Biodegradable Plastic-degrading Activity of Various Species of *Paraphoma*

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Abstract: The fungal strain B47-9, isolated from barley, was previously selected as an effective degrader of various biodegradable plastic (BP) films such as poly(butylene succinate-co-adipate) (PBSA) and poly(butylene succinate) (PBS). The strain has not been identified based on mycological methods because it does not form fruiting bodies, which are the key to morphological identification. Here, we performed molecular phylogenetic analyses of the nuclear ribosomal RNA gene regions and their internal transcribed spacer region of B47-9 and related fungi. The results suggest that B47-9 is closely related to the genus *Paraphoma*. Investigation of the abilities of six strains belonging to the genus *Paraphoma* to degrade BPs indicated that all strains could degrade PBSA and PBS films to varying degrees. Based on our approach, we conclude that strain B47-9 is a species belonging to the genus *Paraphoma*.

Key words: biodegradable plastic film, *Paraphoma*, mulch film, phylogenetic analysis, poly (butylene succinate-co-adipate) (PBSA), poly (butylene succinate) (PBS)

1 INTRODUCTION

In the cultivation of vegetables, covering the soil bed with agricultural mulch films is an indispensable means of maintaining soil temperature and controlling weeds and pests. Generally, mulch film is made of nondegradable synthetic polymer. However, collection of the used film after harvest is labor-intensive, and disposal of the film is costly. To solve these problems, mulch films made of biodegradable plastics (BPs) have been developed that can be plowed into the ground with a tilling machine following use. However, the use of BP mulch film has not been widely adopted because of the difficulty in controlling the degradability under various cultivation environments. A practical BP mulch film would have no significant decrease in performance during the planned useful lifetime, but would degrade rapidly after use. However, it is difficult to develop adequately degradable plastic films. Treatment with a BP-degrading enzyme is one means of accelerating the degradation of used BP mulch films. To obtain such enzymes, BP-degrading microorganisms have been isolated from the natural environment using selective agarose plates containing emulsified poly(butylene succinate-co-adipate) (PBSA). Microorganisms that can degrade emulsified PBSA produce a clear zone around the colony on the selection plate. However, the efficiency of isolation of microorganisms capable of degrading solid BPs is low.

With a focus on the similarities between the structures of the cuticle layer covering the leaves of plants and BPs, we isolated many types of yeast from the surface of rice plants and obtained powerful BP film-degrading enzymes. In addition, the filamentous fungus B47-9 was selected from our collection of fungi isolated from gramineous plants as a high-BP film degrader. A B47-9 culture filtrate with BP-degrading activity was obtained. When the culture
filtrate was sprayed over BP-based mulch film covering the soil bed in a pipe house, the film showed accelerated degradation.

However, the strain B47-9 could not be identified. Although the taxonomy and identification of fungi are based largely on the morphological characteristics of their fruiting bodies and spores, B47-9 does not form such structures. Comparison of the DNA sequence of the internal transcribed spacers ITS 1 and ITS 2 of nuclear ribosomal RNA genes and the 5.8S rDNA gene of B47-9 with those in the DNA Data Bank of Japan (DDBJ) indicated the highest sequence similarity to a strain of Paraphoma chrysanthemicola. The genus Paraphoma was characterized mainly based on consistent thickness of the entire pycnidial wall composed of uniformly shaped and compact cells and well-differentiated setae densely spread over the pycnidial surface. Moreover, based on molecular phylogenetic analysis of the small subunit 18S rDNA (SSU) and large subunit 28S rDNA (LSU) regions, Paraphoma was reconfirmed as a distinct genus.

