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Analysis of microbial communities in local cultivars of astringent persimmon (*Diospyros kaki*) fruits grown in Gyeongnam Province of Korea

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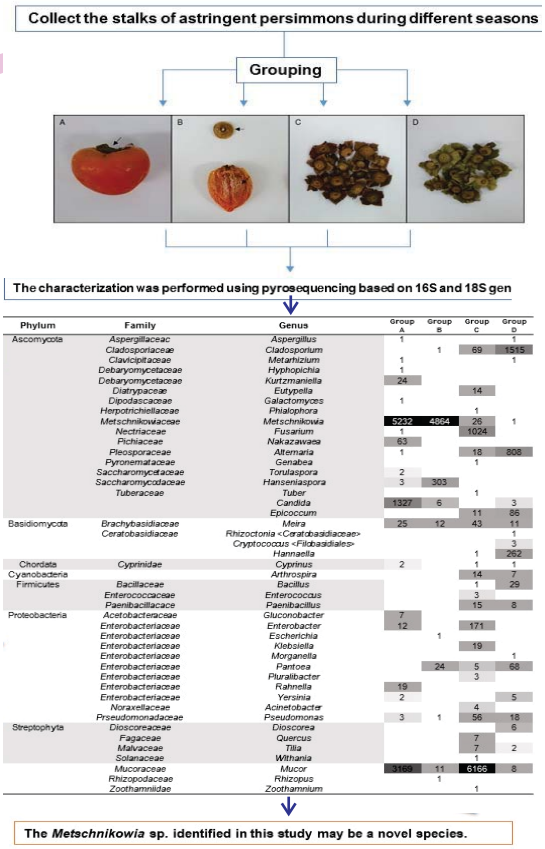
Abstract

Aim : The objective of the present study was to characterize the microbial communities within the stalks of astringent persimmons fruits grown locally in Korea and two commercial herbal products (kaki calyx-Korean & kaki calyx-Chinese, using pyrosequencing based on 16S and 18S genes.

Methodology : 'Gojongsi' sample was collected from Sancheong province, 'Danseongsi' from Sancheong province and 'Bansi' from Miryang province during different seasons (October to December) from Korea Forest Environment Research Institute at Gyeongsangnam-do. Thirteen samples were divided into four groups A, B, C and D. Group A consisted of three kinds of undried stalks collected in October. Group B consisted of three kinds of dried stalks and surfaces of persimmons collected in December. Group C consisted of two Korean herbal medicines (Kaki Calyx) sold in Korea which are commercial products. Group D consisted of two Chinese herbal medicines (Kaki Calyx) sold in China. Thirteen samples cultured in Luria-Bertani broth and potato dextrose broth under specific conditions were collected using sterile streaking sticks and placed into sterile plastic conical tubes.



Phylum	Family	Genus	Group A	Group B	Group C	Group D
Ascomycota	Aspergillaceae	Aspergillus	1			1
	Cladosporiaceae	Cladosporium		1	69	1515
	Clavicipitaceae	Metarhizium	1			1
	Debaryomycetaceae	Hyphopichia	1			
	Debaryomycetaceae	Kurtzmaniella	24			
	Dialypaceae	Eutypella			14	
	Dipodascaceae	Galactomyces	1			
	Herpotrichiellaceae	Phialophora			1	
	Metschnikowiaceae	Metschnikowia	5232	4864	26	1
	Nectriaceae	Fusarium	1		1024	
	Pichiaceae	Nakazawaea	63			
	Pleosporaceae	Alternaria	1		18	808
	Pyronemataceae	Genabea			1	
	Saccharomycetaceae	Torulaspora	2			
	Saccharomycodaceae	Hanseniaspora	3	303		
	Tuberaceae	Tuber			1	
		Candida	1327	6		3
		Epicoccum			11	86
Basidiomycota	Brachybasidiaceae	Meira	25	12	43	11
	Ceratobasidiaceae	Rhizoctonia <Ceratobasidiaceae>				1
		Cryptococcus <Filobasidiales>				3
		Hannaella			1	262
Chordata	Cyprinidae	Cyprinus	2		1	1
Cyanobacteria		Arthrospira			14	7
Firmicutes	Bacillaceae	Bacillus			1	29
	Enterococcaceae	Enterococcus			3	
	Paenibacillacae	Paenibacillus			15	8
Proteobacteria	Acetobacteraceae	Gluconobacter	7			
	Enterobacteriaceae	Enterobacter	12		171	
	Enterobacteriaceae	Escherichia		1		
	Enterobacteriaceae	Klebsiella			19	
	Enterobacteriaceae	Morganella				1
	Enterobacteriaceae	Pantoea		24	5	68
	Enterobacteriaceae	Pluralibacter			3	
	Enterobacteriaceae	Rahnella	19			
	Enterobacteriaceae	Yersinia	2			5
	Noraxellaceae	Acinetobacter			4	
	Pseudomonadaceae	Pseudomonas	3	1	56	18
Streptophyta	Dioscoreaceae	Dioscorea				6
	Fagaceae	Quercus			7	
	Malvaceae	Tilia			7	2
	Solanaceae	Withania			1	
	Mucoraceae	Mucor	3169	11	6166	8
	Rhizopodaceae	Rhizopus		1		
	Zoothamniidae	Zoothamnium			1	

Results : *Metschnikowia* sp. was the dominant microorganism in groups A and B compared with those of groups C and D on the hit map. *Metschnikowia* sp. were detected in group A and B (stalks of herbal medicines without the manufacturing process). *Metschnikowia* sp. was amplified for identification. The results of sequence analysis of 18S rDNA gene showed that there were no matches greater than 97%. Therefore, the *Metschnikowia* sp. identified in this study may be a novel species.

Interpretation : The present study provides information on various microorganisms isolated from nature's fruits and provides a list of microorganisms that can be experimented later.

