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Melon aroma-producing yeast isolated from coastal marine sediment in Maizuru Bay, Japan

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5 **Melon aroma-producing yeast isolated from coastal marine sediment in Maizuru Bay,**

6 **Japan**

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Abstract Researches on marine fungi and fungi isolated from marine environments are not active compared with those on terrestrial fungi. The aim of this study was isolation of novel and industrially applicable fungi derived from marine environments. In this study, 16 fungus-like strains, MS1–MS16, were isolated from coastal marine sediment in Maizuru Bay, Japan, under aerobic culture conditions. Phylogenetic analysis of 18S rRNA gene sequences indicated that 10 isolates belonged to Ascomycota, five isolates belonged to Sordariomycetes, two were Dothideomycetes, and three were Saccharomycetes. Liquid and agar potato dextrose cultures of strains MS1 and MS2 isolated from the coastal sediment released a melon-like aroma. Gas chromatography analysis suggested that strains MS1 and MS2 produce four major chemicals associated with a melon aroma, cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal. The sequence analyses of the 26S rRNA domains 1/2 (D1/D2) and internal transcribed spacer (ITS) regions indicated that strains MS1 and MS2 were phylogenetically identified as *Geotrichum candidum*, a well-known yeast used as a cheese starter. These results suggest the future isolations of novel and functional fungi from marine environments.

Key words Marine fungi· Melon-aroma· Yeast· Coastal sediment· Phylogenetic analysis

Introduction

39

40 Fungi include a variety of industrially useful species such as *Hypocrea jecorina* (*Trichoderma*
41 *reesei*) and *Saccharomyces cerevisiae*. *H. jecorina* produces and secretes a large amount of
42 cellulase, and is a useful industrial enzyme producer. *S. cerevisiae* has long been used as an
43 ethanol producer [1, 2]. Terpene glycosides in grapes were reported to be hydrolyzed to free
44 volatile terpene aroma compounds by yeasts during the aging of wines [3]. Ester levels in
45 Bordeaux red wines were strongly influenced by yeast strains [4]. *Kluyveromyces lactis* and *S.*
46 *cerevisiae* were two potent deacidifying and volatile sulphur-aroma producing
47 yeasts of the cheese ecosystem [5]. *S. cerevisiae* were reported to produce the aroma
48 chemicals of 3-(methylthio)-1-propanol and 3-(methylthio)-propylacetate using L-methionine
49 as sole nitrogen source [6]. Yeasts have a close relationship with flavors and aromas of wines
50 and cheeses.

51 Marine organisms are thought to be excellent bioresources owing to their production of
52 many useful compounds [7]. Approximately 150–200 new compounds are isolated annually
53 from marine fungi [8]. Marine fungi that produce antimicrobial metabolites have been
54 screened [9]. New prenylxanones were detected from the deep-sea sediment-derived fungus
55 *Emericella* sp., which was isolated from the sediment (3,258 m) of the South China Sea [10].
56 New polyketides were detected from the deep-sea sediment-derived fungus *Aspergillus* sp.,
57 which was isolated from the hydrothermal vent (2,255 m, temperature 114 °C) in the

58 southwest Pacific [11]. There may be many uncultured fungal strains that produce useful
59 compounds in marine environments.

60 Isolated marine fungi have been classified into the phyla Chytridiomycota, Oomycota,
61 Basidiomycota, and Zygomycota. A marine chytridial parasitoid of dinoflagellates has been
62 identified as a new genus and species, *Dinomyces arenysensis* [12]. A total of 31 fungi isolates
63 were recovered from seawater and sediment samples from the Pearl River Delta (China), and
64 most belonged to the phyla Ascomycota and Basidiomycota [13]. Ninety-eight fungal strains
65 were isolated from two samples of the marine sponge *Dragmacidon reticulatum* using six
66 different culture media, and 64 distinct fungal ribotypes that belonged to 24 genera of
67 Ascomycota and Zygomycota were obtained [14]. An analysis of internal transcribed spacer
68 (ITS) sequences revealed that 101 phenotypically different fungal isolates obtained from 11
69 sponge samples collected in King George Island, Antarctica belong to the phylum
70 Ascomycota [15]. There may be many uncultured fungal strains representing a wide range of
71 taxa in marine environments.

