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Research Article

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Isolation and Identification of Postharvest Spoilage Fungi from Mulberry Fruit in Korea

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Abstract

BACKGROUND: Spoilage fungi can reduce the shelf life of fresh fruits and cause economic losses by lowering quality. Especially, mulberry fruits have high sensitivity to fungal attack due to their high water content (> 70%) and soft texture. In addition, the surface of these fruits is prone to damage during harvesting and postharvest handling. However, any study on postharvest spoilage fungi in mulberry fruit has not been reported in Korea. This study aimed to examine the spoilage fungi occurring in mulberry fruits during storage after harvest.

METHODS AND RESULTS: In this study, we isolated postharvest spoilage fungi from mulberry fruits stored in refrigerator (fresh fruits) and deep-freezer (frozen fruits) and identified them. In the phylogenetic analysis based on comparisons of the ITS rDNA sequences, the 18 spoilage fungi isolated from mulberry fruits and the 25 reference sequences were largely divided into seven groups that were subsequently verified by high bootstrap analysis of 73 to 100. *Alternaria* spp. including *A. alternate* and *A. tenuissima*, were the most frequently isolated fungi among the spoilage isolates: its occurrence was the highest among the 18 isolates (38.9%).

CONCLUSION: The findings of this study will be helpful for increasing the shelf life of mulberry fruits through the application of appropriate control measures against infection by spoilage fungi during storage.

Key words: Fungi, Internal transcribed spacer sequences, Mulberry fruit, Phylogenetic analysis, Spoilage

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Introduction

Mulberry belongs to the genus *Morus* of the family *Moraceae* and is widely distributed around the world: in Asia, Europe, Africa, and North and South America (Wasano *et al.*, 2009; He *et al.*, 2013). In Korea and some other countries, mulberry fruit has been effectively used in traditional folk medicine to treat aphthae, asthma, colds, coughs, diarrhea, dyspepsia, edema, fevers, as well as to prevent liver damage, strengthen joints, facilitate urine excretion, and reduce blood pressure (Bae and Suh 2007; Qin *et al.*, 2010). Additionally, mulberry fruit has been recognized as a potentially important functional food due to its various biologically active compounds, which include flavonoids (anthocyanin, caffeic acid, gallic acid, rutin, quercetin, isoquercitrin, and kaempferol), anthocyanins, sugars, organic acids, free amino acids, vitamins, and micronutrients (Yang and Tsai 1994; Chu *et al.* 2006; Qin *et al.*, 2010). In addition, mulberry fruits are commercially available

as products of various forms, such as teas, jam, marmalade, frozen desserts, pulp, juice, paste, ice cream, and wine (Pawlowska *et al.*, 2008; Priya, 2012). Moreover, this fruit species is ecologically and commercially important as functional human food and is the sole food source of the silkworm (*Bombyx mori*) (Zhu *et al.*, 2011; Jeong *et al.*, 2014).

Thiyam and Sharma (2013) reported that *Acremonium, Alternaria, Aspergillus, Chalaropsis, Cladosporium, Curvularia, Fusarium, Mucor, Penicillium, Rhizopus,* and *Trichoderma* were common in fruits stored under warm and humid conditions. Using molecular identification through internal transcribed spacer ribosomal DNA (ITS rDNA) regions of fungi isolated from the spoilage fruits, Alwakeel (2013) found that the species *Penicillium* and *Monilinia* commonly caused spoilage of fruits, especially of apples. Molecular techniques were demonstrated to be an effective and easy approach for identification of fungi (Alwakeel, 2013). ITS rDNA regions were used as a primary fungal barcode (Schoch *et al.* 2012). The entire ITS rDNA region in fungi is approximately 600 bp long and contains two variable spacers, ITS 1 and ITS 2, which are separated by the highly conserved 5.8S rRNA gene (White *et al.* 1990).

Spoilage microorganisms can reduce the shelf life of fresh produce during harvesting, postharvest handling, storage, or distribution (Barth *et al.*, 2009; Akhtar *et al.*, 2013). In addition, because the mycotoxins produced by some fungi can cause infections or allergies, the identification of fungal contaminants in fresh fruits is critically important (Tournas and Katsoudas, 2005). Investigations of postharvest spoilage fungi in mulberry fruit have not been reported in Korea. In the present study, we isolated the spoilage fungi from refrigerator (fresh fruits) and deep-freezer (frozen fruits) storage of mulberry fruits after harvest and identified the isolates by analysis of their ITS rDNA sequences.

