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Original

Development of Rapid Identification and Risk Analysis of *Moniliella* Spp. in Acidic Processed Foods

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The number of spoilage incidents in the food industry attributable to a species of the genus *Moniliella* has recently increased, but the risk of food spoilage has not yet been evaluated. The purpose of this study was to develop a method to rapidly identify high-risk species and to conduct a risk analysis study of *Moniliella* spp. Acetic acid resistance of *M. acetoabutens* and ethanol resistance of *M. suaveolens* were higher than for other *Moniliella* species. All examined strains of *M. acetoabutens* developed a high tolerance to acetic acid by being cultured twice in liquid media containing low concentrations of acetic acid. These findings indicate that *M. acetoabutens* and *M. suaveolens* are high-risk species for food spoilage and must be discriminated from other fungi. We developed species-specific primers to identify *M. acetoabutens* and *M. suaveolens* using the polymerase chain reaction (PCR) to amplify the D1/D2 domain of 28S rDNA. The PCR using the primer sets designed for *M. acetoabutens* (Mac_F1/R1) and *M. suaveolens* (Msu_F1/R1) was specific to the target species and did not detect other fungi involved in food spoilage or environmental contamination. This method is expected to be effective for the monitoring of raw materials and components of the food production process.

Key words：Acetic acid resistance / Adaptation / Ethanol resistance / *Moniliella* / Rapid identification.

INTRODUCTION

Heat-resistant fungi, typically ascomycetes, are major causative agents of food spoilage incidents in heated foods (Hosoya et al., 2012, 2014; Nakayama et al., 2010; Yaguchi et al., 2012), while yeast, including the genus *Saccharomyces* and the genus *Pichia* that grow under refrigerated conditions, cause food spoilage incidents in non-heated foods (Betts et al., 1999). Although few spoilage incidents are due to basidiomycetous fungi, such incidents have been reported, as have food spoilage incidents attributed to two species, *Moniliella acetoabutens* and *M. suaveolens* (Carlos et al., 2009). *M. acetoabutens* causes spoilage in foods such as pickles and salad dressing, which are typically unaffected by spoilage due to the presence of acetic acid (Dakin et al., 1968), and it is a major causative agent of secondary contamination in vinegar (Entani and Tsukamoto, 2000). *M. suaveolens* causes the spoilage of foods such as brandy cake and plum wine, which contain alcohol, and breads made in manufacturing environments where alcohol is often sprayed as a disinfectant (Naito, 2000; Tsubouchi, 1997). Novel *Moniliella* spp. have been isolated from the raw materials of other foods such as Malbec and Chardonnay grapes used in wine (Da Rocha Rosa et al., 2002) and from meat processing environments (Thanh et al., 2012). Therefore, the food industry has focused a great deal of attention on the control of the genus *Moniliella*.

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To prevent food spoilage by fungi, high-risk species must be identified (Hitokoto et al., 1987; Morozumi, 1988). The risk of food spoilage by the genus *Moniliella* has not been evaluated to date. To prevent food spoilage incidents due to *Moniliella* spp., high-risk species of this genus must be identified. It is important to identify the presence of these high-risk species when mold is detected in raw materials and in the manufacturing environment. Due to the taxonomic integration of the genus *Trichosporonoides* into the genus *Moniliella*, the number of species in this genus has greatly increased (Carlos et al., 2009), and instances of acetic acid and ethanol resistance have been reported (Naito, 2000; Tsubouchi, 1997). Risk analysis, however, has only been performed for two species, *M. acetoabutens* and *M. suaveolens*, and there have been no reports of the risk levels of other *Moniliella* spp. Moreover, common methods for identifying the species of *Moniliella* rely on morphologic observations, similar to that for ascomycetes (Aou, 1992), and both a lengthy time (minimum of 10 days) and highly trained experts are required to perform this type of identification. Given this background and from the standpoint of microbiologic food safety, the systematic risk evaluation of *Moniliella* spp. and the development of technology to rapidly identify high-risk species are necessary.

The aims of this study were to 1) evaluate the resistance to acetic acid, ethanol, and their combination, and 2) investigate whether *Moniliella* spp. adapt and develop tolerance with repeated culturing on media containing acetic acid and/or ethanol. In addition, to identify high-risk species, we designed specific primers at the species level and used these primers to develop a rapid and versatile identification method.