Introduction

The genus of Persimmon (*Diospyros*) belongs to several plant family Ebenaceae which consists of about 400 species that have been cultivated for many years in Asian counties, including Korea, China and Japan (Hidalgo *et al*., 2012; Guo *et al*., 2006). *Diospyros kaki* Thunb. is one of the cultivated species and is classified into four types; pollination constant non astringent (PCNA), pollination variant non astringent (PVNA), pollination variant astringent (PVA) and pollination constant astringent (PCA) (Parfitt *et al*., 2015). The astringent persimmon is edible after reducing its astringency which occurs with ripening of the fruits or after removal of its high water-soluble tannin content artificially (Li *et al*., 2011; Arnal *et al*., 2003).

Persimmons have various chemical constituents such as carotenoids, tannins, flavonoids, sugars, hydrocarbons, lipids, hydrocarbons, aromatics, terpenoids and steroids (Mallavadhani *et al*., 1998). Astringent persimmons contain water-soluble tannin components has a bitter taste and tannic acid which has been shown to have anti-aging, anticancer and herbivory defense effects (Park *et al*., 2004; Parfitt *et al*., 2015; Kim *et al*., 2011a).

Kaki Calyx is a type of traditional Korean medicine containing persimmons (Nanjing Zhong yi yao da xue *et al*., 2006). In fact, according to Korean medical documents Dong-uibogam [https://en.wikipedia.org/wiki/Dongui_Bogam] and Ben Cao Gang Mu [https://en.wikipedia.org/wiki/ Compendium_ of_ Materia_Medica], the stalks of astringent persimmons are known to be effective in the treatment of bed-wetting, vomiting. Further studies showed that persimmons have been used to treat hiccups, flu, and atherosclerosis and to prevent heart disease (Herbology Editorial Committee of Korean Medicine Schools *et al*, 2004; Heo *et al*., 2001). Persimmons have also been reported to have effects on blood vessels, strengthening of the lungs and alleviation of bronchial diseases (Herbology Editorial Committee of Korean Medicine Schools, 2004; Heo *et al*., 2001; Chinese Pharmacopoeia Commission, 2010). Although genetic diversity and pharmacological studies of astringent persimmons have recently been reported from native provinces (Hwang *et al*., 2010; Choi *et al*., 2016), few studies have attempted to analyze the diversity of microorganisms on astringent persimmons. Thus, this study characterized and identified the microorganisms found on the native cultivars of astringent persimmon using pyrosequencing-a meta-genomic analysis technique.

Astringent persimmons are processed typically to remove its astringency to make it consumable. The level of maturity of astringent persimmons may differ according to the processing environment, and microbiological changes can occur owing to variations in moisture, humidity and light (Candir *et al*., 2009). Therefore, pyrosequencing is a new high-throughput DNA sequencing technique can be used for microbial genome sequencing and discrimination of microorganism strains collected under different conditions (Cummings *et al*., 2013). The high-throughput nature of pyrosequencing makes it possible to save time by profiling large numbers of samples simultaneously (Chun *et al*., 2010). Indeed, in previous studies pyrosequencing has been used to identify and profile microorganisms from astringent persimmons grown under different conditions (Jeon *et al*., 2013; Kim *et al*., 2011b).

The objective of the present study was to characterize the microbial communities within the stalks of astringent persimmon fruits grown locally in Korea and two commercial herbal products (kaki calyx-Korean and kaki calyx-Chinese), using pyrosequencing based on 16S and 18S genes.

Materials and Methods

Sample collection : 'Gojongsi' sample was collected from Sancheong province, 'Danseongsi' from Sancheong province and 'Bansi' from Miryang province collected during different seasons (October and December) from Korea Forest Environment Research Institute at Gyeongsangnam-do. In the present study, thirteen samples were divided into four groups. Group A consisted of three kinds of undried stalks collected in October. Group B consisted of three kinds of dried stalks and surfaces of persimmons collected in December. Group C consisted of two Korean herbal medicines (Kaki Calyx) sold in Korea. Group D consisted of two Chinese herbal medicines (Kaki Calyx) sold in China. Details of the samples has been previously described (Choi *et al*., 2016).

Microorganism preparation and cultures : Thirteen samples were collected into stalks and peels, placed in sterile plastic conical tubes, and cultured in 15 ml of Luria-Bertani broth and potato dextrose broth at 25°C laboratory conditions to compare differences among microorganisms grown in two culture media. Cultured suspensions (200 µl) were transferred to Luria-Bertani agar (LBA; 10.0 g l^{-1} tryptone, 5.0 g l^{-1} yeast extract, 10.0 g l^{-1} sodium chloride, 10 g l^{-1} agar; Difco) and potato dextrose agar (PDA; 4.0 g l^{-1} potato starch, 20.0 g l^{-1} dextrose, 15 g l^{-1} agar; Difco) plates at 25°C for 24 hrs.

DNA extraction, polymerase chain reaction (PCR) and pyrosequencing : Pyrosequencing was carried out following the standard protocol (Buee *et al*., 2009; Serkebaeva *et al*., 2013). Colonies on agar plates were collected using sterile streaking sticks and placed in sterile plastic conical tubes for identification by pyrosequencing. The samples were grinded in a Precellys Grinder (Bertin Technologies, France), centrifuged and washed twice with 1× phosphate-buffered saline (PBS; Biowhittaker, Walkersville, MD, USA). Pellets were stored at -20°C in 1.5-ml E-tubes before DNA isolation.

