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Ganoderma Infecting Citrus in Texas is a Unique Taxon within the *G. lucidum* Complex: Evidence from the ITS Region

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ABSTRACT

Decline and death of citrus trees on sour orange rootstock in Texas is attributed to a member of the *Ganoderma lucidum* species-complex. Internal transcribed spacer (ITS) of this taxon showed high identities with several *Ganoderma* nucleotide sequences previously deposited in the National Center for Biotechnology Information (NCBI) GenBank database. The putative ITS phylogeny generated by comparison with twenty closest *Ganoderma* species showed that a Texas citrus *Ganoderma* isolate described here is a unique taxon. ITS sequence can be used for rapid clinical diagnosis of this fungus in multiple crops.

Keywords: diagnosis, fungi taxonomy, heart rot, internal transcribed spacer, ribosomal DNA, root rot

INTRODUCTION

Ganoderma lucidum (W. Curtis: Fr.) P. Karst., is a wood decay basidiomycete with a wide host range and geographical distribution. This fungus is traditionally used in Asia as medicine for the treatment of cancer, diabetes, and heart diseases. Recently, there is an increased interest in the pharmaceutical properties of *Ganoderma lucidum* (Berger *et al*. 2004). There are some reports on its use in biopulping of wood in the paper industry and for degradation of pollutants such as polychlorinated biphenyls (PCB) and chlorinated phenols (Adaskaveg and Gilbertson 1994).

Several *Ganoderma* species are pathogens that cause heart rot or butt rot in plants, while some are saprophytic. Being a relatively uncommon pathogen, environmental stresses should predispose the citrus trees to *Ganoderma* infection and subsequent damage (Skaria 1990). Nevertheless, *Ganoderma* is sporadically reported causing disease in citrus, such as root rot of sweet lime in Palestine (Reichert 1932) or heart rot in Florida (Knorr 1973). Although it is not a major problem in US citrus, three species were previously reported including *G. applanatum*, *G. brownii*, and *G. lucidum* (Farr *et al*. 1989).

A decline and death of citrus trees on sour orange rootstock in Texas was reported to be associated with a member of the *G. lucidum* complex (Skaria and Farrald 1989; Skaria 1990; Skaria *et al*. 1990). In Texas, *Ganoderma* infection was found in 'Marrs' early orange on sour orange rootstock, Cleopatra mandarin, and Swingle citrumelo (Skaria 1990). The presence of the infected old stumps of the citrus trees that were killed in the 1983 freeze might have contributed to the spread of the fungus to the newly replanted young citrus trees. Removal of the *Ganoderma*-infected citrus trees is the only disease management practice followed in US, however, care should be taken to remove all the old stumps and roots in the vicinity of the tree as they may function as inoculum source to spread the disease.

The application of molecular mycology for taxonomy that started in the late 1970s (de Bertoldi *et al*. 1973) has developed into many sophisticated techniques. Such techniques allow non-taxonomists to make species identifications. One of the widely used techniques is the use of the region of DNA that codes for the production of the ribosomes (Hills and Dixon 1991). Moreover, it was reported that the sequence variation may occur in ITS1, ITS2, and 5.8S regions which showed ITS heterogeneity with in as well as different *Ganoderma* strains (Wang and Yao 2005). In this study, we used the nucleotide sequence of the ITS region to determine its diagnostic value for identification of *Ganoderma* isolated from Texas citrus. The ITS nucleotide sequence data generated in this study is useful in identifying *Ganoderma* and aid in the future studies of Texas citrus *Ganoderma* isolate origin, spread, and adaptation in citrus. Furthermore, the ITS based phylogenetic studies may provide insight in the relationship of Texas citrus *Ganoderma* isolate with other species of Polyporaceae which is useful in disease epidemiological studies in citrus and other plants.

MATERIALS AND METHODS

A total of six *Ganoderma* fruiting bodies from citrus in Mission and Weslaco, TX were used in this study. The genomic DNA was extracted from all these samples and PCR amplifications were performed using BMB-CR/LRO, BMB-CR/5.8S, and 5.8S-R/LRO primers (Vilgalys and Hester 1990) to amplify fragments of ITS1-5.8S-ITS 2, ITS1, and ITS2 regions, respectively.

Fungal material and DNA extraction

Genomic DNA was extracted directly from the fruiting bodies collected from citrus using MasterPure Yeast DNA Purification Kit (Epicentre, Madison, WI) following manufacturer's instructions. Approximately 100 mg fungal tissue was cut from the pore layer, transferred to a chilled mortar, and ground to powder using liquid nitrogen. Further procedure of cell lysis was carried out by adding 300 µL of Yeast Cell Lysis Solution, proteins were precipitated with 150 mL of MPC Protein Precipitation Reagent, and DNA precipitation was done by adding 500 mL of isopropanol. The DNA pellet was washed with 70% ethanol and resuspended in 40 µL of TE buffer.

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Short Communication

Table 1 Gene bank accession numbers and geographical origin for *Ganoderma* and *Coriolopsis* ITS 1 and ITS 2 nucleotide sequences used in multi-alignment and putative phylogenic tree prediction.

