Risk analysis and development of rapid methods for identifying food spoilage fungi

(食品危害カビのリスク評価と迅速同定法の開発)

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Summary

The numbers of food spoilage and health hazard incidents by heat-resistant fungi are increasing. Among the heat-resistant fungi, the genera *Byssochlamys* and *Thermoascus*, which belong to the order Eurotiales and show very close taxonomic affinity, form very high heat-resistant ascospores. These ascospores cannot be sterilized by heating of acidic food. Therefore, microbiologic risk assessment during the production of foods and beverages requires a detailed understanding of the microbiological profile and methods for identification of the genera *Byssochlamys* and *Thermoascus*.

Among the six species belonging to the genus *Byssochlamys*, *Byssochlamys fulva* showed the highest degree of heat resistance, while *Byssochlamys nivea* and *Byssochlamys laguncullariae* possessed the gene encoding isoepoxydon dehydrogenase (idh), an important enzyme in the biosynthesis of patulin, and showed high capacities to produce this mycotoxin.

Thermoascus species showed higher degrees of heat resistance than other heat-resistant fungi belonging to the genera *Byssochlamys*, *Hamigera*, and *Neosartorya*. *Thermoascus aurantiacus* possessed the *idh* gene, but showed no patulin production in potato dextrose broth or Czapek-glucose medium.

These findings indicated that the presence of each of these species or genera in foods is associated with different health risks. Therefore, in the food industry, it is necessary to distinguish among *Byssochlamys, Thermoascus*, and other fungi. The genera *Byssochlamys*

and *Thermoascus* can be identified by sequence analysis of housekeeping genes, such as β-tubulin. However, this method is very difficult in production plants because of the high costs involved and long times required to obtain the results. Therefore, rapid and simple PCR-based methods for genus- or species-level identification and discrimination using specific primer sets have been developed. PCR amplification using primer sets designed for *Byssochlamys* or *Thermoascus* produced products specific for each of these species and did not cross-react with other fungi associated with spoilage of food and environmental contamination. The identification methods were both simple and rapid with extremely high specificity, making them feasible for use in quality management at production plants.

Contents

Part Ⅰ

Evaluation of patulin production and development of a rapid method for identifying and discriminating the genus *Byssochlamys*

Abstract

The heat-resistant fungi belonging to the genus *Byssochlamys* cause spoilage and poisoning of heat-processed acidic foods due to heat-resistant ascospore formation and biosynthesis of the mycotoxin, patulin. This study was performed to characterize the heat resistance and patulin production of four species of *Byssochlamys*, i.e., *Byssochlamys fulva*, *Byssochlamys laguncula*riae, *Byssochlamys nivea*, and *Byssochlamys zollerniae*. *B. fulva* showed significantly greater heat resistance than the other species investigated, and both *B. laguncula*riae and *B. nivea* synthesized patulin. These findings indicated that the presence of each of these species in foods is associated with different health risks. Therefore, a method to identify and distinguish between *Byssochlamys* species is required. However, species-level identification based on morphology alone is difficult. Therefore, species-specific primers were developed to amplify the β-tubulin genes of *B. nivea*, *B. fulva*, *B. laguncula*riae, and *Byssochlamys zollerniae* by polymerase chain reaction (PCR). Amplification using the primer sets designed for *B. fulva*, *B. laguncula*riae, and *B. zollerniae* (B.nivea1F/1R, B.fulva1F/1R, B.lag1F/1R, and B.zol3F/R, respectively) produced PCR products specific for each of these species and did not cross-react with other fungi associated with spoilage of food and environmental contamination. The identification method described here is both simple and rapid with extremely high specificity.

1. Introduction

Fungi generally have poor heat resistance and can be killed in acidic fruit juices by pasteurization. However, heat-resistant fungi form ascospores that allow them to survive at temperatures of up to 75°C for 30 minutes [1)]. *Byssochlamys* spp. are heat-resistant fungi that are widely distributed in soil throughout the world, and can contaminate agricultural crops and food products [2)]. *Byssochlamys* spp. have the most heat-resistant ascospores among the heat-resistant genera reported to date, [3,4)] and are responsible for spoilage of strawberry, pineapple, passion fruit, mango, grape, and citrus fruit juices, as well as other acidic beverages and dairy products [1,5-10)]. In addition, *Byssochlamys* spp. proliferate well under low oxygen conditions, and produce pectinolytic enzymes that alter food characteristics, such as polygalacturonase, and the carcinogenic mycotoxin, patulin [10-12)], thus adversely affecting the quality of processed fruit food products and making these fungi the most harmful organisms in acidic food products.

Control of *Byssochlamys* spp. by pasteurization is difficult, and it is therefore important to prevent contamination by careful monitoring of raw materials and manufacturing environments. The most common identification method relies on morphological examination. However, the high degree of morphological similarity among these fungi [9,13)] poses a problem for such testing, and a polymerase chain reaction (PCR)-based genus-level identification method was therefore developed.

The *Byssochlamys* genus consists of six species: *Byssochlamys nivea*, *Byssochlamys fulva*, *Byssochlamys zollerniae*, *Byssochlamys spectabilis*/*Paecilomyces variotii*, *Byssochlamys laguncullariae*, and *Byssochlamys verrucosa* [14,15]. Among these species, *B. nivea*, *B. fulva*, *B. laguncullariae*, and *B. spectabilis* are causative agents of food spoilage [9,14,15] *B. verrucosa* differs phylogenetically from the other *Byssochlamys* species, and its taxonomic position should therefore be reconsidered [10]. Recently, we designed PCR primer sets for detection of *B. nivea*, *B. fulva*, *B. laguncullariae*, *B. zollerniae*, and *Paecilomyces saturates*, as well as a species-specific primer set for *B. spectabilis* and genus-specific primer sets for *Hamigera* spp., which are heat-resistant fungi that are closely related to *Byssochlamys* spp. Using various combinations of these primer sets, a rapid and convenient PCR-based method was developed for identification of *Byssochlamys* spp. and related fungi at the genus level [10].

The present study was performed to determine the resistance of *Byssochlamys* species to heat treatments typically used in acidic food production, along with patulin production and the risk of patulin production using primers for detection of the gene encoding isoepoxydon dehydrogenase (idh), an enzyme important in the biosynthesis of patulin [12,16,17], between and among the *Byssochlamys* and *Penicillium* genera. Further, new species-specific primers for the β-tubulin gene were designed for *B. nivea*, *B. fulva*, *B. zollerniae*, and *B. laguncullariae*. Accurate risk assessment should involve detection of *Byssochlamys* spp. in raw materials and the food manufacturing environment followed by rapid analysis for precise species-level identification of *Byssochlamys* spp.

2. Materials and Methods

2.1. Fungal strains and culture conditions

Isolates of heat-resistant fungi closely related to the genus *Byssochlamys* were obtained from the Medical Mycology Research Center, Chiba University; the Department of Biotechnology, National Institute of Technology and Evaluation; the Japan Collection of Microorganisms; and the Centraalburean voor Schimmelcultures (Table 1). The *Byssochlamys* spp. listed in Table 2 and other environmental isolates listed in Table 1 were also examined. All strains were grown on potato dextrose agar (Eiken Chemical Co., Tokyo, Japan) at 25°C or 37°C for 2 – 5 days in the dark prior to DNA extraction. For examination of heat resistance, *B. nivea* NBRC 30569, *B. fulva* JCM 12804, *B. laguncullariae* CBS 373.70, and *B. zollerniae* CBS 374.70 were grown on oatmeal agar (Becton Dickinson & Co., Franklin Lakes, NJ) at 30°C for 30 days in the dark.

2.2. Phylogenetic analysis of **Byssochlamys** *and related species*

Fungal DNA was extracted using a Dr. GenTLE High Recovery Kit (Takara Bio, Inc., Ohtsu, Japan) and adjusted to 5 ng/µl in TE buffer. A partial β-tubulin gene region was amplified [14], and the PCR products were labeled using a Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The DNA sequences were determined using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Using the sequences determined here and those from other species of the genus *Byssochlamys* and related species obtained through an ARSA search of the DNA Data Bank of Japan (DDBJ)

(http://arsa.ddbj.nig.ac.jp/html/) and our previously determined sequences, sequence alignment and neighbor joining trees were prepared using ClustalX software (http://clustalx.ddbj.nig.ac.jp/top-.html).

2.3. Heat resistance of **Byssochlamys** *spp. in saline solution and apple juice*

To examine the heat resistance of *Byssochlamys* spp., ascospores were harvested by flooding the surfaces of the plates with 10 ml of 0.1% polysorbate 80 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution and filtering through sterile glass wool into sterile test tubes. The suspensions were vortexed with five sterile glass beads for 3 minutes. The hyphae were pasteurized by heating the suspensions at 65°C for 30 minutes. The ascospore suspensions were diluted with saline solution or 100% apple juice (pH 3.7, 11.2 Brix) into sterile test tubes and subjected to thermal inactivation at 85°C or 80°C for 0.5, 0.75, 1, 2, 4, 6, 8, 10, 20, 30, 40, 60, 90, or 120 minutes. Survivor populations were cultured on potato dextrose agar. The *D*-values of the heat-resistant fungi were calculated [18]. All heat inactivation studies were performed in duplicate.