Genomic DNA was extracted from the colonies using a MagListo Genomic DNA Extraction kit (Bioneer, Korea). The extracted DNA was quantified at 220 nm using a NanoDrop 1000 spectrophotometer (NanoDrop, USA). The DNA extracted from the samples was confirmed by running DNA extracts on 1% agarose gels. Bacterial 16S DNA universal primers 27F (5'-

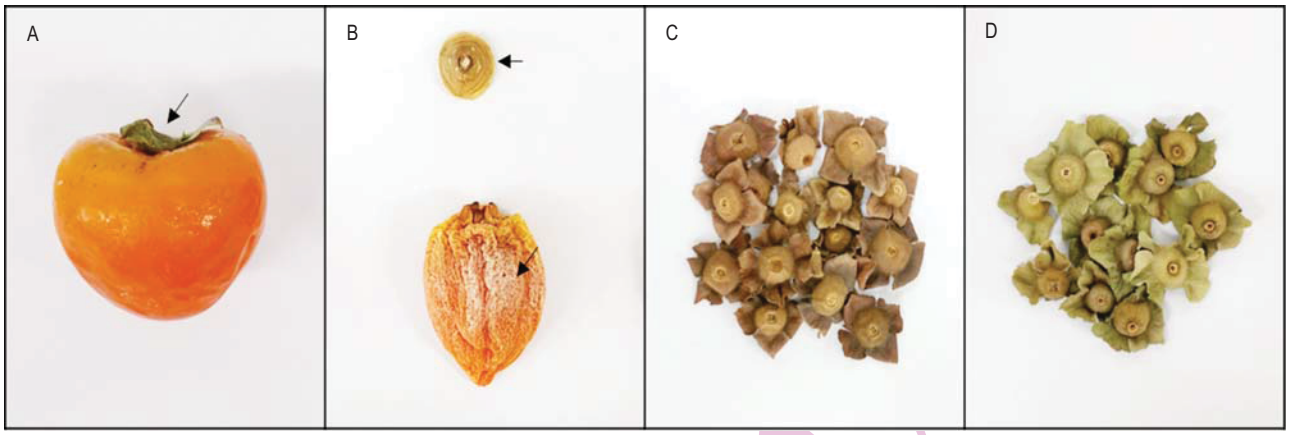


Fig. 1 : Group A: Undried stalk of astringent persimmon; Group B : Dried stalks and surfaces of astringent persimmon; Group C : Korean herbal medicines (Kaki Calyx) and Group D : Chinese herbal medicines

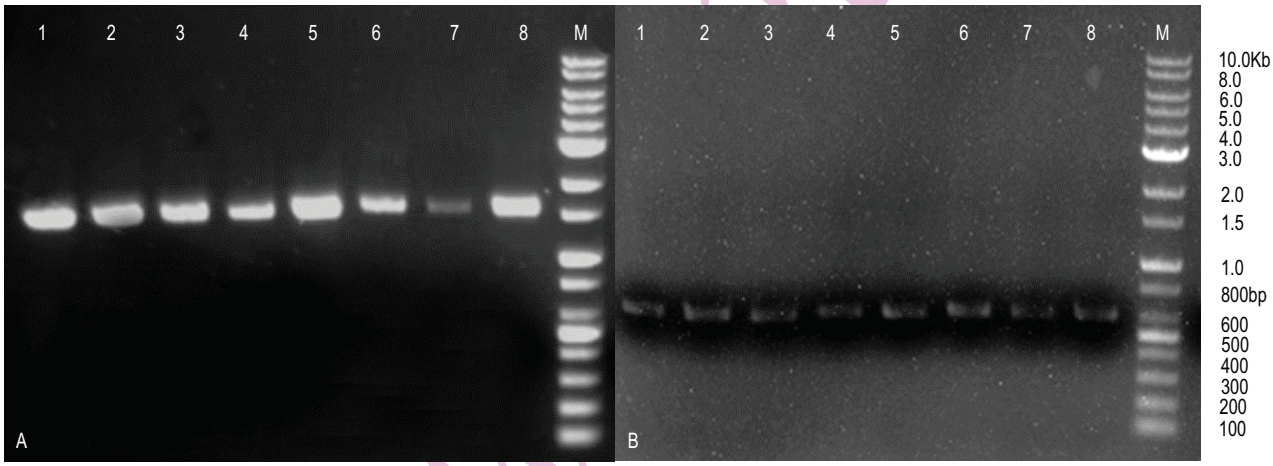


Fig. 2 : Amplification of bacterial 16S rDNA and fungal 18S rDNA by PCR. A1–8: Representative 16S bacterial rDNA PCR products from samples 1–8 (~1.5 Kb). B1–8 : Representative 18S fungal rDNA PCR products from samples 1–8 (~600 bp). *1% agarose gel, 1 µl loading; size marker: Solgent 1 kb plus

