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Molecular Diversity of Fungal Endophytes Isolated from *Garcinia mangostana* and *Garcinia parvifolia*

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***Garcinia* is commonly found in Malaysia, but limited information is available regarding endophytic fungi associated with this plant. In this study, 24 endophytic fungi were successfully recovered from different parts of two *Garcinia* species. Characterization of endophytic fungi was performed based on the conserved internal transcribed spacer (ITS) region sequence analysis and the antimicrobial properties. Results revealed that fruits of the plant appeared to be the highest inhabitation site (38%) as compared with others. *Glomerella* sp., *Guignardia* sp., and *Phomopsis* sp. appeared to be the predominant endophytic fungi group in *Garcinia mangostana* and *Garcinia parvifolia*. Phylogenetic relationships of the isolated endophytic fungi were estimated from the sequences of the ITS region. On the other hand, antibacterial screening showed 11 of the isolates possessed positive response towards pathogenic and nonpathogenic bacteria. However, there was no direct association between certain antibacterial properties with the specific genus observed.**

Keywords: Fungal endophyte, *Garcinia mangostana*, *Garcinia parvifolia*, ITS rDNA, antibacterial

Generally, there are as many as 1 million different fungal species in the world and many endophytic fungi could be part of the undiscovered organisms [8, 24]. Endophytic fungi are defined as endosymbionts that live within the plant intercellular space without causing apparent disease [28], which are commonly found in all species of plants. Endophytic fungi have had increased worldwide attention because of the search for new or raw biologically active compounds. The ability of endophytes to produce a great range of secondary metabolites such as antibiotics, bioinsecticides, fine chemicals, and enzymes had shown some convincing results in combating pathogens and even cancer cell-lines in animal and humans [1, 6, 12, 13, 23].

Despite the omnipresence of endophytic fungi symbiosis with the plant, the extent of their contribution to fungal biodiversity remains unclear [10]. Generally, the biodiversity of endophytic fungi in tropical country is poorly known. As an area with over 15,000 plant species, Malaysia could serve as an important source for the host interaction study of endophytic fungi with tropical rain forest plants [18]. This study was conducted on “Mangosteen” (*Garcinia mangostana*) and “Brunei cherry” (*Garcinia parvifolia*) to explore their alternative biopotential.

Garcinia belongs to the family Clusiaceae, which is native to Asia, Australia, Southern Africa, and Polynesia, and that produces edible fruit. As “queen” of the tropical fruit in Malaysia, *Garcinia mangostana* (Mangosteen) is famous for its sweet, creamy, and fragrant edible flesh [21]. Different parts of *Garcinia mangostana*, such as the hull, bark, and leaf, have been used as a traditional medicine to treat diseases for hundreds of years. In Malaysia, applications of the mangosteen include leaves infusion to the circumcision wound to prevent infection and root decoction for the regulation of female menstruation [15, 17]. Studies based on *Garcinia* species had greatly reviewed its biological potential activity, but analysis based on the endophytic fungi that coexist within the fruit tree is very limited [20, 21]. Therefore, screenings of antibacterial properties from the isolated endophytic fungi were included in this study.

In the present study, we report the identification of isolated endophytic fungi based on internal transcribed spacer (ITS) sequence analysis, and further analysis of the bioproperties among the isolates associated to the part of tree, media, and types of plant using a nonmetric multidimensional scaling approach.

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Materials and Methods

Plant Material

Three *Garcinia mangostana* and *Garcinia parvifolia* trees were selected randomly from the fruit farm of Sungai Rengit Village, Johor, Malaysia during May 2008. The parts of the tree were further classified as leaf, stem, root, fruit, and flower. Selected parts were transported immediately to the laboratory for further endophytic fungi isolation.

Isolation of Endophytic Fungi

In order to eliminate epiphytic microorganisms, each part (leaf, stem, root, fruit, and flower) were subjected to a surface-sterilized procedure. Each part of the samples was thoroughly washed under running tap water, after which the surface was sterilized by submerging the part in 75% ethanol for 2 min, 5.3% sodium hypochlorite for 5 min, and 75% ethanol for 0.5 min, and finally rinsed with sterile distilled water for 1 min. After surface sterilization, the samples were dried on sterile filter paper. Each plant tissue was then cut with a sterile blade into 1-cm segments. The segments were placed on the isolation media, exposing their inner tissue surface. Three medium plates used in this study included potato dextrose agar (PDA), starch casein agar (SCA), and actinomycetes isolation agar (ATC). All plates were incubated at 27°C for up to 3 weeks. Emerging fungi were transferred to fresh medium by the hyphae tips method to obtain the single isolate [3, 7]. Each isolate was kept in a storage tube for future applications.