2.4. Detection of patulin production

B. nivea NBR C31351, *B. fulva* JCM12804, *B. laguncullariae* SUM 3338, and *B. zollerniae* IFM 48424 were inoculated into 100 ml of Czapek-glucose liquid medium (Wako Pure Chemical Industries, Ltd.) and potato dextrose broth (Becton Dickinson & Company) and grown in stationary culture at 25°C for 7 days in the dark. The culture fluid

containing the fungi was then adjusted to pH 3.6 with aqueous acetic acid, extracted with ethyl acetate, and washed with alkali. Patulin was assayed by high-performance liquid chromatography (HPLC) (Nanospace SI-2; Shiseido Co. Ltd., Tokyo, Japan) with a Mightysil RP-18GP column (Kanto Chemical Co. Inc., Tokyo, Japan) and detected at 290 nm. The detection limit for patulin was 0.05 ppm.

2.5. Primer design

Primers for specific amplification of the *idh* gene were designed based on the sequences from 28 strains of 12 *Penicillium* species [19] and 9 strains of *B. nivea* (Accession Nos. DQ322207, DQ322208, DQ322209, DQ322210, DQ322211, DQ322211, DQ322212, DQ322213, and DQ322214). Multiple sequence alignment of 38 strains was performed for conserved gene sequences using DNAsis Pro (Hitachi Software Japan, Tokyo, Japan). Based on these data, *idh*-specific primers were designed corresponding to a site at the 3'-end that was particularly highly conserved in all strains. The following primers were designed for specific amplification of the *idh* gene: idhBP1F (5'-CGCCGATGCATATGGAAGGCGAGAC-3') and idnBP1R (5'-CTGCGCTGCCTTGCAGGGCCC-3').

Four sets of primers were designed to distinguish *B. fulva*, *B. nivea*, *B. laguncullariae*, and *B. zollerniae* by targeting the previously determined partial β-tubulin gene region [14]. Multiple sequence alignments of the partial β-tubulin gene sequences of five *Byssochlamys* species, five *Paecilomyces* species, and two *Hamigera* species were searched for conserved

and polymorphic regions using DNAsis Pro (Hitachi Software Japan). The particularly highly conserved site at the 3'-terminus in the target species was used to design species-specific primers. The following primers were designed for specific amplification of each species: *B. fulva*, B.fulva1F (5'-AACAATTCTACAGGCAGGGC-3') and B.fulva1R (5'-TAGTGGTCGGGTCAGCGGA-3'); *B. nivea*, B.nivea1F (5'-ACAAGAGACAGGAAGAGCCT-3') and B.nivea1R (5'-TTCTTGCCGGCAGCCTAGGA-3'); *B. laguncularіae*, B.lag1F (5'-TCGAGACGTGAGATTGGGAA-3') and B.lag1R (5'-TGTTACCAGCACCGGACTGT-3'); and *B. zollerniae*, B.zol3F (5'-GACCGAACAAGGGACAAAGC-3') and B.zol3R (5'-GTGGTGGGGTCAGCATATAG-3'). The degree of sequence identity between the designed primers and the β-tubulin gene was analyzed by BLAST search (http://www/ncbi.nlm.nih.gov).

2.6. DNA extraction and PCR amplification

Fungal genomic DNA was extracted using Dr. GenTLE (from Yeast) (Takara Bio, Kyoto, Japan) according to the manufacturer's instructions. DNA solutions were diluted to 10 ng/μl with distilled water, with the concentration determined using NanoDrop (LMS, Tokyo, Japan). PCR mixtures were prepared using each primer set, B.fulva1F/R, B.nivea1F/R, B.lag1F/R, B.zol3F/R, or idnBP1F/R primers, and SapphireAmp Fast PCR Master Mix (Takara Bio). All amplifications were performed in a Thermal Cycler Dice (Takara Bio) with initial heat activation at 95°C for 1 minute followed by 35 cycles of

denaturation at 98°C for 5 s, annealing at 59°C for 5 s, and extension at 72°C for 10 s, and a final extension at 72°C for 1 minute. The entire reaction solution (2.5 µl) was applied to a 2% agarose gel and separated electrophoretically at 100 V/45 minutes with buffer consisting of 40 mM Tris-acetate and 1 mM EDTA (pH 8.0). The gels were stained in ethidium bromide for 25 minutes prior to visualization using a UV transilluminator.

Serial dilutions of DNA solution from 10 ng/µl to 10 pg/µl using distilled water of the *B. nivea* NBRC 31351 strain were examined by NanoDrop (LMS). PCR was performed using B.nivea1F/R primers and the limit of detection was measured. The detection limits of B.fulva1F/R, B.lag1F /R, and B.zol3F/R primers were measured in the same way as described for *B. fulva*, *B. laguncullariae*, and *B. zollerniae*.

Nested PCR was performed using primers Bt2a and Bt2b [20] with the template at concentrations of 10 ng/µl to 10 pg/µl, followed by purification of the first PCR product using a High Pure PCR Product purification kit (Roche, Mannheim, Germany). The purified product was subjected to PCR using the appropriate species-specific primer pair as described above.

2.7. Effects of contamination by other fungal DNA

Mixtures of 1 ng of *B. nivea* NBRC 31351 strain DNA and 1 – 1000 ng of *P. citrinum* NBRC 6352 strain DNA were prepared as templates for PCR with the B.nivea1F/R primers. DNA of the NBRC 6352 strain was amplified using a GenomiPhi V2 DNA amplification kit (GE Healthcare, Wauwatosa, WI), and the concentration was adjusted to 1 – 1000 ng/µl

with DNase-free distilled water. Similarly, PCR was performed using the B.fulva1F and B.fulva1R, B.lag1F and B.lag1R, and B.zol3F and B.zol3R primers and template DNA (B.fulva1F/R: *B. fulva* NBRC12804, B. lag1F/R: *B. laguncullariae* CBS 373.70, B. zol3F/R: *B. zollerniae* CBS 374.70, respectively) with amplified *Penicillium* DNA according to the same method.

3. Results

3.1. Phylogenetic analysis of Byssochlamys *and related species*

Figure 1 shows a phylogenetic tree of *Byssochlamys* and related species based on the partial β-tubulin gene sequences. Species of *Byssochlamys*, except for *B. verrucosa*, were clustered together in a monophyletic clade, supported with high bootstrap values. *Byssochlamys verrucosa* was placed in the *Thermoascus* clade, indicating a high degree of relatedness to *Thermoascus* spp. Similar groupings were observed in phylogenetic trees made based on other gene sequences, such as the D2 domain gene (data not shown).

3.2. Heat resistance of Byssochlamys *spp.*

The *D*-values of *B. fulva* were 89.6 and 16.7 minutes at 80°C and 85°C, respectively, in apple juice and 70.6 and 12.3 minutes at 80°C and 85°C, respectively, in saline solution (Table 3). The other *Byssochlamys* spp. showed lower levels of heat resistance than *B. fulva*. For example, *B. nivea* had *D*-values of 2.0 and 1.4 minutes at 85°C in apple juice and saline solution, respectively, and *B. zollerniae* did not form ascospores so showed no resistance to

heat treatment. In each heat treatment, the *D*-value in apple juice was higher than that measured in saline solution.

3.3. Detection of the idh *gene and patulin production*

PCR was performed using the primers idhBPF/R, and the sizes of the PCR products were confirmed by electrophoresis. Among the 18 strains used in this experiment, PCR products of approximately 200 bp were detected for amplification of *Penicillium expansum* (positive control), *B. nivea*, and *B. laguncullariae* (Fig. 2). No PCR products were detected for *P. verrucosum* (negative control), *B. fulva*, or *B. zollerniae* (Fig. 2).

For *B. nivea* NBRC 31351 and *B. laguncullariae* SUM 3338, patulin was detected at levels of 46.0 ppm and 1.3 ppm, respectively, in Czapek-glucose liquid medium. No patulin was detected for *B. fulva* or *B. zollerniae* (Table 4). In potato dextrose broth, *B. laguncullariae* SUM 3338 produced patulin at a level of 34.0 ppm (data not shown).

3.4. PCR using species-specific primers

Twenty-eight strains were subjected to PCR-based assay using the B.fulva1F/1R primer set designed for *B. fulva*, and amplification products of approximately 250 bp were detected for *B. fulva* CBS 146.48^{T} (Fig. 3A) and the six *B. fulva* strains listed in Table 2 (data not shown). No PCR products were obtained for *B. nivea*, *B. laguncullariae*, *B. zollerniae*, *Paecilomyces* spp., *Hamigera* spp., *Talaromyces* spp., or other environmental fungal isolates (Fig. 3A).

PCR with the B.nivea1F/1R primer set yielded products approximately 300 bp in length for *B. nivea* CBS 100.11^{T} (Fig. 3B) and the seven *B. fulva* strains listed in Table 2 (data not shown). No PCR products were obtained for other fungi using these primers (Fig. 3B).

In addition, only *B. laguncullariae* was amplified by PCR using the B.lag1F/1R primer set, yielding a product of 400 bp. No PCR products were obtained for the other species examined (Fig. 3C). Similarly, the B.zol3F/3R primer set yielded a PCR product of 300 bp only for *B. zollerniae*. No PCR products were obtained for the other fungi tested (Fig. 3D). These results indicated the high specificity of these primer sets for each of the target species.

3.5. Detection limits and effects of contamination by other fungal DNA

PCR and nested PCR using primers B.nivea1F/1R with serially diluted *B. nivea* NBRC 31351 template DNA showed detection limits of 0.1 ng/μland 10 pg/μl, respectively (Fig. 4). Similarly, the detection limits for other primers (B.fulva1F/1R, B.lag1F/1R, and B.zol3F/3R) were also 0.1 ng/μl DNA for PCR and 10 pg/μl for nested PCR (data not shown). The specificity of the PCR assay was maintained even if the sample was contaminated 1000-fold with other fungal DNA for all primer sets tested (data for B.nivea1F/1R are shown in Fig. 5).

