

Molecular Phylogenetic Identification of Plant-pathogenic Fungi Isolated from Two Medicinal *Atractylodes* *macrocephala* Koidz Species

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Abstract

Plant-pathogenic fungi are a large and diverse group of organisms which exhibit great importance in agriculture and natural plant communities. In this study, we investigated the taxonomic identities and phylogenetic relationships of pathogenic fungi isolated from two medicinal *Atractylodes macrocephala* Koidz species, Swordlike *A. macrocephala* and Largehead *A. macrocephala*, using the morphological and molecular approaches. Morphological differences among the fungal isolates indicate that diverse distinct morphotypes might be present within the hosts. Forty fungal isolates were selected for further molecular phylogenetic analysis using the internal transcribed spaces (ITS1 and ITS2) of nuclear ribosomal DNA sequence and the intervening 5.8s gene region. While assessing diversity of *Fusarium lateritium* isolate and *Gibberella zeae* species from medicinal *A. macrocephala* using molecular approaches, Based on an 18S rRNA PCR approach, 4 fungal clone types were

detected in medicinal *A. macrocephala*, Our results suggest that *F. lateritium* isolate and *G. zeae* species are the dominant pathogenic fungi in the *A. macrocephala* hosts, and some of these pathogenic fungi exhibit host and tissue specificity at the phylogeny level, fungal clone type A11, B11, D11 belongs to *G. zeae*, while the other C11 clone type belongs to *F. lateritium*. This aspect can be further explored to understand the relationships between plant hosts and their fungal pathogenic. Moreover, we firstly found *F. lateritium* is the dominant pathogenic fungi in *A. macrocephala*.

Keywords: *Atractylodes macrocephala* Koidz, 18srRNA, Fungi, Taxonomy

1. Introduction

Plant-pathogenic fungi show great importance in agriculture and natural plant communities. These pathogenic fungi have evolved different lifestyles. The modes of plant-pathogen interaction also exhibit diversity. Some pathogens can synthesize and secrete toxic metabolites to kill the host cells at infection site and live off the dead tissue, which is referred to as necrotrophy. In contrast, biotrophic fungi do not produce toxins and they require living plants as a source of nutrients. Globally, there are at least one million species of plant-pathogenic fungi (Ganley and Newcombe, 2006). This represents an important genetic resource for biotechnology study. Plant-pathogenic fungi, especially the fungi colonizing medicinal plants, can produce secondary metabolites which have been recognized as potential sources for agricultural, pharmaceutical and industrial uses (Strobel and Daisy, 2003, Hyde and Soyong, 2008). In recent years, plant pathogenic fungi biodiversity, the bioactivity of pathogenic fungi metabolites and the host-pathogen relationship have been received more attention.

In Traditional Chinese Medicine (TCM), Bai Zhu (the rhizomes of *Atractylodes macrocephala* Koidz, a member of the Compositae) is widely distributed in the world, with about 185 species in China and is highly valued for its medicinal properties (LI et al., 2001). *A. macrocephala* can invigorate the spleen, and cure patients with anorexia, excessive perspiration, oedema, splenic asthenia, and abnormal fetal movement (Junhao et al., 1996, Hong et al., 2005). Several recent studies have shown that genetic methods can be successfully used in the studies of pathogenic fungi (Arnold and Lutzoni, 2007; Arnold, 2007; Poulsen et al., 2005; Wang et al., 2005; Morakotkarn et al, 2007; Huang et al, 2008); DNA-based techniques, particularly PCR can detect minute quantities of a pathogen. These methods have provided new opportunities to study and understand, for example, the biology of plant pathogenic fungi, pathogen population structure and dynamics, host- pathogen interactions, etc. These aims have been achieved by optimizing techniques (Mayayo et al., 1999). This mainly based on performing molecular targets, which serve as phylogenetic analysis (Yeo and Wong, 2002). One of targets is the group of genes which encode the nuclear ribosomal RNA (rRNA). Ribosomal DNA (rDNA) is the DNA sequences that direct the synthesis of ribosomal RNA. rDNA is considered a useful marker for phylogenetic studies. The main reason is that its sequences encode multiple-copy loci. The repeated copies are synchronized by concerted evolution (Sugiyama et al., 1999). Furthermore, ribosome are present in all organisms (Van de Peer and De Wachter, 1997), Parts of the molecule are conserved. Therefore, they may serve as reference for evolutionary divergence studies. The 5.8S, 18S, and 25-28S rRNA genes (or rDNAs) are generated from a 35S to 40S precursors, which are spliced out of the transcript. The sequencing of rDNA and internal transcribed spacers (ITS) was applied to fungal taxonomy. Recently, the accumulation of sterile isolates obtained in entophyte surveys obviously improves taxonomic information (Duong et al., 2006; Promputtha et al., 2005; Higgins et al., 2007; Crozier et al., 2006).