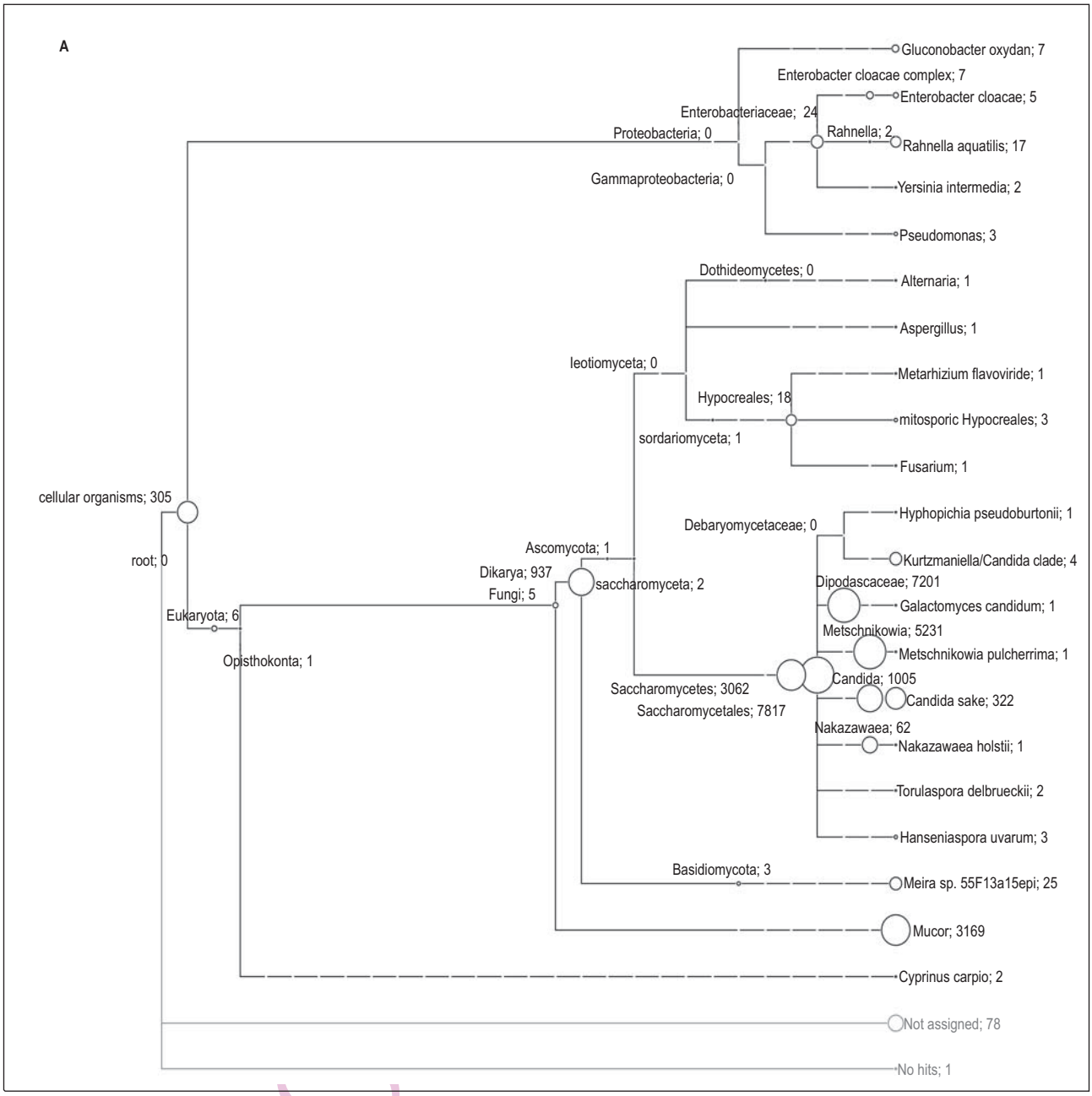
Table 1 : Thirteen samples as 4 different groups used in this study

Sample	Cultivar	Region	Group
Stalk of undried astringent persimmon 1	Gogongsi	Sancheong	
Stalk of undried astringent persimmon 2	Danseongsi	Sancheong	A
Stalk of undried astringent persimmon 3	Bansi	Miryang	
Stalk of dried astringent persimmon 1	Gogongsi	Sancheong	
Stalk of dried astringent persimmon 2	Danseongsi	Sancheong	
Stalk of dried astringent persimmon 3	Bansi	Miryang	B
Surface of dried astringent persimmon 1	Gogongsi	Sancheong	
Surface of dried astringent persimmon 2	Danseongsi	Sancheong	
Surface of dried astringent persimmon 3	Bansi	Miryang	
Stalk of commercial persimmon in Korea 1	Korean herbal medicines	(Kaki Calyx)	C
Stalk of commercial persimmon in Korea 2	Korean herbal medicines	(Kaki Calyx)	
Stalk of commercial persimmon in China 1	Chinese herbal medicines	(Kaki Calyx)	D
Stalk of commercial persimmon in China 2	Chinese herbal medicines	(Kaki Calyx)	

Table 2 : Hit map showing relative abundance of bacterial and fungal groups at genus level for thirteen study samples

