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Phylogenetic analysis of Endophytes from Bitter Melon (*Momordica charantia*) in Guangdong Province

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Abstract

This study aims to isolate and identify taxonomic characterization of endophytic fungi from bitter melon in Guangdong province, China. A total of 1172 endophytic fungi are isolated from roots, stems, leaves, flowers, and fruits of healthy plants, and they are classified to 49 taxa based on morphological and molecular features. The results show that endophytic fungi from bitter melon plants exhibit high biodiversity. *Arthrinium aureum*, *A. marii*, *A. sphaerospermum*, *Corynascus verrucosus*, *Curvularia borreriae* and *C. protuberate* have not been recorded in any plants in China. Basidiomycetous endophytes, such as *Ceratobasidium* sp., and *C. cornigerum*, are reported in this study for the first time in bitter melon. According to the results of phylogenetic analysis, the rDNA ITS (Internal Transcribed Spacer) sequences can successfully separate species, such as *F. solani*, *F. kyushuense*, *C. verrucosus*, *C. globosum*, *E. rostratum*, *C. brachyspora*, *C. verruculosus*, *C. affinis*, *P. bougainvilleicola*, *P. longicolla*, *P. glabrae*, *P. verruculosum*, *P. oxalicum*, *P. citrinum*, *P. chermesinum*, *P. glomerata*, *A. fumigatus* and *A. japonicas*. Some isolates belonging to *A. alternata*, *C. gloeosporioides*, *C. cladosporioides*, *C. brasiliense*, *C. convolutum*, *F. proliferatum*, *F. oxysporum*, *F. verticillioides*, *F. equiseti*, *F. camptoceras* and *Xylaria*, however, require the analysis of others molecular markers to provide better taxonomic resolution. Molecular analyses of rDNA ITS sequences are useful for identification and classification of endophytes of bitter melon, but it is desirable to consider an integrated approach, such as molecular phylogeny, host, colony growth rate, symptoms, and shape of conidia.

Keywords

Endophytic Fungi, *Momordica charantia*, Phylogenetic Analysis

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1. Introduction

Endophytes inter- and/or intra-cellularly colonize healthy living tissues of many host plants, typically without causing any noticeable symptoms of disease [1]-[5]. Endophytic fungi from plants have recently been widely accepted as an important source of drugs [6], and they are believed to be rich in diversity and to provide an excellent potential source of biologically active novel compounds [7] [8]. Until recently, more than 8600 biologically active compounds have been reported from fungi with various usages [9].

Bitter melon (*Momordica charantia*), a medicinal food plant, is known to contain charantin (a steroidal glycoside), vicine (a glycoalkaloid), and polypeptide "p" (a 166 residue insulinomimetic peptide) [10]. Extracts of bitter melon have been found to possess novel bioactive natural products like antibacterial, antifungal, anticancer, antioxidant, antivirus and antidiabetes [11]. The medicinal characteristics of bitter melon may result from the capacity of its endophytes producing biologically active secondary metabolites.

rDNA ITS (Internal Transcribed Spacer) sequences analysis has been widely used in identifying fungi. Sette *et al.* (2006) identified endophytic fungi from coffee plants at least at the genus level, and the results were in accordance with the previous morphological characterization [12]. Chen *et al.* (2008) reported that ITS sequences analysis was especially effective in non-sporulating fungi identification and also reduced the impacts of subjective judgement [13].

The present study aimed to 1) identify endophytic fungi species from bitter melon in Guangdong Province in China, 2) conduct phylogenetic analysis on the ribosomal DNA internal transcribed spacer (ITS) region sequence of endophytes, and 3) collect cultures of endophytic fungi for further screening of the new bioactive compounds.

2. Materials and Methods

2.1. Plant Materials

Samples were collected between 2004 and 2009 from healthy bitter melon plants in five sites in Guangdong Province (Site 1: Huadu Station of the Guangzhou Vegetable Science Institute; Site 2: Forecast Station of Crop Diseases and Pests in Zhaoqing city; Site 3: Shitang town, Renhua county, Shaoguan city; Site 4: Xiangqiao district, Chaozhou city; and Site 5: Ducheng town, Yunan county, Yunfu city). Five healthy bitter melon plants (without visible signs of any disease) were collected from the each of the five sites. Each experimental field had fields of bitter melon at least 100 m^2 large. The samples were placed in an ice-box and processed within 4 h of collection.