DNA Extraction

Molecular methods are useful for evaluating microbial communities' structures and functions; DNA analyses have been applied at different resolution levels for whole communities, bacterial, fungal, and yeast isolates, and clones of specific genes. Therefore, a high quality and quantity of DNA is essential. Each isolated endophytic fungus was cultured using malts extract broth in a shaking incubator at 27°C to obtain solid fungi cell tissue. Endophytic fungi tissues were subjected to a freeze-drying process before genomic DNA extraction. The sodium dodecyl sulfate (SDS) method was applied to extract the genomic DNA. Phenol/chloroform/isoamyl alcohol at 25:24:1 (0.5 ml/g of tissue) and sufficient extraction buffer (100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5 M NaCl, and 1% SDS) were added into the mixture and ground vigorously for 0.5 min with a pestle. Extraction buffer was added (2 ml per 0.5 g of starting tissue) as well as the phenol/chloroform/isoamyl alcohol mixture (1 ml per 0.5 g of starting tissue). After thorough mixing, the mixture was transferred into microcentrifuge tubes and centrifuged at 16,000 ×*g* for 5 min at 25°C. The aqueous phase was transferred to a new tube and mixed with 0.6 volume of isopropanol. The mixture was then incubated at 25°C for 10 min and then centrifuged at 4°C for 15 min at 16,000 ×*g*. The resulted pellet was rinsed with 95% ethanol and then air dried. The dried pellet was resuspended in 340 ml of TE (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0) containing RNase A (20 µg/ml). The mixture was incubated at 37°C for 30 min. After incubation, 0.3 ml of phenol/chloroform/isoamyl alcohol mixture was added to the sample. The sample was mixed and centrifuged at 4°C for 2 min at 16,000 ×*g*. After centrifugation, the supernatant was then transferred to a new tube; 50% vol. 7.5 M ammonium acetate and 2.5 vol. of 100% ethanol were added. Next, the mixture was incubated for 30 min at −20°C and then centrifuged at 4°C for 15 min at 16,000 ×*g*. The resulted pellet of DNA was rinsed with 70% ethanol, air dried, and resuspended in 100 µl of ultra pure water. The quality and quantity of the DNA were determined by a biophotometer (Eppendorf, Hamburg, Germany).

Amplification of Internal Transcribed Spacer (ITS) Sequences

The partial nucleotide base-pair fragment of the internal transcribed spacer rDNA gene from the isolated endophytic fungus was amplified using the polymerase chain reaction (PCR) with universal ITS primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') [26]. The PCR amplification reactions were performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with a total 25 µl of reaction that comprised 20 ng of genomic DNA, 2.5 µl of 10× PCR buffer with 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1.5 µl of 25 mM $MgCl_2$, 1.25 µl of 5% (v/v) DMSO, 1 unit of *Taq* polymerase (Finzymes, Espoo, Finland), and 10 pmol of each primer. A non-template control was included in each run. The cycling parameters were 4 min at 94°C for pre-denaturation, 35 cycles each of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, 1.5 min at 72°C for extension, and a final extension at 72°C for 5 min. The amplified PCR products were resolved by electrophoresis in a 1.5% agarose gel (Bioline, London, U.K.), which was then stained with ethidium bromide (0.5 µg/ml), and the amplicons were visualized under a gel documentation system (Alpha Imager; Alpha Innotech, San Leandro, CA, U.S.A).