Materials and Methods

Isolation of spoilage fungi

Five hundred grams of mulberry fruit samples were randomly collected from four different mulberry varieties (Cheong-Il, Gwasang 2, Su Hyang, and Shim Heung) in the Sericulture and Apicuture Division of the Department of Agricultural Biology, Rural Development Administration, Jeon-Ju, Republic of Korea. After harvest, the samples were placed in separated sterile plastic containers and transferred to the laboratory, where they were kept separately in a refrigerator (fresh fruits) and deep-freezer (frozen fruits) for four days and two weeks, respectively, until further analysis. To isolate spoilage fungi, the fruits were rinsed with sterile water and dried at room temperature. After drying, fruit tissues were taken using sterilized tweezers, placed on potato dextrose agar (PDA; Difco, USA), and incubated at 28°C until fungal proliferation on the medium surface. The fungi grown on the incubated fruit tissues were isolated and transferred onto new PDA, followed by incubation at 28°C.

Genomic DNA extraction

For genomic DNA extraction, the fungi isolated from the mulberry fruits were cultured on cellophane membranes, placed on PDA, and incubated at 28°C for 5–10 days. The cultured fungi were next ground to a fine powder using liquid nitrogen. Genomic DNA was extracted using the CTAB method (Cao *et al.*, 1998). Then, the extracted DNA was purified by phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with one volume of isopropanol. The precipitated DNA was washed sequentially with 70% ethanol and dried. Further, the DNA pellet was dissolved in 60 µL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and treated sequentially with 6 µL 20 mg/mL RNase A by incubation at 37°C for 30 min.

PCR amplification

Extracted DNA was used as a template (adjusted to 100 ng/µL) for PCR amplification of the internal transcribed spacers (ITS) rDNA region. The ITS rDNA region was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). The amplification conditions of the ITS rDNA region were as follows: 5 min initial denaturation at 94°C, followed by 30 cycles of 30-sec denaturation at 94°C, 30 sec primer annealing at 56°C, and 1 min extension at 72°C, and finally 10 min at 72°C for a final extension in a TaKaRa Thermal cycler (TaKaRa, Otsu, Japan). PCR products were detected by electrophoresis on 1.2% agarose gel in 0.5× TAE (Tris–acetic acid–EDTA) buffer, stained with ethidium bromide (EtBr), and visualized under a UV transilluminator. Sequences of PCR products were analyzed by an automated

DNA sequencer (Applied Biosystems, Foster City, CA, USA) at Genocell Total Biotechnology (Yongin, Korea).

Sequence and phylogenetic analysis

For molecular identification of the spoilage fungi isolated from mulberry fruits, the resultant sequences were compared to reference sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov). The sequences of the ITS rDNA regions were aligned for phylogenetic analysis using the program BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The phylogenetic tree was constructed using MEGA7 program (Kumar *et al.*, 2016) based on the neighbor-joining (NJ) method (Saitou and Nei 1987). The confidence levels for the individual branches of the resulting tree were assessed by the bootstrap test (Felsenstein, 1985), in which 1,000 replicate trees were generated from resampled data.

Results and Discussion

Identification of spoilage fungi

A total of 18 spoilage fungi were isolated from refrigerator (fresh fruits) and deep-freezer (frozen fruits) storage of mulberry fruits after harvest and were identified as *Alternaria alternata* (A1, A2, A4, A5, A6, and E1-2), *Alternaria tenuissima* (A3), *Phoma herbarum* (B1 and B2), *Epicoccum nigrum* (C1), *Fusarium tricinctum* (D1), *Fusarium* sp. (E1-1, E2-2, and E2-4), *Penicillium expansum* (E1-3), *Arthrinium* sp. (E2-1 and E2-3), and *Trichoderma atroviride* (E3) (Table 1). The morphologies of the spoilage fungi isolated from mulberry fruits after harvest are illustrated in Fig. 1.

Al-Hindi *et al.* (2011) reported that *Fusarium oxysporum* (banana and grape), *Aspergillus japonicus* (pokhara and apricot), *Aspergillus oryzae* (orange), *Aspergillus awamori* (lemon), *Aspergillus phoenicis* (tomato), *Aspergillus tubingensis* (peach), *Aspergillus niger* (apple), *Aspergillus flavus* (mango), *Aspergillus foetidus* (kiwi), and *Rhizopus stolonifer* (date) were isolated and identified as fruit spoilage fungi. Another study by Tournas and Katsoudas (2005) revealed that the most common spoilage fungi isolated from four berries (blackberries, blueberries, raspberries, and strawberries) were *Alternaria, Botrytis cinerea, Cladosporium, Fusarium, Penicillium* and *Rhizopus*.

