming aerial hyphae without conidial formation (Fig. 4). Matured ascomata are depressed globose, dark brown, 125-130 µm in diameter (Fig. 5). Appendages are mycelioid, subhyaline and thin-walled throughout, interwoven with each other or with mycelia. Asci are 5-6 in an ascoma, 47-68 x 25-39 µm in size with short stalks (Fig. 6). Ascospores are 5-7 in an ascus, oval, 16-23 x 9-14 µm in size. These characters indicate that this fungus belongs to *Erysiphe* section *Erysiphe*.

To obtain some more specimens with ascomata, we kept some soybean individuals in the field and observed ascomata formation twice a month until late January of 2002. Unfortunately, we could not find further formation of ascomata. Most colonies continued to produce conidia vigorously until mid-December. This observation led us to an idea that the colonies with ascomata are derived from a species different from that producing only conidia. To evaluate the possibility, we then carried out molecular analysis of the material with other powdery mildew materials of soybean collected in eastern Asia and the United States.

Molecular phylogenetic analysis
A total of 14 rDNA ITS sequences of powdery mildews are determined in this study, of which 12 are from soybean and two from wild soybean. In the material collected in Mie Prefecture, DNAs were separately extracted from colonies producing conidia or ascomata. The data of the fungi sequenced in this study and their accession numbers are listed in Table 1. Of the 14 ITS sequences, 13 sequences, extracted from conidia-producing colonies, are identical to each other, having 559 nucleotides length in total of ITS1, ITS2, and 5.8S rRNA regions. The DNA extracted from ascomata-forming colony has ITS sequence different from other sequences, having 566 nucleotides length. Identity between the former and latter sequences is 84%.

These sequences were aligned with the sequences of *Erysiphe* sections *Erysiphe* and *Microsphaera* which obtained from DNA databases (Table 2). The alignment data matrix consists of 61 taxa. *Erysiphe nishidana* (Homma) U. Braun & S. Takamatsu (syn. *Uncinula nishidana* Homma) from *Firmiana simplex* (L.) W. F. Wight was used as outgroup taxon based on our unpublished data. Of the 605 aligned characters, 220 sites are variable and 159 sites are phylogenetically informative for parsimony analysis. Using Modeltest (Posada and Crandall 1998) under the likelihood ratio test criterion, we concluded that the Tamura-Nei model (Tamura and Nei 1993), with equal base frequencies, a gamma-distributed rate heterogeneity model (four rate categories, G = 0.9458; Yang 1994) and an estimated proportion of invariant sites (0.4620) was the most appropriate model of evolution for this data set. A heuristic search with this model produced a ME tree with a ME score of 1.11 (Fig. 7). MP analysis found 63 equally parsimonious trees of 561 steps (CI = 0.553, RI = 0.796, RC = 0.440). Strict consensus of the MP trees is shown in Fig. 8.

The 13 powdery mildew sequences from conidia-producing colonies are situated at the most derived position in both ME and MP analyses. They form a clade with *E. pisi* and *E. howeana* U. Braun with high bootstrap supports (89% in ME and 86% in MP) and decay index (3). The genetic distance is 2.4% between soybean fungi and *E. pisi*, and 2.9% between soybean fungi and *E. howeana*. This clade weakly clusters with other legume-parasitic species, *E. lespedezae* Zheng & U. Braun, *E. baeumleri* Magn., and *E. trifolii* Grev. var. *trifolii* in ME tree (less than 50% in bootstrap value), while *E. lespedezae* situates out of the group in MP
The sequence of DNA extracted from ascomata-forming colony is identical to that of *E. glycines* Tai from *Amphicarpaea bractaeta* (L.) Felnald subsp. *edgeworthii* (Benth.) Ohashi var. *japonica* (Oliver) Ohashi, and the fungus situates at the basal position in both ME and MP trees. Although they form a clade with *E. glycines* from *Desmodium podocarpum* DC. subsp. *oxyphyllum* (DC.) Ohashi with high bootstrap support (100%), the genetic distance between the isolates from soybean and *Desmodium* is relatively high (7.8%).

**Discussion**

Of the 14 ITS sequences of soybean and wild soybean powdery mildews determined in this study, 13 sequences extracted from conidia-producing colonies are identical to each other. The remaining one sequence extracted from ascomata-forming colony is distantly related to the above 13 sequences (16% in genetic distance), and identical to the sequences of *E. glycines* from *Amphicarpaea*. Based on the morphological characters of ascomata and the sequence result, we identify the latter isolate as *E. glycines*. Because *E. glycines* belongs to *Oidium* subgenus *Pseudoidium* in its anamorph, this result clearly indicates that two different powdery mildew species sharing *Pseudoidium* anamorph associate with this wide-area outbreak of soybean powdery mildew.