4. Discussion

To analyze the risks associated with *Byssochlamys* spp., we first evaluated the heat resistance of the ascospores of each species. All species and strains examined showed higher *D*-values in apple juice than in saline solution, which was possibly due to the protective effects of the juice components. Of the species examined, *B. fulva* showed the greatest heat resistance. Under standard sterilization conditions involving exposure of vegetative cells to a temperature of 80°C for 10 minutes, three *Byssochlamys* species (*B. fulva*, *B. laguncullariae*, and *B. nivea*) showed *D*-values > 6 indicating that commercial pasteurization is insufficient to kill these organisms. On the other hand, *B. zollerniae* did not form ascospores under our experimental conditions, indicating a very low probability of survival after heat sterilization. These findings indicated that there are marked differences in heat resistance among three species of *Byssochlamys* spp. that have been reported to act as causative agents of food spoilage [9,14,15].

Leuque *et al*. reported a PCR-based method to detect species of *Penicillium* and *Aspergillus* that produce the mycotoxin, patulin [21], but did not discuss patulin production by *Byssochlamys* spp. However, other studies indicated differences in patulin production between *Byssochlamys* species [12,22]. In the present study, PCR using idhPBF/R primer sets did not yield amplification products with *B. fulva* or *B. zollerniae* DNA as the template, suggesting that these species lack the *idh* gene. *B. nivea* and *B. laguncullariae*, both of which are known to possess the *idh* gene, produced patulin. This is the first report of patulin production by *B. laguncullariae* and that the levels of patulin production change

according to the culture conditions, which is extremely important from the perspective of food safety. Moreover, idhBPF/R can be used to evaluate the risk of patulin production by a wide range of fungi.

B. nivea is often detected in food products and produces patulin. *B. laguncullariae* also produces patulin and causes food spoilage [15], and *B. fulva* has a high degree of heat resistance and is the most commonly detected *Byssochlamys* species in food products [23]. Therefore, the development of a rapid method for distinguishing between these and other fungi at the species level is essential. However, identification of *Byssochlamys* strains at the species level is difficult based only on morphological examination.

In this study, species-specific primers were designed based on the β-tubulin gene, which is widely utilized for phylogenetic classification of fungi at the species level [24]. The B.nivea1F/R primer pair and the primer pairs for the other species showed excellent specificity for *B. nivea* and each of the other species, even distinguishing between members of the same genus. Thus, PCR assay with species-specific primers allowed rapid species-level identification of *Byssochlamys* isolates.

As the identification method described here is based on PCR, even dead fungi can be identified as long as the DNA can be extracted. Therefore, the causes of food spoilage can be determined and patulin production can be evaluated, even in cases in which morphologically identifiable fungi cannot be extracted from the food product. This

identification method can be performed in less than 3 hours, and is simple to perform requiring no special expertise.

The method described has high sensitivity of 0.1 ng/µl of DNA, even though the target β-tubulin gene is present in only a single copy. This sensitivity exceeds the detection limit of universal primers for the multicopy 28S rDNA D2 region [14]. Nested PCR could detect 10 pg/µl of DNA, thus further enhancing the detection sensitivity. Moreover, detection was possible even in the presence of 1000-fold excess concentrations of genomic DNA from other fungi. Therefore, in testing of raw materials for food products, specific gene amplification of the four *Byssochlamys* spp. would be possible, even in the presence of other fungi. This identification method has a number of advantages over D1/D2 region sequence analysis, including the use of relatively inexpensive equipment, thus making it feasible for quality management at production plants, and the ability to detect fungi at the species level even in samples containing closely related species.

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Table 1. Fungal isolates used in this study.

Species	Strain No.	Source	β-tubulin gene accession No.	DNA template No.
Byssochlamys fulva	CBS 146.48^{T}	Bottled fruit, UK	FJ389986	1
	IFM 52229	Soil, Brazil	AB539867	2
B. nivea	CBS 100.11^{T}	Unknown source	FJ389999	3
	CBS 133.37	Milk of cow, USA	FJ390000	4
B. laguncullariae	CBS 373.70^{T}	Wood, Brazil	FJ389995	5
B. zollerniae	CBS 374.70^{T}	Wood, Brazil	AY753356	6
Paecilomyces saturatus	CBS 323.34^{T}	Unknown source	AB539868	7
P. saturatus	IFM 57199	Cosmetic material, Japan	FJ390005	8
B. spectabilis / *P. variotii*	CBS 101075^{T}	Heat processed fruit beverage, Japan	AY753372	9
	IFM 52146	Soil, Brazil	AB539869	10
P. variotii var. brunneolus	CBS 370.70^{T}	Wood, Brazil	EU037068	11
P. divaricatus	CBS 284.48^{T}	Mucilage bottle with library paste, USA	FJ389992	12
P. formosus	CBS 990.73B^{T}	Unknown source	FJ389993	13
B. verrucosa	CBS 605.74	Nesting material of bird, Australia	DQ073328	14
Hamiger avellanea	CBS295.48^{T}	Soil, USA	AB539870	15
	NBRC 7721	Unknown source	AB539872	16
H. striata	CBS 377.48^{T}	Canned blueberries, USA	AB539871	17
	NBRC 9162	Soil, Philippines	AB539873	18
Talaromyces byssochlamydoides	CBS 413.71^{T}	Dry soil, USA	AY753374	19
T. emersonii	CBS 393.64^{T}	Compost, Italy	AY766255	20
T. flavus	CBS 310.38^{T}	Unknown source, New Zealand	EU021663	21
T. trachysperumus	CBS 373.48^{T}	Unknown source	AY753371	22
T. wortmannii	CBS 391.48^{T}	Soil, USA		23
	W35	Unknown	AY533533	
Eupenicillium brefeldianum	NBRC 31730^{T}	Soil, Australia		24
Neosartorya fischeri	CBS 544.65^{T}	Mouldy cardboard, Canada	AF057322	25
N. spinosa	NBRC 8782^{T}	Soil, Nicaragua		26
Chaetomium globosum	CBS 148.51	Stored cotton, USA		27
C. funicola	NBRC 6556	Unknown source		28

T; ex type.

Table 2. Other *Byssochlamy* strains.

Byssochlamy fulva	JCM 12804, 12805, 12806,
	NBRC 7901, 31877, 31878
B. nivea	CBS 140.65, 608.71
	NBRC 8815, 8972, 30569, 31351, 31832
B. laguncullariae	SUM 3338

Table 3. Heat resistance of *Byssochlamys* ascospores in apple juice (pH 3.7, 11.2º Brix) or saline solution.

Fungus	Strain	D-value (°C)	
		Heating temperature (°C)	
		80	85
Byssochlamys fulva	JCM12804	89.5	16.7
		70.6	12.3
B. nivea	NBRC31351	15.4	2.0
		13.8	1.4
B. laguncullariae	CBS373.70	5.0	0.4
		4.5	0.4

Table 4. Patulin production of three *Byssochlamys* species in Czapek-glucose broth.

Fungus	Strain	Patulin (ppm)
Byssochlamys nivea	NBRC 31351	46.0
B. laguncullariae	CBS 373.70	1.3
B. fulva	JCM 12804	N.D.
B. zollerniae	CBS 374.70	N.D.

N.D.; Not detected (0.05ppm or less).