In our investigation, *Alternaria alternata* (A4 and E1-2), *Alternaria tenuissima* (A3), *Epicoccum nigrum* (C1), *Fusarium* sp. (E1-1, E2-2, and E2-4), *Penicillium expansum* (E1-3), *Arthrinium* sp. (E2-3), and *Trichoderma atroviride* (E3) were identified in the isolates from fresh mulberry fruits. In addition, *Alternaria alternata* (A1, A2, A5, and A6), *Phoma herbarum* (B1 and B2), and *Fusarium tricinctum* (D1) were identified in frozen mulberry fruits. *Alternaria* and *Fusarium* genus in our study were commonly isolated as spoilage fungi from the fresh and frozen fruits of mulberry. However, *Phoma herbarum* was identified in only two of the isolates from frozen mulberry fruits.

Phylogenetic analysis of the spoilage fungi based on ITS rDNA

The data of the ITS rDNA sequences of the spoilage fungi isolated from mulberry fruits can be seen in Table 1. The PCR product sizes of the ITS rDNA regions were of variable length, from 534 to 608 bp. *Trichoderma atroviride* had the longest ITS rDNA region (607 and 608 bp), whereas *Phoma herbarum* had the shortest one (534 bp). In the phylogenetic analysis, the 18 spoilage fungi isolated from mulberry fruits and 25 reference sequences were largely divided into seven groups (Fig. 2). Each group was verified by high bootstrap analysis of 73 to 100.

Group 1 included seven isolates (A1, A2, A3, A4, A5, A6, and E1-2), three *Alternaria alternata*, (reference sequence CQ-WF-A2, H7, and PAA) and three *A. tenuissima* (reference sequence CS09, GS-BH-A2, and YK12) isolates. The ITS rDNA sequences of isolates A1, A2, A4, A5, A6, and E1-2 showed 100% identity with three *A. alternata* (reference sequence CQ-WF-A2, H7, and PAA), whereas that of the isolate A3 displayed 99.7%–100% identity with the three of *A. tenuissima* (reference sequence CS09, GS-BH-A2, and YK12). The isolate A3 and *A. tenuissima* (reference sequence CS09, GS-BH-A2, and YK12) were clustered into one group together with six other isolates (A1, A2, A4, A5, A6, and E1-2) and *A. alternata* (reference sequence CQ-WF-A2, H7, and PAA). However, *A. alternata* (including isolates A1, A2, A4, A5, A6, E1-2 and the reference sequences CQ-WF-A2, H7, and PAA) and *A. tenuissima* (included isolate A3 and reference sequence CS09, YK12) had different sequence lengths in the ITS rDNA region within 570 and 571 bp, respectively, except for *A. tenuissima* (reference sequence CQ-WF-A2) (570 bp). The isolate C1 in

Table 1. Information and identification for ITS rDNA sequences of the species of spoilage fungi isolated from mulberry fruits