MATERIALS AND METHODS

Test fungal strains and cultures

The fungal strains used in this test were provided by the Centraalbureau voor Schimmelcultures (CBS, English translation: Central Bureau of Fungal Cultures). The test strains used in this study from the genus *Moniliella* were mainly isolated from spoiled foods and food manufacturing environments, and are organized by the CBS (strain numbers are shown in Table 1). These test fungal strains were cultured on potato dextrose agar (Difco Laboratories, Inc.) media at 25℃ in the dark for 3 to 5 days.

TABLE 1. Strains used in this study.

Species name	Strain	Substrate of isolation	DNA template No.		
			Fig.2	Fig.3	Fig.4
Moniliella acetoabutens	CBS 169.66^{T} = IFM 59903^{T}	Sweet fruit sauce, containing 15% sucrose and 3% acetic acid	1	1	1
M. acetoabutens	CBS 170.66 = IFM 59904	Fruit sauce, containing 2.5% acetic acid		2	
M. acetoabutens	CBS 171.66 = IFM 59905	Sweet pickle		3	
M. acetoabutens	CBS 593.68 = IFM 59906	Acetic acid-containing product		4	
M. acetoabutens	CBS 594.68 = IFM 59907	Acetic acid-containing product		5	
M. suaveolens	CBS 126.42^{T} = IFM 60291^{T}	Milk	4		3
M. suaveolens var. *nigra*	CBS 542.78^{T} = IFM 60293^{T}	Black spot on Emmentaler cheese	5		
Moniliella sp.*	CBS 157.58 = IFM 60348	Cheese	2		2
Moniliella sp.*	CBS 221.32 = IFM 60349	Dried leaf			
Moniliella sp.*	CBS 223.32 = IFM 60350	Dried leaf			
M. fonsecae	CBS 10567 = IFM 60299	Flowers of *Byrsonima orbigniana*	12		
M. madida	CBS 240.79^{T} = IFM 60295^{T}	Margarine factory	8		
M. megachiliensis	CBS 190.92^{T} = IFM 60296^{T}	Alfalfa leafcuting bee	9		
M. mellis	CBS 350.33^{T} = IFM 60292^{T}	Honey	6		
M. nigrescens	CBS 269.81^{T} = IFM 60298^{T}	Spoiled melon jam	11		
M. oedocephalis	CBS 649.66^{T} = IFM 60297^{T}	Contents of brood cell of *Apis mellifica*	10		
M. pollinis	CBS 461.67^{T} = IFM 60351^{T}	Pollen in honeycomb	3		
M. spathulata	CBS 241.79^{T} = IFM 60294^{T}	Ghee from buffalo milk	7		

^{T}; ex type.
*; those strains are listed as *M. suaveolens* var. *nigra* on the CBS strain database (http://www.cbs.knaw.nl/Collections/DefaultInfo.aspx?Page=Home).

Acetic acid and ethanol resistance testing

Acetic acid and ethanol resistance of the *Moniliella* species and related species was evaluated based on the minimum inhibitory concentration (MIC) of these test substances on the test strains shown in Table 1. For the acetic acid resistance tests (Fig.1), acetic acid (Sigma-Aldrich Co.) was adjusted to final concentrations of 0.25%, 0.5%, 0.75%, 1.0%, 1.25%, and 1.5% (w/w) in potato dextrose broth (PDB, Difco Laboratories, Inc.) media, and then adjusted to pH 5.0 with sodium hydroxide (Wako Pure Chemical Industries, Ltd.). For the ethanol resistance tests, ethanol (Wako Pure Chemical Industries, Ltd.) was adjusted to final concentrations of 2.5%, 5.0%, 7.5%, 10.0%, and 12.5% (v/w) in PDB. In addition, to determine the MIC of the combination of acetic acid and ethanol against each test fungus, acetic acid at final concentrations of 0.5%, 0.75%, and 1.0% was added to PDB and adjusted to pH 5.0 using sodium hydroxide. Ethanol was then added to the PDB media at concentrations of 1%, 2.5%, 5%, and 7.5% and used for culture of the five *M. acetoabutens* strains (CBS 169.66^T, CBS 170.66, CBS 171.66, CBS 593.68 and CBS 594.68). Media preparations with the addition of no acetic acid and/or ethanol were used as control samples.

