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FULL PAPER

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An original habitat of tempeh molds

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Abstract Tempeh is a traditional Indonesian food made from soybeans fermented with *Rhizopus* species. Some researchers believe the original habitat of the tempeh molds may be closely related to fresh leaves of *Hibiscus* species because these leaves artificially infected with the tempeh molds are used to start tempeh fermentation in cottage-scale factories. To verify this hypothesis, we investigated the occurrence of *Rhizopus* species in *Hibiscus* leaves and identified the isolated *Rhizopus* strains precisely. *Rhizopus oryzae*, one of the tempeh molds, occurred in sample leaves of some *Hibiscus* species with considerable frequency. This result implies that tempeh molds that lived in *Hibiscus* leaves might have fermented soybeans accidentally when used to wrap the cooked soybeans. The original habitat of the tempeh mold could be fresh leaves of *Hibiscus* species.

Key words *Hibiscus* · Original habitat · *Rhizopus oryzae* · Tempeh molds

Introduction

Tempeh is a traditional Indonesian food made from soybeans fermented with fungi of the genus *Rhizopus*. During the fermentation, enzymatic digestion and synthesis increase the amounts of free amino acids, water-soluble nitrogen compounds, free fatty acids, and various kinds of vitamins in the material. These chemical changes bring favorable tastes, flavor, and edibility to soybeans.

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Recently, the nutritional values of tempeh have attracted the attention of many researchers. Therefore, enthusiastic studies have been conducted on efficient preparation of soybeans and improvement of fungal inocula, the fermentation procedures, enzyme production that decreases or eliminates antinutritional constituents, and the nutritional quality of the product (Hachmeister and Fung 1993).

However, the natural habitat of tempeh molds, that is, their origins, have been still obscure. The *Hibiscus* leaves used as the starter of the fermentation should give us a clue about an original habitat of the tempeh molds. In cottage-scale tempeh factories, the workers set *Hibiscus* leaves in a package of finished tempeh so that the leaves are infected with tempeh molds, and these leaves are used as the starter of the fermentation (Djien 1988).

On the basis of this fact, Tubaki et al. (1998) hypothesized that the original habitat of tempeh molds might be closely related to these leaves. Their preliminary investigation showed fungi of the genus *Rhizopus* occurred in fresh leaves of *Hibiscus tiliaceus* L. and *Hibiscus hamabo* Sieb. & Zucc. collected from Bali Is., Indonesia, and coastal regions of middle Japan, respectively.

However, it remains uncertain whether those *Rhizopus* species are tempeh molds because they did not examine the isolates to species level. To verify their hypothesis, we investigated the occurrence of *Rhizopus* species, tempeh molds, in *Hibiscus* leaves collected from tropical areas of Japan. Furthermore, based on the morphological, cultural characteristics, and DNA sequences, we precisely examined *Rhizopus* strains isolated in the present study and in Tubaki et al. (1998). Then, the original habitat of tempeh molds is discussed.

Materials and methods

Distribution of the genus *Hibiscus* in Japan and the sampling sites of *Hibiscus* leaves

In Japan, four woody *Hibiscus* species grow naturally. *Hibiscus hamabo* is distributed in the coastal areas from Miura

Fig. 1. Sampling sites where fresh leaves of *Hibiscus* species were collected in the present study and in Tubaki et al. (1998)

Peninsula to Tokara Islands, *H. tiliaceus* in coastal regions of Ryukyu Islands, and *Hibiscus glaber* Matsumura in Ogasawara islands. *Hibiscus mutabilis* L. grows naturally from the southern part of Kyushu to Ryukyu Islands. In addition to these natural species, horticultural hybrids of *Hibiscus* are raised widely in these regions in Japan.

Fresh leaves of *H. tiliaceus*, *H. mutabilis*, and a horticultural hybrid of *Hibiscus* were collected in Iriomote-jima Is. in February 1998. *H. tiliaceus* leaves were sampled again in Okinawa, Okinawa Pref., in March 1998. The leaves of *H. glaber* were collected in Chichi-jima Is. in March 1998 (Fig. 1).

