Design of a species-specific PCR method for the detection of the heat-resistant fungi *Talaromyces macrosporus* and *Talaromyces trachyspermus*

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Significance and Impact of the Study: Polymerase chain reaction (PCR)-based detection is rapid, convenient and sensitive compared with traditional methods of detecting heat-resistant fungi. In this study, a PCR-based method was developed for the detection and identification of amplification products from *Talaromyces macrosporus* and *Talaromyces trachyspermus* using primer sets that target the isocitrate lyase gene. This method could be used for the on-site detection of *T. macrosporus* and *T. trachyspermus* in the near future, and will be helpful in the safety control of raw materials and in food and beverage production.

Keywords
fungal detection, heat-resistant fungi, isocitrate lyase, PCR, *Talaromyces macrosporus*, *Talaromyces trachyspermus*.

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Abstract
Heat-resistant fungi occur sporadically and are a continuing problem for the food and beverage industry. The genus *Talaromyces*, as a typical fungus, is capable of producing the heat-resistant ascospores responsible for the spoilage of processed food products. Isocitrate lyase, a signature enzyme of the glyoxylate cycle, is required for the metabolism of non-fermentable carbon compounds, like acetate and ethanol. Here, species-specific primer sets for detection and identification of DNA derived from *Talaromyces macrosporus* and *Talaromyces trachyspermus* were designed based on the nucleotide sequences of their isocitrate lyase genes. Polymerase chain reaction (PCR) using a species-specific primer set amplified products specific to *T. macrosporus* and *T. trachyspermus*. Other fungal species, such as *Byssochlamys fulva* and *Hamigera striata*, which cause food spoilage, were not detected using the *Talaromyces*-specific primer sets. The detection limit for each species-specific primer set was determined as being 50 pg of template DNA, without using a nested PCR method. The specificity of each species-specific primer set was maintained in the presence of 1,000-fold amounts of genomic DNA from other fungi. The method also detected fungal DNA extracted from blueberry inoculated with *T. macrosporus*. This PCR method provides a quick, simple, powerful and reliable way to detect *T. macrosporus* and *T. trachyspermus*.

Introduction
The spoilage of thermally processed food and beverage products by heat-resistant fungi has been recognized as problematic in some countries. Heat-resistant fungi can be defined as those capable of surviving temperatures at or above 75°C for 30 or more minutes (Samson et al. 2004). This temperature/time combination is generally effective for inactivating most enzymes, yeasts and the conidia of contaminant fungi. However, heat-resistant fungi are characterized by the production of ascospores that are heat-resistant. Most of these organisms are soil-inhabiting fungi belonging to the genera *Byssochlamys*, *Eupenicillium*, *Hamigera*, *Neosartorya*, *Talaromyces* and *Thermoascus* (Pitt and Hocking 2009; Scaramuzza and Berni 2014).

*Talaromyces* species are among the heat-resistant fungi most frequently isolated from thermally processed fruit...
products. Their ascospores can tolerate high temperatures, high pressures, desiccation and some chemicals, such as ethanol (Reyns et al. 2003; Dijksterhuis and Teunissen 2004; Houbraken et al. 2012). These fungi are the most economically relevant to the food and beverage industry because they commonly cause the spoilage of canned and pasteurized fruit products (Tournas 1994). Talaromyces macrosporus is used as a model organism to study the heat resistance and heat activation of ascospores (Kikoku 2003). Trehalose is contained in the ascospores of this fungus and is degraded to glucose following heat activation. The presence of trehalose in ascospores may act as a stress protectant against dehydration and heat (Dijksterhuis et al. 2002). Talaromyces trachyspermus causes the spoilage of apple juice, heat-processed pineapple products, canned strawberries and tea-based beverages, and has also been isolated from raw materials used to produce pasteurized fruit-based products (Pitt and Hocking 2009; Tranquillini et al. 2017). These fungi are known to produce several types of mycotoxins, including duclauxin and spiculisporic acid (Frisvad et al. 1990).