To evaluate the MIC of acetic acid, ethanol, and their combination, the test fungi were cultured in potato dextrose agar at 25℃ for 5 days, and then the hyphae and conidia were suspended in saline at 10^5 cfu/ml to produce the test fungal solution. Next, 10 µl of each test fungal solution was inoculated in PDB medium containing a reagent and cultured at 25℃ for 7 days. Growth was evaluated visually and the concentration that produced no growth (100% growth inhibition) was recorded as the MIC. The evaluation criteria for measurement of the MIC of acetic acid, ethanol and their combination were applied to determine the resistance to acetic acid alone, ethanol alone, and in combination. All MIC test studies were conducted in duplicate.

Tolerance of *M. acetoabutens* to acetic acid and *M. suaveolens* to ethanol

To evaluate the adaptation and tolerization to acetic acid, five strains of *M. acetoabutens* reported to be acetic acid resistant (CBS 169.66^T, CBS 170.66, CBS 171.66, CBS 593.68 and CBS 594.68) were used. Adaptation culturing of the fungi was performed 0, 1, and 2 times using PDB medium (pH 5.0) containing 1% acetic acid at 25℃ for 7 days, in which all strains could grow. The cultured fungi were centrifuged (5000 g, 10 min), the pellet suspended in 10 ml saline, and the fungi thoroughly mixed using a Vortex-Genie 2 (Scientific Industries, Inc.). After mixing, the fungi were inoculated (10 µl) in PDB medium (pH 5.0) containing acetic acid at concentrations of 1.0%, 1.25%, 1.5%, 1.75%, 2.0%, or 2.5%, and cultured at 25℃ for 7 days. After incubation, the MIC of acetic acid against each fungal strain was determined based on the criteria for fungal growth described for acetic acid and ethanol resistance testing, and a judgment was made on whether adaptation occurred over the course of the culturing.

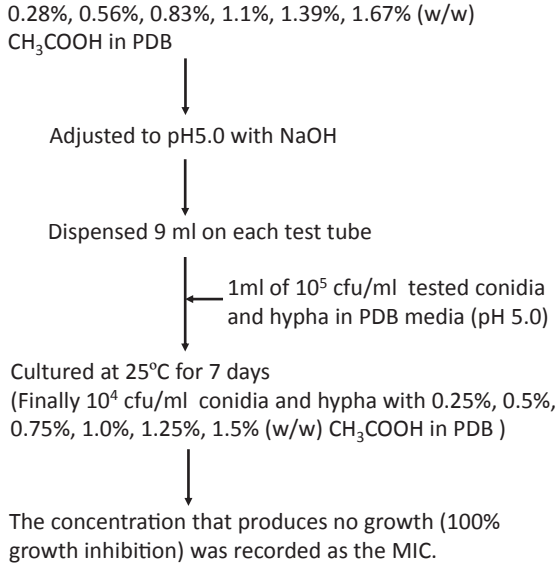

FIG. 1. Resistance test of *Moniliella* strains against acetic acid.

To evaluate the adaptation and tolerization to ethanol, two strains of *M. suaveolens* with reported ethanol resistance (CBS 126.42^T and CBS 350.33) were used. Fungi were cultured 0, 1, and 2 times in PDB medium (pH 5.0) containing 10% ethanol at 25℃ for 7 days. The cultured fungi were centrifuged (5000 g, 10 min), the pellet suspended in 10 ml saline, and the fungi thoroughly mixed using a Vortex-Genie 2 (Scientific Industries, Inc.). After mixing, the fungi were inoculated (10 µl) on PDB medium (pH 5.0) containing ethanol at concentrations of 10%, 12.5%, 15%, 17.5%, or 20%; and then cultured at 25℃ for 7 days. After culturing, the MIC of ethanol against each fungal strain was determined based on the criteria for fungal growth described for the acetic acid and ethanol resistance testing, and a judgment was made on whether adaptation occurred over the course of culturing.