2.2. Fungal Isolation and Cultivation

Surface sterilization was applied as described by Schulz *et al.* (1993) [14]. The surface-sterilized samples of stem, root, flower, and fruit (all) were cut into 5 × 5 mm fragments. A total of 30 fragments from different tissue type per site were prepared. Five fragments from surface-sterilized leaf discs or other organ segments were evenly placed on a 2% potato dextrose agar (PDA) medium. Fifty microgram streptomycin (AMRESCO)/ml were added to repress bacteria. These plates were incubated at 25˚C for 2 months, and examined periodically. Hyphal tips of the developing fungal colonies were transferred onto new malt extract agar (MEA, 2%) plates. The pure endophytic fungal strains were photographed and preserved in the Institute of Plant Pathology, Zhongkai University of Agriculture and Engineering.

2.3. Checking Surface-Sterilization

The method followed Song *et al.* (1999) [15]. A viability test was adopted to test the effectiveness of surface-sterilization [14].

2.4. Identification

Sporulating isolates were identified to genus and possible species using traditional morphological techniques. After subculture of isolates grown on PDA for 1 week at 25˚C, all pure strains were selected for DNA extraction, amplification, and sequencing [16]. DNA samples were checked for purity and integrity by electrophoresis in 1% (w/v) agarose with ethidium bromide (10 mg/mL) in 1 × TAE buffer before storing at 4˚C. Primers ITS4 and

ITS5 [17] constructed for molecular phylogenetic studies were used to amplify the ITS region. PCR was carried out as follows: the reaction mix in a total volume of 25 µl contained in font changed 0.2 mM each of the primer pair, 0.2 mM dNTP, 10 - 50 ng DNA, 0.04 U of Taq polymerase (Promega, WI, USA), and 1 × PCR buffer mix. Samples were incubated in a thermal cycler at 94˚C for 3 min, followed by 35 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min; and finally 72˚C for 7 min.

Phylogenetic analysis was based on blast searches of ITS sequence data in the NCBI GenBank database. Sequences were then aligned to other sequences obtained from the GenBank database with Clustal X 1.83 [18]. Phylogenetic analysis was performed with Mega 4.0 [19] using Kimura 2-parameter model with a transition to transversion ration. Phylogenetic trees were built using the neighbor-joining (NJ) methods [20]. Bootstrap tests were performed using 1000 replicates.

3. Results

3.1. Species Identification

Fungal endophytes were abundant and diverse in healthy plant tissues of bitter melon. Some of the isolates were non-sporulating and therefore difficult to identify decisively based on morphological characters. Based on morphological and molecular features, a total of 1172 endophytes were separated from bitter melon, and they were subsequently classified to 49 taxa (**Table 1**).

3.2. Phylogenetic Analysis

One hundred and eighteen strains, representing 49 taxa, were selected to sequence the internal transcribed spacer region (ITS) of nuclear ribosomal DNA.

Among the *Fusarium*, 30 strains formed five clades (**Figure 1**). Results showed that five strains of *F. solani* were clustered together with reference strains of *F. solani*, forming a unique lineage A1. Results also showed

Table 1. Endophytes of bitter melon in Guangdong province.

Taxon	Sites	Tissues
Ascomycetes		
Alternaria alternata	Huadu	Flowers, leaves
Arthrinium aureum	Huadu	Fruits, leaves
A. marii	Huadu	fruits
A. sphaerospermum	Yunfu	Stems
Aspergillus fumigatus	Zhaoqing	Flowers
Annulohypoxylon atroroseum	Zhaoqing	Leaves
A. nitens	Zhaoqing	Leaves
Ascomycete	Huadu	Fruits
Aureobasidium pullulans	Zhaoqing	Fruits
Bionectria ochroleuca	Chaozhou	Leaves
Botryosphaeria rhodina	Zhaoqing	Stems
B. dothidea	Zhaoqing	Fruits
Chaetomium brasiliense	Huadu	Roots
C. globosum	Huadu	Roots
C. hexagonosporum	Huadu	Leaves
C. madrasense	Huadu	Leaves
Cladosporium cladosporioides	Zhaoqing	Leaves, fruits
	Huadu	Fruits, leaves

