【Note】

Fruit body formation and intra-species DNA polymorphism in Japanese *Wolfiporia cocos* strains

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Abstract

Poria, the dried sclerotium of *Wolfiporia cocos*, is a medicinal mushroom that is widely used in traditional Japanese medicine. The fruit body of *W. cocos* is rarely found in the natural environment in Japan, therefore an optimized technique for fruit body formation is essential for producing new strains through crossbreeding and for biological research. Here, we developed a cultivation technique for fruit body formation of *W. cocos* using three strains collected from different areas of Japan. When mycelia were cultured on sawdust-based medium after liquid medium culture, all strains successfully formed fruit bodies as a brown honeycomb-like structure. Furthermore, we analyzed single nucleotide polymorphisms of the three strains using the STE3-like pheromone receptor protein gene, STE3.2, and found a genetic marker for discriminating one strain from the others. The results are expected to promote extensive studies on crossbreeding and domestic production of *W. cocos*.

Key words：Crude drug, DNA analysis, Fruit body, *Wolfiporia cocos,* Poria

35 ## Introduction

36 The sclerotium of *Wolfiporia cocos,* belonging to Polyporaceae, has been used in
37 traditional Chinese and Japanese medicine [1]. In Kampo medicine, Poria, the dried
38 sclerotium of *Wolfiporia cocos*, has diuretic and sedative effects, and has been widely
39 prescribed in many important formulations. According to a 2018 survey on crude drug
40 usage in Japan, approximately 1,600 tons of Poria are consumed per year, yet Poria is
41 mostly imported from China and Korea [2]. In the 2017 fiscal year, the supply was limited
42 (65 kg) in domestic market and there was no domestic supply in 2018 [2]. *W. cocos* has
43 been widely cultivated in China [1]. On the other hand, previous studies have indicated
44 that the Japanese *W. cocos* strains grow slowly and have a poor ability to form sclerotium
45 compared to Chinese strains [3, 4]. Hence, optimization of the environmental conditions
46 for Japanese strains is necessary for cultivation.

47 As the fruit body of *W. cocos* has not been found in the natural environment, collection
48 techniques of sclerotia often involve traditional methods, such as stabbing an iron spear
49 into the soil, to locate the sclerotia found adhering to the roots of pine in the soil [4]. The
50 collection of *W. cocos* requires hard physical labor, as a result the number of collectors
51 has been decreasing over the years. Therefore, to domestically produce Poria in a stable
52 and sustainable manner, it is desirable to find a strain that is suitable for cultivation in
53 Japan and establish an appropriate cultivation method.

54 Crossbreeding is an approach used to obtain strain diversity. Previously,
55 high-yield-potential strains were developed by crossbreeding Chinese *W. cocos* strains [5,
56 6]. Fruit body formation was induced using a solid medium, and cross-breeding was
57 performed using isolated spores [5-8]. For genetic quality control, the STE3-like
58 pheromone receptor protein gene, STE3.2, was used as an intra-species DNA marker [5,
59 6].

60 In Japan, studies on fruit body formation have been conducted to investigate formation
61 conditions, including irradiation with light [9]. However, the study of fruit body
62 formation was performed using a single strain. In addition, genetic differences between
63 Japanese and Chinese strains have been shown using sequences of the 18S rRNA gene
64 and ITS region and random amplified polymorphic DNA (RAPD) analyses [3, 10].
65 However, intra-species genetic differences among Japanese strains have not been
66 established.

67 In this study, we evaluated the ability of *W. cocos* to form fruit bodies using three
68 strains collected from different areas in Japan. Using a sawdust-based culture, the fruit
69 bodies were observed in the three strains. Furthermore, we analyzed single nucleotide
70 polymorphisms of the three strains using STE3.2 gene sequences and found a genetic
71 marker for discriminating one strain from the others.

72 Material and methods

73 **The strains of *Wolfiporia cocos***

74 Three Japanese strains of *W. cocos*, WP-1, WP-2, and WP-3 (voucher nos. J2020WP01,
75 J2020WP02, and J2020WP03, respectively) were used in this study. The sclerotia of
76 WP-1 (J2020WP01) were collected at Ina, Nagano Prefecture in March 2020. The
77 sclerotia of WP-2 (J2020WP01) were collected at Ina, Nagano Prefecture in November
78 2015. WP-1 and WP-2 were collected as sclerotia that adhered to the roots of Japanese
79 red pine (Pinus densiflora Siebold et Zucc.) in different areas. The sclerotia of WP-3
80 (J2020WP03), which was adherent to the root of Japanese black pine (*Pinus thunbergii*
81 Parl.), were collected in the Ishikawa Prefecture in January 2013. The strains of WP-2
82 and WP-3 were provided from the herbarium of School of Pharmacy, School of
83 Pharmaceutical Sciences, Kanazawa University, Japan (voucher nos. KWCN001 and
84 KWCI001, respectively). The strains were maintained in potato dextrose agar (PDA) at
85 the Medicinal Garden of Josai University.