72 The definition of marine fungi is problematic. Marine Ascomycetes have a wide salinity
73 tolerance, including low-salinity conditions, and are less conditioned by the available
74 substrate [16]. The effects of seawater concentration on hyphal growth and antimicrobial
75 metabolite production in marine fungi have been studied using 0%, 50%, and 100%
76 seawater-based culture medium [9]. The aim of this study was isolation of novel and

industrially applicable fungi derived from marine environments. In this study, fungi were isolated from coastal marine sediment in Maizuru Bay, Japan. Fungi derived from marine sediment habitats were isolated with 100% seawater-based culture medium. Phylogenetic analyses of the 18S rRNA gene, 26S rRNA domains 1 and 2 (26S rRNA D1/D2), and ITS 1/2 regions were conducted to identify the isolated fungal strains.

Materials and methods

Sampling and isolation

A coastal marine surface sediment sample (0–5 cm in depth) was collected using a Smith-McIntyre sediment sampler from a site in Maizuru Bay (35°29'41.1"N 135°22'05.2"E; July 19, 2013) in Kyoto prefecture, Japan. The sediment sample was spread on potato dextrose (PD) agar plates based on seawater, and incubated at 25 °C. Colonies that had a fungus-like appearance were selected for further isolation procedures. The isolation procedure was conducted several times to obtain a pure colony appearance using PDA agar plates. Isolated fungus-like strains were designated strains MS1–MS16.

Phylogenetic analyses

97 Sequence analyses of the 18S rRNA gene, 26S rRNA D1/D2, and ITS regions were conducted.
98 Isolated fungal strains were incubated in PD liquid medium at 30 °C and 120 rpm of shaking
99 for 48 h, and cells were collected by centrifugation ($15,000 \times g$ for 10 min). Total DNA was
100 extracted from fungus cells using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon,
101 OH, USA). DNA fragments of the 18S rRNA gene, 26S rRNA D1/D2, and ITS regions were
102 amplified by polymerase chain reaction with SapphireAmp® Fast PCR Master Mix (Takara
103 Bio, Otsu, Japan).

104 PCR amplification conditions for the 18S rRNA gene were 40 cycles each of 98 °C for 5 s,
105 50 °C for 5 s, and 72 °C for 15 s using the specific primer set NS1 and Fungi18S-R (Table 1)
106 [17]. PCR amplification conditions for the 26S rRNA D1/D2 region were 40 cycles each of
107 98 °C for 5 s, 56 °C for 5 s, and 72 °C for 15 s using the specific primer set NL1 and NL4
108 (Table 1) [18]. PCR amplification conditions for the ITS regions were 40 cycles each of 98 °C
109 for 5 s, 53 °C for 5 s, and 72 °C for 15 s using the specific primer set ITS1 and ITS4 (Table 1)
110 [19].

111 PCR products were purified using the High Pure PCR Product Purification Kit (Roche
112 Diagnostics, Mannheim, Germany), and were sequenced using the Applied Biosystems 3730
113 xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Database searches for
114 similar sequences were conducted using the BLASTN application available at the NCBI
115 website. A multiple sequence alignment was performed using the Clustal Omega program

116 available at the EMBL-EBI website. A phylogenetic tree was constructed using the
117 neighbor-joining method implemented in MEGA6.0 [20] with 1,000 bootstrap replicates.
118 *Smittium imitatum* or *Dipodascus tetrasporeus* was used as an outgroup. The sequences of the
119 18S rRNA gene reported in this study have been deposited in the DDBJ/EMBL/GenBank
120 databases under the accession numbers LC032042 (strain MS1) to LC032051 (strain MS16),
121 26S rRNA D1/D2 region sequences under LC032052 (strain MS1) to LC032053 (strain MS2),
122 and ITS region sequences under LC032054 (strain MS1) to LC032055 (strain MS2).

123

124 **Gas chromatography analysis**

125

126 Isolated strains MS1 and MS2 were incubated in the PD liquid medium based on seawater at
127 30 °C and 120 rpm of shaking for 7 d in 50-ml vials. Head space gases in the vials were
128 injected into a gas chromatography (GC) apparatus of GC-2010 (Shimadzu, Kyoto, Japan).
129 The column was a ZB-WAX (0.53 mm, 30 m, and 1.0 μm). The oven temperature was
130 controlled from 60 °C to 200 °C at a rate of 1 °C min⁻¹. Helium was supplied as a mobile
131 phase at 10 ml min⁻¹. GC analyses of standard chemicals associated with a melon aroma were
132 conducted, including cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and
133 trans,cis-2,6-nonadienal.