Continued

C. oxysporum	Huadu	Fruits
	Tianluhu	Leaves
Colletotrichum gloeosporioides	Chaozhou	Stems, leaves
	Huadu	Stems, roots
C. capsici	Huadu	Leaves
Corynascus verrucosus	Huadu	Roots
Curvularia borreriae	Shaoguan	Roots
C. eragrostidis	Huadu	Flowers
C. lunata	Huadu	Roots
C. protuberata	Shaoguan	Leaves
C. verruculosa	Huadu	Flowers
Exserohilum rostratum	Huadu	Stems, roots
Fusarium camptoceras	Huadu	Leaves
F. equiseti	Shaoguan	Roots, stems
	Huadu	Roots
F. kyushuense	Chaozhou	Roots
	Shaoguan	Stems
	Zhaoqing	Flowers
F. oxysporum	Shaoguan	Roots
	Zhaoqing	Leaves
	Huadu	Roots, stems
F. proliferatum	Shaoguan	Stems, roots
F. semitectum	Huadu	Roots
F. solani	Zhaoqing	Flowers, fruits, leaves
	Huadu	Roots
F. verticillioides	Shaoguan	Stems
Nigrospora oryzae	Huadu	Stems, fruits
N. panici	Huadu	Leaves
Penicillium chermesinum	Zhaoqing	Fruits
P. oxalicum	Huadu	Roots
Pestalotiopsis palustris	Zhaoqing	Leaves
Phoma glomerata	Huadu	Stems
Phomopsis bougainvilleicola	Huadu	Stems
P. glabrae	Huadu	Stems
Rhizopycnis vagum	Huadu	Fruits
Stemphyllium solani	Zhaoqing	Leaves
Xylaria sp.	Zhaoqing	Leaves
Basidiomycetes		
Ceratobasidium sp.	Zhaoqing	Fruits
C. cornigerum	Huadu	Fruits

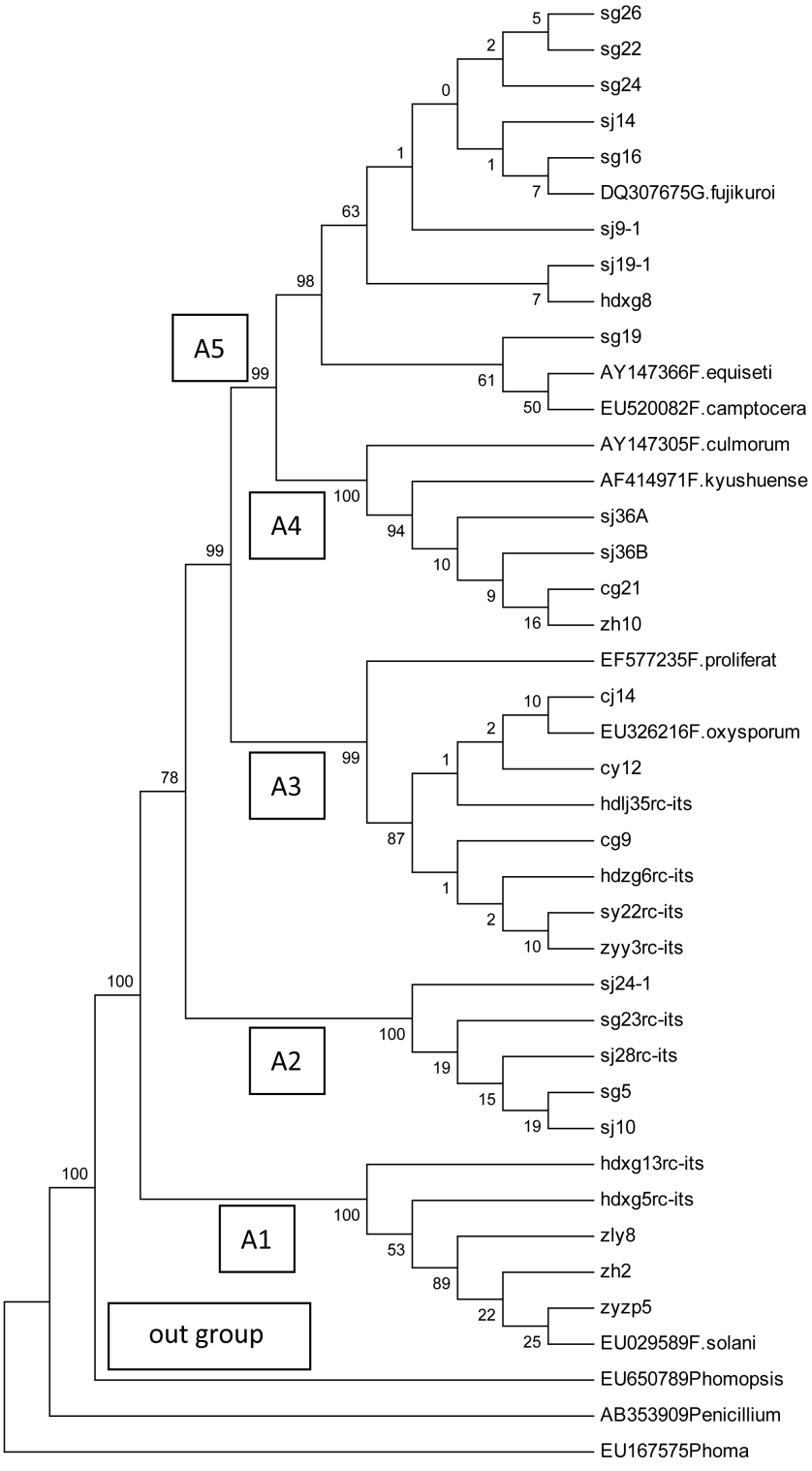

Figure 1. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 38 taxa of *Fusarium*, rooted with *Penicillium marneffei*, *Phomopsis longicolla* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