The ITS region (the whole region spanning ITS1, 5.8S and ITS2) is the most frequently sequenced genetic marker of fungi, and have been proposed as universal fungal DNA barcodes that can be used to delineate species boundaries of fungi, and have been proposed as universal fungal DNA barcodes that can be used to delineate species boundaries of fungi. The B47-9 genome was sequenced in our laboratory using the Illumina platform Illumina Miseq System (Illumina K.K., Tokyo, Japan), and the scaffold sequences were assembled using the sequence reads from both paired-end and mate pair libraries, which will be reported elsewhere. The sequences of SSU and LSU and ITS regions were identified by alignment of these genes from B47-9 against genomic scaffold sequences using FASTA version 36.3.6d with the default parameters without using a sequence mask. Multilocus analysis of sequence data of the SSU and LSU was performed. Target regions of the partial SSU, LSU rDNA, and ITS were analyzed using fungal specific primers NS1 and NS4 for partial SSU rDNA, LROR, and LR7 for partial LSU rDNA and ITS5 and ITS4.

The phylogeny of the morphologically similar Coelomycetes was determined utilizing the sequence data of the SSU and LSU regions. The sequence data of the SSU and LSU genes for the 37 strains examined in this paper were revised by Aveskamp et al. and downloaded from the DDBJ/EMBL/GenBank databases. A phylogenetic tree was constructed from sequences of the SSU and LSU genes combined by the maximum likelihood (ML) method using shotgun searches with RAxML version 8. The search was repeated until the maximum likelihood was identified. Base composition homogeneity tests were conducted using Kakusan. The reliability of the inferred tree was estimated by bootstrap analysis with 1000 replicates.

Sequence data of the ITS region for the 23 strains were also revised by Aveskamp et al. and downloaded from the DDBJ/EMBL/GenBank databases. The ITS region sequences of six MAFF strains were obtained as described in our previous report. Phylogenetic analyses of the ITS region were performed for each dataset based on similar tree topologies obtained. Neighbor joining (NJ) distance analysis was conducted with MEGA Ver.5 with Kimura 2-parameter substitution models. The robustness of the trees was evaluated by 1000 bootstrap replications.

### 2 EXPERIMENTAL PROCEDURES

#### 2.1 Fungal strain

Strain B47-9 was isolated from barley in May 2006 in Tsukuba, Ibaraki, Japan. B47-9 was deposited in the National Institute of Technology and Evaluation (NITE, Japan) and NITE Patent Microorganisms Depositary (accession number: NITE P-573). Six strains belonging to the genus Paraphoma (Table 1) were deposited in the National Institute of Agrobiological Sciences, Japan, (NIAS)Genebank.

### Table 1 Fungal strains degrading biodegradable plastics examined in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Date of sampling</th>
<th>Source locality</th>
<th>Isolation source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAFF244948</td>
<td>Paraphoma chrysanthemicola</td>
<td>April, 2014</td>
<td>Chikusei, Ibaraki</td>
<td>Glycine soja</td>
</tr>
<tr>
<td>MAFF244949</td>
<td>Paraphoma chrysanthemicola</td>
<td>April, 2014</td>
<td>Tsukuba, Ibaraki</td>
<td>Glycine soja</td>
</tr>
<tr>
<td>MAFF244950</td>
<td>Paraphoma chrysanthemicola</td>
<td>April, 2014</td>
<td>Tsukuba, Ibaraki</td>
<td>Glycine soja</td>
</tr>
<tr>
<td>MAFF244952</td>
<td>Paraphoma radicina</td>
<td>April, 2014</td>
<td>Tsukuba, Ibaraki</td>
<td>Glycine soja</td>
</tr>
<tr>
<td>MAFF244953</td>
<td>Paraphoma radicina</td>
<td>April, 2014</td>
<td>Tsukuba, Ibaraki</td>
<td>Glycine soja</td>
</tr>
<tr>
<td>MAFF245116</td>
<td>Paraphoma chrysanthemicola</td>
<td>July, 2014</td>
<td>Nayaro, Hokkaido</td>
<td>Atractylodes lancea</td>
</tr>
<tr>
<td>B47-9</td>
<td>Unidentified</td>
<td>May, 2006</td>
<td>Tsukuba, Ibaraki</td>
<td>Hordeum vulgare</td>
</tr>
</tbody>
</table>