2. Materials and Methods

2.1 Plant Material

Healthy plants of medicinal *A. macrocephala* growing in Baoxin was sampled from October 2010 to June 2011. A typical symptom of diseased *A. macrocephala* plants were collected from Baoxin, West Sichuan China. Fresh samples were taken to the laboratory and treated within 8 hours. Digital photographs were taken of each sample, and all the samples were deposited in School of Biological Sciences, Research Center for Pathogenic fungi, Sichuan Agricultural University.

2.2 Isolation of Pathogenic Fungi

A total of 30 samples of both roots and stems from the *A. macrocephala* were first washed in running water. The roots and stems were cut into pieces (5mm in length). Surface sterilization and isolation of Pathogenic fungi followed a modified procedure as described by Schulz et al. (1993), and the details of the procedure were also given in Huang et al. (2007).

2.3 DNA Extraction, Amplification and Sequencing of Fungal ITS

A total of 2 representative isolates of various morphological Pathogenic fungi isolated from each *A. macrocephala* species were selected for molecular identification. Fungal genomic DNA was prepared with the NucleoSpin® DNA extraction Kit (Macherey- Nagel, Düren, Germany). The fungal mycelia grew on PDA for 5-10 days at 25°C and were suspended in 400 µl of buffer C1. About 10 mg of fungal mycelia were scraped from fresh culture with a sterile nipper. All the extraction procedure accorded to the instructions which was given in the kit user's manual. The extracted DNA pellet was kept at -20° C .PCR reactions were carried out in a PTC-100™ thermal cycler (MJ Research, Inc., Watertown, MA, USA) with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Amplified fragment includes ITS1, 5.8S and the ITS2 of rDNA. Amplification was performed in a 25 µl reaction mixture containing 2 x Taq PCR Master Mix 12.5µL, 2 µl primes IST1-F and ITS4-R primers, 3µl of fungal genomic DNA and mini-pure water 7.5µL. The PCR product was purified with High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Germany). Direct DNA sequencing was performed using primers ITS1 (5'-GCATCGATGAAGAACGCAGC-3') and ITS5 on an ABI 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems, Inc.).

2.4 DNA Sequence Assembly and Alignment

Sequence similarity searches were performed for each of the 30 representative fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov>). The ITS1-5.8S-ITS2 sequences of pathogenic fungi isolates were aligned with the sequences of selected reference taxa using the ClustalW and the alignment was inspected and adjusted manually where necessary. The nucleotide sequences of reference taxa along with their GenBank accession numbers are listed in Table 2. GenBank accession numbers of the 10 representative pathogenic fungi sequences from this study and their top BLAST match sequences are given in Table 3.

2.5 Phylogenetic Analysis

Phylogenetic analysis was conducted based on both the ITS and 5.8S gene data using both maximum parsimony (MP) and neighbor joining (NJ) approaches. MP and NJ searches were carried out using MEGA*4.0.2 All characters were equally weighted and unordered. Alignment gaps were treated as missing data. MP analysis was conducted using a heuristic search with treebisection-reconnection (TBR) branch swapping and 100 random addition sequence replicates. Statistical support for the internal branches was estimated by bootstrap analysis based on 5,000 replications. NJ trees were constructed based on the total character differences and bootstrap values were calculated from 1,000 replications.

