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PCR–based fingerprinting and identification of contaminative fungi isolated from rye breads

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Abstract. Fungi are the most frequent cause of microbial spoilage in baked products, including rye bread. As the baking process destroys fungal spores in bread, the post–processing is the main source for mould contamination. Rapid detection methods are needed to track down the origin of the contamination source. In the present research we used a combined molecular approach consisting of PCR–fingerprinting with an M13 primer and further identification of each genotype by amplification and sequencing of the Internal Transcribed Spacer region, the β–tubulin gene and the D1/D2 region of the large subunit of the 28S rDNA. Different rye breads from five bakeries were stored plastic–packed for one month and the fungal colonies with unique morphology were isolated from the bread surfaces. Based on random amplified polymorphic DNA analysis using M13 primer 50 fungal isolates were clustered into eight groups and identified as *Aspergillus chevalieri*, *Aspergillus flavus/oryzae*, *Aspergillus niger*, *Aspergillus tubingensis*, *Penicillium citrinum*, *Penicillium corylophilum, Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* species. Sequencing of the β–tubulin gene and the ITS region showed an equal efficiency for the identification of *Penicillium* species, whereas only the sequence of the β–tubulin gene allowed us to identify most isolates from the genus *Aspergillus* including closely–related black–spored *Aspergillus* species. Yeasts were identified at the species level based on the sequences of the Internal Transcribed Spacer region and the D1/D2 region.

Key words: Mould identification, Yeast identification, PCR–fingerprinting, ITS region, D1/D2 region, β–tubulin gene, rye sourdough breads.

INTRODUCTION

Fungi are the most frequent cause of microbial spoilage in baked products (Legan, 1993; Saranraj & Geetha, 2012). According to Malkki & Rauha (2000) up to 5% of the total bread produced yearly worldwide is lost due to fungal deterioration. Fungal spores are killed by heat treatment during the baking process and thus, contamination occurs post baking through the air and by direct contact with processing equipment during cooling, slicing, and packaging (Knight & Menlove, 1961; Legan, 1993).

Dough acidification is an important step in traditional rye bread technology. Sourdough, a mixture of water and flour fermented by lactic acid bacteria (LAB), is routinely used as acidifier (Kulp & Lorenz, 2003). Low pH (3.5–4.8) of classical rye breads combined with metabolites produced by LAB during fermentation process bio–

protect against some bacterial spoilage (Schnürer & Magnusson, 2005; Gerez et al., 2009; Dalie et al., 2010). Nevertheless, rye breads can be contaminated by a range of different moulds. Species from the *Penicillium* and *Aspergillus* genera seem to predominate (Lund et al., 1996). *Penicillium verrucosum*, *Aspergillus ochraceus*, *Aspergillus bombycis*, and many other representatives of these two genera are well known mycotoxin producers, including aflatoxins B1, B2, G1, G2, and ochratoxin A (Schmidt, 2003; Varga et al., 2011). Dich et al. (1979) found an aflatoxin producing *Aspergillus flavus* in spoiled rye bread and ochratoxigenic *A. ochraceus* was isolated from mouldy bread in Italy (Visconti & Bottalico, 1983). Thus, in addition to economic losses, public health problems cannot be excluded (Legan, 1993; Bento et al., 2009; Duarte et al., 2009; Gerez et al., 2014).

Only a few papers describing contaminative fungi isolated from rye breads have been published and all of them are based only on morphological identification of fungal isolates (Dich et al., 1979; Spicher, 1985; Lund et al., 1996). However, recent studies revealed that phenotypic–based classification of closely–related mould species can be confusing and lead to misidentification (Samson et al., 2007; Silva et al., 2011; Jang et al., 2012). In addition, morphological identification cannot be used for contamination source tracking at strain level. Instead, DNA based methods should be used for it. Information regarding effectiveness of different molecular markers for identification of food contaminating fungi is so far limited (Le Lay et al., 2016; Garnier et al., 2017). Presently, there is no single molecular marker or method advised for fungal identification or genotyping (Vanhee et al., 2010; Araujo, 2014). Sequencing of Internal Transcribed Spacer (ITS) region, D1/D2 region of 28S subunit, β–tubulin, actin, calmodulin and RPB2 gene regions are used for identification (Vanhee et al., 2010; Araujo, 2014).