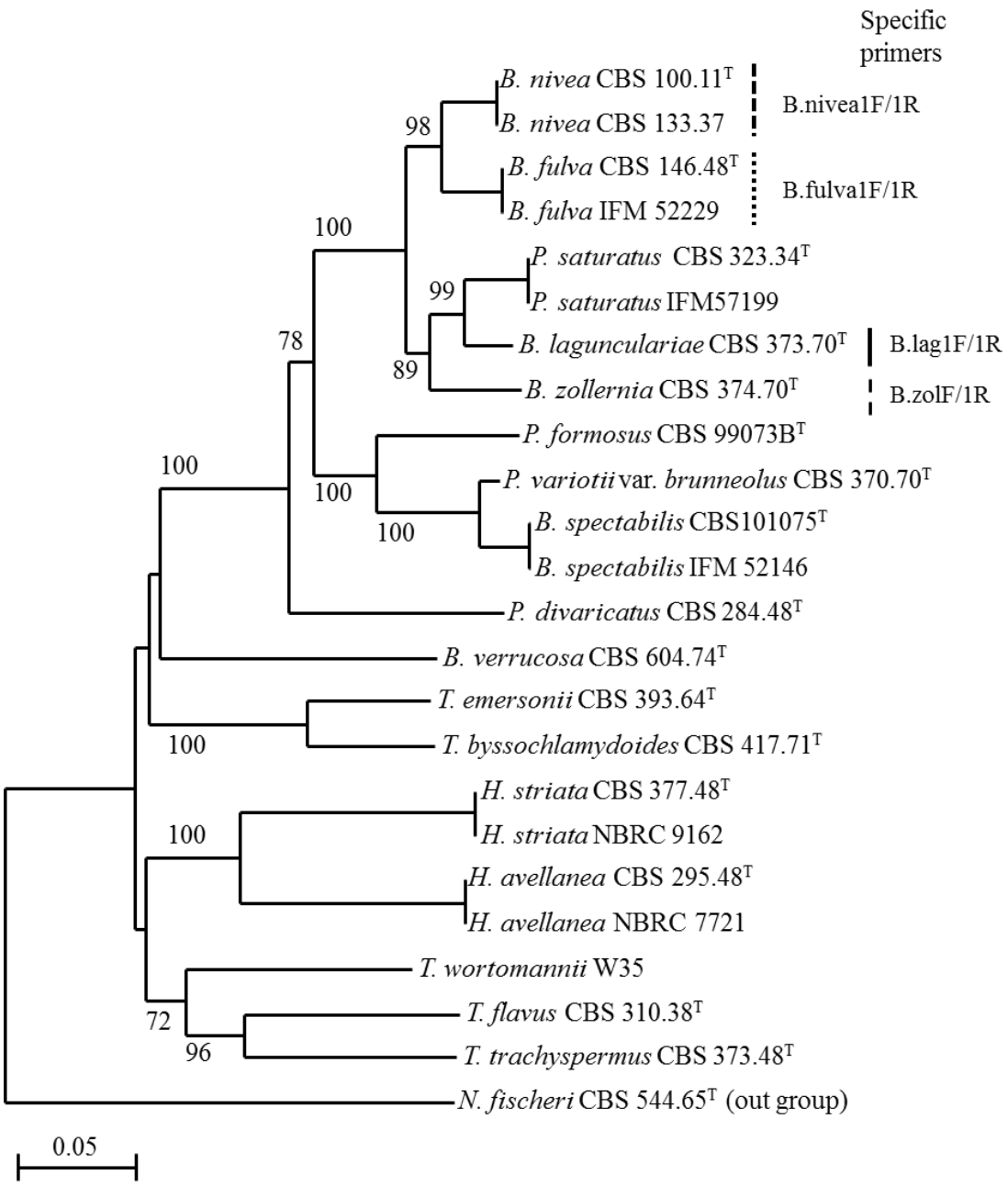

Figure 1. Neighbor joining tree based on the partial β-tubulin gene sequences for *Byssochlamys* and related species. Bootstrap samplings based on 1000 samplings supporting the internal branches with probability ≥ 50% are shown.

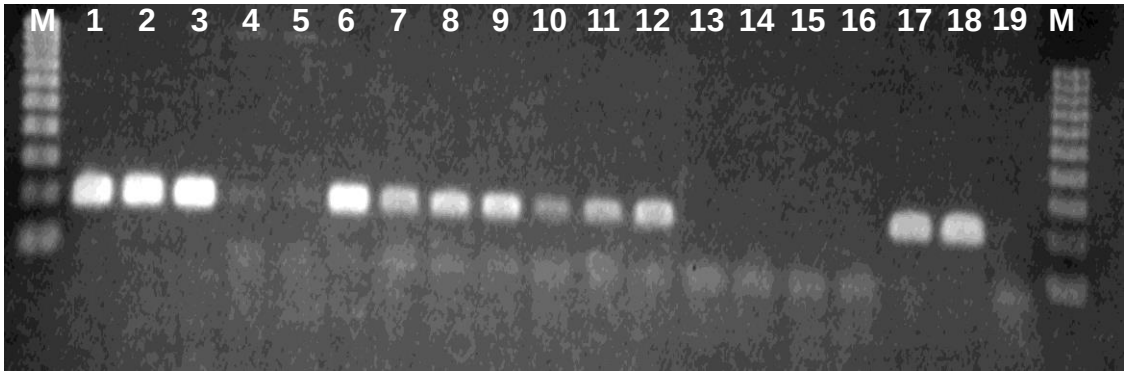

Figure 2. PCR amplification using *Penicillium* and *Byssochlamy*s DNA with idhBP1F/R primers. Numbers indicate the genomic DNA template subjected to PCR. M, 100-bp ladder; 1, *P. expansum* CBS 325.48; 2, *P. expansum* IFO 8800; 3, *P. expansum* IFM 58916; 4, *P. verrucosum* CBS 603.74 (negative control); 5, *P. verrucosum* CBS 115508 (negative control); 6, *B. nivea* NBRC 8815; 7, *B. nivea* NBRC 8972; 8, *B. nivea* NBRC 30569; 9, *B. nivea* NBRC 31351; 10, *B. nivea* NBRC 31832; 11, *B. nivea* CBS 140.65; 12, *B. nivea* CBS 608.71; 13, *B. fulva* NBRC 7901; 14, *B. fulva* NBRC 31877; 15, *B. fulva* NBRC 31878; 16, *B. fulva* JCM 12804; 17, *B. laguncul*ariae CBS 373.70; 18, *B. laguncul*ariae SUM 3338, 19, *B. zollerniae* CBS 374.70.

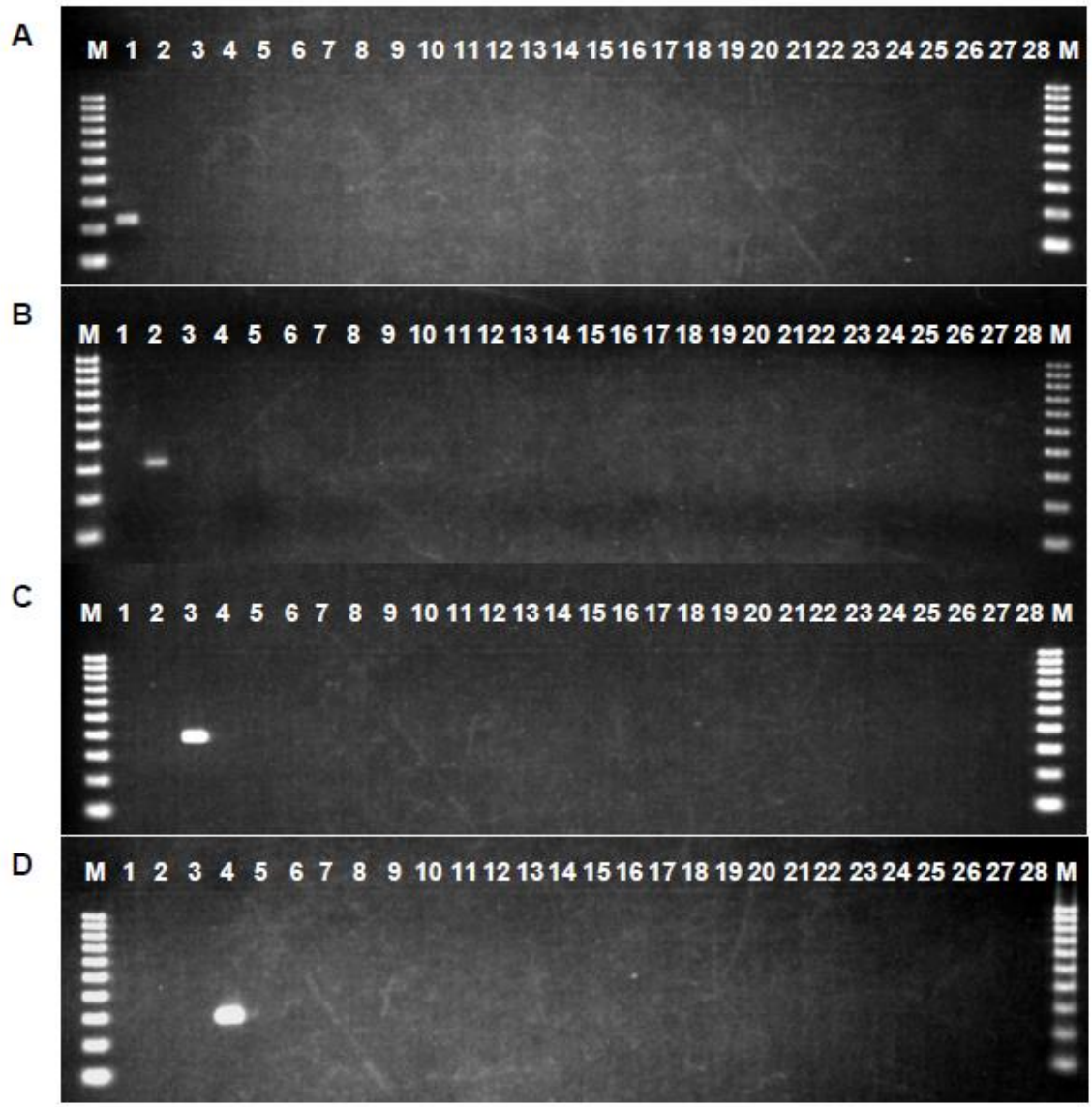

Figure 3. PCR amplification using species-specific primers B.fulva1F/R (A), B.nivea1F/R (B), B.lag1F/R (C), and B.zol3F/R (D) to identify *B. nivea*, *B. fulva*, *B. laguncullariae*, and *B. zollerniae*, respectively. Numbers at the top of each lane (1 – 28) correspond to the DNA templates shown in Table 1.

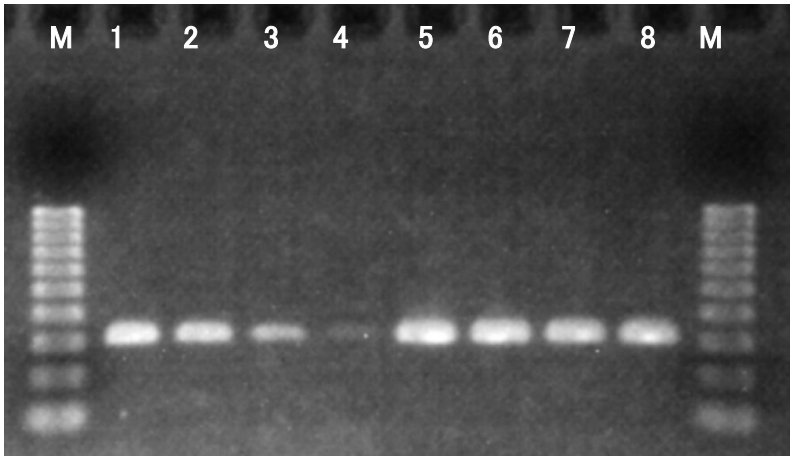

Figure 4. Detection limit of B.nivea1F/R primers using DNA of *B. nivea* NBRC31351. The numbers at the top of lanes 1 – 4 refer to 1st PCR only, and those at the top of lanes 5 – 8 refer to nested PCR. Amounts of DNA: 10 ng (1, 5), 1 ng (2, 6), 100 pg (3, 7) and 10 pg (4, 8). M: 100-bp ladder.

