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Identification and Taxonomy of the Endophytic Fungi and Potential Pathogens in *Citrus* Plants

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The genus *Citrus* (Aurantioideae, Rutaceae) is the sole source of the citrus fruits of commerce showing high economic values. However, plant disease and insect pests are always threatening citrus plants resulting in destructive losses in fruit yields. Although some pathogen agents of disease have been well known, until now, there have not been efficient methods for disease prevention and cure. In the present work, we collected 30 healthy citrus plants covering 23 *Citrus* species as object of study, and investigated their species of endophytic fungi and potential pathogens from different environmental situations. Based on the sequence analysis of nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) region, 7 fungus species were determined and identified as potential pathogens, and additional 3 fungus/bacterial species were undetermined and considered as endophytic fungus/bacteria having no obvious pathogenic tendency. We suggested that potential pathogens on plants need to accumulate to certain amounts and induce disease symptom until development of certain stages or there is some endophytic fungi able to inhibit the growth of pathogens. This work would provide an efficient and accurate method to identify fungus species, and help the early detection and prevention of plant disease, and instruct the disease control using biological methods.

Key words: *Citrus*, endophytic fungi, fungal diversity, pathogens.

Citrus is one of the most important fruit crops in the world with production in over 100 countries[1]. It is widely grown in the tropical, subtropical, and borderline subtropical areas, with total global production reaching 7.4 million metric tons just during 2009-2010[2, 3]. However, citrus species are often puzzled by abiotic and biotic diseases, inducing destructive losses in fruit yields[4-6]. Among them, abiotic stresses are caused by natural disaster or severe climate alter, while biotic stresses are mostly caused by fungi or bacteria. One of the most widespread and devastating diseases is Huanglongbing (HLB), which is caused by *Candidatus liberibacter*[7]. Another disease, citrus bacterial canker disease (CBCD) results in canker of leafs or barks, which is caused by bacterial species, *Xanthomonas campestris*[8]. Citrus chlorotic dwarf disease (CCDD) resulting in leaf chlorosis is transmitted by the

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bayberry whitefly *Parabemisia myricae* Kuwana[9, 10]. Although many literatures on the disease symptoms of citrus plants are available, such as leaf spots, chlorosis, and canker[5, 11], until now, there have not been efficient methods for control, prevention or cure.

The occurrence of citrus diseases is considered to be closely associated with their environmental factors, such as climate, geographic situation, and even their environmental fungi (resulting in endophytic fungi or called parasitic fungi). Endophytic fungi live their entire life cycle inside plant tissues without causing apparent symptoms of infection, and form association with all plants[12-14]. They have certain relationships with growing environment and geographic situation[15], just as said that one place has their own people, able to extend in animals (like panda), plants and even fungi. If their environment alters, it might influence species diversity of endophytic and parasitic fungi[16]. Pathogens also belong to some kind of bacteria or fungi. The coexistence of plants, endophytic and parasitic fungi and pathogens forms competitive relations with each other, because all kinds of fungi compete for one same ecological niches[17, 18]. On the other hand, the existence of endophytic and parasitic fungi could improve the interactions and defense systems of plants with potential pathogens. Some researchers found that fungal endophyte species might grow into pathogens in some phase of life cycle[19]. Thus, monitoring and characterizing fungal endophytic communities and their interactions is crucial to understanding fungal diseases of the host plant. In addition, early detection of pathogens would instruct and help the efficient disease control of the host plants. Thus, identifying of fungal endophytic communities is also the prerequisite and requirement for best management practice.

Some of fungal pathogens of disease have been well known, such as HLB's pathogen, *Candidatus liberibacter*, while the others have not yet, especially some endophytic fungi[20]. Although according to the disease diagnoses of citrus plants, we could initially estimate which kind of diseases plants fall and which pathogens plants are infected by, however, the diagnostic techniques are time consuming and subject to methodological and experimental errors[21], and difficult to use for those less frequently infected diseases[22]. In recent years, highly sensitive biochemical techniques have been developed. These techniques allow disease diagnosis in few hours, however, these techniques are sometimes expensive for reagents and instruments and professional training is required. The limitation of these techniques results in difficult extensive use. Based on these shortcomings, the manipulation-simple, price-acceptable, more efficient and accurate techniques are required to develop. Recently, molecular identification techniques based on PCR amplification of representative fragments and then DNA sequence analysis have been widely used for species discrimination. The application of these molecular marker techniques is to examine and analyze the genome-wide variability. Among them, the internal transcribed spacer (ITS) region of 18S-28S nuclear ribosomal DNA (nrDNA) is mostly widely used for phylogenetic studies[23]. It allows high nucleotide variability of ITS sequence, easily PCR amplification, and high primer universality[24, 25]. This region has been used for phylogenetic studies of microbe, plants, and even animals[26-28]. If this molecular identification technique is used for fungal species discrimination from environmental citrus plant materials, it would achieve efficient and accurate estimation of species of endophytic fungi/pathogens and early detection of potential pathogen-infected citrus plants. In this study, we collected 30 *Citrus* populations consisting of 23 *Citrus* species and detected the existence instance of their endophytic fungi. Our objectives were to: (1) examine the assemblage of endophyte fungi of 30 *Citurs* populations; (2) conduct a comparative study of fungal endophyte communities in *Citurs* populations distributed in different geographic situations; (3) study the relations of endophytic fungus species and their geographic situations; (4) assess the possible interactions among these endophytes.