ITS Region Sequencing and Phylogenetic Analysis

The gel section with desired band was carefully excised under UV light and subjected to extraction using an RBC gel extraction kit (Realbiotech, Banqiao City, Taiwan). Recovered products were analyzed by sequencing (First-base, Seri Kembangan, Malaysia). The sequence obtained from each isolate was further analyzed using BLAST from the National Center of Biotechnology Information (NCBI) Web site. Sequences obtained were subjected to Clustal W analysis using DNASTAR software version 5.05 for the phylogenetic analysis. Analyzed sequences were uploaded to NCBI. Additionally, nonmetric multidimensional scaling (NMDS) was performed for all endophytic fungi, as well as their respective community dynamics using PRIMER v6 (PRIMER-E, Plymouth, U.K.) software based on data obtained from the antimicrobial activity, part of trees, media of isolation, type of plants, and species isolated.

Well Diffusion Assay

Antibacterial activity was measured using a well diffusion method. Isolated endophytic fungi were first cultured with the malt extract broth for 20 days and the broth suspension was then filtered for further application. Selected pathogenic bacteria (*Staphylococcus aureus*, *Listeria monocytogenes, Bacillus subtilis*, *Aeromonas hydrophila,* and *Streptococcus faecalis*) and nonpathogenic bacteria (*Agromyces lapidis* and *Bacillus megaterium*) were cultured overnight at 30°C−37°C in Tryptic Soy Broth to an optical density (OD) of 0.6 at 620 nm. A total of 100 ml of bacteria culture was spread evenly on Mueller−Hinton plates for further assessment. Multi wells with 6-mm diameter were created on the plates and 20 µl of the endophytic fungi filtered suspension was then added into the well. The assay was conducted in triplicate and repeated two times. The plates were incubated at 30°C−37°C accordingly for 24 h. Antibacterial activity was determined by measuring the inhibition zone of the fungi suspension against selected pathogens.

RESULTS AND DISCUSSION

Isolation and Distribution of Endophytic Fungi

A total of 24 endophytic fungi that comprised 10 different genera were successfully recovered from the isolation process, which included four isolates from *Garcinia mangostana,* and 20 from *Garcinia parvifolia.* In this study, 46% (11/24) of the isolated endophytic fungi were derived from PDA, followed by SCA (33%) and ATC (21%) (Fig. 1). SCA and ATC were included in this study despite the conventional isolation by PDA, to increase the chances of endophytic fungi recovery for diversity assessment. However, the frequency of endophytic fungi isolated from *Garcinia mangostana* was lower as compared with *Garcinia parvifolia*. The low recovery rate of endophytic fungi from *Garcinia mangostana* might be due to the lack of essential specific growth substances such as plant raw material in the isolation medium. According to Mungo *et al*. [16], isolation media that contain a blend of different parts of the plant raw material exhibit different efficiencies in fungi isolation.

Each part of the plant such as the leaf, stem, root, fruit, and flower, were collected and subjected to the endophytic isolation for diversity comparison. As shown in Fig. 1, 38% of endophytic fungi were isolated from the fruit, followed by stem (29%), leaves (25%), and flower (8%), and there was none from the root. The reason for low colonization by endophytic fungi in the root is unclear, but it could reflect the differences in microbial and physiological conditions in the different plant parts [25]. Besides this, these results may be related to the possible available specific growth-promoting factors present in the different plant parts [16]. On the other hand, some researchers [14, 19] suggested that endophytic fungi show a certain degree of tissue recurrence or specificity.

The diversity indices allow a quick preliminary assessment of the data to identify extreme trends [4]. Hence, a disparity in evenness amongst endophytic fungi communities reflects the inhabitation profile of certain specific endophytes. The calculated diversity indices (Table 1) reflect the richness of phylotypes in *Garcinia parvifoli.* Besides this, active isolates (endophytic fungi that demonstrated antibacterial activity) were group together, as I in Fig. 2, when analyzed by NMDS. Two homogenous groups, namely II and III (Fig. 2), were distinguished from the rest at 40% similarity. Group II comprised isolates that derived solely from the stem of the plants, whereas Group III consisted of endophytic fungi isolated from the fruit part. Further analysis is required to investigate the factors associated with the inhabitation of certain endophytic fungi on specific parts of the plants.