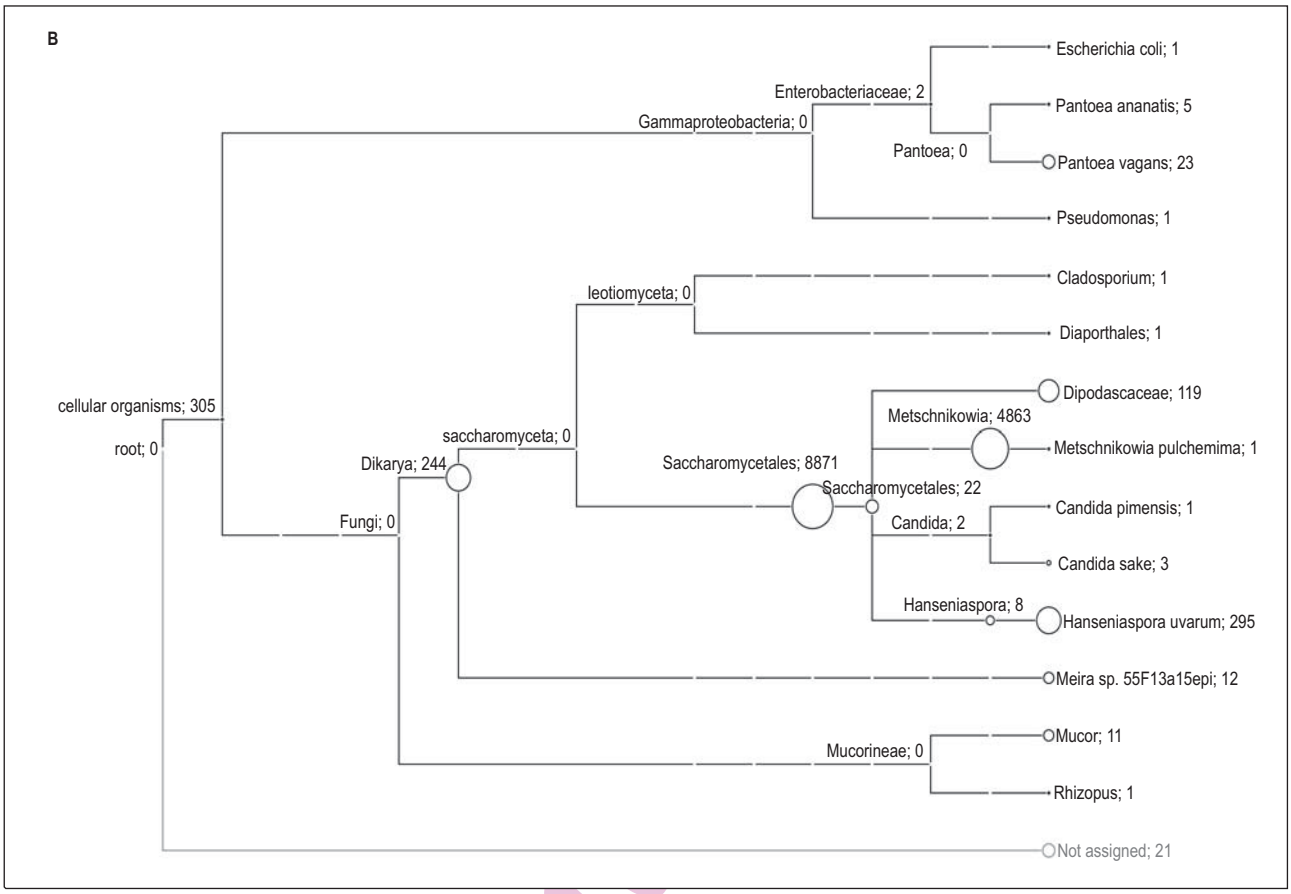
Phylum	Family	Genus	Group A	Group B	Group C	Group D
Ascomycota	*Aspergillaceac*	*Aspergillus*	1			1
	Cladosporiaceae	*Cladosporium*		1	69	1515
	Clavicipitaceae	*Metarhizium*	1			1
	Debaryomycetaceae	*Hyphopichia*	1			
	Debaryomycetaceae	*Kurtzmaniella*	24			
	Diatrypaceae	*Eutypella*			14	
	Dipodascaceae	*Galactomyces*	1			
	Herpotrichiellaceae	*Phialophora*			1	
	Metschnikowiaceae	*Metschnikowia*	5232	4864	26	1
	Nectriaceae	*Fusarium*	1		1024	
	Pichiaceae	*Nakazawaea*	63			
	Pleosporaceae	*Alternaria*	1		18	808
	Pyronemataceae	*Genabea*			1	
	Saccharomycetaceae	*Torulaspora*	2			
	Saccharomycodaceae	*Hanseniaspora*	3	303		
	Tuberaceae	*Tuber*			1	
		Candida	1327	6		3
		Epicoccum			11	86
Basidiomycota	*Brachybasidiaceae*	*Meira*	25	12	43	11
	Ceratobasidiaceae	*Rhizoctonia <Ceratobasidiaceae>*				1
		Cryptococcus <Filobasidiales>				3
		Hannaella			1	262
Chordata	*Cyprinidae*	*Cyprinus*	2		1	1
Cyanobacteria		*Arthrospira*			14	7
Firmicutes	*Bacillaceae*	*Bacillus*			1	29
	Enterococcaceae	*Enterococcus*			3	
	Paenibacillacace	*Paenibacillus*			15	8
Proteobacteria	*Acetobacteraceae*	*Gluconobacter*	7			
	Enterobacteriaceae	*Enterobacter*	12		171	
	Enterobacteriaceae	*Escherichia*		1		
	Enterobacteriaceae	*Klebsiella*			19	
	Enterobacteriaceae	*Morganella*				1
	Enterobacteriaceae	*Pantoea*		24	5	68
	Enterobacteriaceae	*Pluralibacter*			3	
	Enterobacteriaceae	*Rahnella*	19			
	Enterobacteriaceae	*Yersinia*	2			5
	Noraxellaceae	*Acinetobacter*			4	
	Prseudomonadaceae	*Pseudomonas*	3	1	56	18
Streptophyta	*Dioscoreaceae*	*Dioscorea*				6
	Fagaceae	*Quercus*			7	
	Malvaceae	*Tilia*			7	2
	Solanaceae	*Withania*			1	
	Mucoraceae	*Mucor*	3169	11	6166	8
	Rhizopodaceae	*Rhizopus*		1		
	Zoothamniidae	*Zoothamnium*			1	

Index color																			
Range of Count	0	1	2	3	4	5	6	7	8	9	10	10~49	51~100	101~500	501~1,000	1,001~2,000	2,001~3,000	3,001~4,000	4,001~5,000	5,001~6,000	6,001~



AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') were used to amplify 1.5 Kb of 16S DNA. The 600-bp 18S DNA gene was amplified using fungal 18S DNA universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR was performed with an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: bacterial 16S DNA (initial denaturation at 95°C for 15 min; 35 cycles of 95°C for 20 sec, 50°C for 40 sec and 72°C for 1.5 min; and a final extension step at 72°C for 5 min) and fungal 18S DNA (initial denaturation at 95°C for 15 min; 35 cycles of 95°C for 20 sec, 53°C for 40 sec, 72°C for 1.5 min; and a final extension step at 72°C for 5 min).