No.	Isolate ID	Species and strain number	ITS rDNA			Reference
			Identification	Identity (%)	Length (bp)	
1	A1[a]		*A. alternata*	No.8-13: 100, 100, 100, 99.8, 99.8, 99.8	570	This study
2	A2[a]		*A. alternata*	No.8-13: 100, 100, 100, 99.8, 99.8, 99.8	570	This study
3	A3[b]		*A. tenuissima*	No.8-13: 99.8, 99.8, 99.8, 100, 99.7, 100	571	This study
4	A4[b]		*A. alternata*	No.8-13: 100, 100, 100, 99.8, 99.8, 99.8	570	This study
5	A5[a]		*A. alternata*	No.8-13: 100, 100, 100, 99.8, 99.8, 99.8	570	This study
6	A6[a]		*A. alternata*	No.8-13: 100, 100, 100, 99.8, 99.8, 99.8	570	This study
7	E1-2[b]		*A. alternata*	No.8-13: 100, 100, 100, 99.8, 99.8, 99.8	570	This study
8		*A. alternata* CQ-WF-A2			570	GenBank: KF308898; direct submission
9		*A. alternata* H7			570	Bukovska *et al.* (2010)
10		*A. alternata* PAA			570	GenBank: MH221088; direct submission
11		*A. tenuissima* CS09			571	GenBank: KX015987; direct submission
12		*A. tenuissima* GS-BH-A2			570	GenBank: KF308854; direct submission
13		*A. tenuissima* YK12			571	GenBank: MF405157; direct submission
14	B1[a]		*P. herbarum*	No.16-19: 100, 99.8, 100, 100	534	This study
15	B2[a]		*P. herbarum*	No.16-19: 100, 99.8, 100, 100	534	This study
16		*P. herbarum* GX8-2			534	GenBank: KU324835; direct submission
17		*P. herbarum* QL72-1			534	GenBank: AB369456; direct submission
18		*P. herbarum* XGC-31			534	Zhang *et al.* (2017)
19		*Phoma* sp. 3 TMS-2011			534	Shrestha *et al.* (2011)
20	C1[b]		*E. nigrum*	No.21,22: 100, 100	544	This study
21		*E. nigrum* ARSL 071114.11			544	Jelic *et al.* (2016)
22		*E. nigrum* BLE13			544	Botella and Diez (2010)
23	D1[a]		*F. tricinctum*	No.27-29: 99.8, 88.7, 88.7	561	This study
24	E1-1[b]		*Fusarium* sp.	No.27-29: 88.6, 100, 100	546	This study
25	E2-2[b]		*Fusarium* sp.	No.27-29: 88.6, 100, 100	546	This study
26	E2-4[b]		*Fusarium* sp.	No.27-29: 88.6, 100, 100	546	This study
27		*F. tricinctum* ELRF 6			561	Lakshman *et al.* (2017)
28		*Fusarium* sp. CS01			546	GenBank: KX015979; direct submission
29		*Fusarium* sp. 15 YS-2008			546	GenBank: EU594570; direct submission
30	E1-3[b]		*P. expansum*	No.31-34: 100, 100, 100, 100	584	This study
31		*P. expansum* ATCC 7861			584	Haugland *et al.* (2004)
32		*P. expansum* KUC1909			584	Jang *et al.* (2011)
33		*P. expansum* NRRL 35231			584	Dombrink-Kurtzman (2007)
34		*P. expansum* NRRL 6069			584	
35	E2-1[b]		*Arthrinium* sp.	No.37-39: 99.8, 99.8, 99.8	580	This study
36	E2-3[b]		*Arthrinium* sp.	No.37-39: 99.8, 99.8, 99.8	580	This study
37		*A. arundinis* CBS 106.12			580	Crous and Groenewald (2013)
38		*Arthrinium* sp. CS06			580	GenBank: KX015984; direct submission
39		*Arthrinium* sp. wb558			580	Buzina *et al.* (2003)
40	E3[b]		*T. atroviride*	No.40-43: 100, 99.8, 99.7	607	This study
41		*T. atroviride* NFCF005			607	Yun *et al.* (2016)
42		*T. atroviride* NFCF028			607	
43		*T. atroviride* LY357			608	Pu *et al.* (2013)

[a] spoilage fungi isolated from frozen mulberry fruit. [b] spoilage fungi isolated from fresh mulberry fruit.