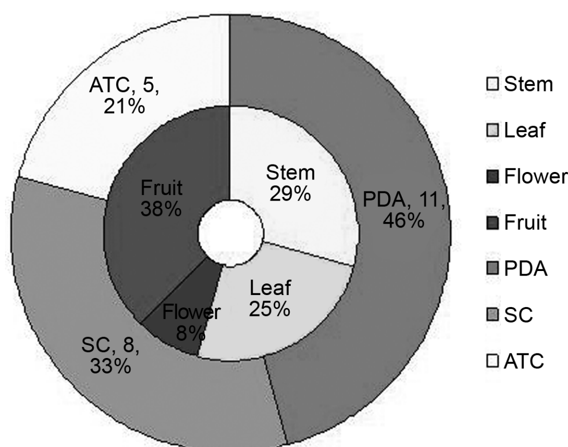

Fig. 1. Pie chart of isolated endophytic fungi based on isolation media and section.

DNA Extraction and Molecular Identification of Endophytic Fungi by ITS rDNA Sequences

In this study, the most common and distinct SDS-based method with slight modification was adapted for an effective and efficient fungi genomic DNA extraction. Freeze-drying of the mycelia that will greatly weaken the cell wall as compared with common silica sand and the liquid nitrogen grinding technique allow better genomic DNA extraction. Additionally, this method eliminates laborious and time-consuming steps [9, 23]. The quantity and quality of DNA obtained ranged from 16 ng/μl to 217 ng/μl and 1.29 to 1.84 $OD_{260/280}$, respectively. All of them were suitable for PCR amplification.

The ITS is a nonfunctional RNA sequence located in structural ribosomal RNAs (rRNA). Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because of its high copy number of rRNA genes, which allows easy amplification even from small quantities of DNA, and it possesses a high degree of variation even between closely related species [11, 29]. Amplified ITS sequences of the isolated endophytes were subjected to BLAST search, as shown in Table 1, and yielded high homology with the respective species in the GenBank database from NCBI. Molecular identification, which was based on sequence analysis, revealed that the isolated endophytic fungi were greatly attributed to 10 genera, *Aspergillus*, *Diaporthe*, *Fusarium*, *Curvularia*, *Glomeralla*, *Guignardia*, *Hypocrea*, *Phoma*, *Phomopsis*, and *Xylariales*, which indicates the diversity of endophytic fungi associated with the *Garcinia parvifolia* and *Garcinia mangostana*. All of the isolated endophytes were found to be categorized under Ascomycota and none from the Basidiomycota phylum, in concurrence with observation reported by Crozier *et al.* [3] and Qi *et al.* [22].

The sequences of some nearest neighbors of the endophytic fungi isolates were retrieved from GenBank (NCBI) to construct the phylogenetic tree. Based on the phylogenetic tree, as shown in Fig. 3, two main clusters, A and B, started to branch at 98.6% dissimilarities. There were 12

Table 1. Endophytic fungi isolated from *Garcinia mangostana* and *Garcinia parvifolia* with antimicrobial activity.