86

87 **Fruit body formation**

88 The solid medium consisted of the following components: peptone 2 mg/ml (Kyokuto
89 Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), Bacto Yeast Extract (Thermo Fisher
90 Scientific) 2 mg/ml, glucose (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) 20
91 mg/ml, $MgSO_4$ (FUJIFILM Wako Pure Chemical Co.) 1 mg/ml, KH_2PO_4 (FUJIFILM

92 Wako Pure Chemical Co.) 1 mg/ml, K_2HPO_4 1 mg/ml, agar (FUJIFILM Wako Pure
93 Chemical Co.) 20 mg/ml. Incubation was conducted using a 200 ml Erlenmeyer flask
94 with 30 ml solid medium. *W. cocos* mycelia, which were maintained in PDA medium,
95 were picked up using a 200 μl tip, seeded into the solid medium, and incubated at 25 °C
96 for 30 d with light shielding. The flask was capped with aluminum foil and maintained
97 under humid conditions. After 30 d, an approximately 3 cm long scratch was made on the
98 solid medium using a sterile blade. The scratched medium was further incubated at 25 °C
99 for 30 d. During this period, the medium was kept in darkness but exposed to indoor
100 lighting for an hour every three days. After 30 days, the number of fruit bodies per
101 medium for each strain was counted.

102 The sawdust-based culture was performed as follows: *W. cocos* mycelia were seeded
103 into 250 ml liquid medium in a 300 ml Erlenmeyer flask. The liquid medium consisted of
104 24 mg/ml Potato Dextrose Broth (Becton Drive, Franklin Lakes, New Jersey, USA), 20
105 mg/ml glucose, 3 mg/ml $MgSO_4 \cdot 7H_2O$ (FUJIFILM Wako Pure Chemical Co.), 3 mg/ml
106 KH_2PO_4 (FUJIFILM Wako Pure Chemical Co.), and 10 μg/ml vitamin B_1. Liquid-based
107 culture was performed at 25 °C on a shaker (120 rpm) for 30 d. The sawdust-based
108 medium consisted of $(NH_4)_2SO_4$ (FUJIFILM Wako Pure Chemical Co.) 8 g, $CaCl_2$
109 (FUJIFILM Wako Pure Chemical Co.) 2 g, vitamin B_1 (Tokyo Chemical Industry Co.,
110 Ltd., Tokyo, Japan) 0.05 g, K_2HPO_4 6 g, KH_2PO_4 6 g, glucose 30 g, and H_2O 900 ml per

111 red pine sawdust 1 kg. The sawdust-based medium (60 g) was dispensed into a bottle
112 capped with aluminum foil and autoclaved at 120 °C for 60 min. After 30 d of
113 liquid-based culture, the cultured medium (50 ml) was added to the sawdust-based
114 medium in a bottle. The surface of the sawdust-based medium was covered with
115 autoclaved gravel stones (200 g). The transitioned medium was cultured at 25 °C for 30
116 additional days. During this period, the medium was kept in darkness, but exposed to
117 indoor lighting for an hour every three days. Each treatment included three replicates, and
118 two to three flasks and bottles were used per replicate.

119

120 **Spore isolation and nuclear staining**

121 The fruit bodies were placed onto a solid plate dish and cultured at 25 °C. A single colony
122 on the lid was suspended with 60 µl H_2O. Nuclei were stained with Hoechst 33342
123 (1:1000 dilution; Dojindo, Kumamoto, Japan). The spores were monitored using
124 BZ-X700 (Keyence, Osaka, Japan).