Mycological observations

To search occurrences of tempeh fungi, we obtained 20 disks of 6mm diameter from each sample of collected leaves. These disks were washed according to the methods described by Tokumasu (1980). To suppress bacterial growth after plating, these disks were dried on a sterilized filter paper. Each dried disk was laid down onto a Miura agar plate (Miura and Kudo 1970) and incubated at 25°C. We isolated sporulated fungi from the leaf disks for a week and identified them.

The frequency of occurrence of each fungus was calculated by the following equation: frequency of occurrence (%) = the number of disks from which the fungus was detected/the total number of disks in each examination $\times 100$.

The isolated *Rhizopus* strains were grown on potato dextrose agar (PDA; 3.9% Nissui potato dextrose agar) and malt agar (MEA; 2% Difco malt extract and 2% agar) and examined for morphological and cultural characteristics.

PCR amplification and DNA sequencing

Isolated *Rhizopus* species were grown in malt extract broth (2% Difco malt extract, 0.1% Difco peptone, and 2% glucose). The genomic DNA was extracted from a small amount of the mycelium by using Isoplant (Nippon Gene,

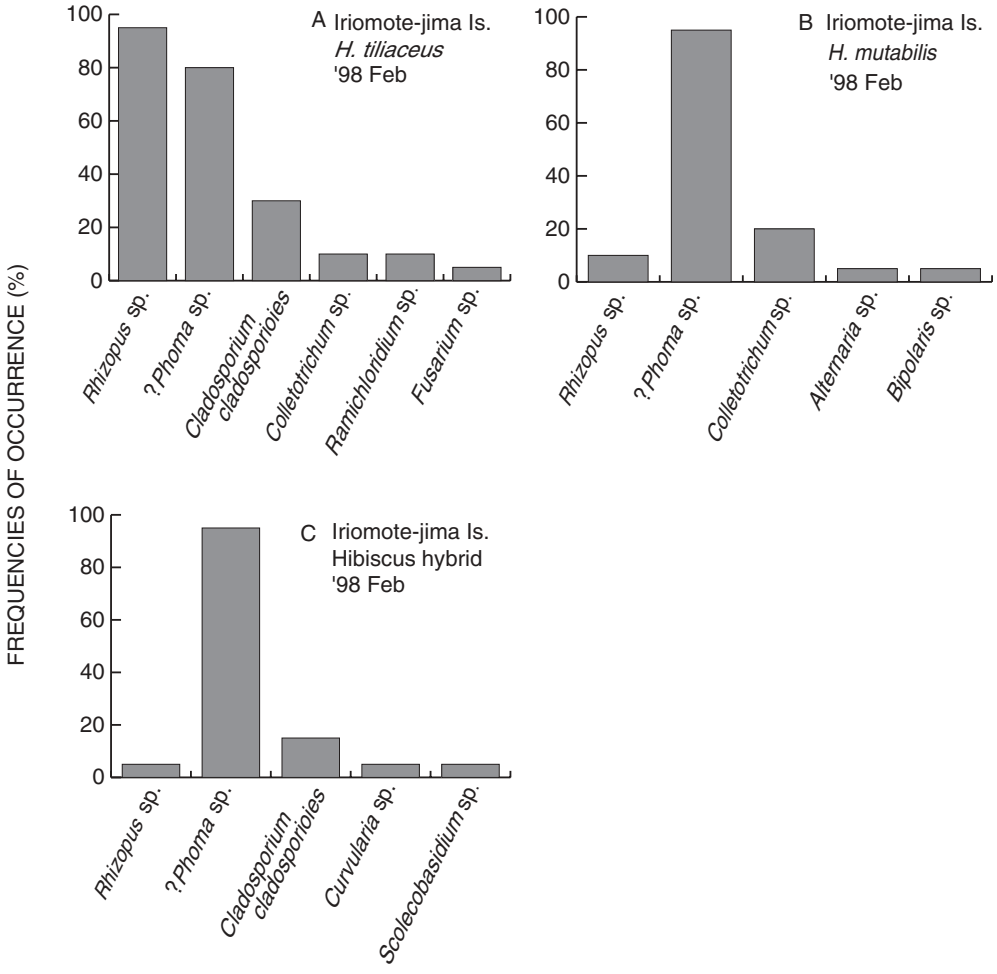

Fig. 2. Frequencies of occurrence of dominant fungi in samples of *Hibiscus* leaves collected at Iriomote-jima Is.