The aims of our research were to isolate contaminative fungi from rye breads supplied by different Estonian bakeries and to test the effectiveness of different molecular markers in identifying common rye bread contaminative fungi. Discrimination level of random amplified polymorphic DNA (RAPD) analysis using M13 primer followed by sequencing of β–tubulin gene, ITS region and D1/D2 region of 28S subunit for different fungal species was evaluated in order to compose fast and reliable protocol for identification and tracking mould contaminations in baking industry.

MATERIALS AND METHODS

Bread samples and fungi isolation

Ten sliced sourdough rye breads produced by five different Estonian bakeries, designated A, B, C, D and E were purchased. Loaves were made without any preservatives and packed into plastic bags. The presence of fungal colonies on the surface of each loaf was evaluated visually after one, two, three, and four weeks of storage at room temperature. At the end of the fourth week all fungal colonies with different morphology were isolated from the surface of each bread onto Sabouraud Dextrose agar (10.0 g l^{-1} peptone, 12.0 g l^{-1} agar, 40.0 g l^{-1} D–glucose, pH = 5.3). These fungi were then cultivated at 25 °C for 7 days and isolated to obtain monosporal cultures. Pure cultures were cultivated on Yeast Extract Sucrose agar (YES) (yeast extract 20 g l^{-1},

sucrose 150 g l^{-1}, $MgSO_4*7H_2O$ 0.5 g l^{-1}, agar 20 g l^{-1}) at 25 °C for 7 days and stored at + 4 °C for further use.

DNA extraction from fungal cultures

The pure fungal cultures were cultivated in Sabouraud Dextrose broth (10.0 g l^{-1} peptone, 40.0 g l^{-1} D–glucose, pH = 5.3) at 25 °C for 5 days. DNA from the resulting fungal biomass was extracted according to Azevedo et al. (2000).

Random amplified polymorphic DNA analysis using M13 primer

A PCR reaction was performed in 25 µl volumes containing 100 ng of fungal DNA, 40 pmol of M13 primer (5'–GAGGGTGGCGGTTCT–3') (Meyer et al., 1999) (Microsynth AG, Switzerland) and 5 µl of 5x HOT FIREPol® Blend Master Mix (Solis BioDyne, Estonia). The PCR was performed as it was described by Meyer et al. (1999) with some modifications. Initial hold at 95 °C for 15 minutes was followed by 35 cycles of 94 °C for 20 seconds, 50 °C for 1 minute, and 72 °C for 20 seconds with a final extension at 72 °C for 6 minutes. The amplified DNA was then analyzed on 15 g kg^{-1} agarose gel at 70 V for 45 minutes and visualized under UV light.

Identification of fungal isolates

The ITS region was amplified using ITS1 (5'–TCCGTCGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') (Kwiatkowski et al., 2012) (Microsynth AG, Switzerland) primer pair. D1/D2 region of the 28S rDNA was amplified using forward primer NL–1 (5′–GCATATCAATAAGCGGAGGAAAAG–3') and reverse primer NL–4 (5′–GGTCCGTGTTTCAAGACGG–3') (Kwiatkowski et al., 2012) (Microsynth AG, Switzerland). For each primer set PCR reactions were performed in 50 µl volumes containing 100 ng of fungal DNA, 40 pmol of each primer, and 10 µl of 5x HOT FIREPol®Blend Master Mix (Solis BioDyne, Estonia) with the remaining volume consisting of ultrapure water. Amplifications of ITS region and D1/D2 region were performed according to Kwiatkowski et al. (2012) with minor modifications. Amplification of ITS region was performed with initial denaturation at 95 °C for 15 minutes followed by 30 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 1 minute and a final extension at 72 °C for 6 minutes. For the amplification of D1/D2 region after initial denaturation at 95 °C for 15 minutes 30 cycles of 94 °C for 15 seconds, 55 °C for 30 seconds and 68 °C for 2 minutes with a final extension at 68 °C for 5 minutes were performed.