125

126 **Single nucleotide polymorphism (SNP) analysis**

127 DNA samples were extracted using NucleoSpin Plant II (Takara, Shiga, Japan) according
128 to the manufacturer's instructions. Genomic DNA was quantified using a NanoDrop 1000
129 Spectrophotometer (Thermo Fisher Scientific, Loughborough, UK). The STE3.2 gene

130 was amplified by polymerase chain reaction (PCR) in a final volume of 50 μl containing
131 2×PCR buffer 25 μl, 2 mM dNTPs 10 μl, 0.5 μM each of forward and reverse primers,
132 KOD FX Neo (1.0 U/μl) 1 μl and genomic DNA (20 ng). PCR conditions were as follows:
133 initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for
134 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 1 min, followed by a 4 °C hold.
135 Forward (5′-3′, AAT CGC TTT CTC GTT CAT CG) and reverse (5′-3′, AAG GCA CAC
136 CTC AAC AAC TG) primers were used to amplify the STE3.2 gene [11]. The PCR
137 products were electrophoresed on 1% agarose gels and purified using the FastGene
138 Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). PCR products were sequenced
139 using the forward or reverse primer and BigDye® Terminator v1.1 (Applied Biosystems,
140 USA) on a 310 DNA genetic analyzer (Applied Biosystems) according to the
141 manufacturer's protocol. The sequencing data were aligned and analyzed using
142 Molecular Evolutionary Genetics Analysis (MEGA) version 6.

143

144 ## Results and discussion

145 **Fruit body formation**

146 Fruit body formation was evaluated using the three *W. cocos* strains collected in Japan
147 (Table 1). We first evaluated the fruit body formation using a solid medium cultivation
148 method adapted from a previously reported study, with minor modifications [5]. Three
149 fruit bodies among seven flasks were observed in WP-1, as white honeycomb-like
150 structures. The morphology of the fruit body found in our study is similar to that of the
151 described fruit body in the previous study [5]. No fruit bodies were observed in either
152 WP-2 or WP-3. The previous report showed that the probability of fruit body formation is
153 approximately 20% under optimized conditions [5]. We next tested a sawdust-based
154 culture for efficient formation of the fruit body. Mycelia were first seeded into a liquid
155 medium for 30 d, then transferred to a sawdust-based medium. All strains successfully
156 formed fruit bodies with the sawdust-based culture. The ratio of fruit bodies per bottle in
157 each strain was 1.20 (WP-1), 1.75 (WP-2), and 0.30 (WP-3), respectively. The fruit body
158 formation ratio of the WP-3 strain was relatively low compared to that of the WP-1 and
159 WP-2 strains. The difference in the ability to form fruit bodies might be caused by
160 intra-species phylogenetic differences. All fruit bodies cultivated using the
161 sawdust-based culture had a brown honeycomb-like structure (Fig. 1b). We next isolated
162 spores from the fruit body of WP-1 formed in the sawdust-based culture and observed

163 them under a microscope. Spores were visualized by fluorescent nuclear staining (Fig.
164 1c). These results showed that we succeeded in fruit body formation using the three
165 Japanese strains.

166

167

168 **Table 1** Fruit body formation

Strain	Solid medium culture		Sawdust-based culture	
	Number of fruit bodies (flasks)	Ratio of fruit bodies per flask	Number of fruit bodies (bottles)	Ratio of fruit bodies per bottle
WP-1	3 (7)	0.43	12 (10)	1.20
WP-2	0 (7)	0	14 (8)	1.75
WP-3	0 (7)	0	3 (10)	0.30

169

170 **Intra-species polymorphism of *Wolfiporia cocos* strains**

171 Next, we performed SNP analysis to discriminate the intra-species Japanese strains. A
172 previous study indicated that sequencing STE3.2 regions are useful for discriminating
173 intra-species strains and can be used as a crossbreeding marker [6]. Therefore, we
174 sequenced and aligned the STE3.2 partial coding regions (Table 2). Although no
175 differences were observed between the STE3.2 regions of WP-1 (DDBJ/EMBL/GenBank

176 database accession no. LC672030) and WP-2 (accession no. LC672031), there were five
177 nucleotide differences between WP-1/WP-2 and WP-3 (accession no. LC672032). Of the
178 five SNPs, three nucleotides of WP-1 and WP-2 (nucleotide positions 196, 226, and 658)
179 were degenerate nucleotides. Two nucleotide at position 54 and 737 clearly discriminated
180 between WP-1/WP-2 (T) and WP-3(C).