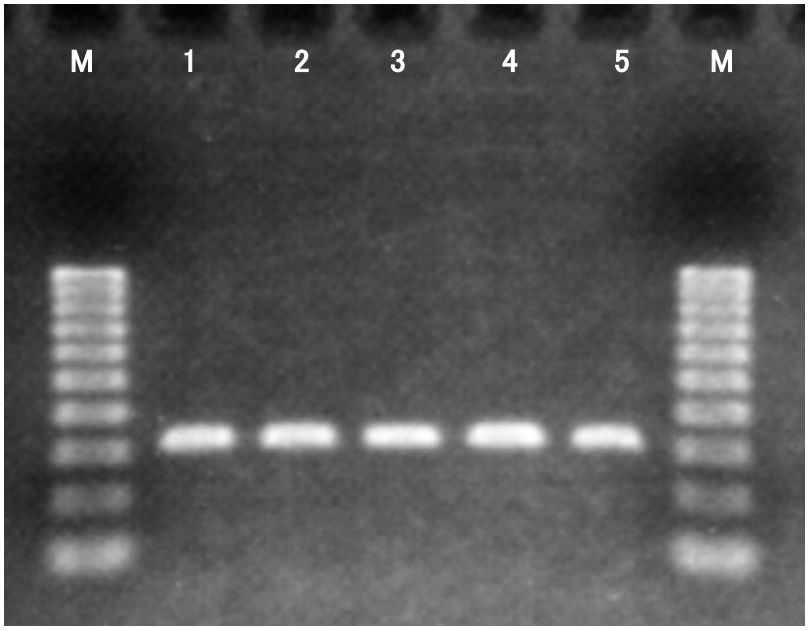

Figure 5. PCR amplification using genomic DNA of *B. nivea* and *P. citrinum* with B.nivea1F/R primers. The numbers indicate the amounts of DNA. M, 100-bp ladder; 1, 1 ng of *B. nivea*; 2, 1 ng of *B. nivea* and 1 ng of *P. citrinum*; 3, 1 ng of *B. nivea* and 10 ng of *P. citrinum*; 4, 1 ng of *B. nivea* and 100 ng of *P. citrinum*; 5, 1 ng of *B. nivea* and 1000 ng of *P. citrinum*.

Part Ⅱ

Evaluation of patulin production and development of a rapid method for identifying and discriminating the genus *Thermoascus*

Abstract

Despite the increasing incidence of spoilage in the food industry due to ascomycetous fungus species belonging to the genus *Thermoascus*, there have been no previous studies regarding the risks of such food spoilage. Our analyses indicated that *Thermoascus* species have higher degrees of heat resistance than other heat-resistant fungi belonging to the genera *Byssochlamys*, *Hamigera*, and *Neosartorya*. Both *Thermoascus aurantiacus* and *Byssochlamys verrucosa* possess the *idh* gene, but showed no patulin production in potato dextrose broth or Czapek-glucose medium. Therefore, in the food industry, it is necessary to distinguish between *Thermoascus* and other fungi. In this study, a rapid and highly sensitive PCR-based method for detecting *Thermoascus* at the genus level was developed, and is expected to be useful for surveillance of raw materials used in food production.

1. Introduction

Food spoilage due to heat-resistant fungi was first reported in canned foods in the 1930s, and numerous incidents of fungal spoilage of processed fruit juices and beverages have since been reported [1-4]. Asexual fungi have hyphae and conidia that are susceptible to heat, and these fungi can typically be killed by treatment at 70°C for 10 minutes. However, fungi belonging to the genera *Byssochlamys*, *Neosartorya*, *Hamigera*, and *Thermoascus* of the order Eurotiales (Plectomycetes) form ascospores that are highly heat-resistant, which allows these species to survive heat sterilization and to be present in processed acidic beverages [3].

As implied by the genus name, *Thermoascus* spp. can grow at high temperatures. The major species belonging to this genus are *Thermoascus crustaceus*, *Thermoascus thermophilus*, *Thermoascus aurantiacus*, and *Thermoascus aegyptiacus* [5,6]. These fungi have been detected in various agricultural products, including maize stored in sub-Saharan Africa and olives and olive cake in Morocco [7,8], as well as in food-related environments [6,9]. The incidence rates of spoilage incidents in various processed tea and fruit juice products due to this genus are increasing. In addition, the production of high levels of amylase and cellulase by *Thermoascus* spp. can markedly alter food product properties, and the high thermostability of these enzymes makes them difficult to inactivate with heat treatment [10,11]. With the exception of one report by King et al. [12] indicating that *Thermoascus aurantiacus* ascospores survived at 88°C for 60 minutes, there have been no reports regarding the risks associated with this genus in the food industry. Therefore, the growth, heat resistance, and formation temperature range of ascospores of *Thermoascus* spp. were analyzed to establish risk analysis data for each

species belonging to this genus. Furthermore, species belonging to *Thermoascus* and the closely related genus *Byssochlamys* were shown to produce the mycotoxin, patulin. To evaluate the risk of patulin production, homology analysis was performed for the gene encoding isoepoxydon dehydrogenase (idh), an important enzyme involved in patulin production [13-15]. Detailed risk analysis based on patulin production was performed for various *Thermoascus* species grown in culture medium.

The detection and identification of fungi are generally based on morphological examination, but there is a great deal of interest in the development of more rapid and versatile methods for these purposes. Due to a lack of genetic data, however, no methods for rapid detection of *Thermoascus* species have been established. We successfully developed a PCR-based method for genus- and species-level detection and identification of *Byssochlamys* and *Neosartorya* [16-18].

This study was performed to evaluate the risks of food spoilage associated with *Thermoascus* species, and it was shown to be necessary to distinguish *Thermoascus* spp. from other heat-resistant fungi. Next, various genes used in phylogenetic classification of fungi were analyzed, new specific primers targeting gene sequence patterns specific to *Thermoascus* were designed, and a PCR-based method was established for the rapid detection of *Thermoascus* at the genus level and to distinguish these organisms from other fungi.

2. Materials and Methods

2.1. Phylogenetic analysis of **Thermoascus** *and related species*

Fungi were cultured on potato dextrose agar (PDA) medium (Eiken Chemical Co., Tokyo, Japan) in the dark at 25°C or 37°C for 7 days. Fungal DNA was extracted using a Dr. GenTLE High Recovery Kit (Takara Bio, Inc., Ohtsu, Japan) and adjusted to 5 ng/µl in TE buffer. The region encoding the large subunit of the RNA polymerase II gene (RPB1) was amplified, and the PCR products were labeled using a Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), according to the method of Samson et al. [19]. The DNA sequences were determined using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Using the sequences determined here and those from other species of the genus *Thermoascus* and related species obtained through an ARSA search of the DNA Data Bank of Japan (DDBJ) (http://arsa.ddbj.nig.ac.jp/html/) and our previously determined sequences, sequence alignment and neighbor joining trees were prepared using ClustalX software (http://clustalx.ddbj.nig.ac.jp/top-.html).

2.2. Growth and ascospore formation of **Thermoascus** *spp.*

To evaluate the potential for food spoilage due to individual *Thermoascus* spp. over a range of temperatures, the growth and ascospore formation were examined for the following test fungi: *T. aegyptiacus* IFM 61569, *T. aurantiacus* IFM 57325, *T. crustaceus* IFM 57326, and *T. thermophilus* IFM 59664. Each strain was inoculated onto oatmeal agar medium (Difco, BD, Sparks, MD) or PDA medium (Eiken Chemical Co., Tokyo, Japan) in triplicate and cultured over temperatures ranging from 20°C to

60°C for 14 days. Growth temperature range was assessed by visual inspection, and ascospore formation temperature range was assessed by microscopic examination.

2.3 Heat resistance of **Thermoascus** *ascospores*

T. aegyptiacus IFM 61569, *T. aurantiacus* IFM 57325, and *T. crustaceus* IFM 57326 were cultured at 35°C, while *T. thermophilus* IFM 59664 was cultured at 30°C on oatmeal agar medium (Difco) in the dark for 30 days. The ascocarps were collected in centrifuge tubes containing 0.1 M phosphate buffer (pH 7.0), and the hyphae were removed with sterilized gauze. After centrifugation (700 × *g*, 10 min), the supernatant was discarded, and the pellets were washed three times with 0.1 M phosphate buffer (pH 7.0).

Standard glucose-tartrate solution (glucose, 16 g; DL-tartaric acid, 0.5 g; distilled water (DW), 100 ml; adjusted to pH 3.6) was used as a model acidic beverage. The ascospores were adjusted to 10^6 spores/ml in standard glucose-tartrate solution, and each species was tested for heat resistance using the thermal death time (TDT) test tube method [20]. To assess survival, germination rate was determined by counting the number of germinated ascospores in triplicate [21]. A survival curve was plotted from the number of surviving fungi, and the *D*-value (min) was calculated.