Phylogenetic examination and detection using specific primers for *M. acetoabutens* and *M. suaveolens*

The genomic DNA was extracted using PrepMan Ultra Reagent (Applied Biosystems) kits according to the manufacturer's instructions. The concentration of the extracted DNA solution was measured using NanoDrop (LMS Co., Ltd.) and adjusted to 5 ng/µl

using ultraPURE distilled water (Invitrogen). The base sequence of the D1/D2 domain of each species was searched in the DNA Data Bank of Japan (DDBJ:http://www.ddbj.nig.ac.jp/). For species not registered in the database, the D1/D2 domain was amplified and sequenced. The D1/D2 domain was amplified by polymerase chain reaction (PCR) using the primer pair NL1, 5'-GCATATCAATAAGCGGAGGAAAAG-3' and NL4, 5'-GGTCCGTGTTTCAAGACGG-3' (Kurtzman and Robnett, 1997), and illustra™ PuReTaq Ready-To-Go PCR Beads (GE Healthcare) with 35 cycles of denaturation at 95℃ for 1 min, annealing at 53℃ for 1 min, and elongation at 72℃ for 1 min. PCR products were labeled using BigDye terminator Ver. 1.1 (Applied Biosystems), and the sequences were determined using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Alignment analysis of the obtained base sequences was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html), and neighbor-joining analysis was performed using NJPLOT (http://pbil.univ-lyon1.fr/software/njplot.html) to generate a phylogenetic tree for members of the genus *Moniliella* based on the D1/D2 domain.

Based on the D1/D2 domain sequence determined for members of the genus *Moniliella*, the base sequences of *M. acetoabutens* and *M. suaveolens* and species-specific primers were determined. The homology of the D1/D2 domain was calculated by multiple sequence alignment analysis using ClustalW. We designed the species-specific primers based on the criteria of conserving the 3' end of the extension in *M. acetoabutens* and *M. suaveolens* and selecting base sequences with little similarity to other fungi.

The extracted DNA and species-specific primers were used for PCR, which was performed using a Premix Taq PCR Kit (Takara Bio Inc.) with amplification on the Thermal Cycler Dice (Takara Bio Inc.) following the manufacturer's instructions. The amplification conditions were heat denaturation at 97℃ for 10 min, followed by 30 cycles of 97℃ for 1 min, 61℃ for 1 min, and 72℃ for 1 min. The PCR product (2 µl) and 0.5 µl of 5×Nucleic Acid Sample Loading Buffer (Bio-Rad Laboratories) were mixed well, the samples were electrophoresed on 2% agarose gel (135 V, 25 min), the DNA was stained with SYBR Safe (Invitrogen), and the presence or absence of the amplified target DNA was confirmed.

To assess the detection sensitivity of the species-specific primers designed for *M. acetoabutens* and *M. suaveolens*, genomic DNA of the target fungi was serially diluted (10 ng to 10 pg), and the first PCR and nested PCR were performed using 1 µl of diluted DNA solution (final concentrations, 0.33 ng/µl to 0.33 pg/µl). The PCR was performed using the same conditions described above. For the nested PCR, the first PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics), and then PCR was performed again using the same primer pair and conditions described above. The PCR products were electrophoresed on 2% agarose gel (135 V, 25 min), the DNA was stained with SYBR Safe (Invitrogen), and the presence or absence of amplified DNA was confirmed.

RESULTS

Acetic acid and ethanol resistance of *Moniliella* spp.

Table 2 shows the MIC of acetic acid and ethanol against each *Moniliella* spp. All five tested strains of *M. acetoabutens* (CBS 169.66^T, CBS 170.66, CBS 171.66, CBS 593.68 and CBS 594.68) exhibited high resistance to acetic acid and grew in the presence of 1.25% acetic acid. *M. suaveolens* CBS 126.42^T and *M. suaveolens* var. *nigra* CBS 542.78^T, and related species *M. spathulata* CBS 241.79^T grew in the presence of 0.75% acetic acid, but no other fungi showed any growth in the presence of 0.75% acetic acid. For ethanol resistance, *M. suaveolens* CBS 126.42^T and *M. suaveolens* var. *nigra* CBS 542.78^T was used for testing, and both strains grew at an ethanol concentration of 10%, demonstrating very high ethanol resistance. All other species of the genus *Moniliella* had an MIC of 7.5%

TABLE 2. Minimum inhibitory concentration (MIC) of acetic acid and ethanol.