Classification and identification of microorganisms : Purified PCR products were used to construct gene libraries and read nucleotide sequences using a GS Junior Titanium Sequencing Kit (Roche, USA). Sequence reads obtained using a pyrosequencer were analyzed as previously described (Diouf *et al*., 2015). After using pyrosequencing, sequence analysis and taxonomy were carried out. Obtained sequences from GS Junior were compared



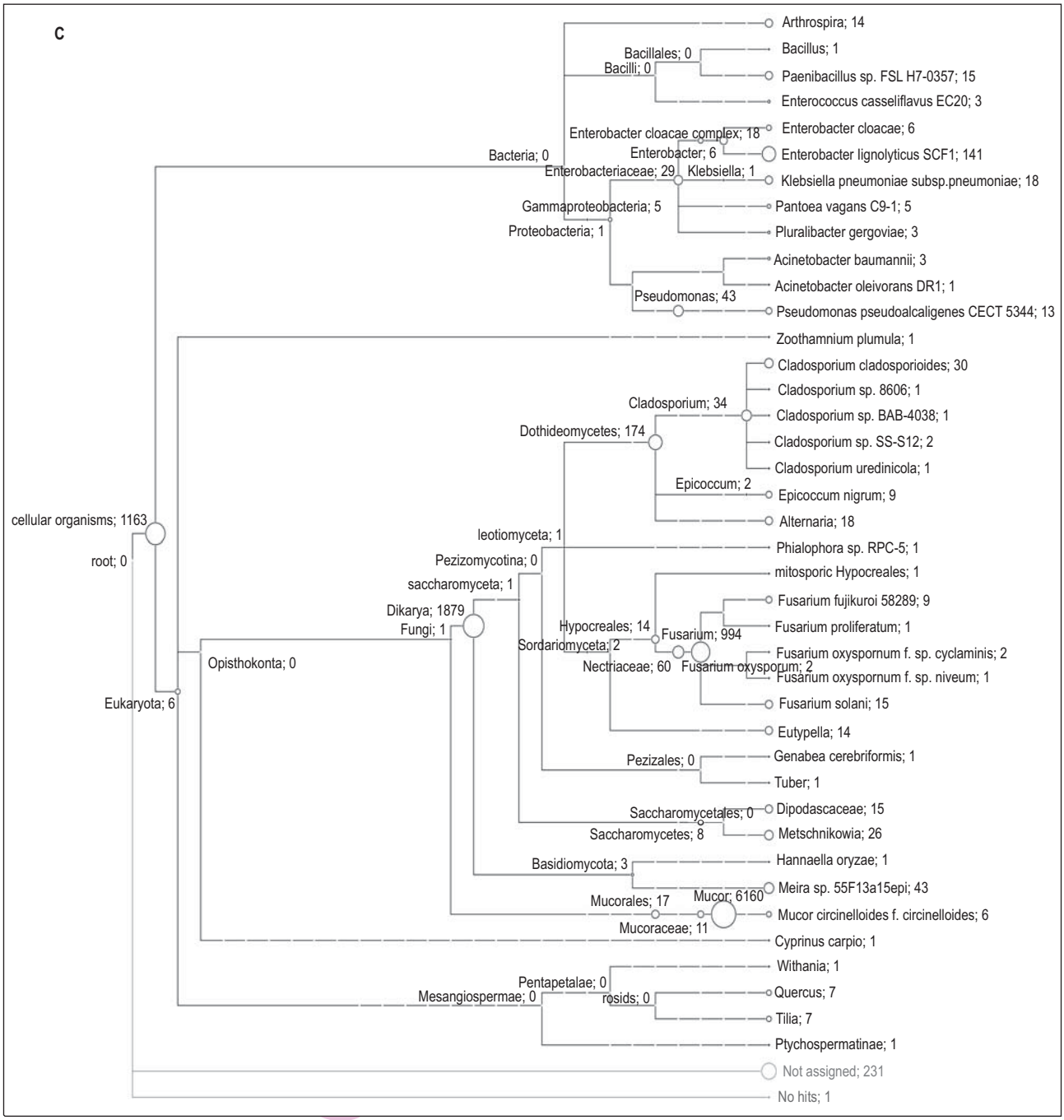
with 16S and 18S rDNA gene sequences from the non-redundant sequence database using BLAST Alignment Search Tool (V2.2.30+) at the National Center of Biotechnological Information (NCBI) to determine their approximate taxonomic identifications and investigate microbial diversity. Each sequence was then classified by taxonomy using NCBI taxonomy in the MEGAN Program (V5.10.1), and the results were used to perform taxonomic classification. NCBI taxonomy provided 660,000 or more taxa. Species were classified according to the hierarchy of super-kingdom, kingdom, phylum, class, order, family, genus and species. Phylogenetic trees of rooted tree form were completed by assigning all the sequences to each node. Each group of identified microorganisms was presented according to ITS taxonomy counting heat maps using Microsoft Excel.

Results and Discussion

16S rDNA for bacteria and 18S rDNA for fungi were amplified by PCR using 27F and 1492R primers (PCR product of approximately 1.5 Kb) or ITS1 and ITS4 primers (PCR product of approximately 600 bp; Fig. 2) for identification using pyroseqeuncing. Stalks from specific localities in Korea were analyzed for diversity. The viable cell counts for the main microbial groups in stalks is shown using a hit map (Table 2). Microorganism diversity was abundant in the stalks of undried astringent persimmons, stalks and surfaces of dried astringent persimmons, and stalks of commercial persimmons (group A, group B, group D and group C, respectively). The most abundant bacterial orders were found in stalks of commercial persimmons, followed by those in stalks of undried astringent persimmons and stalks and surfaces of dried astringent persimmons, whereas the most abundant fungal orders were found in the stalks of undried astringent persimmons, followed by the stalks and surfaces of dried astringent persimmons and stalks of commercial persimmons.