*2.4. Evaluation of patulin production in culture and by detection of the isoepoxydon dehydrogenase (***idh***) gene*

Evaluation of the presence or absence of the *idh* gene in members of the *Thermoascus* genus and related species was performed using the following

region-specific primers corresponding to functional domains[16]: idh2444 (5'-ATGCACATGGAAGGCGAGAC-3') and idh2887 (5'-CAAVGTGAATTCCGCCATCAACCAAC-3'). PCR mixtures consisting of 1 μl of 5 ng/μl DNA, 1 μl of each primer (10 pmol), 22 μl of DW, and 25 μl of SapphireAmp Fast PCR Master Mix (Takara Bio Inc.) were subjected to 35 cycles of denaturation at 98°C for 5 s, annealing at 59°C for 5 s, and extension at 72°C for 10 s.

The PCR products were electrophoresed on a 2% agarose gel for 45 minutes at 100 V, and the presence or absence of bands and band sizes were evaluated. The PCR products were purified using High Pure PCR Product Purification Kits (Roche, Mannheim, Germany), and labeled with idh2444 or idh2887 primers using a Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The base sequence was determined using an ABI PRISM 3130 (Applied Biosystems). Homology between the *idh* genes of members of the *Thermoascus* genus and *Byssochlamys nivea* (which produces patulin) was examined using DNASIS Pro (Hitachi), and patulin production potential was evaluated.

Patulin production was evaluated in the culture media of *Thermoascus* spp. cultures. The fungi tested were *T. thermophilus* IFM 60075, *T. crustaceus* IFM 60077, *T. aurantiacus* IFM 57325, *Byssochlamys verrucosa* IFM 48423, and *B. nivea* NBRC 57325. Fungi were inoculated into 100 ml of Czapek-glucose medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or potato dextrose broth (PDB) (Difco) and cultured in the dark at 35°C for 7 days without agitation. The culture broth containing each fungus was adjusted to pH 3.6 with aqueous acetic acid, extracted with ethyl acetate, and washed with alkali. Patulin was quantified by high-performance liquid

chromatography (Nanospace SI-2; Shiseido, Tokyo, Japan) fitted with a Mightysil RP-18GP column, (Kanto Kagaku, Tokyo, Japan) with detection at 290 nm. The limit of detection for patulin was 0.05 ppm.

2.5. Design of **Thermoascus** *genus-specific primers*

For the *Thermoascus* spp. listed in Table 1, the homology of the RPB1 gene was calculated by multiple alignment analysis (DNASIS Pro; Hitachi Software, Tokyo, Japan). Conserved sequences in *Thermoascus* were identified and used to design genus-specific primers with conservation at the 3' end in each *Thermoascus* spp., selection of base sequences with little similarity to fungi of other genera, T_m of 60°C ± 2°C, about 20 bases in length, and about 50% GC content as conditions for primer target regions. Based on these criteria, *Thermoascus* genus-specific primers were also designed: RPB_F2 (5'-ATCTGCCGGCGTGATGTGTTCCTG -3') and RPB_R2 (5'-GTTGTGCAGAAGCCAGTTGACC-3'). A search for homology of the designed base sequences was performed using the Basic Local Alignment Search Tool (http://www/ncbi.nlm.nih.gov).

2.6. Evaluation of **Thermoascus** *genus-specific primers*

To evaluate the specificity of the designed primer set, DNAs from *Thermoascus* spp. cultures and other related fungi isolated frequently from food processing environments (Table 1) were subjected to PCR with these primers. DNA was extracted using a Gen TLE High Recovery Kit (Takara Bio, Inc.), stored at –20°C, and used in assays over a period of 6 months. PCR was performed with mixtures consisting of 1 μl of 5 ng/μl DNA, 1 μl of each primer (10 pmol), 22 μl of DW, and 25 μl of SapphireAmp Fast PCR

Master Mix (Takara Bio, Inc.), and amplification was carried out by heating at 97°C for 10 minutes followed by 30 cycles of 97°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute.

PCR products were electrophoresed on 2% agarose gels for 45 minutes at 100 V, and the presence or absence of bands and band sizes were evaluated. To determine the detection sensitivity of the designed primers, genomic DNA from *T. crustaceus* IFM 60232 was serially diluted (10 ng – 10 pg), and first PCR and nested PCR were performed. For nested PCR, the first PCR products were purified using High Pure PCR Product Purification Kits (Roche, Mannheim, Germany), and PCR was performed again under the same conditions described above. These PCR products were also electrophoresed on 2% agarose gels for 45 minutes at 100 V, and the presence or absence of bands and band size were evaluated.

The effects of contamination by other fungal DNA on the *Thermoascus* detection method were also examined. *T. crustaceus* IFM 60232 DNA (1 ng) and *P. citrinum* NBRC 6352 DNA (1 – 1000 ng), was amplified using a GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Wauwatosa, WI), and added to PCR mixtures using the RPB_F2/R2 primer pair.

To evaluate detection of *Thermoascus* in beverages, *T. crustaceus* IFM 60232 ascospores (10 cfu) were added to 500 ml of an acidic isotonic beverage (pH 3.4). This was then filtered using a Microfil V filtration device (Merck Millipore, Billerica, MA), and cultured on PDA medium (Difco) at 30°C for 22 hours. DNA was then extracted

using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems), and PCR was performed under the conditions described above.

3. Results

3.1. Phylogenetic analysis of Thermoascus *and related species*

Figure 1 shows a phylogenetic tree of *Thermoascus* and related species based on the RPB1 gene sequences. All species of *Thermoascus* clustered together on the tree. *Byssochlamys verrucosa* was placed in the *Thermoascus* clade, indicating a high degree of relatedness to *Thermoascus* spp. Similar groupings were observed in phylogenetic trees made based on other gene sequences, such as the β-tubulin gene (data not shown).

3.2 Growth and ascospore formation of Thermoascus *spp.*

Table 2 shows the temperature range over which growth and ascospore formation were observed for each species on oatmeal medium. *T. aegypticus*, *T. crustaceus*, *T. thermophilus*, and *B. verrucosa* grew between 25°C and 50°C. *T. aurantiacus* showed growth and ascospore formation over a higher temperature range of 33°C to 57°C. The highest growth temperature observed for *B. verrucosa* was 53°C, which was above the temperature ranges for other *Byssochlamys* spp. Ascospore formation was observed at 25°C to 35°C for *T. crustaceus*, while *T. aegypticus* and *T. thermophilus* showed ascospore formation at temperatures that were 5°C – 10°C higher. *B. verrucosa* did not form ascospores under the present culture conditions.

3.3. Heat resistance of ascospores formed by **Thermoascus** *spp.*

Table 3 shows the heat resistance of *Thermoascus* spp. ascospores. The ascospores of *T. aegyptiacus* IFM 61569 and *T. thermophilus* IFM 59664, IFM 60075 did not germinate after heat treatment at 90°C. Heating at 90°C provided the best germination of these ascospores, and therefore only the *D*-values of these strains at 90°C were determined, and z-values were not calculated. The ascospores of *T. aurantiacus* IFM 57325 and NBRC 31693 did not germinate after heating at 90°C. Inactivation of ascospore germination by heating was observed in the range 80°C to 85°C, and therefore *D*-values at 80°C, 83°C, and 85°C were determined and the z-values were subsequently calculated. The *D*-value for *T. crustaceus* IFM 57326 could not be determined accurately, but survival was confirmed even after heating at 90°C for 90 minutes.

3.4. Detection of the **idh** *gene and patulin production*

Figure 2 shows the results of PCR using the *idh* gene-specific primers, idh2444 and idh2887. A 400-bp PCR product was detected for *B. nivea*, which is known to produce patulin. Signals identical in size to those seen for *B. nivea* were also detected for *T. aurantiacus* and *B. verrucosa* and showed 99.9% base sequence identity and complete identity at the amino acid level. However, *T. aegyptiacus, T. crustaceus*, and *T. thermophilus* yielded no PCR products with these primers for the *idh* gene.

Table 4 presents the results regarding production of patulin in the culture media for *Thermoascus* spp. and related species. The levels of patulin production were 55.6 ppm in PDB and 42.8 ppm in Czapek-glucose medium for *B. nivea*, which possesses the *idh*

gene. However, despite homology with the *B. nivea idh* gene, *T. aurantiacus* and *B. verrucosa* showed no patulin production in PDB or Czapek-glucose medium. No patulin production was detected for other fungal species that were suggested to lack the *idh* gene.

3.5. Evaluation of **Thermoascus** *genus-specific primers*

PCR was performed using the RPB_F2 and RPB_R2 primer set, and the presence or absence as well as the sizes of PCR products were evaluated by electrophoresis. Amplification products about 400 bp in length (Fig. 3), consistent with the primer design, were detected for all *Thermoascus* species and strains listed in Table 1. Similar findings were detected for *B. verrucosa*, which is phylogenetically very closely related to the *Thermoascus* genus. However, no amplification products were detected for other *Byssochlamys* or *Hamigera* species, or other fungi associated with spoilage in food products (Fig. 3). In addition, gene amplification products of about 400 bp were produced by PCR with DNA extracted from *T. aurantiacus* that had been added to an acidic beverage.

Furthermore, as multiple fungal species are frequently detected in food processing environments, the ability of this PCR assay to detect DNA from *Thermoascus* spp. in samples containing DNA from other fungi was also examined. Briefly, aliquots of 1 ng of DNA from *T. crustaceus* IFM 60232 and 1 – 1000 ng of DNA amplified from *P. citrinum* NBRC 6352 were added to PCR mixtures and amplified. The results indicated that this PCR assay was capable of detecting *Thermoascus* DNA even in the presence of a 1000-fold excess of *P. citrinum* DNA.