134

Results

Biodiversity of fungous strains isolated from the marine sediment collected at Maizuru

Bay

Fig. 1

Sixteen fungus-like strains were isolated from the Maizuru Bay marine sediment sample using

PD agar plates under aerobic conditions at 25 °C (Fig. 1). Phylogenetic analysis using 18S

rRNA gene sequences indicated that 10 isolates (MS1–MS3, MS6, MS8–MS12, and MS16)

belong to Ascomycota (Fig. 2). Strains MS1–MS3 belong to Saccharomycetes, strains MS10

and MS16 belong to Dothideomycetes, and strains MS6, MS8, MS9, MS11, and MS12

belong to Sordariomycetes. Strains MS1–MS3 were related to species in the genus

Fig. 2

Geotrichum, strains MS10 and MS16 were related to species in the genera *Fenestella* and

Pyrenochaeta, and strains MS6, MS8, MS9, MS11, and 12 were related to species in the

genus *Hypocrea*. Sequence similarities for the 18S rRNA gene were 95.9% and 98.5%

between strain MS1 and *G. candidum* (X69842) and between strain MS2 and *G. candidum*

(X69842), respectively.

Identifications of the volatile substances corresponding to culture aroma

Fig. 3

Strains MS1 and MS2 produced a melon-like aroma on agar PD plates and in liquid PD

culture medium. The cells of strains MS1 and MS2, related to the genus *Geotrichum*, had a yeast-like form (Fig. 3). The cell length of strain MS1 was 7–9 μm , and that of strain MS2 was 7–13 μm . Gas chromatograms of strains MS1 and MS2 are shown in Fig. 4. GC analysis indicated that strains MS1 and MS2 produce cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal in the liquid PD culture. GC chromatograms show that there are additional volatile compounds in the head space gases of strains MS1 and MS2. The chromatograms of strains MS1 and MS2 indicate a similar pattern.

Fig. 4

Fingerprinting of the yeast strains producing the melon aroma

Phylogenetic analysis of strains MS1 and MS2 based on the 26S rRNA D1/D2 region sequence indicated that these two strains form an monophyletic clade with *G. candidum* CBS178.71, *G. candidum* CBS607.85, *G. candidum* CBS11628, and *G. candidum* CBS11616 (Fig. 5). Sequence similarities for the 26S rRNA D1/D2 region were 99.3% and 99.6% between MS1 and *Galactomyces candidum* CBS11616 (JN974264) and between MS2 and *G. candidum* CBS11616 (JN974264), respectively. *Geotrichum phurueaensis*, *Galactomyces pseudocandidum*, *Geotrichum europaeum*, and *Galactomyces geotrichum* form an monophyletic clade. *Galactomyces reessii* and *Galactomyces citri-aurantii* form an monophyletic clade. The sequence analyses of strains MS1 and MS2 based on the 26S rRNA

174 D1/D2 region showed that these two strains were phylogenetically identified as *G. candidum*
175 (Fig. 5).

Fig. 5

176 Sequence analysis of the ITS region showed that strain MS1 forms a clade with *G.*
177 *candidum* Tom1 and *G. candidum* 282A (Fig. 6). Sequence similarities for the ITS region
178 were 99.6% and 99.6% between strain MS1 and *G. candidum* Tom1 (KF298071) and between
179 strain MS1 and *G. candidum* 282A (KF669518), respectively. The sequence of strain MS1
180 was relatively similar to those of *G. candidum* L19PB, *G. candidum* L13PC, and *G. candidum*
181 L20B. Strain MS2 formed a clade with *G. candidum* Thu1. The sequence similarity for the
182 ITS region was 98.6% between strain MS2 and *G. candidum* Thu1 (KF298070). The
183 sequence of strain MS2 was relatively similar to those of *G. candidum* Gou1, *G. candidum*
184 Que1, *G. candidum* Mah2, *G. candidum* CBS178.71, and *G. candidum* CBS11176. Strains
185 MS1 and MS2 were not closely related to *G. reessii*, *G. citri-aurantii*, or *G. pseudocandidum*.
186 The sequence analyses of strains MS1 and MS2 based on the ITS region indicated that these
187 two strains were phylogenetically identified as *G. candidum* (Fig. 6).