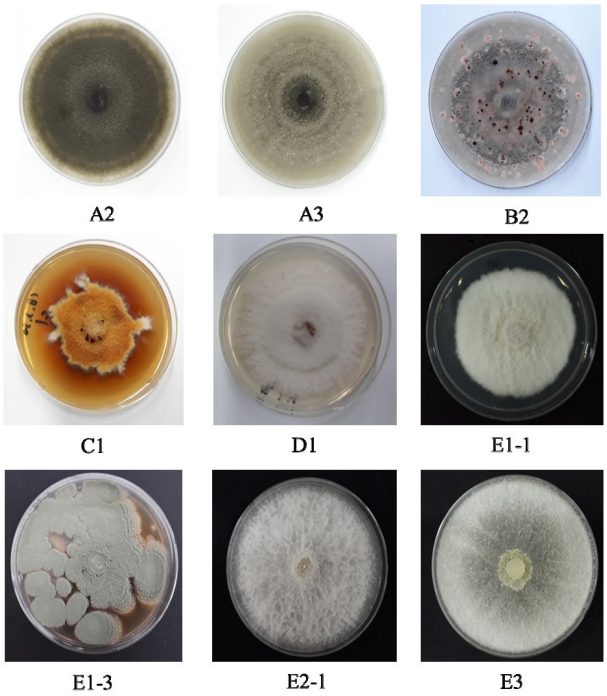

Fig. 1. Morphology of the spoilage fungi isolated from mulberry fruit after harvest. (A2) *Alternaria alternata*; (A3) *Alternaria tenuissima*; (B2) *Phoma herbarum*; (C1) *Epicoccum nigrum*; (D1) *Fusarium tricinctum*; (E1-1) *Fusarium* sp.; (E1-3) *Penicillium expansum*; (E2-1) *Arthrinium* sp.; (E3) *Trichoderma atroviride*.

group 2 exhibited 100% identity with two *Epicoccum nigrum* sequences (reference sequence ARSL 071114.11 and BLE13). Further, the two isolates (B1 and B2) in group 3 had 99.8%–100% identity with *Phoma* sp. (reference sequence TMS-2011), and three *Phoma herbarum* (reference sequence GX8-2, QL72-1, and XGC-31). Group 4 included the isolate E1-3 and four *Penicillium expansum* (reference sequence ATCC 7861, KUC1909, NRRL 35231, and NRRL 6069) with 100% identity. The isolates E2-1 and E2-3 in the group 5 showed 99.8% identity with *Arthrinium arundinis* (reference sequence CBS 106) and two *Arthrinium* sp. (reference sequence CS06 and wb558). Group 6 included the isolate E3 and three *Trichoderma atroviride* (reference sequence NFCF005, NFCF028, and LY357) with 99.7%–99.8% identity. Furthermore, group 7 was divided into *Fusarium tricinctum* (included the isolate D1 and reference sequence ELRF 6) and *Fusarium* sp. (included isolates E1-1, E2-2, E2-4 and reference sequence 15 YS-2008, CS01). The isolate D1 was with 99.8% identity with *Fusarium tricinctum* (reference sequence ELRF 6). In addition, the isolates E1-1, E2-2, and E2-4 had 100% identity with two *Fusarium* sp. sequences (reference sequence 15 YS-2008 and CS01).

Alternaria sp. (including *A. alternate* and *A. tenuissima*) was the most frequently isolated species among the spoilage fungi with the highest occurrence of 38.9% among all 18 isolates, followed by *Fusarium* sp. (*F. tricinctum*) (22.2%), *Phoma herbarum*, and *Arthrinium* sp. (11.1%), whereas *Epicoccum nigrum*, *Penicillium expansum*, and *Trichoderma atroviride* had the least occurrence (5.6%). Tournas and Katsoudas (2005) found *Alternaria* in 46% of the blueberry and 8% of the strawberry samples they analyzed. They also isolated *Fusarium* sp. from 22%, 13%, 25%, and 8% of the blackberry, blueberry, raspberry, and strawberry samples, respectively. In another study, *Aspergillus niger*, *Rhizopus stolonifera*, *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Alternaria alternata*, *Penicillium digitatum* and *Geotrichum candidum* were isolated from spoiled tomato fruits, and their percentages of occurrence were confirmed to be 47.27, 16.36, 12.73, 3.64, 10.91, 5.45, and 3.64, correspondingly (Onuorah and Orji, 2015).

Tournas and Katsoudas (2005) suggested that the lower contamination level in blueberries was owing to impenetrability to most fungi due to smoother and harder fruit skin compared with that of other berries. However, various microorganisms can easily attach and invade the inner tissues of berry fruits, such as blackberries, raspberries, and strawberries, due to their significantly thinner surface, numerous indentations, and fiber-like protuberances. Thus, these fruits have a higher susceptibility to injury and breakage of the epidermis. They also suggested that the high *Alternaria* contamination of blueberries may be partially explained by the activities of faster-growing fungi, such as *Rhizopus* sp. Likewise, among the spoilage fungi isolated from mulberry fruits in our examination, the higher *Alternaria* sp. contamination might have been related to the faster growth rate of this fungus compared to those of other spoilage fungi.