Traditional methods for fungal identification are mainly based on phenotype, which requires an expertise in analysing their morphology. These methods are often time-consuming and laborious, and discrimination beyond the genus level is difficult. Recently, matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) has been used to characterize clinically relevant fungi (Cassagne et al. 2011), and spoilage-related fungi have been identified using Fourier transform infrared spectroscopy (Shapaval et al. 2013, 2017). However, spectroscopic techniques have high set-up and additional consumables costs, and require highly skilled personnel.

Polymerase chain reaction (PCR) has advantages over traditional methods for identifying fungi that cause food spoilage. Species-specific primers have been designed and proven useful in detecting heat-resistant fungi, including members of the genera Bysochlamys, Hamigera, Neosartorya and Thermoascus (Nakayama et al. 2010; Yaguchi et al. 2012; Hosoya et al. 2014). For the genus Bysochlamys, PCR assays using species-specific primers developed for the β-tubulin gene detected amplicons specific for B. fulva, B. lagunculariae, B. nivea and B. zollerianae (Hosoya et al. 2012). In addition, specific PCR methods were used for the accurate detection of medical and plant pathogenic fungi, such as Aspergillus species and Fusarium oxysporum f. sp. raphani (Arancia et al. 2016; Kim et al. 2017). A PCR method using specific primers and DNA extracted from soil samples successfully detected Alternaria solani in soil (Gu et al. 2017).

The objectives of this study were to develop a relatively simple, rapid and specific PCR-based method to detect T. macrosporus and T. trachyspermus using primer sets targeting the gene encoding isocitrate lyase, which is a unique enzyme in the glyoxylate cycle. This assay could be used in the food and beverage industry.

**Results and discussion**

**Amplification of a partial sequence of the isocitrate lyase gene**

The PCR products amplified with the primers Icl-F and Icl-R (Table 1) were confirmed to be approximately 650 bp of T. macrosporus JCM22818 and JCM22819, and T. trachyspermus NBRC31360 and NBRC31757 (data not shown). The amplicons were direct-sequestenced, and the deduced products of these DNA amplicons showed high sequence similarities with the isocitrate lyase proteins of other ascomycete fungi (Fig. S1).

In recent years, DNA microarray-based methods for the detection and identification of fungi related to the food and beverage industry have been developed and used for multiplex detection (Ishiki et al. 2014; Aoyama and Miyamoto 2016). The partial sequences of the isocitrate lyase gene from T. macrosporus and T. trachyspermus (Fig. S2) would be useful as probes on a DNA microarray to detect and identify each species.

**Specificity test**

Based on the alignments of the partial sequences of the isocitrate lyase gene, the species-specific primers, Icl-Tmac-F and Icl-Tmac-R for T. macrosporus, and Icl-Ttra-F and Icl-Ttra-R for T. trachyspermus, were designed.

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**Table 1 Primers used for polymerase chain reaction amplifications**

| Primer name | Sequence (5’ to 3’) | Purpose |
|-------------|---------------------|---------|
| Icl-F       | AAGCGMCCKTYYACNGCNAGCARAT | Amplification of a partial sequence of the isocitrate lyase gene |
| Icl-R       | CCRGCCATRTGNCRCAYTTYTTNGT | Specific detection of Talaromyces macrosporus |
| Icl-Tmac-F  | GTTGGACATTTACACAGCTG | Specific detection of Talaromyces trachyspermus |
| Icl-Tmac-R  | TTTTGTCTTGGTAAGAGTAGTG | |
| Icl-Ttra-F  | GCCGATTTGAACTTGTACACG | |
| Icl-Ttra-R  | GGAGTTGTATACGCTCCTCT | |