The β–tubulin gene sequences were amplified using Bt2a (5'– GGTAACCAAATCGGTGCTGCTTTC–3') and Bt2b (5'– ACCCTCAGTGTAGTGACCCTTGGC–3') (Zampieri et al., 2009) primer pair (Microsynth AG, Switzerland). PCR protocol was modified from Silva et al. (2011). The 50 µl of PCR mixture contained 20 ng of fungal DNA, 40 pmol of each primer, and 10 µl of 5x HOT FIREPol®Blend Master Mix (Solis BioDyne, Estonia) with the remaining volume consisting of ultrapure water. The mixture was subjected to the following amplification program: initial hold at 95 °C for 15 minutes; followed by 35 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds; followed by a final extension at 72 °C for 5 minutes.

PCR products were purified with GeneJET PCR Purification kits (Thermo ScientificTM, Tartu, Estonia) and sequencing of the fragments was carried out at a commercial facility (Estonian Biocenter, Tartu, Estonia). The sequences obtained were compared with the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, USA).

RESULTS AND DISCUSSION

The growth of fungal colonies on sourdough rye breads obtained from bakeries B, C and D was observed during the first week of storage, whereas breads from bakeries A and E got spoiled at the end of third and fourth week, respectively (Table 1). In total, 50 colonies were picked up from studied breads and the monosporal cultures were obtained. Detected fungal morphological biodiversity varied among breads produced by different bakeries. Two fungal morphotypes were detected on A_{bakery} (A1, A111) and C_{bakery} breads (C111, C121) and three morphotypes on breads from B_{bakery} (B111, B123, B213) (Fig. 1). The most morphologically diverse fungal community was observed on D_{bakery} breads (D131, D121, D221, D231, Fig. 1), whereas in samples from E_{bakery} only one type of fungi was detected (E1, Fig. 1).

Table 1. Identified fungal species in bread samples from five Estonian bakeries (A, B, C, D and E) and the duration of mould–free shelf life

Representatives of isolated fungal strains	RAPD pattern	Presence in fungal population of each bakery				
		A	B	C	D	E
Penicillium corylophilum A111, C111	RAPD I	+	–	+	–	–
Wickerhamomyces anomalus A1	RAPD II	+	–	–	–	–
Aspergillus niger B213, D231	RAPD III	–	+	–	+	–
Aspergillus flavus/oryzae B111, D131	RAPD IV	–	+	–	+	–
Aspergillus tubingensis B123, C121	RAPD V	–	+	+	–	–
Aspergillus chevalieri D121	RAPD VI	–	–	–	+	–
Saccharomyces cerevisiae E1	RAPD VII	–	–	–	–	+
Penicillium citrinum D221	RAPD VIII	–	–	–	+	–
Mould–free shelf life, weeks		< 3	< 1	< 1	< 1	> 3

Most colonies isolated from A_{bakery} breads in the end of the storage period were of the white variety and grew to about 4 cm in diameter on YES agar without any clear signs of sporulation (A111, Fig. 1). A minority of isolates produced smaller colonies on YES agar without forming mycelium (A1, Fig. 1). Upon microscopic examination of A1 type isolates we detected the presence of budding yeast–like cells.

Moulds isolated from B_{bakery} breads produced large pale – yellow colonies with yellow and white outlines (B111, Fig. 1) or large ivory colonies with pale– and dark–brown conidia respectively and rich sporulation (B123, B213, Fig. 1). A part of moulds isolated from C_{bakery} breads created colonies similar to A111 isolates (C111, Fig. 1), whereas others formed large ivory colonies with pale–brown spores in the middle and were rather similar to B123 isolates (C121, Fig. 1). Moulds isolated from D_{bakery} breads produced large pale–yellow colonies similar to B111 isolates (D131, Fig. 1), small (about 15 mm in diameter) white colonies with grey and yellow edges (D121, Fig. 1), small white–grey colonies without any signs of sporulation (D221, Fig. 1) and large

ivory colonies with dark–brown conidia in the middle similar to B213 isolates (D231, Fig. 1). Finally, fungi isolated from E_{bakery} breads created small ivory colonies with smooth matte surface on YES agar (E1, Fig. 1) and their microscopic examination revealed yeast–like cells.