MATERIALS AND METHODS

Plant materials

Tirty *Citrus* plant materials consisting of 23 *Citrus* species, provided by Department of Horticulture, Kangwon National University, Korea, were investigated in the present study. Fresh mature and healthy leaves were sampled from these *Citrus* plants and immediately stored in liquid nitrogen

condition. The fresh leaf tissues were used for DNA extraction. Their specimens and relevant information listed here have been deposited in the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). The NCBI GenBank accession numbers of endophytic fungi/ pathogens of *Citrus* plants investigated in this study was also shown in Table 1. Among them, 17 *Citrus* plants (No. 11-27 of Table 1) were long cultivated in Jeju Island, including *C. natsudaidai*, *C. obovoidea*, *C. tachibana*, *C. grandis*, *C. leiocarpa*, *C. tangerina*, *C. ichangensis*, *C. nippokoreana*, *C. aurantium*, *C. pseudogulgul*, *C. benikoji*, *C. erythrosa*, *C. sunki*, and *C. platymamma*. Other *Citrus* plants were cultivated in other geographic situations.

Isolation of DNA, PCR amplification and sequencing

DNA extractions were performed by using the modified cetyltrimethylammonium bromide (CTAB) method[29]. The ITS1-5.8S-ITS2 region was amplified using universal primers ITS1 and ITS4 in 20 µl PCR reaction[30] PCR amplification was conducted using this set of primers with the following program: 35 cycles of denaturation at 95°C for 1 min, annealing 54°C for 1 min, and a final extension step at 72°C for 1 min. The amplification products were checked by electrophoresis through 1.0% agarose gel, and then purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) or Gel Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at MACROGENE Advancing through Genomics (Korea, http:// dna. macrogen. com/kor/).

Sequence editing and analogue

For editing and assembly of the complementary strands, the software program DNAMAN version 6.0 (Lynnon Biosoft Corporation, USA, www.lynon.com) was used. Analogue of our sequences and nucleotide sequence comparisons were detected with Basic Local Alignment Search Tool (BLAST) network services against databases (http://www.ncbi. nlm. nih.gov/). The multiple sequence alignment of ITS1-5.8S-ITS2 region was also performed using DNAMAN version 6.0 software, to detect single nucleotide polymorphisms.

RESULTS AND DISCUSSION

PCR amplification

PCR amplification of nrDNA ITS region was performed using ITS universal primer sets, ITS1 and ITS4 [30]. Due to a very broad range of primer universality, each DNA samples extracted from 30 environmental *Citrus* plant materials investigated in this study generated 1~3 bands with ~100 bp variation in length. The main and larger fragments were from plant DNAs, while the shorter ones were from endophytic fungi, and pathogens from environmental conditions. Among all *Citrus* plant materials, seven samples had only plant DNA amplification (Table 1), that might be caused by experimental errors or little coexistence with fungi. Easy amplification of nrDNA ITS region is one advantage, simultaneously one disadvantage. For the amplification with relative large variation, the sequencing and following analysis could be performed. If the fragments amplified from environmental materials are narrowly varied, species-specific primers should be newly designed. In the present study, ~100 bp length variation of amplified fragments was enough to separate with each other and perform the sequencing.