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Table 2 Fungal strains used and their resulting polymerase chain reaction (PCR) amplicons

| Species                  | Strain no.       | PCR primer set | DNA template no. |
|--------------------------|------------------|----------------|-----------------|
| *Talaromyces macrosporus* | JCM22818<sup>T</sup> | +              | 1               |
|                          | JCM22819         | –              | 2               |
|                          | NBRC7132         | +              | 25              |
|                          | NBRC30070        | –              | 26              |
| *Talaromyces trachyspermus* | NBRC31360     | –              | 3               |
|                          | NBRC31757<sup>T</sup> | –              | 4               |
|                          | NBRC6440         | –              | 27              |
|                          | NBRC106931       | –              | 28              |
| *Byssoclamys fulva*      | NBRC31767<sup>T</sup> | –              | 5               |
| *Byssochlamys nivea*     | JCM12806         | –              | 6               |
| *Byssochlamys verrucosa* | JCM22713<sup>T</sup> | –              | 7               |
| *Byssochlamys zollerniae* | JCM12808<sup>T</sup> | –              | 8               |
| *Eupenicillium brefeldianum* | NBRC31730     | –              | 9               |
| *Eupenicillium lapidosum* | NBRC6100<sup>T</sup> | –              | 10              |
| *Eurotium amstelodami*   | NBRC33018<sup>T</sup> | –              | 11              |
| *Eurotium chevalieri*    | JCM1568<sup>T</sup> | –              | 12              |
| *Eurotium herbariorum*   | JCM1575<sup>T</sup> | –              | 13              |
| *Eurotium repens*        | JCM1580<sup>T</sup> | –              | 14              |
| *Eurotium rubrum*        | JCM22942<sup>T</sup> | –              | 15              |
| *Hamigera avellanea*     | NBRC31667<sup>T</sup> | –              | 16              |
| *Hamigera striata*       | NBRC6106<sup>T</sup> | –              | 17              |
| *Neosartorya fischeri*   | JCM1740<sup>T</sup> | –              | 18              |
| *Neosartorya glabra*     | NBRC8789<sup>T</sup> | –              | 19              |
| *Neosartorya spinosa*    | NBRC8779<sup>T</sup> | –              | 20              |
| *Paecilomyces variotii*  | NBRC100534<sup>T</sup> | –              | 21              |
| *Thermoascus aurantiacus* | JCM12816       | –              | 22              |
| *Thermoascus crustaceus* | NBRC9129        | –              | 23              |
| *Thermoascus thermophilus* | NBRC9643      | –              | 24              |
| *Talaromyces bacillisporus* | NBRC31150     | –              | 29              |
| *Talaromyces byssachlamydoides* | JCM12813     | –              | 30              |
| *Talaromyces derxii*     | NBRC32181<sup>T</sup> | –              | 31              |
| *Talaromyces emersonii*  | NBRC31232<sup>T</sup> | –              | 32              |
| *Talaromyces galapagensis* | NBRC101902<sup>T</sup> | –              | 33              |
| *Talaromyces helicus*    | NBRC31751<sup>T</sup> | –              | 34              |
| *Talaromyces leycettanii* | NBRC31193<sup>T</sup> | –              | 35              |
| *Talaromyces luteus*     | NBRC31753<sup>T</sup> | –              | 36              |
| *Talaromyces mimosinus*  | NBRC31754<sup>T</sup> | –              | 37              |
| *Talaromyces rotundus*   | NBRC31756<sup>T</sup> | –              | 38              |
| *Talaromyces spectabilis* | JCM12815       | –              | 39              |
| *Talaromyces thermophilus* | NBRC31798<sup>T</sup> | –              | 40              |
| *Talaromyces wortmannii* | NBRC7738        | –              | 41              |
| *Chaetomium globosum*    | NBRC6347        | –              | 41              |
| *Deveriesia thermodurans* | BFF228         | –              | n.d.            |
| *H. striata*             | BFF75           | –              | n.d.            |
| *Penicillium digitatum*  | NBRC7758        | –              | –               |

JCM, Japan Collection of Microorganisms, RIKEN BioResource Center; NBRC, Biological Resource Center, National Institute of Technology and Evaluation; <sup>T</sup>, ex type; +, detection; –, no detection; n.d., not determined.
using each primer set did not produce amplicons of the appropriate sizes from the other strains tested (Table 2 and Fig. S3a,b). Each primer set showed excellent specificity for *T. macrosporus* or *T. trachyspermus*. Thus, PCR with species-specific primers enabled *Talaromyces* detection and identification at the species level.