Species name	Strain No.	MIC of acetic acid (%)	MIC of ethanol (%)
Moniliella acetoabutens	CBS 169.66^T	1.5	7.5
M. acetoabutens	CBS 170.66	1.5	7.5
M. acetoabutens	CBS 171.66	1.5	7.5
M. acetoabutens	CBS 593.68	1.5	7.5
M. acetoabutens	CBS 594.68	1.5	7.5
M. suaveolens	CBS 126.42^T	1.0	12.5
M. suaveolens var. *nigra*	CBS 542.78^T	1.0	12.5
M. fonsecae	CBS 10567	0.5	7.5
M. madida	CBS 240.79^T	0.75	7.5
M. megachiliensis	CBS 190.92^T	0.5	7.5
M. nigrescens	CBS 269.81^T	0.5	7.5
M. oedocephalis	CBS 649.66^T	0.75	7.5
M. pollinis	CBS 461.67^T	0.5	7.5
M. spathulata	CBS 241.79^T	1.0	7.5
Penicillium citrinum	IFM 47483	0.5	2.5

^{T}; ex type.

ethanol, exhibiting very high ethanol resistance at the genus level compared to a 2.5% MIC for *Penicillium citrinum* IFM 47483, a common mold ubiquitous in the environment.

The MIC of ethanol for five strains of *M. acetoabutens* CBS 169.66^{T}, CBS 170.66, CBS 171.66, CBS 593.68 and CBS 594.68 at each acetic acid concentration (combined effects of acetic acid and ethanol) is shown in Table 3. The addition of acetic acid tended to decrease the MIC of ethanol of these strains. In the presence of 0.5% acetic acid, the MIC of ethanol was 7.5% for all fungal strains. As the acetic acid concentration increased, however, the resistance of the CBS 169.66^{T} strain significantly increased compared to that of the other fungal strains.

TABLE 3. MIC (%) of ethanol in the presence of acetic acid for five strains of *Moniliella acetoabutens*.

Strain No.	Concentration of acetic acid (%)		
	0.5	0.75	1.0
CBS 169.66^{T}	7.5	7.5	5
CBS 170.66	7.5	5	2.5
CBS 171.66	7.5	5	2.5
CBS 593.68	7.5	5	2.5
CBS 594.68	7.5	5	2.5

^{T}; ex type.

Adaptation and tolerization of the genus *Moniliella* to acetic acid

Adaptation and tolerization testing results using acetic acid for the five strains of *M. acetoabutens* CBS 169.66^{T}, CBS 170.66, CBS 171.66, CBS 593.68 and CBS 594.68 are shown in Table 4. In the absence of culturing or culturing only one time on media containing 1% acetic acid, the MIC for all fungal strains was 1.5%, but culturing two times on media containing 1% acetic acid led to weak growth of all strains with 1.5% acetic acid. In particular, for the CBS 169.66^{T} strain, growth was observed even at 1.75% acetic acid. On the other hand, after repeated cultureing of *M. suaveolens* CBS 126.42^{T} on media containing 10% ethanol, there was no growth at 15% for either strain tested. No adaptation or tolerization was observed (data not shown).

Phylogenetic analysis of the genus *Moniliella*

New sequences of *M. suaveolens* var. *nigra* CBS 157.58, CBS 221.32, and CBS 223.32 were deposited to the DNA Data Bank of Japan with the accession numbers LC004102, LC004103, and LC004104, respectively. The phylogenetic tree of 13 reported species of the genus *Moniliella* (Thanh et al., 2013) and 4 *M. suaveolens* var. *nigra* based on the D1/D2 domain is shown in Fig.2. *M. suaveolens* CBS 126.42^{T} (AF335524) and *M. suaveolens* var. *nigra* CBS 542.78^{T} (AF335520) were identical at 99.1% (560/565 bp) of sequence. Moreover, there were no major differences in the

TABLE 4. Adaptation and tolerization to acetic acid for the five strains of *Moniliella acetoabutens*.