Fig. 6

189 Discussion

190
191 Sixteen fungus-like colonies were isolated from Maizuru Bay surface sediment. Ten isolates
192 belong to Ascomycota based on a phylogenetic analysis. Representatives of the genera

193 *Penicillium* and *Hypocrea* were the most diverse and abundant fungi isolated from marine
194 sponges [14]. In the present study, 5 strains in the genus *Hypocrea* were isolated from coastal
195 sediment. *Hypocrea* may be one of the most frequent genus isolated from marine
196 environments. Isolated marine fungi have previously been classified as belonging to the phyla
197 Chytridiomycota, Oomycota, Ascomycota, Basidiomycota, and Zygomycota [13,14]. The
198 presently isolated fungi from the coastal marine sediment do not show substantial
199 phylogenetic divergence.

200 Phylogenetic 18S rRNA gene sequence analysis showed that strains MS1 and MS2 were
201 related with the genus *Geotrichum*, phylum Ascomycota, class Saccharomycetes, order
202 Saccharomycetales, family Endomycetaceae. The 26S rRNA D1/D2 and ITS sequence
203 analyses indicated that strains MS1 and MS2 were phylogenetically identified as *G. candidum*.
204 *G. candidum* is known as a plant pathogenic fungus and is used for rind formation during
205 Camembert cheese production [21, 22]. *Geotrichum candidum* refers to an anamorph and
206 *Galactomyces candidus* refers to a teleomorph [19, 23]. In the present study, strain MS1 is
207 designated *Geotrichum candidum* MS1, and strain MS2 is designated *Geotrichum candidum*
208 MS2. There could be confusion regarding whether *Galactomyces candidus* and *Galactomyces*
209 *candidum* is the appropriate species name. The original species names used in the associated
210 references or gene databases are used in this paper.

211 Liquid cultures and agar plates of strains MS1 and MS2, respectively, produced a

212 melon-like aroma. Volatile analysis indicated that the melon aroma resulted from acetones,
213 non-acetone esters, sulfur-containing compounds, alcohols, and aldehydes [24]. Important
214 chemicals known to cause a melon aroma are cis-3-hexen-1-ol, cis-6-nonenal,
215 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal [24, 25]. GC analysis suggested that strains
216 MS1 and MS2 produce these four major melon-aroma chemicals. *G. candidum* isolated from
217 sludge of an aerated pilot-scale bubble column was reported to produce a pineapple-like
218 aroma [26]. It produces ethyl esters of acetic acid and butyric acid, methyl-3-butan-1-ol, and
219 methyl-2-propan-1-ol with glucose [26]. Strains MS1 and MS2 produce cis-3-hexen-1-ol,
220 cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal; therefore, different strains of
221 *G. candidum* could produce different kinds of compounds associated with fruity aromas.
222 Production of volatile compounds by *Geotrichum fragrans* using cassava wastewater as
223 substrate has also been reported [27]. The newly isolated fungal strains of MS1 and MS2
224 might be applicable for melon-aromatic food.

225 *G. candidum* directly and positively contributes to cheese ripening and flavor development
226 of many soft and semi-hard cheeses [28, 29]. In Camembert cheese production, *G. candidum*
227 grows on the outside of the cheese and contributes to the formation of a rind [22, 30]. ITS
228 region sequence analysis suggested that strain MS1 is similar to *G. candidum* Tom1. Strain
229 Tom1 was isolated from hard cheese, Tomette des Alpes, in France [31]. ITS region sequence
230 analysis suggested that strain MS2 is similar to *G. candidum* Thu1. Strain Thu1 was isolated

231 from soft cheese, Thurgauer weinkäse, in Switzerland [31]. ITS sequences of *G. candidum*
232 strains L13PC, L19PB, L20BK, and 282A were reported from cheese-related studies
233 (unpublished data recorded in DDBJ/EMBL/GenBank). Strains MS1 and MS2 are closely
234 related to *G. candidum* strains isolated from cheese; therefore, there is a possibility of using
235 the presently isolated two yeast strains for cheese ripening. *Galactomyces* Ferment Filtrate is
236 used to produce cosmetics such as facial treatment essence, clear lotion, and masks. It is
237 reported to have markedly increased caspase-14 expression [32]. Caspase-14 expression
238 prevents epidermal UVB damage and water loss [33]. Phylogenetic information regarding this
239 *Galactomyces* sp. is not available; therefore, further research is necessary to determine
240 whether the ferment filtrates of strains MS1 and MS2 have similar activity.