Owing to longer reads, the 16S and 18S rDNA gene-based clone libraries allowed us to carry out a comparative analysis of bacterial and fungal communities at a higher level of phylogenetic resolution. This approach generated sequences from four sampling groups, and a phylogenetic tree with taxonomic positions of different bacterial and fungal operational taxonomic units (OTUs) was constructed using the maximum likelihood method implemented in MEGAN (Fig. 3).

The microbial community composition of group A at the phylum level was dominated by Ascomycota (67.26% of reads) and Zygomycota (32.01% of reads). Ascomycota (99.02% of



reads) was predominant in the stalks and surfaces of dried astringent persimmons. Group C consisted of Zygomycota (80.26% of reads), Ascomycota (15.16% of reads) and Proteobacteria (3.36% of reads), whereas the stalks of commercial persimmons consisted of Ascomycota (84.89%), Basidiomycota (9.74%), Proteobacteria (3.23%) and Firmicutes (1.30%). It was reported that seasonal climatic change can affect serveral microbial community and shift their phenology to adapt to seasonal events (Fuchs *et al*., 2017) .

Mucor sp. and *Candida* sp. were also abundant in stalks of undried astringent persimmons. *Candida* sp. are frequently found in critically ill patients (Rello *et al*., 1998). Abbas *et al*. (2002) reported isolation and characterization of extracellular lipase from *Mucor* sp. strain from palm fruits. *Mucor* sp. from stalks of astringent persimmons can also be used as a source material for extracellular lipase. *Cladosporium* sp. was a dominant genus identified in stalks of commercial persimmons. Some studies have reported the isolation of antifouling and

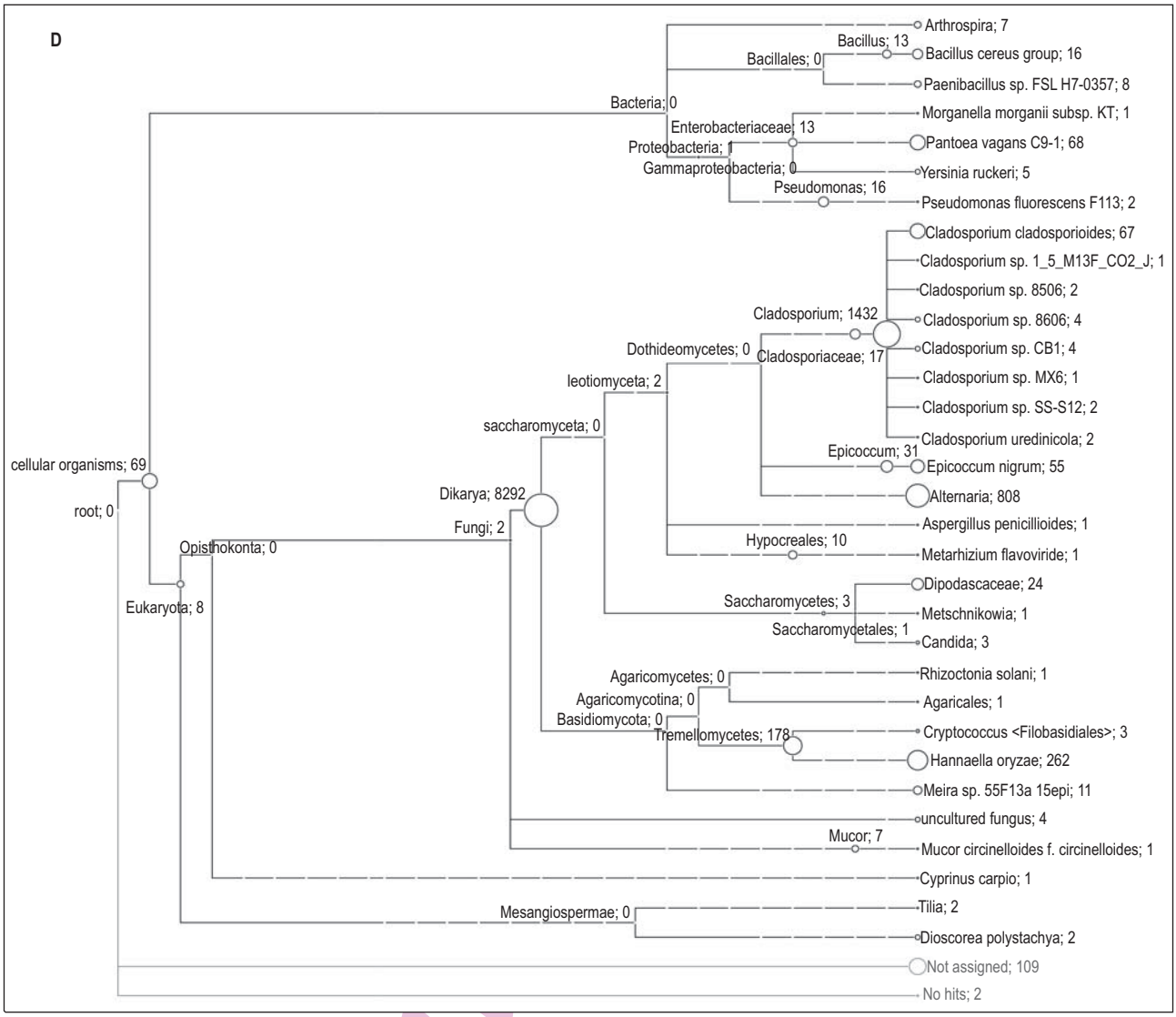


Fig. 3 : Phylogenetic tree with taxonomic positions of different bacterial and fungal operational taxonomic units (OTUs) detected in persimmons samples by sequencing of 16S rDNA and 18S rDNA. The tree was constructed by maximum likelihood method implemented in MEGAN (V5.10.6). A : Stalks of undried astringent persimmons; B : Stalks and surfaces of dried astringent persimmons; C : Stalks of Korean commercial persimmons and D : Stalks of Chinese commercial persimmons

antibacterial compounds from a marine fungus identified as *Cladosporium* sp. (Qi *et al*., 2009). Additionally, *Mucor* sp. and *Fusarium* sp. were the dominant genera in the stalks of commercial persimmons. Fungi of *Fusarium* sp. are plant pathogens and source of important mycotoxins in animal and human health (Placinta *et al*., 1999). Lee *et al*. (2014) reported identification of Fusarium-derived mycotoxins in rice.