**2.2 Molecular phylogenetic analyses**

The B47-9 genome was sequenced in our laboratory using the Illumina platform Illumina Miseq System (Illumina K.K., Tokyo, Japan), and the scaffold sequences were assembled using the sequence reads from both paired-end and mate pair libraries, which will be reported elsewhere. The sequences of SSU and LSU and ITS regions were identified by alignment of these genes from B47-9 against genomic scaffold sequences using FASTA version 36.3.6d with the default parameters without using a sequence mask. Multilocus analysis of sequence data of the SSU and LSU was performed. Target regions of the partial SSU, LSU rDNA, and ITS were analyzed using fungal specific primers NS1 and NS4 for partial SSU rDNA, LROR, and LR7 for partial LSU rDNA and ITS5 and ITS4.

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2.3 BP film-degrading ability on agarose plates

Evaluation of the BP film-degrading activities of six Paraphoma strains and B47-9 was performed as described in our previous report[6]. Two types of BP mulch film were used as the substrates: poly(butylene succinate) (PBS; Bionolle 1001G) and PBSA (Bionolle 3001G), both of which have an average molecular weight of 20 to 25 \times 10^4 and thickness of 20 \mu m and are colored black with carbon powder. These products were obtained from Showa Denko K. K. (Tokyo, Japan).

The PBSA or PBS films were cut into squares measuring 2 \times 2 cm, sterilized with 70% ethanol, and air-dried on a clean bench. Four pieces of the cut films were placed on fungal minimum medium agarose plates side by side, forming a larger square with a distance of 2 mm between two adjacent cut films. The agarose grown with the test strain was sliced into cubes (2 \times 2 \times 2 mm) and inoculated at the center of the four cut films mounted in a dish. The cut films were removed from the agar plates after incubation at 28°C for 7 days; their images were scanned using a transparency scanner, and their degradation ratios were evaluated by comparing the luminance of the 4 cm² area of the residual film with that of the fresh film as described previously[6].

3 RESULTS AND DISCUSSION

3.1 Molecular phylogenetic analyses

The ML tree was calculated based on two gene sequences of SSU and LSU (accession nos. LC126020 and LC126021, respectively) of the strain B47-9, with 37 strains downloaded from the DDBJ/EMBL/GenBank databases (Fig. 1). In the phylogenetic tree, the strain B47-9 and P. chrysanthemicola, P. fimeti, and P. radicina formed a clade. The results indicated that B47-9 and P. chrysanthemicola are closely related.

Six MAFF strains including four P. chrysanthemicola strains and two P. radicina strains were obtained from NIAS Genebank. The DNA sequences of their ITS region were analyzed and deposited in DDBJ as listed in Table 2. The phylogenetic relationships of the strain B47-9 ITS region with those of MAFF strains and 23 strains obtained from the DDBJ/EMBL/GenBank databases were calculated based on NJ analysis (Fig. 2). Based on these results, strain B47-9 and P. chrysanthemicola strains were also placed in the same clade. The results of NJ analysis with the ITS region were congruent with those of ML analysis of two combined loci, SSU and LSU (Fig. 1). Both results showed that strain B47-9 grouped with the genus, Paraphoma including P. chrysanthemicola, P. radicina, and P. fimeti.

We previously named the BP-degrading enzyme of strain B47-9 "Paraphoma-related fungus cutinase-like enzyme."
and demonstrated its ability to degrade various BP films, including PBS, PBSA, and poly(ε-caprolactone), as well as poly(butylen adipate-co-terephthalate) (PBAT) and poly(DL-lactic acid)\(^1\). Selection of suitable culture conditions using a jar fermenter resulted in a 20-fold increase in BP-degrading enzyme production by strain B47-9\(^2\). For technical use of microorganisms, taxonomic information is needed to determine the influence of these organisms on humans, animals, and the environment. According to the taxonomic information, it may also be possible to obtain better BP-degrading enzyme producers from the same group. The strain B47-9 was isolated from