In Korea, mulberry fruits are harvested only from mid-May to early July. After harvest, the mulberry fruits are commonly kept under frozen storage as they can lose their commercial value because of the rapid ripening processes and deterioration in quality after harvest (Hu *et al.*, 2014). Moreover, surface damage of mulberry fruits can easily occur at harvesting and postharvest handling. In addition, these fruits are perishable after harvest due to their

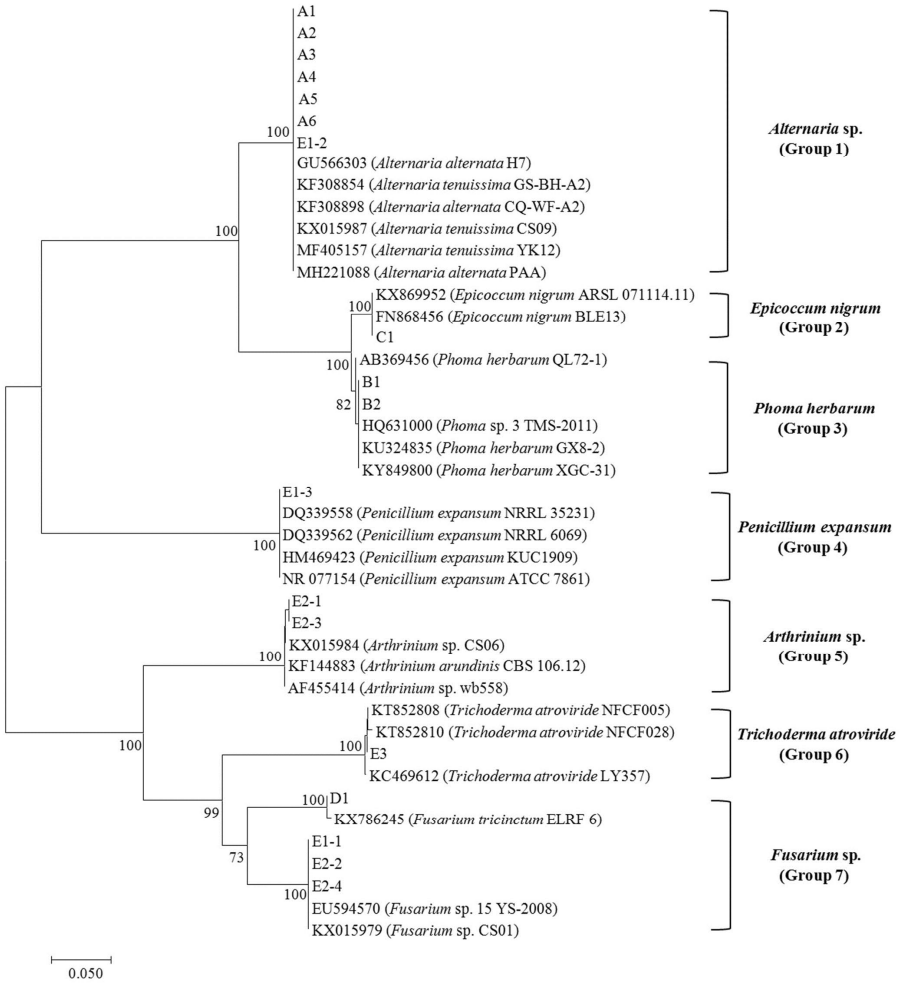

Fig. 2. Phylogenetic relationships based on the ITS rDNA regions of the spoilage fungi isolated from mulberry fruits. This tree was obtained by neighbor-joining (NJ). The numbers at the branch nodes represent the bootstrap values obtained from 1,000 replications.

high water content (over 70%), soft texture, and high sensitivity to fungal attacks (Ercisli and Orhan, 2007; Wang *et al.*, 2013). More specifically, postharvest microbial spoilage of mulberry fruits has caused economic losses by lowering their quality and reducing the shelf life.

Many researchers (Tournas and Katsoudas, 2005; Al-Hindi *et al.*, 2011; Thiyam and Sharma, 2013; Etebu and Benjamin, 2014; Rahi *et al.*, 2017) have attempted to isolate the fungi causing postharvest spoilage in fruits, identify the morphological or molecular components responsible, and find out a cost-effective control practice for the isolated fungal strain. The high prevalence of the spoilage fungi demands that appropriate control measures against infection should be employed to maintain freshness and high quality of mulberry fruits. However, little is known about the spoilage fungi of postharvest mulberry fruits in Korea, and there is a lack of proper control measures. Thus, this study could provide valuable basic information that will facilitate the increase of the shelf life of mulberry fruits through appropriate control measures against infection from spoilage fungi during storage.

Note

The authors declare no conflict of interest.

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