Toyama, Japan). This extract was treated as the template DNA for polymerase chain reaction (PCR).

Polymerase chain reactions were performed by using TaKaRa Ex Taq DNA polymerase (Takara Bio, Shiga, Japan) in iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The internal transcribed spacer (ITS) regions were amplified using originally designed primers, RH1 and RH2 (5′-CACCGCCCGTCGCTACT and 5′-GCTAGGCCAA ACAGGTTCCAA), under the following thermal conditions: an initial incubation of 94°C for 5 min, 25 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 30 s, and a final extension period of 72°C for 5 min. After electrophoresis, the amplified DNA was extracted from an agarose gel by using a Geneclean Kit (Bio-101, Carlsbad, CA, USA). The extracted DNA was cloned on pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into JM109-competent cells. After an insert check, the competent cells were grown in Terrific broth and the plasmid vector was collected by using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany).

The collected plasmid DNA was labeled using a Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA) with M13 forward and M13 reverse RID 800 Infrared Labeled Dye primers (Aloka, Tokyo, Japan). DNA sequences of both directions were determined by Sanger method (Sambrook and Russell 1989) with DNA Analyzer Gene Readir 4200 (Li-Cor, Lincoln, NE, USA). The raw sequence data were edited and aligned using GENTYX-MAC ver. 8.0.

Results

Fungi occurring in *Hibiscus* leaves

Figure 2 shows the frequencies of occurrence of dominant fungi that were isolated from samples of *Hibiscus* leaves during the survey at Iriomote-jima Is. in February 1998. *Rhizopus* sp. occurred with a high frequency of 95% in a sample of *H. tiliaceus* (Fig. 2A). Lower frequencies of 10% and 5% were observed in a sample of *H. mutabilis* leaves and a sample of *Hibiscus* hybrid leaves, respectively (Fig. 2B,C).

In these samples, a *Phoma*-like species was another dominant fungus. The frequencies of occurrence of this fungus ranged from 95% in the samples of *H. mutabilis* and *Hibiscus* hybrid (Fig. 2B,C) to 80% in *H. tiliaceus* leaves (Fig. 2A).