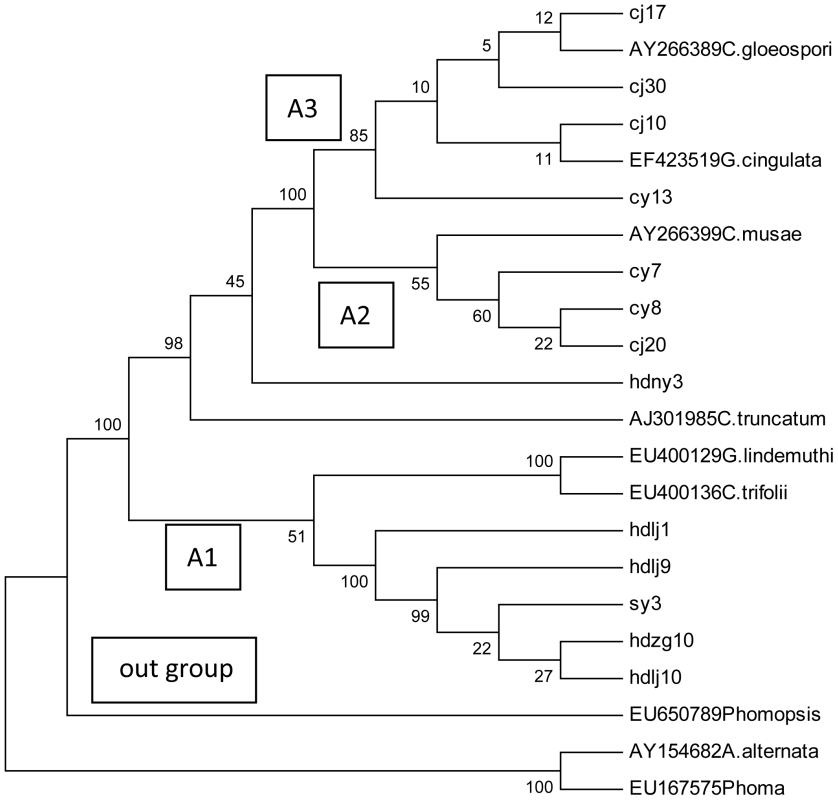

Figure 2. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 19 taxa of *Colletotrichum*, rooted with *Alternaria alternata*, *Phomopsis longicolla* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

that three strains of *F. proliferatum*, one strains of *F. oxysporum*, and one strains of *F. verticillioides* were clustered together, forming lineage A2. Seven strains of *F. oxysporum* were clustered together with reference strains of *F. oxysporum* and *F. proliferatum*, forming a unique lineage A3. Four strains of *F. kyushuense* were clustered together with reference strains of *F. kyushuense* and *F. culmorum*, forming a unique lineage A4. Four strains of *F. equiseti* and five strains of *F. camptoceras* were clustered together with reference strains of *F. equiseti*, *F. camptoceras* and *Gibberalla fujikuroi*, forming lineage A5.

Among the *Colletotrichum*, 13 strains formed three clades (**Figure 2**). Results showed that five strains of *C. gloeosporioides* were clustered together with reference strains of *Glomerella lindemuthiana* and *C. trifolii*, forming a unique lineage A1. Results also showed that three strains of *C. gloeosporioides* were clustered together with reference strains of *C. musae*, forming lineage A2. Four strains of *C. gloeosporioides* were clustered together with reference strains of *C. gloeosporioides* and *G. cingulata*, forming a unique lineage A3.

Among the *Cladosporium*, 12 strains formed three clades (**Figure 3**). Results showed that one strain of *C. cladosporioides* formed a unique lineage A1. Six strains of *C. cladosporioides* were clustered together with reference strains of *C. chlorocephalum*, *C. oxysporum*, *C. colocasiae*, *C. tenuissimum*, *C. cladosporioides* and *C. gossypiicola*, forming lineage A2-1. Five strains of *C. cladosporioides* were clustered together, forming a unique lineage A2-2.