Adaptation culture times	Strain No.	Concentraion of acetic acid (%)			
		1.5	1.75	2.0	2.5
0	CBS 169.66^{T}	−	−	−	−
	CBS 170.66	−	−	−	−
	CBS 171.66	−	−	−	−
	CBS 593.68	−	−	−	−
	CBS 594.68	−	−	−	−
1	CBS 169.66^{T}	−	−	−	−
	CBS 170.66	−	−	−	−
	CBS 171.66	−	−	−	−
	CBS 593.68	−	−	−	−
	CBS 594.68	−	−	−	−
2	CBS 169.66^{T}	±	±	−	−
	CBS 170.66	±	−	−	−
	CBS 171.66	±	−	−	−
	CBS 593.68	±	−	−	−
	CBS 594.68	±	−	−	−

^{T}; ex type. −: No growrh. ±: Weak growth compared to the control.

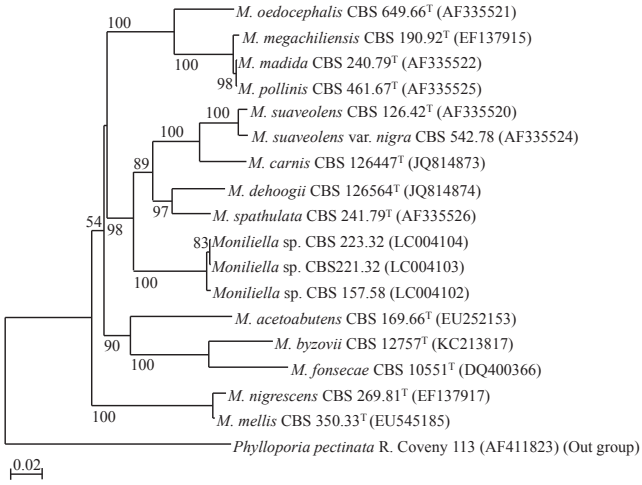

FIG. 2. Neighbor-joining tree from sequences of the D1/D2 domain of 28S rDNA on *Moniliella*. Each number indicates the percentage of bootstrap samplings derived from 1000 samples, supporting the internal branches of 50% or higher.

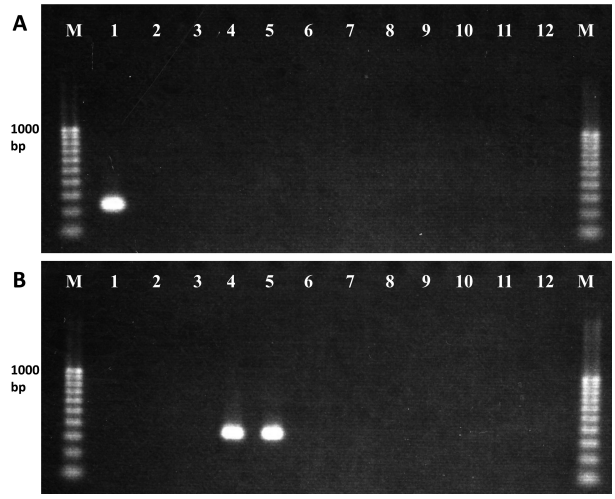

FIG. 3. PCR amplifications using the primer sets designed for *Moniliella acetoabutens* (Mac_F1/R1, A) and *M. suaveolens* (Msu_F1/R1, B). Numbers indicate the genomic DNA templates (1 to 12) shown in Table 1. M: 100 bp-ladder.

morphologic characteristics (de Hoog, 1979). On the other hand, *M. suaveolens* var. *nigra* CBS 157.58, CBS 221.32, and CBS 223.32 were separated from *M. suaveolens* CBS 126.42^T and other species, *M. spathulata* CBS 241.79^T, *M. dehoogii* CBS 126564^T, and *M. carnis* CBS 126447^T were placed between three strains of *M. suaveolens* var. *nigra* CBS 157.58, CBS 221.32 and CBS 223.32, and the type strain CBS 126.42^T. Their branches were supported by high bootstrap values. Thus, it is appropriate to consider these strains as distinct species from *M. suaveolens*, and we treated them as *Moniliella* sp.