Serial No.	Taxa	ITS 1 and ITS2	Geographical origin
1	Texas citrus *Ganoderma* isolate	EU147278 and EU147279	USA
2	*Coriolopsis caperata*	EU030178.1	Central America
3	*G. lucidum*	Z37051.1	Taiwan
4	*G. lucidum*	FJ501561.1	China
5	*Hericium erinaceum*	EU520249.1	China
6	*Ganoderma* sp.	AY636069.1	India
7	*G. lucidum*	AY636059.1	India
8	*G. boninense*	X78749.1	Taiwan
9	*Ganoderma* sp.	AY508882.1	India
10	*G. pseudoferreum*	FJ392284.1	China
11	*G. pseudoferreum*	FJ392283.1	China
12	*G. lucidum*	EU021462.1	Taiwan
13	*G. lucidum*	EU021460.1	Taiwan
14	*G. lucidum*	EU021459.1	Taiwan
15	*G. lucidum*	AY636068.1	India
16	*G. philippii*	AJ627584.1	Indonesia
17	*G. lucidum*	Z37048.1	Taiwan
18	*G. lucidum*	X78743.1	Taiwan
19	*G. lucidum*	X78745.1	Taiwan
20	*G. lucidum*	X78744.1	Taiwan
21	*G. pseudoferreum*	FJ392285.1	China

Polymerase chain reaction

PCR was performed in a total volume of 25 µL containing 50 ng DNA, 2 mL 10X PCR buffer (Tris HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7), 0.2 mM each of dNTPs, 2 µM each of either primers BMB-CR (5'-GTACACACCGCCCGTCG-3')/5.8S (5' -CGCTGCGTTCTTCATCG-3'), 5.8S-R (5'-TCGATGAAGAACGCAGC-3')/LRO (5'-GCTTAAGTTCAGCGGGT-3') or BMB-CR/LRO (Vilgalys and Hester 1990), and 1U Hot star *Taq* DNA polymerase (Qiagen Inc., Valencia, CA). The reaction was incubated in the DNA engine dyad Peltier thermal cycler (Bio-Rad Laboratories, Hercules, CA). PCR temperatures were as described by Vilgalys and Hester (1990). The PCR products were run on 1% agarose gels prepared in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide and visualized under ultraviolet (UV) light.

DNA sequencing

The 427, 360, and 769 bp fragments amplified with primers BMB-CR/5.8S, 5.8S-R/LRO, and BMB-CR/LRO were cut from the gel, purified using Qiaquick Gel Extraction kit (Qiagen Inc.), cloned into pCR4-TOPO vector (Invitrogen Corp., Carlsbad, CA), and sequenced (MWG-Biotech Inc, High Point, NC). The DNA sequences were compared to the GenBank database for homology. Twenty ITS1 and ITS2 nucleotide regions showing high identities with Texas citrus *Ganoderma* nucleotide sequences were aligned using ClustalW (v1.4) multiple alignment (Thomson *et al*. 1994), distances were calculated using Phylip's DNADIST (Felsenstein 1993), and putative phylogenetic tree was prepared using the Fitch-Margoliash method (Fitch and Margoliash 1967).

RESULTS AND DISCUSSION

The basidiocarp of Texas citrus *Ganoderma* was circular to oval shaped with a yellow to dark brown, smooth margin, and laccate pileus surface (**Fig. 1**). These morphological characters may indicate that this isolate belong to *G. lucidum* complex. Primers BMB-CR/5.8S, 5.8S-R/LRO, and BMB-CR/LRO amplified ITS 1, ITS 2, and ITS 1-5.8S-ITS 2 regions, respectively yielding 427, 360, and 769 bp amplicons from all the isolates (**Fig. 2**). The ITS 1 and ITS 2 nucleotide sequences from all these isolates were 100% identical. The nucleotide sequences were also deposited in the Genebank (**Table 1**).

A homology search for the ITS 1 and ITS 2 nucleotide sequences of Texas citrus *Ganoderma* isolate at GenBank database showed high identities to several members of *Ganoderma* nucleotide sequences. We also analyzed the multiple alignments of the ITS 1 and ITS 2 sequences of Texas *Ganoderma* citrus isolate with 20 closest polypore members (**Table 1**) through ClustalW (v1.4) multiple alignment. The distances were calculated using Phylip's DNADIST, and a putative phylogenetic tree was prepared using the Fitch-Margoliash method (**Fig. 3**). The tree topologies indicate that the *Ganoderma* infecting Texas citrus is

Fig. 1 Basidiocarps of *Ganoderma* on a grapefruit symptomatic tree in Texas. These were the typical fruiting bodies sampled for this study.