Plant	Sample	Media	Part[a]	ITS ID	Species richness	Pielou's evenness	Shannon index	NCBI Accession No.	Relative sequence		Antibacterial well diffusion[b]
									Accession No.	% Similarity	
Garcinia parvifolia	3 GP	PDA	L	*Guignardia camelliae*				GQ352474	FJ462743.1	99%	-
	5 GP	PDA	T	*Aspergillus aculeatus*				GQ352493	EU833205.1	99%	-
	36 GP	PDA	S	*Hypocrea virens*				GQ352475	EF442081.1, EF442078.1	100%	-
	37 GP	PDA	S	*Phomopsis* sp. VegaE3-31				GQ352477	EU002931.1	99%	-
	40 GP	PDA	S	*Phomopsis* sp. BFM-L45				GQ352478	AB369484.1, AB369483.1	98%	-
	45 GP	PDA	T	*Phomopsis* sp. TSM-2005-06				GQ352480	DQ235672.1	98%	-
	47 GP	PDA	S	*Phomopsis* sp. EXMQ-4				GQ352481	FJ233186.1	99%	-
	56 GP	PDA	F	*Fusarium equiseti*				GQ352485	FJ459976.1	99%	*Staphylococcus aureus* (9), *Agromyces lapidis* (6), *Listeria monocytogenes* (7), *Bacillus megaterium* (5), *Bacillus subtilis* (5)
	77 GP	SC	S	*Phoma* sp. GAH7				GQ352490	FJ950743.1	98%	*Agromyces lapidis* (8), *Listeria monocytogenes* (6)
	84 GP	PDA	S	*Phoma moricola*	3.672	0.895	2.224	GQ352491	AB470906.1, AB470868.1 FJ462764.1, FJ462755.1 EU732730.1	99%	-
	106 GP	SC	T	*Curvularia affinis*				GQ352486	EF187909.1	99%	*Staphylococcus aureus* (6)
	133 GP	SC	S	*Phomopsis* sp. GX9-1C				GQ352483	FJ037768.1	97%	-
	141 GP	SC	S	*Fusarium equiseti*				GQ352488	FJ459976.1	99%	*Bacillus megaterium* (12)
	144 GP	SC	T	*Diaporthe phaseolorum*				GQ352487	AY577815.1	99%	*Staphylococcus aureus* (6)
	157 GP	SC	L	*Xylariales* sp. NR-2006-D55				GQ352489	DQ480357.1	99%	-
	162 GP	SC	S	*Guignardia vaccinii*				GQ352495	EU686803.1, EU167584.1 EU273524.1, AY601899.1	99%	*Listeria monocytogenes* (9), *Aeromonas hydrophila* (10), *Bacillus megaterium* (11), *Bacillus subtilis* (10)
	170 GP	PDA	L	*Guignardia vaccinii*				GQ352496	EU686803.1, EU167584.1 EU273524.1, AM403717.1 AY601899.1	99%	-

Table 1. Continued.

Plant	Sample	Media	Part[a]	ITS ID	Species richness	Pielou's evenness	Shannon index	NCBI Accession No.	Relative sequence: Accession No.	Relative sequence: % Similarity	Antibacterial well diffusion[b]
Garcinia parvifolia	179 GP	ATC	T	*Phomopsis* sp. EXMQ-4	3.672	0.895	2.224	GQ352484	FJ233186.1	99%	-
	184 GP	ATC	F	*Fusarium* sp. Dzf18				GQ352492	FJ770065.1, FJ715505.1 FJ605243.1, AB369482.1 AB369259.1, EU543261.1 EU285554.1	100%	*Staphylococcus aureus* (10), *Agromyces lapidis* (9), *Bacillus subtilis* (7)
	190 GP	ATC	L	*Guignardia vaccinii*				GQ352497	EU686803.1, EU167584.1 EU273524.1, AY601899.1	100%	*Staphylococcus aureus* (10), *Listeria monocytogenes* (9), *Aeromonas hydrophila* (7), *Bacillus megaterium* (11), *Bacillus subtilis* (10), *Streptococcus faecalis* (9)
Garcinia mangostana	54 GM	PDA	T	*Glomerella cingulata*	0	-	0	GQ352494	AB219013.1, AB219007.1 AB218999.1, AB218995.1 AY245021.1, AB233342.1 AB042315.1	100%	-
	116 GM	SC	L	*Glomerella cingulata*				GQ352476	AB219013.1, AB219007.1 AB218999.1, AB218995.1 AY245021.1, AB233342.1 AB042315.1	99%	*Agromyces lapidis* (7)
	146 GM	ATC	T	*Glomerella cingulata*				GQ352479	AB042317.1	99%	*Agromyces lapidis* (5)
	150 GM	ATC	L	*Glomerella cingulata*				GQ352482	AB219013.1, AB219007.1 AB218999.1, AB218995.1 AY245021.1, AB233342.1 AB042315.1	99%	*Agromyces lapidis* (5)

[a]Part of tree: L, Leaf; S, Fruit; T, Stem; F, Flower.