241 The present study confirmed that marine environments harbor a diversity of unknown fungi
242 with unique features, and further physiological researches on marine fungi are very important
243 for applied and environmental microbiology. Considering industrial application in Japan, it is
244 advantageous that the fungal strains of MS1 and MS2 with melon-aroma were newly isolated
245 from Japanese territorial sea.

246

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251

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343 protects against epidermal UVB photodamage and water loss. Nat Cell Biol 9:666–674
344

345

346 Table 1 List of primers used in this study

Primer name	Sequence (5'-3')	Amplification target	Reference
NS1	GTAGTCATATGCTTGTCTC	18S rRNA gene	17. Chen <i>et al.</i> 2011
Fungi18S-R	GATCCCTAGTCGGCATAGTT	18S rRNA gene	17. Chen <i>et al.</i> 2011
NL1	GCATATCAATAAGCGGAGGAAAAG	26S rRNA gene D1/D2 region	18. Yalçın <i>et al.</i> 2014
NL4	GGTCCGTGTTTCAAGACGG	26S rRNA gene D1/D2 region	18. Yalçın <i>et al.</i> 2014
ITS1	TCCGTAGGTGAACCTGCGG	IST region	19. Hong <i>et al.</i> 2004
ITS4	TCCTCCGCTTATTGATATGC	IST region	19. Hong <i>et al.</i> 2004

347

Figure legends

Fig. 1 Photographs of fungus-like colonies of strains MS1-MS16 isolated from the Maizuru Bay marine sediment.

Fig. 2 Phylogenetic tree including 10 isolated fungal strains from the Maizuru Bay marine sediment and authentic fungal strains based on the 18S rRNA gene sequences. The tree was constructed using the neighbor-joining algorithm with bootstrap analyses (1,000 replicates) using MEGA6. Accession numbers were shown in parentheses. The scale bar represents 0.05 of estimated sequence divergence. *S. imitatum* was used as an outgroup.

Fig. 3 Photographs of the cells of strain MS1 (a) and strain MS2 (b). These were photographed at magnification of 1,000 times by an optical microscope.

Fig. 4 Gas chromatograms of culture head gases for strains MS1 (a) and MS2 (b). Four chemicals, cis-3-hexen-1-ol, cis-6-nonenal, 3,6-nonadien-1-ol, and trans,cis-2,6-nonadienal, known as the main components of melon aroma were used for external standards.

Fig. 5 Phylogenetic tree for isolated strains MS1 and MS2 generated from 26S rRNA gene D1/D2 region sequences. The tree was constructed using the neighbor-joining algorithm with bootstrap analyses (1,000 replicates) using MEGA6. *Gal.* means genus *Galactomyces*, and *Geo.* means genus *Geotrichum*. Accession numbers were shown in parentheses. The scale bar represents 0.01 of estimated sequence divergence. *D. tetrasporeus* was used as an outgroup.

Fig. 6 Phylogenetic tree for isolated strains MS1 and MS2 generated from ITS region

373 sequences. The tree was constructed using the neighbor-joining algorithm with bootstrap
374 analyses (1,000 replicates) using MEGA6. *Gal.* means genus *Galactomyces*, and *Geo.* means
375 genus *Geotrichum*. Accession numbers were shown in parentheses. The scale bar represents
376 0.02 of estimated sequence divergence. *D. tetrasporeus* was used as an outgroup.

377

Fig. 1 Sutani et al.

Fig. 2 Sutani et al.

a

b

Fig. 3 Sutani et al.

Fig. 4 Sutani et al.

Fig. 5 Sutani et al.

Fig. 6 Sutani et al.