3. Results

3.1 Isolation and Morphological Grouping of Endophytic Fungi from Two Medicinal *A. macrocephala*

Morphological identification of the 40 fungal isolates from two medicinal *A. macrocephala* was first carried out according to colony or hyphal morphology of the fungal culture, characteristics of the spores, and reproductive structures if discernible (Barnett et al., 1998). Based on these features, the 36 pathogenic fungi could be classified into 2 different morphological taxa (Table 1). Most of the fungal isolates (25 isolates) were obtained from host Swordlike *A. macrocephala*, 14 isolate isolated from its root, 9 isolates from stems. 15 fungal isolates were isolated from host Largehead *A. macrocephala*, 8 from its roots and 5 from stems. Using the traditional morphological techniques, only some of the fungal isolate could be identified to the genus level. Among them, whilst cultural techniques are unsuitable for detection of fungi that are slow growing, or non-culturable in vitro and the choice of the medium may select the species recovered and some fungal isolates (10%) lacking sporulating structures failed to speculate.

3.2 Molecular Identification and Phylogenetic Analysis of Representative Pathogenic Fungi

In addition to the morphological characterization, molecular analyses were carried out to confirm the identification of 2 representative fungal isolates from the two medicinal *A. macrocephala*. Sequence analysis of the ITS consensus sequences (509–535 bp) of the isolate pathogenic fungi were identified in Figure 1.

The sequences from fungal clone types A11, B11, D11 and C11 were further examined through BLAST and FASTA searches and through direct comparisons with potentially related taxa. The highest match was to a *F. lateritium* isolate HMA-1 sequence with the next closest matches to *F. lateritium* (AF310980.1) and *F. tricinctum* (AB470878.1) species. These 2 genera are possibly closely related members of the *Fusarium*, and so the clone type C11 fragment was aligned against the 5 best matches from that order, some further representative species of these genera and representative sequences of the other related fungal orders that were included in the 10 best

matches. The 10 protein-coding genes for each of the fungi were considered as a single unit that was used to construct neighbor-joining (NJ) and maximum parsimony (MP) phylogenetic tree and *Ustilaginoidea virens* (AB092953.1) was used as an outgroup accepting the fact that amino acid sequences are more informative for analyzing sequences as divergent as the reference genes (Bullerwell et al., 2003, Gray et al., 1999) (Fig. 2 and Fig. 3). A phylogenetic tree (Fig. 2) recovered clone type C11 and the 1 best matches in a distinct group within a larger *F. lateritium*, and some of these were also represented by marine genera. These were linked to, but distinct from, a group formed largely of species that were pathogens of other fungi. So *F. lateritium* is the dominant Pathogenic fungi firstly detected in *A. macrocephala*. And the *F. lateritium* grouping was well supported by a terminal bootstrap value of 100%. The distance-based phylogenetic reconstruction also supported this grouping based on the small evolutionary distances within the *F. lateritium* Isolates within this group had differences ranging from 0% to 3% and evolutionary distance less than 3.8% (Table 2, Table 3).

Sequences from clone types A11, B11 and D11 showed 98% similarity over 100% of the sequence to a range of fungal genera that are all placed in the *G. avenacea*. In addition, clone A11, B11 and D11 showed 100% similarity over 94% of the sequence to 1 sequence from *F. culmorum* (AY147313.1). The sequences were included in a phylogenetic analysis with similar sequences from a range of *G. avenacea* species (Fig. 3). Clone types clone A11, B11 and D11 were recovered together with the *G. zeae* (JF303858.1) sequence in a distinct clade. Bootstrap confidence levels were high for these groupings, but generally low across this dataset.

4. Discussion

In this study, we examined the genetic diversity of *F. spp.* and *G. zeae* Spp. isolates derived from medicinal *A. macrocephala* in Research Center for Pathogenic fungi, Sichuan Agricultural University. Toward this end, we studied the feasibility of using the ITS region for the detection and identification of *F. spp.* and *G. zeae* spp. to the species level. In addition, we compared this molecular classification technique to classification based on examination of morphologic characteristics.

4.1 Diversity of Plant-pathogenic Fungal in This Study

In this study, 40 Plant-pathogenic fungal isolates were obtained from Swordlike *A. macrocephala* and Largehead *A. macrocephala*, for fungal identification, we relied on a combination of traditional and molecular methods. Four of the representative morphological isolates were further identified with molecular phylo-genetic analysis of ITS1-5.8S-ITS2 sequences. Generally, there was a good agreement between morphological and ITS-sequence based approaches. The Plant-pathogenic fungal communities associated with the two *A. macrocephala* species showed high species diversity. In addition, fungal species compositions were distinct in different tissues. These fungi could be identified to species, genus, or family level, mainly including *F. lateritium* and *G. zeae*. *G. zeae* was the most frequent Plant-pathogenic fungal either within or among the hosts, consistent with the findings reported in other studies of *G. zeae* (Yun et al., 2000, Wildermuth et al., 1997). In this study, two Plant-pathogenic fungal isolates of morphological were isolated from stems and roots of *A. macrocephala*. *G. zeae* is common Plant-pathogenic fungal in many species (Lee et al., 2009, Pereyra and Dill-Macky, 2008) and we reported the phylogenetic significance of morphological character in the taxonomy of *G. zeae* species. However, *F. lateritium* is the dominant Pathogenic fungi firstly detected in *A. macrocephala*.