[b]The value in brackets refers to the inhibition zone diameter in millimeter

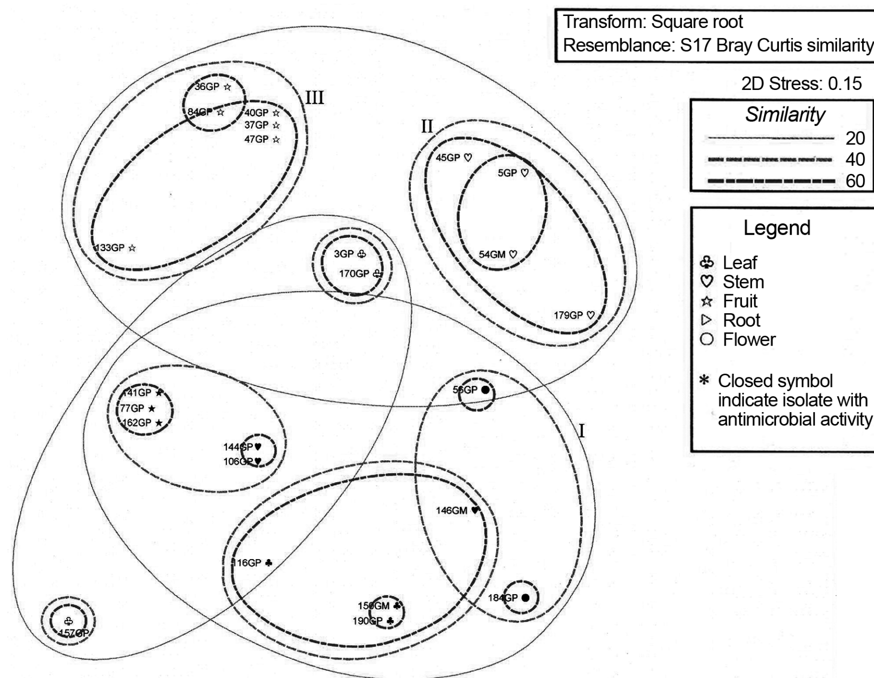

Fig. 2. Nonmetric multidimensional scaling ordination plot according to the isolate's dynamics.
Groups were derived from cluster analyses at 20%, 40%, and 60% similarity with ordination two-dimensional stress values of 0.15. Groups from cluster analyses (hierarchical agglomerative clustering with group-average linkage based on Bray−Curtis similarities) were superimposed on the NMDS plot with the X axis as MDS 1 and the Y axis as MDS 2.

isolates clustered within cluster A that possessed identical sequence alignment from the retrieved neighbor sequence. Twelve endophytic fungi (157GP, 190GP, 170GP, 162GM, 150GM, 116GM, 146GM, 144GP, 179GP, 133GP, 141GP, and 184 GP) possessed less then 1% of nucleotide dissimilarity to *Xylariales* sp, *Guignardia* sp., *Glomerella* sp., *Diaporthe* sp., *Phomopsis* sp., and *Fusarium* sp., respectively. Both *Diaporthe* sp. and *Phomopsis* sp. are the same species, and isolate 179GP was clustered with the *Diaporthe* sp. group. Generally, fungi are classified primarily based on the structures associated with sexual reproduction, with asexually reproducing fungi (anamorphs) having separate nomenclature from their sexual states (teleomorphs). Previous study had claimed *Diaporthe* sp. as the teleomorph stage of *Phomopsis* sp. and this had greatly explained the interchangeable nomenclature [2, 5, 27]. Results from the alignment of the ITS rDNA regions indicated that a high degree of length variations, deletions, and insertion occurs in the ITS region for the isolates from cluster B when compared with the sequences obtained from the GenBank (results not shown). This finding had greatly revealed the uniqueness of our isolated endophytic fungi.

Screening of Endophytic Fungi for Selected Antimicrobial Activity by Agar Well Diffusion

The results of antimicrobial activity against selected plant and human pathogens by the suspensions of endophytic fungi are shown in Table 1. Eleven isolates (45.8%) displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 5 to 12 mm (Table 1). Nearly half (45.4%) of the active isolates displayed a remarkable inhibition towards selected human pathogens, such as *Staphylococcus aureus*, and this finding was concurrence with the result of Phongpaichit *et al.* [21]. Two isolates, 56GP and 190GP, identified as *Fusarium equiseti* and *Guignardia vaccinii*, respectively, possessed the most antibacterial activities.

On the other hand, 28% of the active endophytic fungi were found to be originated from the stem and fruit, whereas 18% were from the flower. Homogenous active isolates as clustered in Group I were found to be distinguished from the rest at 60% similarity, using NMDS (Fig. 2). This finding suggests that most of the active isolates share almost 60% of the clustering properties, such as media of isolation, antibacterial activities, part of trees, type of plants, and species. Further additional analysis is required to investigate which properties are significantly correlated among these active isolates.