Since ascomata formation seems to be rare on soybean, it is possible that *E. glycines* is not original pathogen of soybean and incidentally infects to the plant. However, formation of ascomata having mycelioid appendages was observed also in Okayama Prefecture, Japan, in 2000 (Y. Nomura and K. Fujioka, personal communication). Sawada et al. (1982) reported serious outbreak of soybean powdery mildew in Tokyo and identified the causal fungus as *E. pisi* sensu Homma (1937). We cannot directly compare the *E. pisi* of Sawada et al. (1982) with our isolate because the specimen observed by Sawada et al. (1982) is not available. He described that his soybean isolate was very similar to isolate from *Amphicarpaea* in both teleomorphic and anamorphic characters (Sawada 1984). Because *E. glycines* is included in *E. pisi* sensu Homma (1937), the causal fungus of the outbreak in Tokyo is likely to be *E. glycines*. 

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glycines. These evidences suggest that E. glycines is an original pathogen of soybean powdery mildew.

The identical ITS sequence between isolates from soybean and Amphicarpaea suggests that the pathogen came from Amphicarpaea, although we have to conduct inoculation test to confirm it. Erysiphe glycines of Amphicarpaea is commonly found under forests in Japan. Conidia of E. glycines may easily arrive to soybean field and infect soybean if cultivars of soybean are susceptible to the fungus. A question as to why powdery mildew has not been popular on soybean until recent outbreak is, however, still remaining to be solved. Because many reports (Demski and Phillips 1974; Grau and Laurence 1975; Dunleavy 1977; Mignucci and Chamberlain 1978; Mignucci and Lim 1980; Lohnes and Nickell 1994; Hasama et al. 2000) indicate that the soybean powdery mildew is cultivar specific, the question might be addressed by difference of susceptibility of soybean cultivars to the fungus.

The 13 anamorphic specimens have ITS sequences identical to each other and similar anamorphic characters. Because this fungus is commonly isolated from every place of outbreak of soybean powdery mildew, this may be the major causal fungus of recent outbreak of the disease. Molecular phylogenetic analysis indicates that pea powdery mildew, E. pisi, is the closest relative of this fungus. However, there is 2.4% genetic distance between the soybean fungus and E. pisi. We further compared the sequence of the soybean powdery mildew with 143 ITS sequences of fungi belonging to Erysiphe sections Erysiphe and Microsphaera, including 30 sequences from legume parasitic species (unpublished data). There is no sequence identical to the sequence of soybean fungus, which suggests that the fungus is unique to soybean and wild soybean, and was introduced from outside of Japan.

The causal fungus of soybean powdery mildew is known as Erysiphe (Microsphaera) diffusa in the United States (McLaughlin 1977). Erysiphe diffusa has dichotomously branched appendages on its ascomata, which conspicuously differs from E. glycines having mycelioid appendages (Braun 1987). Powdery mildews having dichotomously branched appendages have long been classified as the genus Microsphaera, and those having mycelioid appendages as the genus Erysiphe. However, Microsphaera and Erysiphe (section Erysiphe) share Pseudoidium anamorph and cannot be differentiated from each other by anamorphic
characters. Molecular phylogenetic analyses (Takamatsu et al. 1999) indicated that Microsphaera and Erysiphe (section Erysiphe) are grouped together in a clade, and cannot be separated from one another in phylogeny. Braun and Takamatsu (2000) proposed to combine the genera Erysiphe (section Erysiphe), Microsphaera, and Uncinula into a single genus Erysiphe based on the molecular phylogenetic studies (Takamatsu et al. 1999; Saenz and Taylor 1999; Mori et al. 2000) and scanning electron microscopy of conidia (Cook et al. 1997).

It is possible that the second species of the soybean powdery mildew is *E. diffusa* based on the following reasons: 1) the ITS sequences of isolates in eastern Asia are identical to the sequences of the two isolates collected in the United States, 2) anamorphic characters of the present fungus (straight or occasionally flexuous conidiophore at the base, and conidial shape and size) are in good agreement with the description of *E. diffusa* (Braun 1987). However, it cannot be confirmed yet because the specimens collected in the United States do not have ascomata. We thus do not know whether these US materials are *E. diffusa* or not. Materials having ascomata of *E. diffusa* are essential to confirm the species name.

In conclusion, we showed that two *Erysiphe* species associate with the recent outbreak of soybean powdery mildew in eastern Asia: one species having only conidial stage and another having perfect stage. It has been reported that the soybean powdery mildew has strict cultivar specificity (Demski and Phillips 1974; Grau and Laurence 1975; Dunleavy 1977; Mignucci and Chamberlain 1978; Mignucci and Lim 1980; Lohnes and Nickell 1994; Hasama et al. 2000). Because the specificity may differ between the fungal species, the causal fungus should be identified to investigate reactions of soybean cultivars to powdery mildew. The two species can simultaneously infect a single individual or a single leaf of soybean. Separate or simultaneous infection of these two species may cause various different reactions of soybean cultivars. Hasama et al. (2000) investigated reactions of soybean cultivars to powdery mildew in fields in 1998 and 1999, and reported that the results significantly differed between the years. This difference might be attributed in species responding to outbreak of the disease during the two years.