The detection sensitivity of PCR assay using RPB_F2 and RPB_R2 was approximately 100 pg of template DNA for first PCR. However, with nested PCR, amplification products were detected even with only 10 pg of template DNA. These results indicated that the detection sensitivity of this assay using RPB_F2 and RPB_R2 on first PCR was 100 pg, which was improved 10-fold by nested PCR (Fig. 4).

4. Discussion

In this study, the risks of food spoilage associated with several species belonging to the genus *Thermoascus* were evaluated. These species showed greater degrees of heat resistance compared to other heat-resistant fungi, i.e., *B. fulva* (D_{85} = 12.3 min [16]) and *N. fischeri* (D_{85} = 10 – 35 min [1]), which have highly heat-resistant ascospores. Indeed, *T. aegyptiacus* showed the highest degree of heat resistance (D_{90} = 56.2 min) among the species included in the analysis. In addition, all species of *Thermoascus* examined were able to grow more than at 50°C. As standard sterilization conditions involve exposure of vegetative cells to a temperature of 80°C for 10 minutes, the *D*-value of > 6 for *Thermoascus* indicated that commercial pasteurization is not sufficient to kill these organisms and achieve complete sterilization. It is widely recognized within the food industry that *Thermoascus* spp. are difficult to control, and therefore accurate detection of these species, at least at the genus level, in raw materials and in the manufacturing environment would be important for controlling and preventing contamination. Therefore, the development of a rapid method for detecting *Thermoascus* spp. is required. However, *Thermoascus* spp. have a similar type of sclerotioid cleistothecium to some species of *Eupenicillium*, and the order *Thermoascaceae* is composed of two closely related clades, i.e., *Thermoascus* and *Byssochlamys* [5], making it difficult to

detect and distinguish members of the genus *Thermoascus* using traditional morphology-based methods.

Samson et al. noted that *B. verrucosa* is misidentified as belonging to the genus *Byssochlamys*, which was subsequently confirmed by the clustering of this species with *Thermoascus* on a phylogenetic tree based on the RPB1 [5)] and β-tubulin [17,22)] gene sequences. Furthermore, *B. verrucosa* can grow even at 53°C, a temperature at which other *Byssochlamys* spp. cannot grow. However, *B. verrucosa* has not been reclassified as belonging to the genus *Thermoascus*. Therefore, a new rapid PCR-based method for detecting and distinguishing *Thermoascus* spp. and *B. verrucosa* from other fungi was developed using the RPB1 gene and genus-specific primer set, RPB_F2/R2.

B. nivea and *B. laguncullariae*, which are related to the genus *Thermoascus*, were shown to possess the *idh* gene and produce patulin [16)], and *Byssochlamys* and some species of *Thermoascus* were shown to have the same anamorph (*Paecilomyces*) [5)]. As *T. aurantiacus* was shown to be toxic to chicken embryos and weanling rats [23)], it was hypothesized that *Thermoascus* spp. may carry a risk of patulin production. Both *T. aurantiacus* and *B. verrucosa* were shown here to possess the *idh* gene, and each showed 99.9% identity with that of *B. nivea* at the nucleotide level. However, *T. aegyptiacus*, *T. crustaceus*, and *T. thermophiles* did not possess the *idh* gene, and formed a monophyletic clade separate from *T. aurantiacus* and *B. verrucosa* in the phylogenic tree (Fig. 1). In this study, despite abundant fungal growth, patulin was not detected in PDB or Czapek-glucose liquid medium. This was due to a defect in a gene other than *idh* involved in patulin biosynthesis, such as 6-MSA synthase [24)], or deactivation of IDH (Puel et al., 2007). These findings indicated that the risk of patulin

production by *Thermoascus* spp. is very low. As this genus is used to produce heat-resistant enzymes, this study is important not only for food safety but also for industrial enzyme production.

Using the PCR-based detection method discussed here, even dead fungi can be detected if the DNA can be extracted. Therefore, the causative organisms of food spoilage can be determined, even in cases in which morphologically identifiable fungi are not isolated from the food product. Furthermore, this new detection method is unaffected by acidic beverages. As this method can be performed rapidly with high sensitivity and requires no special expertise, it is expected to be beneficial for the surveillance of raw materials in food production.

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Table 1. Strains used in this study.

Species	Strain No.	GenBank accession No.	DNA template No.	RPB_F2/R2 detection
Thermoascus aegyptiacus	IFM 61569^{T} = NHL 2914^{T}	AB849502	4	+
Th. aurantiacus	IFM 57325 = NBRC 6766			+
	IFM 60076		3	+
	IFM 60078 = CBS 256.34			+
	IFM 60080 = CBS 100054			+
	NBRC 31693			+
	CBS 396.78	JN121671		None examined
Th. crustaceus	IFM 57326 = NBRC 9129			+
	IFM 60077^{T} = CBS 181.67^{T}	JN121591	2	+
	IFM 60232 = CBS 374.62			+
Th. thermophilus	IFM 59664 = SUN47			+
	IFM 59665 = SUN49			+
	IFM 60075^{NT} = CBS 528.71^{NT}	JN121697	1	+
Byssochlamys verrucosa	IFM 48423^{T} = IAM 13423^{T}	JN680311	5	+
B. fulva	IFM 51213^{T} = CBS 132.33^{T}	AB849503	13	-
B. nivea	IFM 51243^{T} = CBS 100.11^{T}	JN121511	12	-
B. spectabilis	IFM 52963^{T} = CBS 101075^{T}	JN121554	11	-
Hamigera avellanea	IFM 52957^{isoT} = CBS295.48^{isoT}	JN121632	15	-
H. striata	IFM 52958^{NT} = CBS 377.48^{NT}	JN121665	14	-
Rasamsonia emersonii	IFM 52961^{T} = CBS 393.64^{T}	JN121670	10	-
Talaromyces flavus	IFM 52962^{NT} = CBS 310.38^{NT}	JN121639	6	-
Ta. trachyspermus	IFM 52964T = CBS 373.48T	JN121664	7	-
Ta. wortmanii	IFM 53866^{T} = CBS 91.48^{T}	JN121669	8	-
Ta. luteus	IFM 53168^{T} = CBS 348.51^{T}	JN121656	9	-
(Out group)				
A. fumigatus	DAOM 215394	JN985124		

^{T}; ex type, ^{NT}; neotype.

Table 2. Growth and ascospore formation temperature of members of the genus *Thermoascus* and *Byssochlamys verrucosa.*

Species	Strain	Temperature (°C)	
Thermoascus aegypticus	IFM 61569	Growth	20-55
		Ascospore forming	25-40
T. aurantiacus	IFM 57325	Growth	35-57
		Ascospore forming	35-40
T. crustaceus	IFM 57326	Growth	25-50
		Ascospore forming	25-35
T. thermophilus	IFM 59664	Growth	20-53
		Ascospore forming	20-45
Byssochlamys verrucosa	IFM 48423	Growth	20-53
		Ascospore forming	Not forming

Table 3. Heat resistance of strains of genus *Thermoascus* based on *D* and z-values.

Species	Strain	D-value（min）				z-value (°C)
		Heating temperature (°C)				
		80	83	85	90	
Thermoascus aegyptiacus	IFM 61569	—	—	—	56.2	—
T. aurantiacus	IFM 57325	57.1	13.2	10.8	—	5.2
T. aurantiacus	NBRC 31693	288	50.1	16.2	—	4.0
T. thermophilus	IFM 59664	—	—	—	21.3	—
T. thermophilus	IFM 60075	—	—	—	20.5	—

—: not evaluated

Table 4. Patulin production of genus *Thermoascus* and related species

Species	Strain	Medium	Patulin (ppm)
Thermoascus thermophilus	CBS 528.71	CzL[a]	N.D.
		PDB[b]	N.D.
T. crustaceus	IFM 60077	CzL	N.D.
		PDB	N.D.
T. aurantiacus	IFM 57325	CzL	N.D.
		PDB	N.D.
Byssochlamys verrucosa	IFM 48423	CzL	N.D.
		PDB	N.D.
Byssochlamys nivea	NBRC 31351	CzL	42.8
		PDB	55.6

[a]; CzL = Czapek-glucose liquid. [b]; PDB = Potato dextrose broth.
N.D.; not ditected.