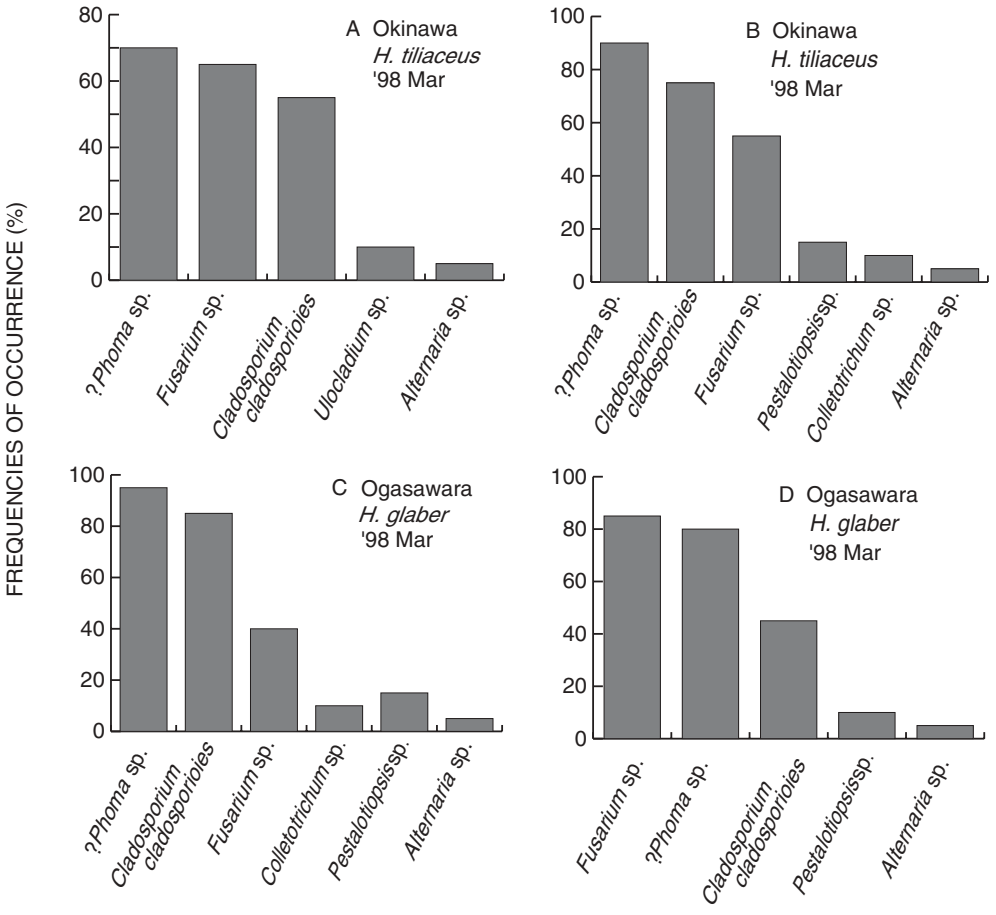

Fig. 3. Frequencies of occurrence of dominant fungi in samples of *Hibiscus* leaves collected at Okinawa and Chichi-jima Is.

Cladosporium cladosporioides (Fres.) de Vries and *Colletotrichum* sp. occurred with considerable frequency in some samples. *Ramichloridium* sp., *Fusarium* sp., *Alternaria* sp., *Bipolaris* sp., *Curvularia* sp., and *Scolecobasidium* sp. occurred infrequently.

To reconfirm the presence of *Rhizopus* sp. in *Hibiscus* leaves, we sampled other *H. tiliaceus* leaves and *H. glaber* leaves in two locations each in Okinawa Is. (Fig. 3A,B) and Chichi-jima Is. (Fig. 3C,D), respectively. However, we could not find *Rhizopus* species at all from those samples. In those leaves, the dominant species were *Phoma*-like species, *Fusarium* sp. and *Cladosporium cladosporioides*, which occurred with remarkably high frequencies. Other than these species, *Ulocladium* sp., *Alternaria* sp., *Pestalotiopsis* sp., and *Colletotrichum* sp. also occurred with relatively low frequencies.

Taxonomic characteristics of isolated *Rhizopus* strains

Table 1 summarizes the morphological and cultural characteristics of six strains of *Rhizopus* sp. isolated from *Hibiscus* leaves. The sporangiophores of these strains on MEA were sometimes more than 1 mm in height, and the diameters of sporangia of those on PDA and MEA ranged from 30 to 220 μm. They did not grow at 45°C but grew at 36°C on PDA and MEA. According to a revision of the genus *Rhizopus* (Schipper 1984), *Rhizopus* with these characteristics belongs to *Rhizopus* (*Rh.*) *oryzae* Went & Prinsen Geerl.

For the precise identification of these strains, we determined their DNA sequences in ITS1, 5.8S rDNA, and ITS2 regions (Table 2). The total length in these regions was 564 bp in each strain of PCNB1276, PCNB1278, PCNB1279, PCNB1280, and PCNB1284, and 565 bp in PCNB1289. PCNB1276, PCNB1279, PCNB1280, and PCNB1284 had the same sequence for all those examined here. PCNB1278 had As at 143rd and 193rd in its ITS2 region whereas the other strain had Ts at these positions. PCNB1289 had one more G at 76th in its ITS 1.