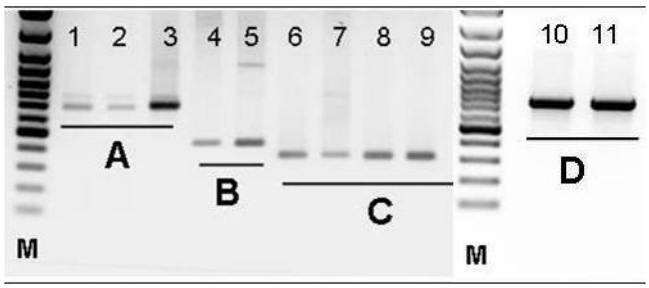

Fig. 2 PCR. Agarose gel showing polymerase chain reaction products amplified with primers BMB-CR/LRO (**A**) and (**D**), BMB-CR/5.8S (**B**), and 5.8S-R/LRO (**C**), yielding 769 bp (ITS1-5.8S-ITS 2), 427 bp (ITS1), and 360 bp (ITS2) amplicons, respectively. M = 100 bp DNA ladder.

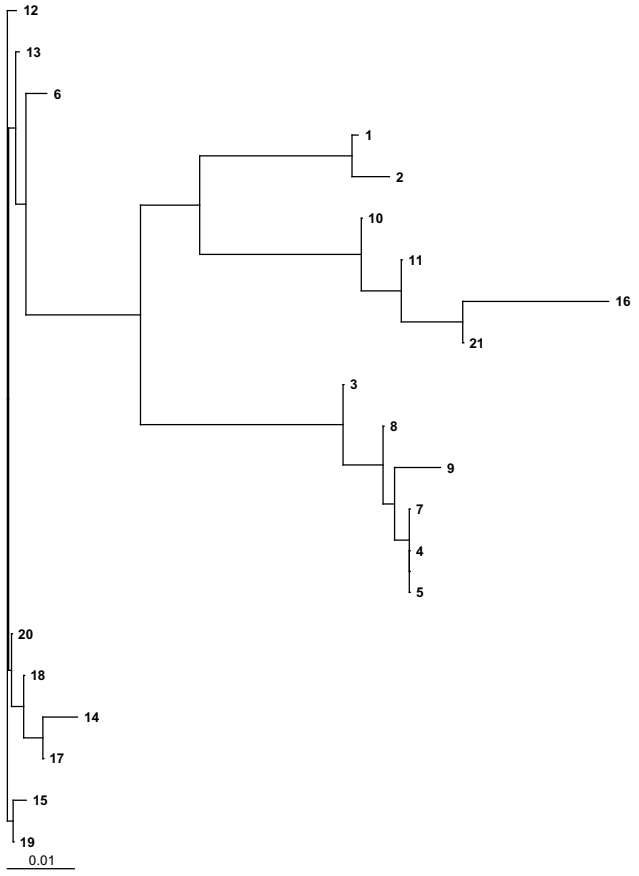

Fig. 3 Putative phylogenetic tree (Fitch-Margoliash method) predicted for Texas citrus *Ganoderma* isolate based on multiple nucleotide sequence alignment of ITS 1 and ITS 2 regions of rDNA with 20 closest members of *Ganoderma*. Identities of the isolates are given in **Table 1**.

a new species with in *G. lucidum* complex. The ITS phylogeny clearly identified distinct clades, with one clade containing Texas citrus *Ganoderma* isolate and *Coriolopsis caperata* (synonymous with *Datronia caperata*) and the other containing *G. philippii*. This may indicate that Texas citrus *Ganoderma* isolate and *Coriolopsis caperata* are closely related taxa and might have evolved from a common ancestor. Recently, a phylogenetic study of *C. caperata* based on ITS sequences was reported from mangrove forests of Central America (Bergemann *et al*. 2009). *C. caperata* is also wide spread in non-mangrove tropical forests with a broad host ranges (Lindblad 2000). We observed a very low level of nucleotide sequence difference in the ITS 1 and ITS 2 of Texas citrus *G. lucidum* compared to other *Ganoderma* which may indicate a recent divergence from related taxa (Moncalvo *et al*. 1995a; Gottlieb *et al*. 2000).

More than 250 *Ganoderma* species have been described (Ryvarden 1991). *G. lucidum* is the most cosmopolitan member of the genus *Ganoderma* which is polyphyletic depending on geographical origin. Also, the morphological characters to distinguish them are highly variable and overlapping, which makes the identification of species within this genus difficult. Conversely, molecular data of ITS regions and 26S rDNA have proven to be highly useful in the taxonomic classification of *Ganoderma* and correlated well with the morphological classification (Moncalvo *et al*. 1995a, 1995b). Phylogenetic analysis based on ITS DNA sequence was successfully used in classification of Australian *Ganoderma* isolates (Smith and Sivasithamparam 2000) and South American collections of *Ganoderma* (Gottlieb and Wright 1999; Gottlieb *et al*. 2000). However, a very low level of nucleotide sequence divergence in the rDNA among *Ganoderma* and a highly variable sequence variation among species suggest that extreme care should be taken in interpreting data inferring *Ganoderma* taxonomic grouping (Moncalvo *et al*. 1994, 1995a; Smith and Sivasithamparam 2000). It is important to consider morphological, cultural, and molecular data together in determining the phylogenetic classification of *Ganoderma*. However, our ITS sequence data may provide a rapid, clinical diagnosis of this fungus. It is a valuable tool in the identification process of *Ganoderma* that attack different host plants.

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