Sequence analogue

Each DNA fragment was sequenced and analogized using the BLAST on NCBI server (http:/ /www.ncbi.nlm.nih.gov/). The identity of our sequencing results is above 93% to 99% compared to sequence sources of existent plant and fungus species from GenBank database. The results of species discrimination were shown in Table 1, remarked following relevant NCBI GenBank accession numbers. Seven determinate fungus species (including *Cladosporium* spp., *Colletorichum* spp., *Leptosphaerulina* spp., *Guignardia* spp., *Sporobolomyces* spp., *Phoma* spp., and *Tilletiopsis* spp.) and three undetermined fungus/bacterial species were detected from our DNA samples (Table 1). In order to understand the communities of endophytic fungi and pathogens, the phylogenetic tree was constructed based on the DNA alignment using DNAMAN software version 6.0 (Fig. 1). There was clear clade between the exclusive bacterial species from kk-22 material (JQ990194) and fungus species (all other samples),

sharing 33% identity rate. Among the endophytic fungi and pathogens, two main clades consisting of eight sub-clades were divided: the uncultured fungi from kk-13, p-19, p-13, kk-53, kk-74, and p-74 formed one sub-clade; *Sporobolomyces koalae* from kk-80 formed one; *Tilletiopsis pallescens* from kk-106 and p-106 formed one; *Cladosporium* spp. from kk-30, kk-69, kk-98, p-69, kk-77, and kk-66 formed one; *Colletotrichum* spp. from kk-70, p-71, kk-72, and kk-71 formed one; *Guignardia* spp. from kk-80 formed one; *Leptosphaerulina australia* from kk-75 and kk-76 formed one; *Phoma* spp. from

Table 1. Taxon of Citrus plants investigated in this study and their specimen voucher, NCBI GenBank Accession Number, and endophytic fungi and pathogens.

No.	Species	Tribe	Specimen voucher	NCBI submission	Endophitic fungi and pathogens
1	*Citrus kinokuni*	*Sinocitrus*	kk-8	-	
2	*Citrus unshiu*	*Sinocitrus*	kk-13	JQ990191	uncultured fungus
3	*Citrus unshiu*	*Sinocitrus*	p-13	JQ990192	uncultured fungus
4	*Citrus medica var. sarcodactylis*	*Citrophorum*	p-19	JQ990193	uncultured fungus
5	*Citrus sinensis*	*Aurantium*	kk-22	JQ990194	uncultured bacterial
6	*Citrus hassaku*	*Sinocitrus*	kk-28	-	
7	*Citrus grandis*	*Cephalocitrus*	p-29	-	
8	*Citrus hybrid*	-	p-30	JQ990195	*Cladosporium* spp.
9	*Citrus spp.*	-	p-53	JQ990196	uncultured fungus
10	*Citrus limon*	*Citrophorum*	kk-55	-	
11	*Citrus natsudaidai*	*Sinocitrus*	kk-57	-	
12	*Citrus obovoidea*	*Sinocitrus*	kk-66	JQ990197	*Cladosporium* spp.
13	*Citrus tchibana*	*Sinocitrus*	kk-69	JQ990198	*Cladosporium* spp.
14	*Citrus tchibana*	*Sinocitrus*	p-69	JQ990199	*Cladosporium* spp.
15	*Citrus grandis*	*Cephalocitrus*	kk-70	JQ990200	uncultured fungus
16	*Citrus leiocarpa*	*Sinocitrus*	kk-71	JQ990201	*Colletotrichum gloeosporidoides*
17	*Citrus leiocarpa*	*Sinocitrus*	p-71	JQ990202	*Colletotrichum gloeosporidoides*
18	*Citrus tagerina*	*Sinocitrus*	kk-72	JQ990203	*Colletotrichum gloeosporidoides*
19	*Citrus ichang papeda*	*Papedocitrus*	kk-73	-	
20	*Citrus nippokoreana*	*Sinocitrus*	kk-74	JQ990204	uncultured fungus
21	*Citrus nippokoreana*	*Sinocitrus*	p-74	JQ990205	uncultured fungus
22	*Citrus aurantium*	*Sinocitrus*	kk-75	JQ990206	*Leptosphaerulina australis*
23	*Citrus pseudogulgul*	*Sinocitrus*	kk-76	JQ990207	*Leptosphaerulina australis*
24	*Citrus benikoji*	*Sinocitrus*	kk-77	JQ990208	*Cladosporium cladosporioides*
25	*Citrus erythrosa*	*Sinocitrus*	kk-78	JQ990209	unclutured fungus
26	*Citrus sunki*	*Sinocitrus*	kk-79	-	
27	*Citrus platymamma*	*Sinocitrus*	kk-80	JQ990210 JQ990211 JQ990212	*Guignardia* spp. *Sporobolomyces koalae* *Phoma* spp.
28	*Citrus unshiu*	*Sinocitrus*	kk-98	JQ990213	uncultured fungus
29	*Poncitus trifoliata*	*Poncitus*	kk-106	JQ990214	*Tilletiopsis pallescens*
30	*Poncitus trifoliata*	*Poncitus*	p-106	JQ990215	*Tilletiopsis pallescens*

- means indeterminate or non-detection.

kk-80 formed one; the first three sub-clades formed one clade, sharing 49% identity rate with the other clade composed of the later five sub-clades.