In the DNA data bank of Japan (DDBJ), we could find 112 DNA sequences that referred to those of ITS1, 5.8S rDNA, and ITS2 regions of *Rh. oryzae* and its synonyms. The sequences of six *Rhizopus* strains in the present study were more than 98% homologous to those of 104 strains and remarkably different from those of 8 strains. However, these 8 strains with 632 bp in ITS1, 5.8S rDNA, and ITS2 regions had sequences highly homologous to those of the *Rhizopus microsporus* group. Consequently, we identified the present 6 strains as *Rh. oryzae*.

When Schipper (1984) published her revision, she regarded *Rh. oryzae* as the synonym of *Rhizopus arrhizus* Fisher in Rabenh. However, she retained the name of *Rh. oryzae* because she could not access to the type material of

Table 1. The morphological and cultural characteristics of the isolated *Rhizopus* strains

Strain	Substratum	Origin	Sporangiophore on MEA (mm)	Sporangia on PDA (μm)	Sporangia on MEA (μm)	Growth at 36°C	Growth at 45°C
PCNB1276[a]	A fresh leaf of *Hybiscus hamabo*	Hachijou-jima Is.	>1	(40) 56–76 (95)	(53) 72–110 (150)	+	−
PCNB1278[a]	A fresh leaf of *Hybiscus hamabo*	Hachijou-jima Is.	>1	(35) 110–130 (150)	(50) 120–150 (220)	+	−
PCNB1279[a]	A fresh leaf of *Hybiscus hamabo*	Shimoda	>1	(30) 130–150 (170)	(70) 120–135 (210)	+	−
PCNB1280[a]	A fresh leaf of *Hybiscus hamabo*	Shimoda	>1	(50) 110–125 (170)	(55) 110–135 (165)	+	−
PCNB1284	A fresh leaf of *Hybiscus tiliaceus*	Iriomote-jima Is.	>1	(60) 120–135 (160)	(60) 125–150 (195)	+	−
PCNB1289[a]	A fresh leaf of *Hybiscus tiliaceus*	Bali Is.	>1	(35) 75–132 (150)	(55) 98–135 (155)	+	−

MEA, malt extract agar; PDA, potato dextrose agar
[a]Strains collected by Tubaki et al. (1998)

Table 2. The summary of sequences of ITS 1, 5.8S rDNA, and ITS 2

Strain	Accession number	Number of base pairs	Position of different base		
			76th in ITS 1 of PCNB1289	143rd in ITS2	193rd in ITS2
PCNB1276	AB109754	564	–	T	T
PCNB1278	AB109755	564	–	A	A
PCNB1279	AB109756	564	–	T	T
PCNB1280	AB109757	564	–	T	T
PCNB1284	AB109758	564	–	T	T
PCNB1289	AB126323	565	G	T	T

ITS, internal transcribed spacer

Rh. arrhizus and because she was not sure of the identity between *Rh. oryzae* and *Rh. arrhizus*. One year later, Ellis (1985) showed 97% complementarity of DNA between *Rh. oryzae* and *Rh. arrhizus*. Then, he reduced *Rh. oryzae* to a synonym under *Rh. arrhizus*, taking account of the years when the names were published. Thus, we should use the species name of *Rh. arrhizus.* However, as the name of *Rh. oryzae* has been widely used, we retained the name of *Rh. oryzae* in this article for convenience.