Metschnikowia sp. was the dominant microorganism present in group A and B as compared with those of groups C and D on the hit map. Group C and D were the products of Korean herbal medicines (Kaki Calyx) and Chinese herbal medicines. *Metschnikowia* sp. were detected in group A and B (stalks of herbal medicines without the manufacturing process). Consistent with this, *Metschnikowia* sp. has varied habitats, including flowers, surfaces of plants and fruits (Álvarez-Pérez *et al*., 2016).

Notably, stalks of astringent persimmons without processing contained *Metschnikowi*a sp., whereas stalks of processed persimmons did not contain *Metschnikowia* sp. Thus, the process used to make commercial stalk products could affect *Metschnikowia* sp. *Metschnikowia* sp. was amplified for identification in detail. The size of the *Metschnikowia* sp. PCR product was approximately 377 bp (Fig. 4). The results of sequence analysis of 18S rDNA gene showed that there were no matches greater than 97%. Generally, if genomic DNA

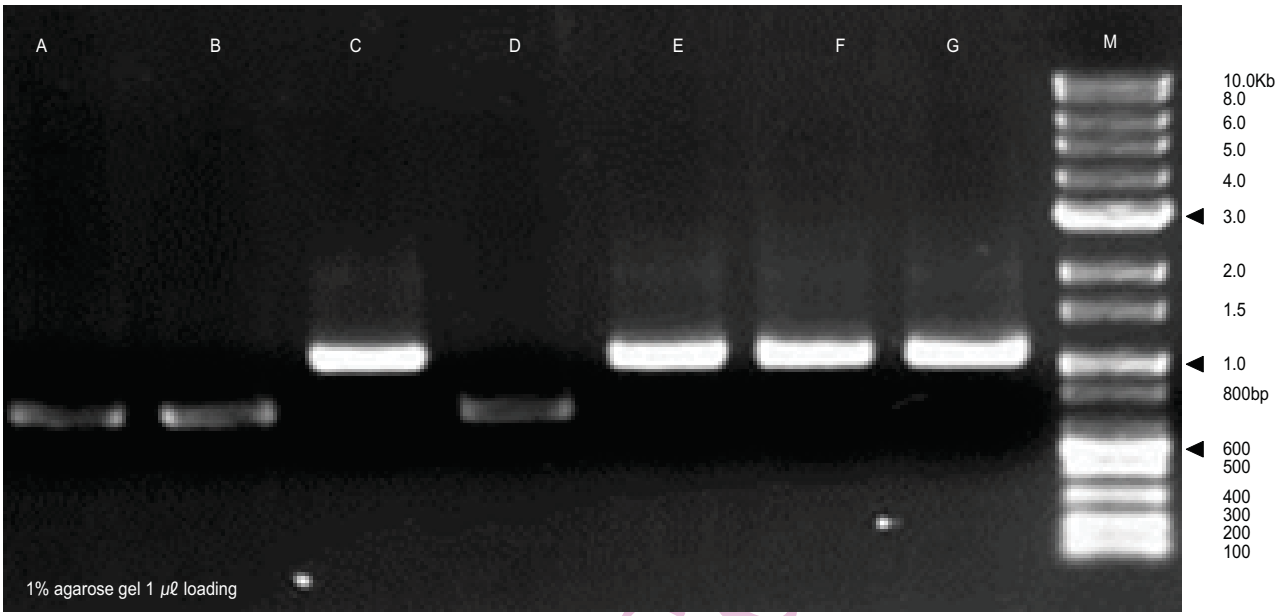


Fig. 4 : Amplication of fungal 18s RNA by PCR A, B, D: PCR products of Hanseniaspore uvarum (original size : 700bp, total size: 1011bp), C, E-G: PCR products of Metschnikowia sp. (original sixe 377bp, total size 637bp)

relatedness is less than 70%, the strain is considered to be an independent species based on the microorganism taxonomic system (Wayne *et al*., 1987). If the 16S rRNA sequence similarity is less than 97%, the genomic DNA relatedness is also less than 70%, as demonstrated by statistical analysis (Stackebrandt and Goebel, 1994). Therefore, the *Metschnikowia* sp. identified in the present study may be a novel species.

Metchnikowia pulcherrima strains have been reported to have biocontrol potential against blue mold of apple (Janisiewicz *et al*., 2011). In fact, microorganisms can be the most effective alternative method apart from use of fungicides, and hence the discovered *Metschnikowia* sp. in this study can also be a potential candidate in biocontrol application. Also various species of *Metschikowia* sp. including like *Metschnikowia koreensis* sp. nov. and *Metschnikowia persici* sp. nov. have been reported to be newly found in flowers and fruits of plants (Hong *et al*., 2001; Wang *et al*., 2017).

The findings of this study provide a foundation for the identification of useful microorganisms that may be beneficial for astringent persimmons. To the best of our knowledge, this is the first report demonstrating the presence of *Metschnikowia* sp. within the microbial community of astringent persimmons. In addition, only three cultivars from Gyeongnam province were used. Further studies need to be conducted using cultivars from other localities in the next study. In recent years, studies on the antimicrobial activity using microorganisms isolated from soil or plants have been carried out (Abid *et al*., 2016). This article also provides useful basic information on various microorganisms isolated from nature's fruits and provides a list of microorganisms on which experiments can be carried out in future.

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