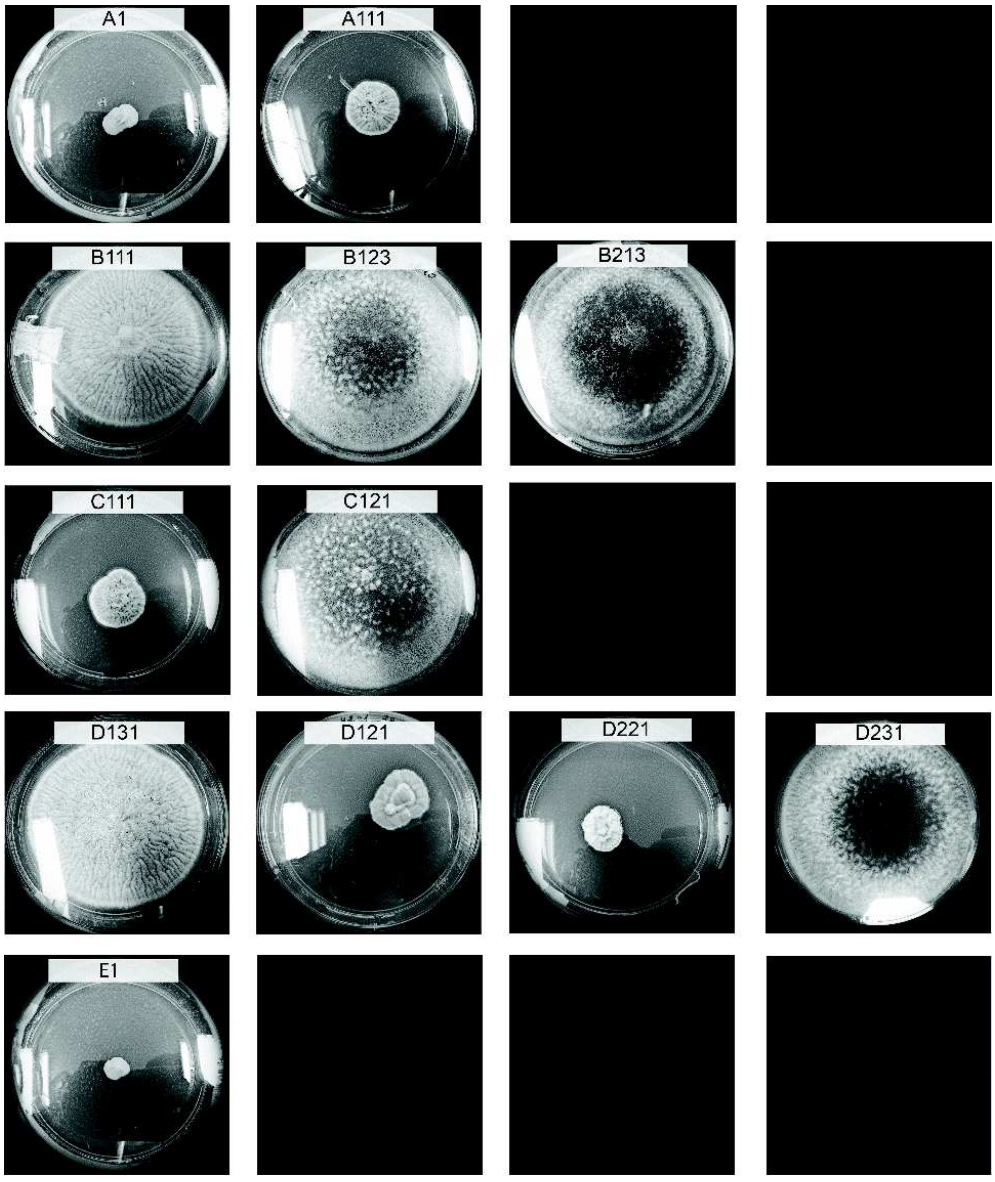

Figure 1. Fungal morphotypes obtained from breads originating from bakeries A (A1; A111); B (B111; B123; B213); C (C111; C121); D (D131; D121; D221; D231) and E (E1).

All 50 fungal isolates were genotyped by RAPD–PCR using M13 primer. Eight reproducible RAPD patterns with clear banding profiles were obtained (Fig. 2). Several RAPD patterns were bakery–specific (RAPD II, RAPD VI–VIII, Table 1), whereas representatives of other RAPD patterns were found on breads produced by different bakeries (Table 1). It is considered that biodiversity of contaminating fungi is related to the frequency of fungal infections in bakeries (Lund et al., 1996). Indeed, in our study the direct correlation between the numbers of moulds with different RAPD patterns and duration of shelf–life of rye bread was observed. Samples from A_{bakery} and E_{bakery}, where only yeasts or one mould species were detected, had the longest shelf–life among all bread samples evaluated in this study (Table 1).