**Sensitivity test**

To investigate the detection sensitivity of each primer set, a dilution series of the template DNA, ranging from 5 ng to 500 fg, was prepared. PCR with each primer set showed that the intensity of the expected band became fainter as the amount of template DNA decreased and became indiscernible at 5 pg of template DNA (Fig. 1a, b). The PCR detection limit for each species-specific primer set was determined to be 50 pg of template DNA, without using a nested PCR method.

Some studies have developed nested PCR methods to improve PCR sensitivity. Nested PCR can increase the sensitivity of primers by at least 10-fold (Nakayama et al. 2010; Yaguchi et al. 2012; Hosoya et al. 2014). Using a nested PCR method, we will develop highly effective assays for the detection of *T. macrosporus* and *T. trachyspermus*. Facilitating the detection of heat-resistant fungi will result in more effective on-site management by removing contaminated raw materials from food and beverage production.

**Effects of contaminating DNA from other fungi**

To assess the effects of contaminating DNA from other fungi, 50 pg of *T. macrosporus* JCM22818 DNA and the serially diluted DNA of other fungi were mixed and used as the template for PCR assays. The extracted DNAs from *Chaetomium globosum* NBRC6347 and *Penicillium digitatum* NBRC7758 were used as the contaminating DNA. The specificity and sensitivity of the PCR assay were maintained even in samples contaminated 1,000-fold with DNA from other fungi (Fig. 2a,b). Similar results were obtained for the PCR assay of *T. trachyspermus* NBRC31757 (Fig. 2c,d). *C. globosum* has reported resistance to peracetic acid, which is used in the sanitization of manufacturing environments in the food and beverage industry (Nakayama et al. 2013). *P. digitatum* is a fungal necrotroph that causes a common citrus postharvest disease known as green mould (Marcet-Houben et al. 2012). Our results suggest that a PCR method based on the amplification of the isocitrate lyase gene is highly reliable, even in cases of contamination by other fungi, which commonly occurs in raw materials. Thus, it is expected to be beneficial to monitoring for contaminant detection along food production lines and in final products.

**Detection of *T. macrosporus* in blueberry**

*Deveriesia thermodurans* BFF228 and *Hamigera striata* BFF75 were used for co-inoculations with *T. macrosporus* JCM22818 of blueberry. *D. thermodurans* BFF228 and *H. striata* BFF75, which were isolated from frozen blueberry, are heat-resistant fungi (Kikoku et al. 2008). The genomic DNA from blueberry contaminated with heat-resistant fungi was extracted and used in the PCR-based detection assay. The expected band was amplified from blueberry inoculated with *T. macrosporus* JCM22818 (Fig. 3a). In contrast, no PCR products were amplified from the non-inoculated blueberry, *D. thermodurans* BFF228 and *H. striata* BFF75 (Fig. 3b). Thus, the species-specific PCR method developed in this study could be valuable for the detection of *T. macrosporus* from contaminated blueberry.

The rapid detection and identification of heat-resistant fungi are critically important to evaluate the risk of food spoilage. The PCR-based method presented here is very simple, fast, stable and sensitive when compared with the traditional methods of fungal identification. Thus, it is expected to be extremely beneficial to assessing the purity of raw materials in the food and beverage manufacturing environment. The PCR-based method could be applied to establish quality and biosecurity markers for raw materials and final products in the processing industries.

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**Figure 1** Polymerase chain reaction sensitivity. Detection limits for the species-specific primers using template DNA from *Talaromyces macrosporus* JCM22818 (a) and *Talaromyces trachyspermus* NBRC31757 (b). Each lane contains different DNA amounts, as follows: 5 ng (Lane 1), 500 pg (Lane 2), 50 pg (Lane 3), 5 pg (Lane 4) and 500 fg (Lane 5). M, 100-bp DNA ladder.
Materials and methods

Fungal strains and culture conditions

*T. macrosporus*, *T. trachyspermus* and other fungal species used in this study are listed in Table 2 as are the confirmed specificities of the species-specific primers. These strains were grown on potato dextrose agar (Difco, Detroit, MI) or in potato dextrose broth (Difco) for 1 week at 25–30°C prior to DNA extraction.