181

182 **Table 2** Single nucleotide polymorphism among three strains

	54*	196	226	246	472	559	658	737	761
WP-1	T	G/T	A/G	G	G	C	C/T	G	C
WP-2	T	G/T	A/G	G	G	C	C/T	G	C
WP-3	C	G	G	G	G	C	T	A	C
STE.3.2 gene*	C	G	G	C	T	T	C	G	T

183 *Nucleotide number is described according to the STE.3.2 gene (KF044360)

184

185 In this study, the fruit body formation method using sawdust-based culture was newly
186 established, and the three strains successfully formed fruit bodies in all bottled mediums.
187 Generally, methods of fruit body formation involves induction by irradiation with light
188 and temperature control using solid agar medium [5-8]. Compared with the methods, our
189 method using sawdust-based culture may be particularly useful for strains with slow

190 mycelium growth. In our method, cultured liquid medium was added to the
191 sawdust-based medium and further cultured. A previous study performed a two-stage *W.*
192 *cocos* culture under shaking and static conditions [12]. When mycelia were cultured
193 under shaking conditions and then incubated under static conditions, *W. cocos*
194 accumulated triterpenoids, which are major components of *W.* ***cocos*** sclerotium [12]. In
195 contrast, there were no or a few triterpenoids when shaking was continued [12]. Another
196 study also demonstrated that mycelia increased under shaking conditions and
197 accumulated metabolites under static conditions [13]. Therefore, by adding cultured
198 liquid medium using our procedure, sawdust-based medium could be rapidly filled with
199 mycelia, resulting in efficient formation of fruit body.

200 DNA analysis of the STE3.2 gene showed that we were able to discriminate one strain
201 (WP-3) from the others (WP-1 and WP-2). These results indicate that crossbreeding of
202 WP-1 and WP-2/WP-3 can be authorized by the sequence at nucleotide position 54 and
203 737. However, there were no genetic differences between WP-1 and WP-2 STE3.2 genes,
204 which might indicate that strain variety for these two is associated with the collection
205 areas and/or conditions. The collection areas of WP-1 and WP-2 were different but
206 located in the same prefecture (Nagano Prefecture), and WP-1 and WP-2 were collected
207 from the roots of red pine. Whereas, WP-3 was collected from the Ishikawa prefecture
208 and was collected from black pine roots. Our data showed that both WP-1 and WP-2

209 succeeded in forming fruit bodies with high efficiency compared to WP-3. The success
210 rate of fruit body formation might be affected by genetic factors. *W. cocos* grows naturally
211 all around Japan. Collecting *W. cocos* strains with genetic diversity and establishing
212 crossbreeding technology could lead to the development of strains suitable for cultivation
213 in Japan.

214 Acknowledgments

215 We are grateful to Dr. Yohei Sasaki and Dr. Hirokazu Ando, Kanazawa University for
216 kindly providing *W. cocos* mycelia. We also thank Dr. Masayuki Mikage and Dr.
217 Yukihiro Nomura for providing useful information.

218

219 Competing interests

220 The authors declare no competing financial interests.

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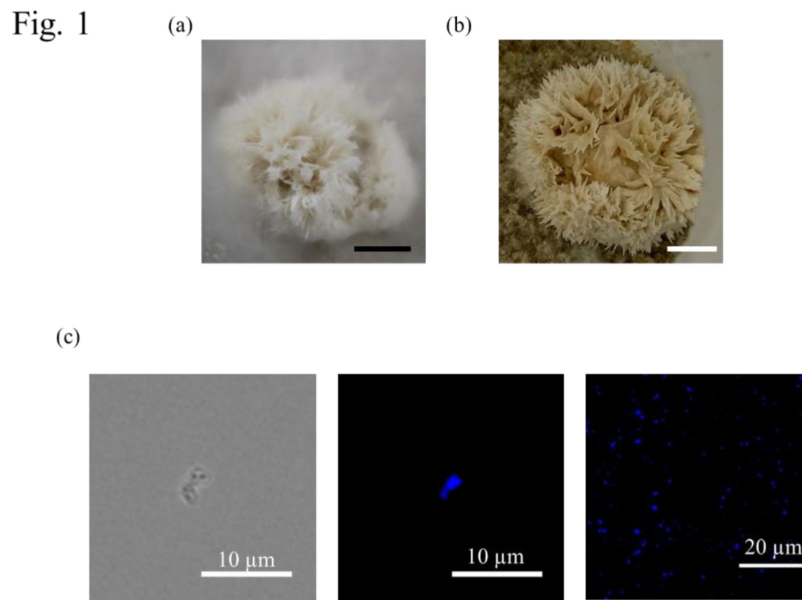
266

267 **Figure legend**

268 **Fig. 1 (a, b)** Fruit body formation. (a) Fruit body by solid medium culture (WP-1) (b)

269 Fruit body by sawdust-based culture (WP-1). The scale bar is 0.5 mm (c) Spores observed

270 using microscope. **(c)** Bright-field image and nuclei staining image.



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