Evaluation of specific primers for the genus *Moniliella*

Species-specific primers for *M. acetoabutens* and *M. suaveolens* were designed: (*M. acetoabutens*: Mac_F1, 5'-GTGCTTGGCCTGGCTTGTG-3' and Mac_R1, 5'-GGCAGACACCTGGAGAACGA-3'; *M. suaveolens*: Msu_F1, 5'-CGTGCTTGGTCTGGCCTG-3' and Msu_R1, 5'-GGCTATAACACTCCCCCCAGA-3'). PCR was performed using Mac_F1/R1 and Msu_F1/R2, and the presence or absence of PCR-amplified products and their sizes were confirmed by electrophoresis. With PCR using Mac_F1/R1 and *M. acetoabutens* DNA, we detected amplification products with a size of ~200 bp, as expected from the primer design (Fig.3). No amplification products were observed, however, for other *Moniliella* spp. or other species closely related to food spoilage (Fig.3).

With PCR using Msu_F1/R2 and DNA of *M. suaveolens* CBS 126.42^T and *M. suaveolens* var. *nigra* CBS 542.78^T, we detected clear amplification products of ~300 bp (Fig.4). No gene amplification products were

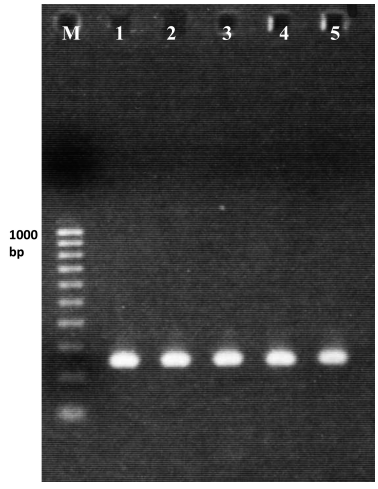

FIG. 4. PCR amplifications using the primer set designed for *Moniliella acetoabutens* (Mac_F1/R1, A). Numbers indicate the genomic DNA templates (1 to 5) shown in Table 1. M: 100 bp-ladder.

observed, however, for other *Moniliella* spp., including *Moniliella* sp. CBS 157.58, CBS 221.32, and CBS 223.32, or other species closely related to food spoilage (Fig.3). PCR using these two primer sets, Mac_F1/R1 and Msu_F1/R2, did not detect other species involved in food spoilage or environmental contamination (Fig.5).

With PCR using the *M. acetoabutens*-specific primer set Mac_F1/R1 and *M. acetoabutens* CBS169.66^T strain DNA, PCR products from the first PCR were detected with the use of 100 pg of template DNA, but no PCR products were detected with 10 pg. With nested PCR, however, PCR products were detected even with

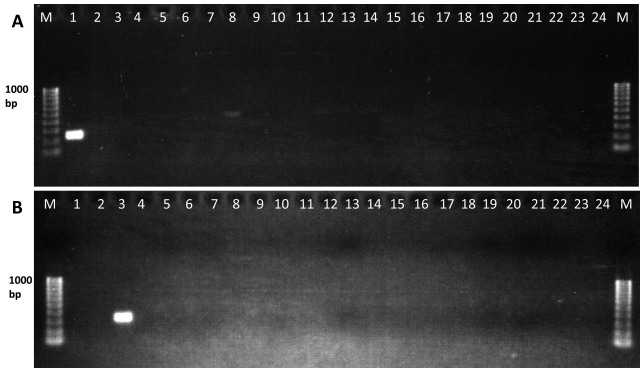

FIG. 5. PCR amplifications using the primer sets designed for *Moniliella acetoabutens* (Mac_F1/R1, A) and *M. suaveolens* (Msu_F1/R1, B). Numbers indicate the genomic DNA templates (1 to 3) shown in Table 1; 4, *Asperguillus niger* IFM 55890^{T}; 5, *A. flavus* IFM 48054^{NT}; 6, *A. nidulans* IFM 57839^{T}; 7, *Byssochlamys spectabilis* IFM 52963^{T}; 8, *B. fulva* IFM 51214^{T}; 9, *B. nivea* IFM 48422^{T}; 10, *Talaromyces flavus* IFM 52962^{NT}; 11, *T. macrosporus* IFM 48407 ; 12, *T. trachysperumus* IFM 52964^{T}; 13, *T. wortmannii* IFM 53866^{NT}; 14, *T. luteus* IFM 53168^{NT}; 15, *Hamigera avellanea* IFM 52957; 16, *H. striata* IFM 52958; 17, *Eupenicillium brefeldianum* IFM 42321^{NT}; 18, *Penicillium griseofulvum* IFM 47730^{NT}; 19, *Alterraria alternata* IFM 56020; 20, *Aurerobasidium pullulans* IFM 41411; 21, *Chaetomium globosum* IFM 40871; 22, *Fusarium oxysporum* IFM 50002; 23, *Trichoderma viride* IFM 41604; 24, *Cladosporium cladosporioides* IFM 46166^{NT}; M, 100 bp-ladder. ^{T}; ex type. ^{NT}; ex neotype.