Discussion

Rhizopus microsporus v. Tiegh. var. *oligosporus* (Saito) Schipper & Stalpers (=*Rhizopus oligosporus* Saito) is the principal fungus used for the tempeh preparation in Indonesia. Hesseltine (1965) isolated 40 strains of *Rhizopus* from Indonesian tempeh, which would produce an acceptable product when soybeans were fermented with these pure cultures. Among these strains, 25 strains belonged to *Rh. oligosporus*, 11 strains to *Rh. oryzae* and its synonyms, and 4 strains to *Rhizopus stolonifer* (Ehren.) Vuill. Also, in another survey, Hesseltine (1991) isolated 17 *Rhizopus* strains from commercial tempeh. Among them, 16 strains belonged to *Rh. oligosporus*. Djien (1988) also surveyed which molds were used for tempeh production by collecting tempeh samples from markets in the various regions in Indonesia. He showed that most molds that produce tempeh of good quality were *Rh. oligosporus*. At the present, 1 strain, *Rh. oligosporus* NRRL 2710, is the recommended strain for producing tempeh commercially (Wang 1981).

However, other *Rhizopus* species are also capable of fermenting soybeans to tempeh. Actually, the aforementioned 11 *Rhizopus* stains isolated by Hesseltine (1965) were *Rh. oryzae*, and its synonyms. Djien (1988) experimentally showed *Rh. stolonifer*, *Rh. oryzae*, and *Rh. arrhizus* could make complete tempeh. Yuan and Jong (1984) described ATCC 48108 isolated from tempeh as a new species, *Rhizopus azygosporus* Yuan & Jong, which is also available for tempeh production. Schwertz et al. (1997) reexamined the morphological and physiological characteristics of a strain of *Rh. microsporus* var. *oligosporus*, which has been used to ferment nondehulled soybean and to degrade toxic compounds in cereals, and newly identified this strain as *Rhizopus microsporus* v. Tiegh. var. *chinensis* (Saito) Schipper & Stalpers. These findings suggest that some *Rhizopus* species have an exact potential to ferment soybeans to tempeh.

We could not isolate *Rhizopus* from all the samples of *Hibiscus* leaves. However, it is certain that *Rh. oryzae* occurs in the leaves of *Hibiscus* species that inhabit a broad area from middle and south Japan to Southeast Asia because all the six strains randomly examined in the present study belonged to *Rh. oryzae*, although they were isolated from four different locations that were remarkably remote from each other (see Table 1).

In Bali Is., Indonesia, Tubaki et al. (1998) isolated *Rhizopus* species with high frequencies of more than 90% from the fresh leaves of *H. tiliaceus* and dried those leaves used as the starter of tempeh fermentation. They stated that some *Rhizopus* species other than *Rh. microsporus* var. *oligosporus* also lived in those leaves. One of these *Rhizopus* species would be *Rh. oryzae* because PCNB1289, an isolate from Bali Is., was identified as *Rh. oryzae*.

In the past, *Hibiscus* leaves might have been be used to wrap cooked soybeans. *Rhizopus oryzae* dwelling in those leaves would have fermented soybeans into an easily digestible and good-tasting food, producing the original form of tempeh. The original habitat of tempeh molds could be fresh leaves of *Hibiscus* species.

Several fungi other than *Rhizopus* sp. were isolated from *Hibiscus* leaves in the present study. Most of them are endophytic fungi, or epiphytic fungi that can penetrate into living plant tissue. In leaf tissues of various plants, such fungi as *Phyllostica* spp., *Phoma* spp., *Phomopsis* spp., and *Colletotrichum* spp. are common (Petrini 1986, 1991; Carroll 1990; Okane et al. 1998, 2001). Except for *Rhizopus* species, as we roughly identified the isolates, we have possibly lumped fungi such as *Phyllostica* spp., *Phoma* spp., and *Phomopsis* spp. together as *Phoma*-like species. *Colletotrichum* sp. also occurred in three samples although their frequencies were relatively low, 10% to 20%.

In addition to these fungi, *Fusarium* sp., *Alternaria* sp., and *C. cladosporioides* occurred in most samples. Carroll (1990) pointed out that some avirulent strains of *Fusarium* persist endophytically in crop plants. The *Fusarium* sp. isolated in the present study may be able to live in *Hibiscus* leaves endophytically. Okane et al. (1998) also reported *Cladosporium* spp. and *Alternaria altenata* exist endophytically in the leaves of ericaceous plants, although the frequencies of occurrence of both fungi were low in the present study.

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