4.2 Taxonomy and Phylogeny of Plant-pathogenic Fungal Isolates in This Study

The major polygenetic relationships were congruent based on MP, NJ, and except for minor differences in the placement of some small clades, which had not affected fungal strain identification.

All the 36 Plant-pathogenic fungal were obtained from host *A. macrocephala*, which accounted for 90% of its total fungal isolates. Of the four isolates, A11, B11, C11 and D11 were selected for molecular identification; only one or two nucleotides were different in their ITS1-5.8S-ITS2 sequences. A11, B11 and D11 were similar in morphological characteristics, and the three isolates from the stems, Sequences alignment showed that they were 100% identical to many closely related species of *G. spp.*, with *G. zeae* as the most frequently matched species. In the present study, *G. zeae* isolates obtained from roots and stems identical in their ITS1-5.8S-ITS2 sequences, suggesting that they may belong to the same fungal taxa. As indicated by their sequence homology to the corresponding GenBank accessions, the two isolates from stems (A11 and D11) and the one from roots (B11) might belong to *G. zeae*. This study showed that *G. zeae* species are the most frequent Plant-pathogenic fungal in *A. macrocephala*. In our phylogenetic analysis, most of the three representative fungal isolates could be identified as *G. zeae*, which were sexual phases of *Gibberella*. However, clone type C11 and the 1 best match in a distinct group within a larger *F. lateritium*, and some of these were also represented by marine genera. These were linked to, but distinct from, a group formed largely of species that were pathogens of other fungi. So *F. lateritium* is the dominant Pathogenic fungi firstly detected in *A. macrocephala* in our research.

4.3 The Use of Molecular Technique in Identifying Plant-pathogenic Fungal

Traditionally, microscopic and cultural techniques are often available for detecting and enumerating fungal spores. However, the methods are time-consuming and laborious. They require skilled and highly specialized expertise. Furthermore, microscopy is often fallible for identification of non-descript spores. Endophytic fungi are assessed by rolling the surface-sterilized plant tissue samples, and subsequently isolation of fungal endophytes which grow in samples placed onto culture media (Bills et al., 1996, Devarajan and Suryanarayanan, 2006). However, cultural techniques are inappropriate for detection of slow growing or non-culturable fungi in vitro (Lacap et al., 2003). Fungi that emerge from these samples can be identified by means of phenotypic (morphological) or genotypic (molecular) characters. In contrast, molecular techniques are sensitive and specific for identifying microorganisms. They can be used for the taxonomic classification and identification of microbial strains (Sette et al., 2006). The internal transcribed spacer (ITS) has been used in numerous systematic studies at genus and species levels of a wide array of plant taxa (Alice and Campbell, 1999, Sang et al., 1995). ITS-1 and ITS-2 are two internal spacers which locate between genes encoding the 18S, 5.8S and 26S nuclear ribosomal RNA (nrRNA) subunits. In addition, the 5.8S nrRNA is referred to as the nrDNA ITS region (Baldwin, 1992). The length of ITS-1 and ITS-2 are less than 300 bp. The length of 5.8S subunit is almost invariant. It is around 160 bp within angiosperms. This makes the entire ITS region less than 700 bp. Therefore, this is easy for sequencing and analysis. ITS region belongs to the nrDNA family, which undergoes rapid concerted evolution. Its rapid concerted evolution, small size and ease of amplification made the nrDNA ITS region as a phylogenetic standpoint and also promote accurate reconstruction of species relationship. Many studies revealed a phylogenetic relation among different species (Baldwin et al., 1995). Based on DNA analysis, problems associated with taxonomic identification of mycelia sterilia could be solved. However, molecular analysis alone also has limitations. It cannot overcome the problem of over-isolating fast growing fungal species at the expense of slow growing taxa, nor isolating species that will not grow in culture (Duong et al., 2006, Hyde and Soyong, 2008). The use of ITS sequences also has limitations in phylogenetic analysis. Because the noncoding ITS sequence is fast evolving with many variable characters, it is usually difficult to achieve a perfect sequence alignment at high taxonomic levels. Moreover, it has been shown that 20-30% of sequences downloaded from GenBank for comparative analysis may not be accurate in their identification (Nilsson et al., 2006, Hyde and Soyong, 2008). This inaccuracy in the database may have contributed to some of the unexpected observations in our study, such as *F. lateritium* and *G. zeae* all having their accessions scattered across the phylogenetic tree, whereas some different species (e.g., species of *F. culmorum* and *G. zeae*) being clustered within the same clade. Further studies using different gene sequences can be conducted to resolve this type of difficulties in the phylogenetic analysis of fungi.