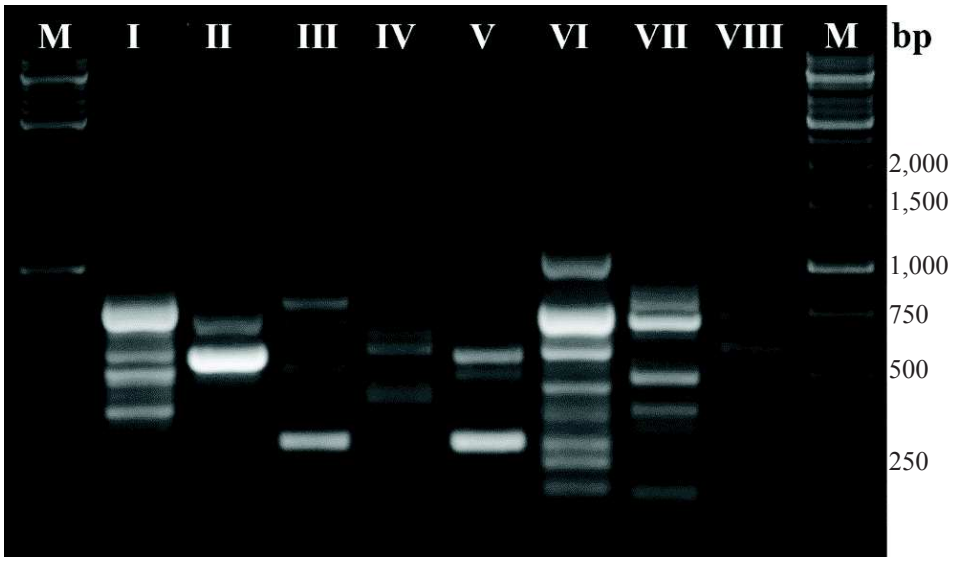

Figure 2. Eight RAPD patterns of fungal isolates obtained by RAPD–fingerprinting with M13 praimer (see the Table 1 for more detailed information). M: 1 kb DNA ladder.

The representative isolates of different RAPD types were identified based on sequences of the ITS region, the β–tubulin gene, or the D1/D2 region of the large subunit of the 28S rDNA as *Aspergillus chevalieri, Aspergillus flavus/oryzae, Aspergillus niger, Aspergillus tubingensis, Penicillium citrinum, Penicillium corylophilum, Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* species (Annex 1). None of the molecular markers used were suitable for identification of all fungal isolates either due to low discriminative capacity towards a certain species or a continuous failure of amplification/low quality of sequences (Annex 1). In case of isolates belonged to RAPD pattern I sequencing of the β–tubulin gene and ITS region yielded 100% identical sequence data for *Penicillium corylophilum*/*Penicillium obscurum* or *Penicillium corylophilum/Penicillium obscurum/Penicillium chloroleucon* respectively (Annex 1). All identified species belonged to *Penicillium* section *Exilicaulis*. However, as the result of the recent revision of this section by Visagie et al. (2016), both *P. obscurum* and *P. chloroleucon* were considered as synonyms for *P. corylophilum* species. Thus, RAPD I isolates can be identified as *P. corylophilum* based on sequences of β–tubulin gene and

ITS region. This mould species is widely found in cereals and damp buildings (Magan, Arroyo & Alfred, 2003; McMullin, Nsiama & Miller, 2014) and has been previously reported as dominant species in contaminated rye breads made without chemical preservatives (Lund et al., 1996).

Another species of genera *Penicillium* was represented by isolates belonging to RAPD VIII pattern and was identified as *Penicillium citrinum* by all three molecular markers used (Annex 1). There is no data concerning the contamination of rye bread by *P. citrinum* species. However, *P. citrinum* was found in wheat flour and bread in the USA (Bullerman & Hartung, 1973). This species is widely found in soil and plants (Houbraken & Samson, 2011), and thus it may contaminate the bakery environment via flour particles that spread through the air and also by landing on equipment used for slicing and packaging.