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>DDBJ accession no.</th>
<th>Degradation area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAFF244948</td>
<td>Paraphoma chrysanthemicola</td>
<td>LC126022</td>
<td>44.1 30.4</td>
</tr>
<tr>
<td>MAFF244949</td>
<td>Paraphoma chrysanthemicola</td>
<td>LC126023</td>
<td>34.9 35.0</td>
</tr>
<tr>
<td>MAFF244950</td>
<td>Paraphoma chrysanthemicola</td>
<td>LC126024</td>
<td>32.3 17.5</td>
</tr>
<tr>
<td>MAFF244952</td>
<td>Paraphoma radicina</td>
<td>LC126025</td>
<td>59.4 47.0</td>
</tr>
<tr>
<td>MAFF244953</td>
<td>Paraphoma radicina</td>
<td>LC126026</td>
<td>67.5 65.5</td>
</tr>
<tr>
<td>MAFF245116</td>
<td>Paraphoma chrysanthemicola</td>
<td>LC126027</td>
<td>6.8 0.4</td>
</tr>
<tr>
<td>B47-9</td>
<td>Unidentified</td>
<td>AB693768</td>
<td>99.2 95.0</td>
</tr>
</tbody>
</table>

Fig. 2 Neighbor joining tree of the rDNA-ITS region of strain B47-9 and species belonging to Paraphoma, Phoma, Pyrenochaeta, and related genera. Numbers on the branches represent the percentage of congruent clusters in bootstrap trials repeated 1000 times when the values were greater than 50%.
barley. Although *P. chrysanthemicola* was originally isolated from *Chrysanthemum morifolium*, *P. chrysanthemicola* MAFF244948, 244949, and 244950 examined here were isolated from *Glycine soja*, and MAFF245116 was isolated from *Atractylodes lancea*. *Paraphoma radicina* MAFF 244952 and MAFF 244953 examined here were isolated from *Glycine soja*. Therefore, all of the *Paraphoma* strains tested were isolated from plants. With the exception of MAFF 245116, all of the source plants were collected within Ibaraki prefecture, Japan.

### 3.2 Evaluation of enzyme-based BP mulch film degradation

Because strain B47-9 has strong BP degradation ability, the capabilities of six MAFF *Paraphoma* strains were tested. Similar to strain B47-9, all of the MAFF strains tested showed the ability to degrade emulsified PBSA on selective agarose plates. In addition, all of the tested strains degraded films composed of PBSA and PBS (Fig. 3). Strain B47-9 reduced the area of PBSA film by 99.2% and that of the PBS film by 95% (Table 2). On the other hand, the other strains degraded PBSA film by 6.8% to 67.5% and PBS film by 0.4% to 65.5% (Table 2). When we previously tested 1227 strains of phylloplane fungi of various morphologies, only 55 strains (4.5%) were selected as capable of degrading emulsified PBSA. Of these 55 strains, 43 (78.2%) and 37 (67.3%) degraded PBSA and PBS, respectively, on the same selective agarose plates. The observation that all of the strains belonging to the

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**Fig. 3** Images of degraded PBSA films (left) and PBS films (right) removed from agarose plates with colonies of the fungal strains examined. a: MAFF244948, b: MAFF244949, c: MAFF244950, d: MAFF244952, e: MAFF244953, f: MAFF245116, g: B47-9, h: Control.
genus Paraphoma investigated in this study had the ability to degrade BP was of great interest as potential sources of strong BP degraders. In future, we intend to investigate the capacities of fungal strains belonging to the genera Phoma and Pyrenochaeta, which are closely related to the genus Paraphoma, to degrade polymeric compounds.

4 Conclusion
For establishment of a technique to accelerate degradation of used BP agricultural mulch films using BP-degrading filamentous fungi, we found the source of strong degrader. Practical application of these microorganisms to large fields will lead to the development of low-cost, environmentally friendly techniques.

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