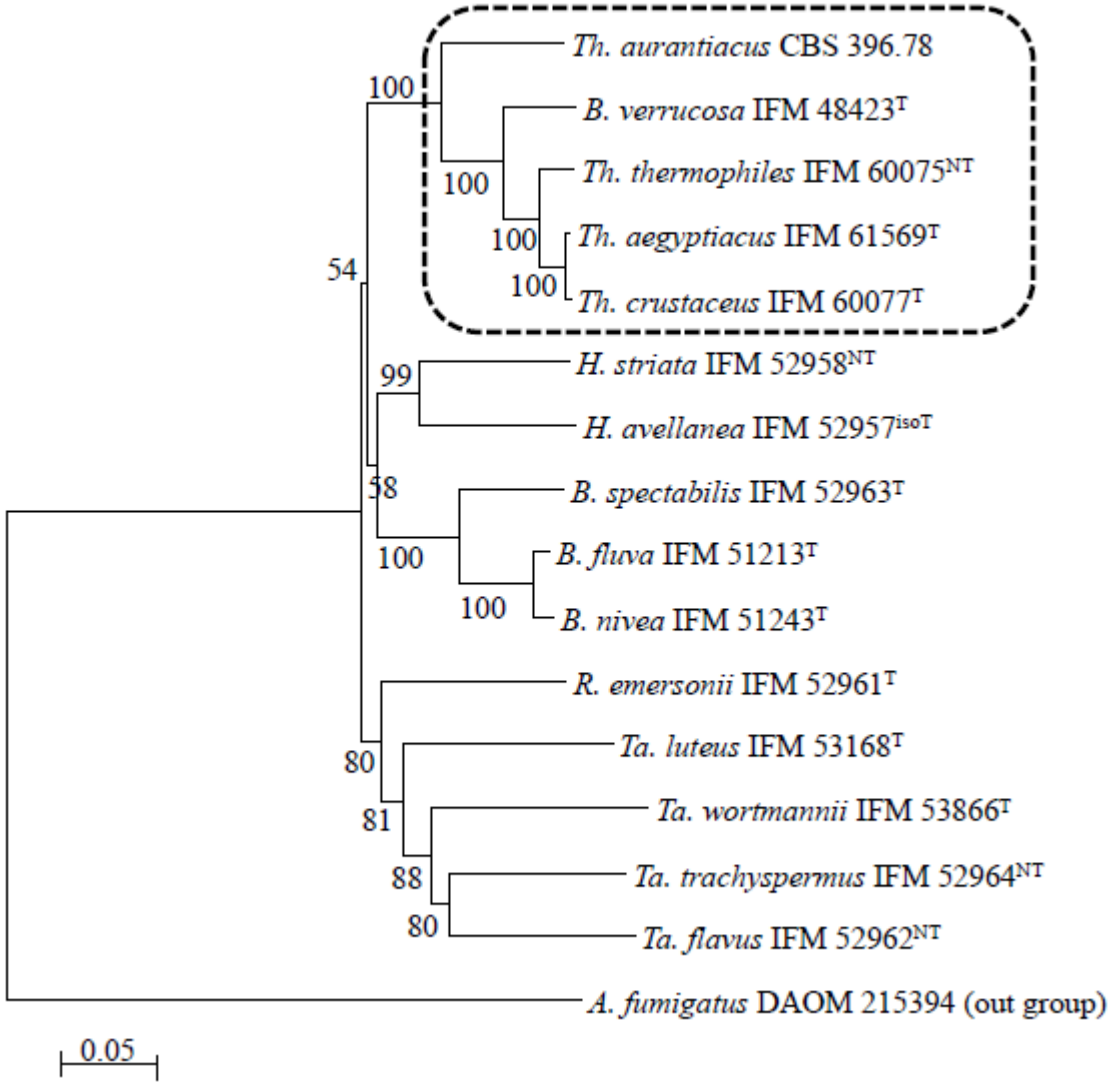

Fig. 1. Neighbor joining tree based on RPB1 gene sequences for *Thermoascus* and related species. Bootstrap values based on 1000 samplings supporting the internal branches with a probability of ≥ 50% are shown.

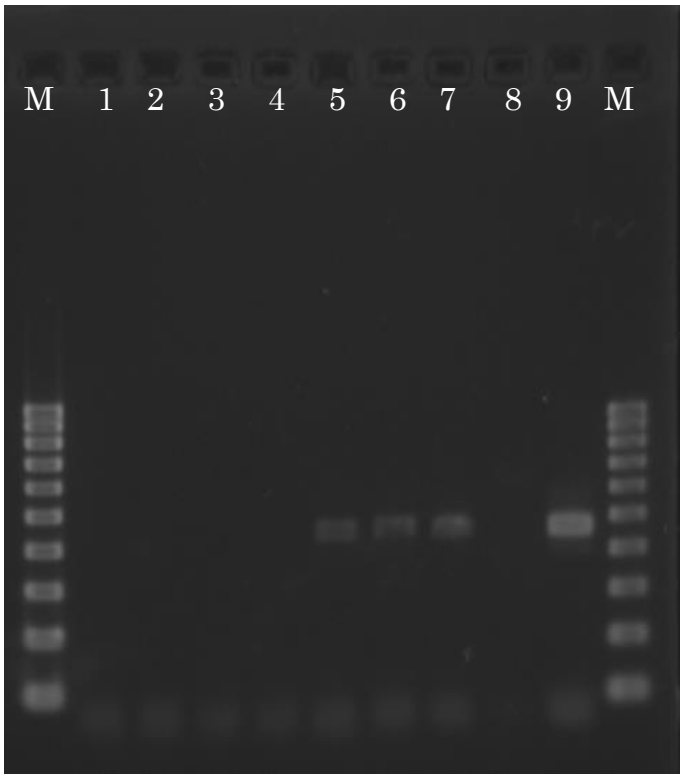

Fig. 2. PCR amplification to detect the *idh* gene using DNA of genera *Thermoascus* and *Byssochlamys* with primer set idh2444 and idh 2887. Numbers on the lanes correspond to the following markers and genomic DNA templates: M, 100-bp ladder; 1, *Th. thermophilus* IFM 60075; 2, *Th. thermophilus* IFM 59665; 3, *Th. crustaceus* IFM 60077; 4, *Th. crustaceus* IFM 57326; 5, *Th. aurantiacus* IFM57325; 6, *Th. aurantiacus* IFM60076; 7, *Byssochlamys nivea* NBRC 57325; 8, *Th. aegyptiacus* IFM 61569 and 9, *B. nivea* IFM 48423.

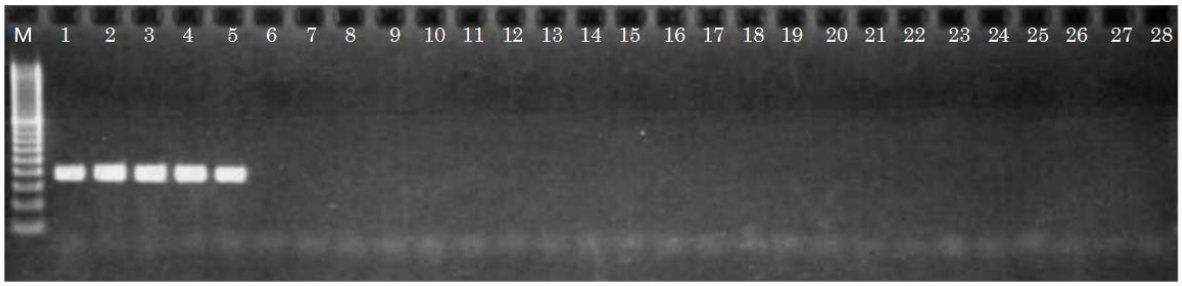

Fig.3. PCR amplification using the primer set, RPB_F2 and RPB_R2, to detect strains in the genus *Thermoascus* and *B. verrucosa*. Numbers indicate the genomic DNA templates (1 – 15) shown in Table 1: 16, *Neosartorya fischeri* IFM 57324; 17, *N. spinosa* IFM 47025; 18, *Aspergillus fumigatus* IFM 47042; 19, *A. niger* IFM 55890; 20, *A. flavus* IFM 48054; 21, *Eupenicillium brefeldianum* IFM 42321; 22, *Penicillium griseofulvum* IFM 49451; 23, *Alternaria alternata* IFM 56020; 24, *Aureobasidium pullulans* IFM 41411, 25, *Chaetomium globosum* IFM 40869; 26, *Fusarium oxysporum* IFM 50002; 27, *Trichoderma viride* IFM 51045; 28, *Cladosporium cladosporioides* IFM 46166; M, 100-bp ladder.

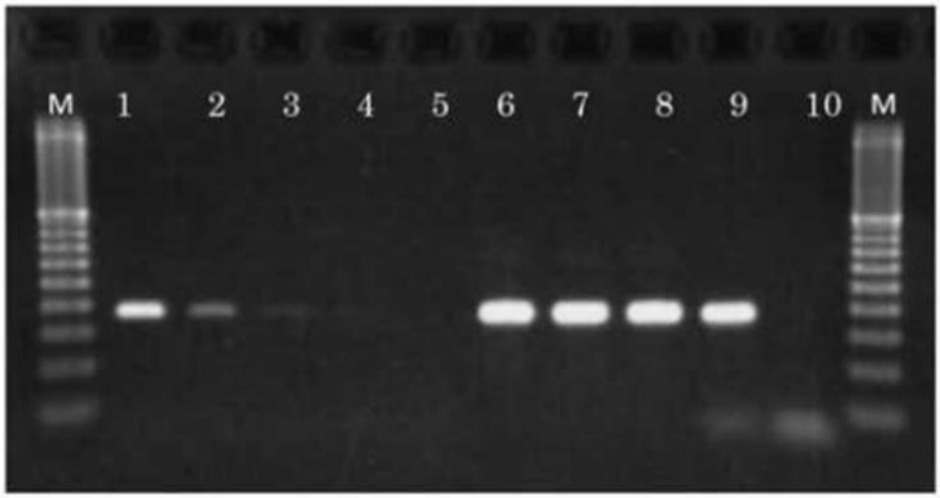

Fig. 4. Detection limit of the PCR with the RBP_F2/R2 primer set: using *T. crustaceus* IFM 60232 DNA. Lane numbers 1 – 4 refer to first PCR only and 5 – 8 refer to nested PCR. The amounts of DNA used in the PCR mixtures were as follows: 10 ng (1, 6), 1 ng (2, 7), 100 pg (3, 8) and 10 pg (4, 9), 1 pg (5, 10). M: 100 bp ladder.

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