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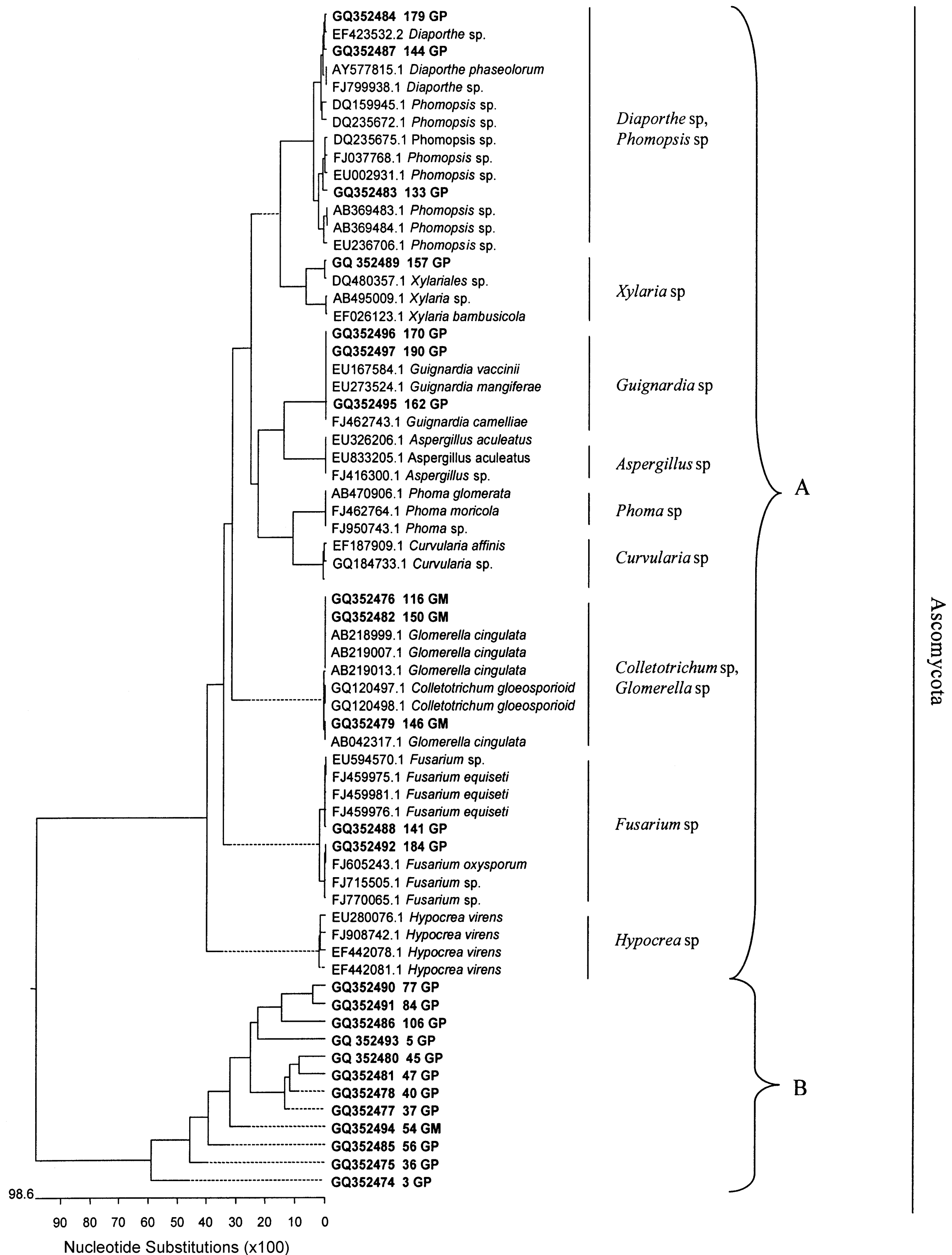

Fig. 3. Phylogenetic tree inferred from the nearest neighbor of fungal endophytes presented in this study, based on the maximum parsimony method.

laboratory facilities in the Department of Biomedical Science, Faculty of Medicine and Health Sciences.

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