The most easy and effective method to differentiate the two species of soybean powdery mildew...
mildews may be molecular techniques using PCR. Because there is 16% sequence divergence between the two species, it will be easy to design PCR primers to separate these species. Alternatively, differentiation based on anamorphic characters may be also possible, although not easy. Sawada (1984) reported that branching angle of hyphae is different between *E. pisi* on pea and soybean fungus. Further detailed observation of the fungi might make possible to differentiate the species based on anamorphic features.

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Legends of Figures

Figs. 1-3. Scanning electron micrographs of the anamorph of soybean powdery mildew fungus. 1 Conidiophores arising from the upper part of mother cells, straight or occasionally flexuous at the base of foot-cells, producing conidia singly followed by 1-3 cells. 2 Appressoria on hyphae, formed opposite in pairs, multilobed to moderately lobed. 3 Conidia with oval, ellipsoid, and cylindric shape, producing *polygoni*-type germ tubes. Bars 1-3 20 µm

Figs. 4-6. Teleomorph (Ascomata) of soybean powdery mildew fungus found in Mie Prefecture, Japan. 4 Matured dark-brown ascomata embedded in colonies forming aerial hyphae. 5 An ascoma having mycelioid appendages. Bar 100 µm 6 Asci and ascospores. Bar 50 µm

Fig. 7. A minimum-evolution (ME) tree based on ITS data for 14 isolates from soybean and wild soybean, 39 taxa of *Erysiphe* sections *Erysiphe* and *Microsphaera*, and one outgroup taxon. Model parameters: equal base frequencies with rate heterogeneity; gamma shape parameter = 0.9458; proportion of invariable sites = 0.4620; six rate categories; Tamura-Nei model (Tamura and Nei 1993) with transformation parameters \([\text{A-C]} = 1.0000, [\text{A-G}] = 2.8140, [\text{A-T}] = 1.0000, [\text{C-G}] = 1.0000, [\text{C-T}] = 3.9310, [\text{G-T}] = 1.0000\). Percent bootstrap support (1000 replications) is indicated above or below nodes. Solid circle indicates isolate from legume plant.

Fig. 8. Strict consensus of 63 equally parsimonious trees based on ITS data for 14 isolates from soybean and wild soybean, 39 taxa of *Erysiphe* sections *Erysiphe* and *Microsphaera*, and one outgroup taxon. Percent bootstrap support (1000 replications) and decay indices are shown above and below nodes, respectively. The consistency index (CI) is 0.553; the retention index (RI) is 0.796; and the rescaled consistency index (RC) is 0.440. Solid circle indicates isolate from legume plant.
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不完全世代属である Oidium 属 Pseudoidium 亜属の病原菌によるダイズうどんこ病の多発
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分類学的、系統学的配置は不明のままであった。我々はうどんこ病に罹病したダイズの1個
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**Table 1.** Isolate, location, year of collection and database accession number of the rDNA ITS sequences of soybean and wild soybean powdery mildew used in this study.

<table>
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<th>Database accession no.(^b)</th>
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<td>2001</td>
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**Glycine max** (soybean)

**Glycine soja** (wild soybean)

SMK15414 | Chunchon, Korea | 1998 | AB078812 |
MUMH1162 | Gifu, Japan     | 2000 | AB078813 |

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\(^a\) Herbarium specimens at the following herbaria: MUMH = Mie University Mycological Herbarium, Japan; SMK = Mycological Herbarium, Department of Agricultural Biology, Korea University.

\(^b\) The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank Database under the respective accession number.
Table 2. Fungal materials and sequence database accession numbers used for phylogenetic analysis

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Host plant</th>
<th>Isolate$^a$</th>
<th>Country of origin</th>
<th>Database accession no.$^b$</th>
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<tbody>
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<td><em>E. friesii</em> var. <em>dahurica</em></td>
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Table 2. - Continued -

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<th>Fungus</th>
<th>Host plant</th>
<th>Isolate(^a)</th>
<th>Country of origin</th>
<th>Database accession no.(^b)</th>
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\(^a\) MUMH = Mie University Mycological Herbarium; TPU = Herbarium of Toyama Prefectural University;
YNMH = Yukihiko Nomura Mycological Herbarium; VPRI = Plant Disease Herbarium, Institute for Horticultural Development, Victoria, Australia; UC = University of California Herbarium.

\(^b\) DDBJ, EMBL, and GenBank database accession number of the nucleotide sequence data.

\(^c\) Unpublished sequence provided by J. Cunnington.