Representatives of RAPD III, RAPD IV, RAPD V and RAPD VI patterns belong to the *Aspergillus* genera and were identified at species level only based on β–tubulin gene sequence (Annex 1) including closely–related black–spore *Aspergilli* species *Aspergillus tubingensis* (RAPD V) and *Aspergillus niger* (RAPD III). The latter are the most difficult groups to identify using morphology–based methods (Varga et al., 2000; Varga, Frisvad & Samson, 2011; Jang et al., 2012). Whereas *A. niger* is frequently reported as bread contaminant (Legan, 1993; Lund et al., 1996; Saranraj & Geetha, 2012), there is no data regarding the contamination of rye bread by *A. tubingensis*. RAPD VI pattern was composed by *Aspergillus chevalieri*, a xerophilic mould growing on food with water activity down to 0.65 like rolled oats, chocolate, some dried fruits and nuts (Pomeranz, 1991). Its telemorph, *Eurotium chevalier*, has been detected as a contaminant of milk bread rolls (Le Lay et al., 2016). RAPD IV group also belonged to *Aspergillus* genera but it was not identified at species level. Sequences of both β–tubulin gene and D1/D2 region have the similar identity to the sequences of both *Aspergillus flavus* and *Aspergillus oryzae* species (Annex 1). These are very closely–related species, which are genetically almost identical (Chang & Ehrlich, 2010; Amaike & Keller, 2011) but have different economic impact. While most *A. flavus* strains are aflatoxigenic and infect preharvest and postharvest seed crops, representatives of *A. oryzae* species have been widely used for preparation of traditional fermented foods and beverages. Genome sequence data supports the view that *A. flavus* and *A. oryzae* are the same species with the latter representing a domesticated clade of *A. flavus* (Amaike & Keller, 2011). Although Nikkuni et al. (1998) showed that these two species could be distinguished based on ITS region sequence, Jang et al. (2012) reported that sequences of all targeted regions (ITS, D1/D2 region and β–tubulin gene) were not variable enough to distinguish *A. flavus* from *A. oryzae.*

Isolated morphotypes A1 and E1, which were microscopically identified as yeasts, clustered into RAPD II and RAPD VII patterns, respectively (Table 1). Representatives of RAPD II pattern were identified as *Wickerhamomyces anomalus* based on sequences of ITS and D1/D2 regions while amplification of β–tubulin gene repeatedly failed. All three selected primer pairs performed equally well for the identification of RAPD VII as *Saccharomyces cerevisiae* (Annex 1). In the study performed by Lund et al. (1996) significant part of fungi isolated from spoiled rye breads belonged to yeast species causing surface spoilage of baked products known as ‘chalk moulds’. In our trial yeasts were isolated only from samples obtained from bakeries A and E. Whereas only single cases of contamination by *S. cerevisiae* have been previously described (Spicher, 1985),

Wickerhamomyces anomalus together with *Endomyces fibuliger* and *Hyphopichia burtonii* yeast species belongs to the most frequently reported cause of 'chalk mould' bread defect (Lund et al., 1996; Deschuyffeleer et al., 2011).

Although the ITS region is considered as the universal barcode for fungal identification (Schoch et al., 2012) in our study its discriminative capacity was insufficient for identification of most fungal isolates belonging to *Aspergillus* genera. Garnier with co–authors (2017) noticed its limited taxonomic resolution for *Penicillium* and *Cladosporium* species. Thus, the β–tubulin gene should be recommended as a primary molecular marker for identification of fungi associated with rye breads. Preliminary clustering of fungal isolates with RAPD–PCR appeared to be an efficient way to reduce the sequencing expenses. Additional studies should be performed to evaluate the efficiency of RAPD–PCR fingerprinting with M13 primer to track fungi contamination source at strain level.

CONCLUSIONS

1. Fungi isolated from mouldy rye breads mainly belonged to *Aspergillus* and *Penicillium* species.
2. The β–tubulin gene sequence has higher taxonomic resolution for identification of mould isolates belonging to *Aspergillus* genera comparing to ITS region and D1/D2 region.
3. Clustering of fungal isolates with RAPD–PCR using M13 primer followed by identification based on the β–tubulin gene sequence can be recommended as a protocol for identification and tracking mould contamination in baking industry.

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