10 pg of template DNA. In addition, the detection limits were similar for the *M. suaveolens* specific primer set Msu_F1/R1 (data not shown).

DISCUSSION

We first evaluated the resistance to ethanol and acetic acid, which are commonly used as preservatives, in most species of *Moniliella*, which are causative agents of food spoilage or found frequently in spoiled foods. *M. suaveolens* CBS 126.42^{T} and *M. suaveolens* var. *nigra* CBS 542.78^{T} were able to grow even in cultures containing 10% ethanol, conditions under which other species do not grow. *M. acetoabutens* CBS 169.66^{T}, CBS 170.66, CBS 171.66, CBS 593.68 and CBS 594.68 had extremely high acetic acid resistance and the MIC of ethanol did not decrease in the presence of 0.5% acetic acid. Interestingly, the MIC of acetic acid increased for all *M. acetoabutens* strains tested by the second round of culture in liquid medium containing 1.0% acetic acid, a phenomenon called adaptation or tolerization. This is the first report regarding the adaptation or tolerization of *M. acetoabutens*. Given that food is stored under acidic conditions for long periods, *M. acetoabutens* will adapt and develop the ability to grow in acidic foods. Therefore, our findings led us to conclude that *M. acetoabutens* and *M. suaveolens* (including *M. suaveolens* var. *nigra*) are high-risk fungi in acidic foods. There is no noticeable difference in the phylogeny and morphology between *M. suaveolens* and *M. suaveolens* var. *nigra*, and their MICs of acetic acid and ethanol are identical. It is accordingly needed to re-examine the taxonomic position of *M. suaveolens* var. *nigra*.

Both *M. acetoabutens* and *M. suaveolens* form chlamydospores, which have high heat resistance (Aou, 1992), and *M. acetoabutens* exhibits some degree of resistance to preservation agents, such as sorbic acid (Dakin et al., 1968). Our findings and those of previous studies indicated that *M. acetoabutens* and *M. suaveolens* pose a high risk for food contamination and must be distinguished from other fungi to achieve appropriate control in the food industry.

Control technologies for food spoilage include the use of thermal pasteurization, preservation agents, and filtering. Thermal treatment may lead to deterioration in taste, consumers tend to want food products that avoid the overuse of preservation agents, and filtering is impossible for foods that contain solid components, such as salad dressing. Therefore, we consider the prevention of contamination to be the most practical way of preventing food spoilage. If contamination by *M. acetoabutens* and *M. suaveolens* occurs, identification of these organisms is very important, because microbiologic risks depend on the species of the causative agent. Conventional morphologic identification methods (Aou, 1992), however, are time-consuming and require a high skill level, and identification methods based on molecular genetics require very costly equipment. Moreover, contamination with multiple species DNA interferes with sequence analysis for the target species.

We developed species-specific primers to identify *M. acetoabutens* and *M. suaveolens* based on PCR-based amplification of the D1/D2 domain. The D1/D2 sequence of the novel species differed by 15% or more from any related species (Rosa et al, 2009), and it was therefore suitable for the identification of the species of *Moniliella*. PCR using the primer sets designed for *M. acetoabutens* (Mac_F1/R1) and *M. suaveolens* (Msu_F1/R1) produced species-specific PCR products. The sensitivity using Mac_F1/R1 was 100 pg of DNA for the first PCR, but improved to 10 pg with nested PCR. These results indicate that detection using species-specific primers has very high sensitivity. PCR using these two primer sets did not detect other fungi involved in food spoilage or environmental contamination. Using this PCR method, even dead fungi can be identified if the DNA can be extracted. Therefore, the causative organisms of food spoilage can be determined, even in cases

in which fungi that could be identified based on morphologic criteria are not isolated from the food product. As this method can be performed rapidly with high sensitivity and requires no special expertise, it is expected to be beneficial for the monitoring of raw materials and food production processes.

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