5. Conclusion

In summary, the ITS region provides a sufficient genetic scaffolding to detect and reliably differentiate *F. spp.* and *G. zeae* spp. isolated in medicinal *A. macrocephala*. In recent years, DNA-based technology is widely used. We can detect and classify the isolated fungal precisely and rapidly using the DNA-based technology. The nrDNA ITS sequence has already been proved the usefulness in phylogenetic analysis of *F. lateritium* isolates and *G. zeae* species according to the present study. These studies also provide substantial basis for future phylogenetic and evolutionary analysis among the groups belonging to the family *F. spp.* and *G. zeae* spp.. Our results will be helpful to further study the biogeographical and molecular evolutionary, their phylogenetic origins and intraspecific population.

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Table 1. Number and morphological taxonomic identification of the Pathogenic fungi isolated from two *A. macrocephala* species

Cultivated variety	Swordlike <i>A. macrocephala</i>		Largehead <i>A. macrocephala</i>	
	Stem	Root	Stem	Root
Fungal taxon				
<i>F.lateritium</i> isolate	6	8	3	5
<i>G. zeae</i>	3	6	2	3
Other	0	2	1	1

Table 2. Morphological identification, GenBank accession numbers and top BLAST match sequences of the fungal isolates from *A. macrocephala* included in phylogenetic analysis

Fungal strain	Morphological identification	GeneBank accession No.	Reference accession No.	coverage	Max ident
A11,B11,D11	<i>G.zeae</i>	EU255799.1	<i>Gavenacea</i> isolate FA09	97%	99%
C11	<i>F. lateritium</i> isolate	GU480949.1	<i>F.lateritium</i> isolate HMA-1	97%	99%

Table 3. Species and GenBank accession number used in the study

Species	GeneBank accession No	Species	GeneBank accession No
<i>Gavenacea</i>	HQ020475.1	<i>G. zeae</i>	HQ176433.1
<i>G. avenacea</i>	HQ020473.1	<i>G.zeae</i>	GQ466391.1
<i>F. tricinatum</i>	AB369452.1	<i>G. zeae</i>	HQ176432.1
<i>F. lateritium</i>	GU480949.1	<i>G. zeae</i>	GU327636.1
<i>F. lateritium</i>	AF310980.1	<i>G. zeae</i>	JF303858.1
<i>G. avenacea</i>	EU255803.1	<i>G. zeae</i>	EU255799.1
<i>F. tricinatum</i>	AB470878.1	<i>F. sp.</i>	FJ614630.1
<i>F.sp.</i> ZL-GT	HQ832677.1	<i>F. sp.</i>	HQ630964.1
<i>F. oxysporum</i>	AB470850.1	<i>F. culmorum</i>	AY147313.1
<i>U. virens</i> U16	AB092953.1	<i>U. virens</i> U16	AB092953.1