In sub-clade level, some sequence could not be estimated to certain fungus species according to simplex BLAST result, e.g. uncultured fungus from kk-98 in *Cladosporium* spp. sub-clade, and uncultured fungus from kk-70 in *Colletotrichum* spp. sub-clade. However, through the phylogenetic analysis, the sequences showed high identity with sequences which had been identified to determinate fungus species. Based on this result, we boldly forecasted that uncultured fungi from kk-98 and kk-77 were similar to *Cladosporium* spp. and *Colletotrichum* spp., respectively.

Species discrimination of endophytic fungi

Sometimes, the fungus species exhibiting specific functions or leading to diseases were easily found and investigated as focus of current researches, while those having no distinct functions were easy to ignore. Most endophytic fungi were considered as this poorly known group. Even, we did not definitude their species affiliation situation, like the uncultured fungus sub-clade composed of kk-13, p-19, p-13, kk-53, kk-74, and p-74 (Fig. 1). However, these endophytic fungi might form closely community with other endophytic fungi and pathogens, operate to fungal ecological balance, pathogen inhibition, and even disease control. Identifying and charactering endophytic fungus species are also an important work.

Another uncultured fungus from kk-78, in this study, showed 76% identity rate with *Leptosphaerulina australia* and *Phoma* spp. and form one sub-clade with them, indicated that this fungus species affiliation was similar to pathogenic *Leptosphaerulina australia* and *Phoma* spp. Close attentions on growth stage of this uncultured fungus from kk-78 were needed, in case it changed to pathogenic. In addition, the exclusive bacterial species from kk-22 was probably from environmental bacterial, which had close relation with geographic situation of plant materials.

Species discrimination of pathogens

Citrus plants are facile to infect with various diseases, so the pathogens have a variety of species. In this study, seven determinate fungus

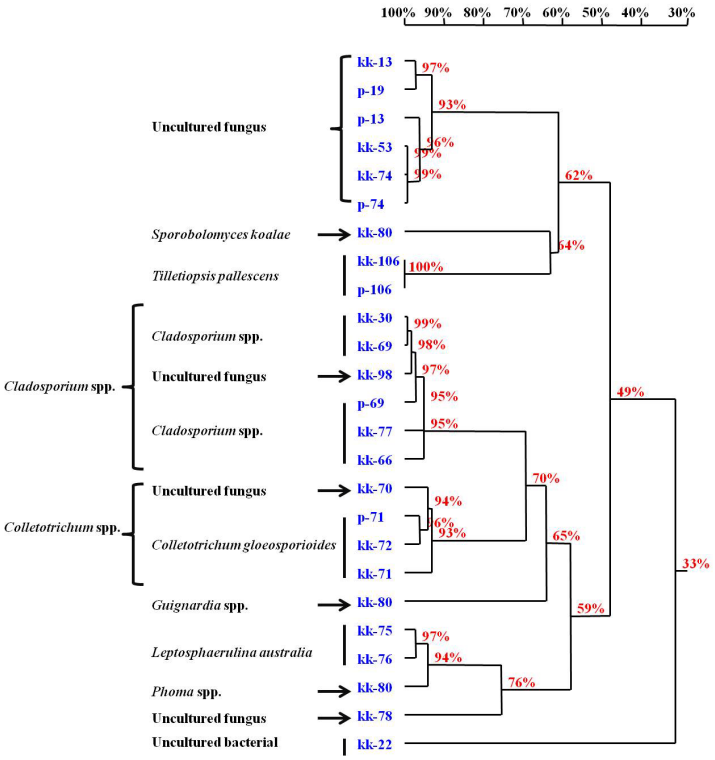

Fig. 1. Phylogenetic tree of endophytic fungi and pathogens detected from 30 citrus plant materials constructed by nrDNA ITS region sequence

species were detected, including *Cladosporium* spp. (*Cladosporium cladosporioides*), *Colletotrichum gloeosporioides*, *Leptosphaerulina australia*, *Guignardia* spp., *Sporobolomyces koalae*, *Phoma* spp., and *Tilletiopsis pallescens* (Table 1, Fig. 1). As described previously, the fungal community of endophytic fungi and pathogens might have close relations with geographic situations, our result showed evidence: *Citrus* plant materials collected form Jeju Island, South Korea were detected to carry pathogens, while other plant materials collected from other geographic situations were difficult to detect fungal DNA.