Genomic DNA extraction

Genomic DNA was extracted from mycelia using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp., Irvine, CA).

PCR amplification

The primers Icl-F and Icl-R (Table 1) were designed based on known isocitrate lyase gene sequences from other fungi (Dunn et al. 2009) and used to amplify partial *T. macrosporus* and *T. trachyspermus* isocitrate lyase gene sequences using the Quick Taq HS DyeMix (Toyobo, Osaka, Japan). The PCR conditions were as follows: 94°C for 2 min and 35 cycles of 94°C for 30 s, 50°C for 30 s and 68°C for 30 s, followed by 68°C for 5 min. The column-purified amplicons were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with an ABI Prism 310 genetic analyzer (Applied Biosystems). The sequences were then used as queries in BLAST algorithm-based searches of the NCBI databases to detect similar nucleotide and protein sequences. The partial isocitrate lyase gene sequences have been deposited in the DDBJ (accession numbers LC310931 and LC310932 for *T. macrosporus* JCM22818 and JCM22819 respectively, and accession numbers LC310933 and LC310934 for *T. trachyspermus* NBRC31360 and NBRC31757 respectively). Primers were designed for
species-specific amplifications based on the alignment of multiple partial isocitrate lyase gene sequences using ClustalW. The *T. macrosporus* primers were designated Icl-Tmac-F and Icl-Tmac-R, while the *T. trachyspermus* primers were designated Icl-Ttra-F and Icl-Ttra-R (Table 1). The PCR conditions for the detection of the *T. macrosporus* or *T. trachyspermus* isocitrate lyase gene were as follows: 94°C for 2 min, 30 cycles at 94°C for 30 s, 53°C (for *T. macrosporus*) or 50°C (for *T. trachyspermus*) for 30 s and 68°C for 20 s, followed by 68°C for 5 min. The PCR products were analysed by 1.3% agarose gel electrophoresis. All PCR experiments were performed three times using DNA from independent biological replicates.

Detection limits

*T. macrosporus* JCM22818 genomic DNA was serially diluted from 5 ng µl⁻¹ to 500 fg µl⁻¹ in distilled water. PCR was completed using the Icl-Tmac-F and Icl-Tmac-R primers and the serially diluted DNA samples to determine the detection limit for *T. macrosporus*. The detection limit for *T. trachyspermus* NBRC31757 was similarly estimated using the Icl-Ttra-F and Icl-Ttra-R primers.

Effects of contaminating DNA from other fungi

Mixtures of 50 pg of *T. macrosporus* JCM22818 genomic DNA and 50 pg to 50 ng of genomic DNA extracted from *C. globosum* NBRC6347 and *P. digitatum* NBRC7758 were prepared as templates for PCR using the Icl-Tmac-F and Icl-Tmac-R primers. The fungal genomic DNA was serially diluted from 50 ng µl⁻¹ to 50 pg µl⁻¹ in distilled water. The effects of contaminating DNA from other fungi on the detection of *T. trachyspermus* NBRC31757 was similarly assessed using the Icl-Ttra-F and Icl-Ttra-R primers.

Detection of *T. macrosporus* in blueberry

The frozen blueberry was purchased from Seven-Eleven Japan Corp. (Tokyo, Japan). Fully thawed blueberry was washed with 70% ethanol for 10 min and left to air-dry for 15 min. The sterilized blueberry was inoculated using a modification of the procedure described by Tryfinopoulou et al. (2015). Instead of inoculating with conidia, pieces (2 mm × 2 mm) of growing mycelia from potato dextrose agar cultures of each heat-resistant fungi were used to inoculate the blueberry, which were then incubated at 25°C for 1–3 weeks. They were then homogenized, and the pellets were used for DNA extractions using a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp.). The extracted DNA from the inoculated and non-inoculated blueberry were used to specifically detect *T. macrosporus*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Alignment of multiple partial isocitrate lyase protein sequences.

**Figure S2.** Positions of primers for the specific detection of *Talaromyces macrosporus* and *Talaromyces trachyspermus*.

**Figure S3.** Specific detection of *Talaromyces macrosporus* and *Talaromyces trachyspermus*.