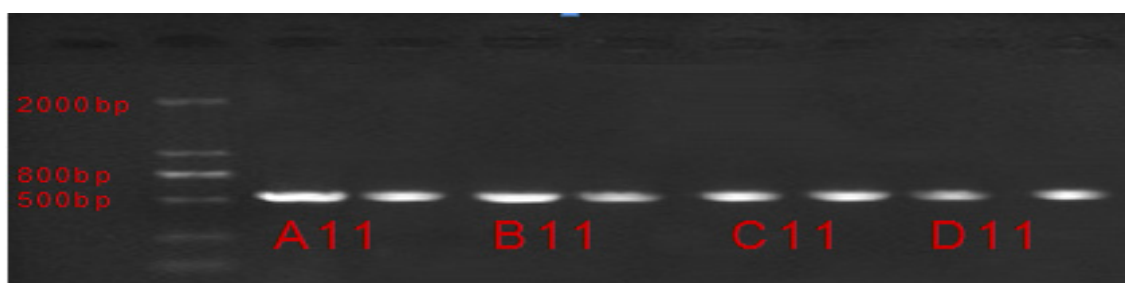


Figure 1. nrDNA ITS region amplified from *A. macrocephala* using IST1-F and ITS4-R primers (Biogene, USA)

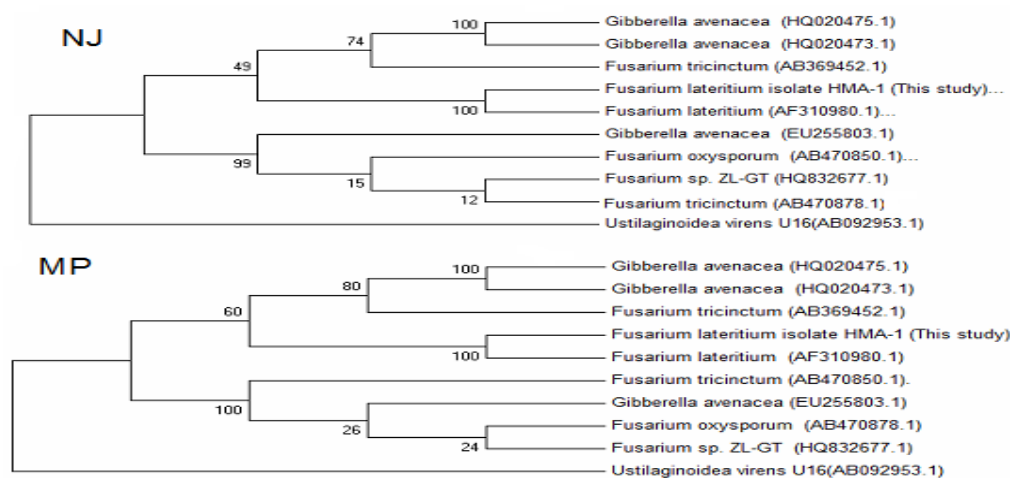


Figure 2. Maximum likelihood tree showing the relationship between fungal clone sequence type C11 and available sequences from databases

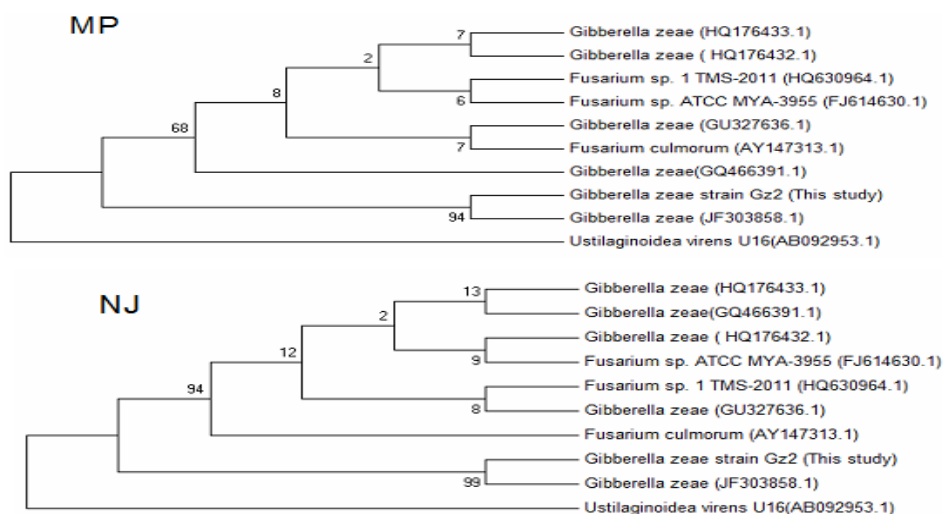


Figure 3. Maximum likelihood tree showing the relationship between fungal clone sequence types A11, B11 and D11 and available sequences from databases