Detected *Cladosporium* spp. commonly found in nature, have previously been found in *Citrus* species[31-37]. *Cladosporium elegans*, *Cladosporium citri* have been isolated from *Citrus* spp.[31, 32]. Undetermined *Cladosporium* species have been isolated from rotten fruit of *C. junos*[33], and *C. unshiu*[34]. *Cladosporium herbarunm* has been isolated from *C. unshiu*, *C. junos*, and *Fortunella* spp. fruits[35]. For *Cladosporium cladosporioides*, this species has been isolated from *C. reticulate* in Yunnan[36], from debris of *Citrus* spp. in Trinidad[37], resulting in sooty spots in citrus plants. There were six plant materials investigated in this study detectable in *Cladosporium* spp. (Table 1, Fig. 1). Thus, more administrations and attentions should be paid on these plant materials, in order to instruct early prevention and control of disease.

Detected *Colletotrichum* spp. could cause anthracnose disease and postharvest decay on many tropical, subtropical, and temperate fruits, such as citrus fruits. *Colletotrichum acutatum* has been considered to as the pathogen agent of postbloom fruit drop[38]. *Colletotrichum gloeosporioides* has been isolated from *Citrus* spp., as the pathogen agent of postharvest anthracnose. Ten strains of *Colletotrichum* spp. have been isolated from *C. reticulate*[39]. In the present work, four plant materials were found parasitic by *Colletotrichum* spp. (Table 1, Fig. 1). Supervising the stage of growth and development of pathogens in these plant materials was considered crucial.

Tilletiopsis spp. has evidence of inhibiting powdery mildew in cucumber by synthesizing antifungal protein[40, 41]. Using this biological effect of *Tilletiopsis* spp., this fungus was even used for biological control of *Botrytis cinerae* in grape and strawberry[42]. In this study, *Tilletiopsis pallescens* was isolated from two citrus plants, kk-106 and p-106. We boldly predicate that the reason why there is no other pathogen detected in both citrus materials is the existence of endophytic fungi *Tilletiopsis pallescens* having inhibitive effect.

The citrus plant material of kk-80 was detected to exhibit three fungus species, including *Guignardia* spp., *Sporobolomyces koalae*, and *Phoma* spp. (Table 1, Fig. 1). Among them, *Guignardia* spp. is a common parasitic fungus species, sometimes, they might result in black spot disease in citrus plants, e.g. by *Guignardia citricarpa*[43], and dieback disease in Pinaceae plants and citrus plants, e.g. by *Guignardia laricina*[6], while sometimes, they show bioactive antifungal effect[44]. *Sporobolomyces koalae* is an anamorphic yeast, which has not been suspected to be pathogenic. *Phoma* spp. are commonly found to be parasitic in plants, and suspected to induce stalk spot or leaf spot disease in various species of plants[45].

Communities of endophytic fungi and pathogens and potential application

Whether endophytic fungi and pathogens could live with healthy citrus plants? The answer is positive. The *Citrus* plant materials investigated in this study was from healthy citrus trees, however, the affirmative result of fungal detection fully illuminated the probability of coexistence of healthy plants with endophytic fungi and pathogens. In this study, the healthy *Citrus* tree, kk-80 showed a positive evidence of the coexistence of endophytic fungi (*Guignardia* spp. and *Sporobolomyces koalae*) and pathogens (*Phoma* spp.). The results suggested that pathogens only running up to a certain amount could produce disease, while in the absence of a comparative amount, they were only potentially pathogenic or not pathogenic. Thus, it is a good time for early detection of citrus disease and prevention management before pathogens parasitic in citrus plants proliferate to reach a certain amount. However, the reason of citrus tree, kk-80 still keeping healthy might have another explanation. It was that the existence of *Guignardia* spp. having biological antifungal effects might inhibit the growth of pathogen,

Phoma spp., and keep the balance of amounts of antifungal *Guignardia* spp. and pathogenic *Phoma* spp. This work provides an efficient and accurate protocol of identifying endophytic fungus and pathogen species based on sequence analysis of nrDNA ITS region, which makes early detection of citrus disease easy and time-saved. This result could apply for instructing disease prevention management of citrus trees and other commercial crops.

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