Among the *Alternaria*, 8 strains formed one clade (**Figure 4**). Results showed that eight strains of *A. alternata* were clustered together with reference strains of *A. alternata*, *A. longipes*, *A. arborescens*, *A. tenuissima*, forming lineage A.

Among the *Corynascus* and *Chaetomium*, 6 strains formed three clades (**Figure 5**). Results showed that one strain of *C. brasiliense*, one strain of *C. convolutum*, and one unidentified strain were clustered together with reference strains of *C.* sp, forming lineage A1. One strain of *C. verrucosus* was clustered with reference strains

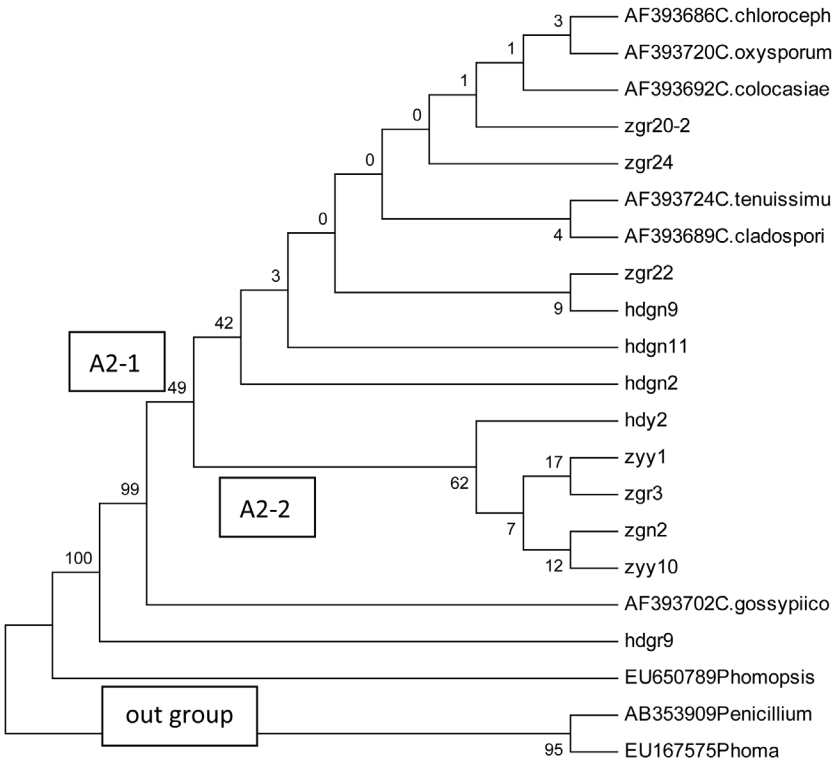

Figure 3. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 18 taxa of *Cladosporium*, rooted with *Penicillium marneffei*, *Phomopsis longicolla* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

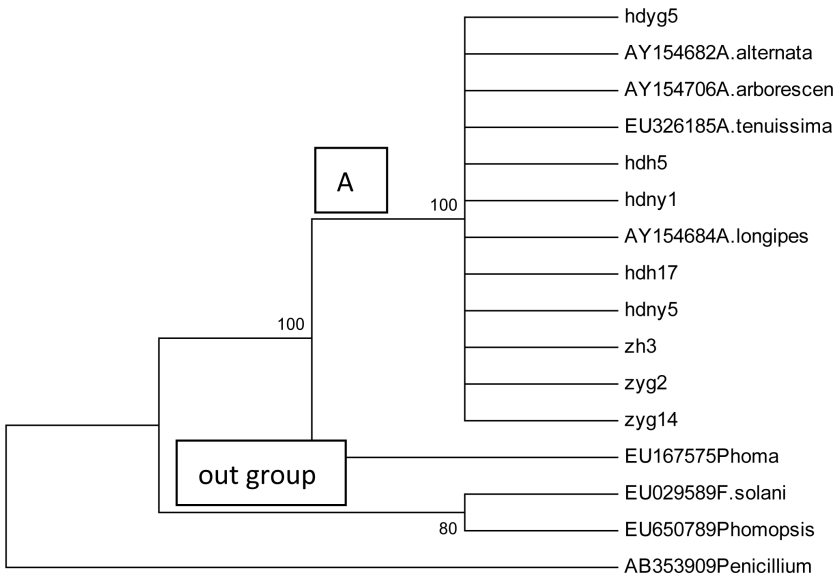

Figure 4. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 12 taxa of *Alternaria*, rooted with *Fusarium solani*, *Penicillium marneffei*, *Phomopsis longicolla* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

of *C. verrucosus*, and *C. kuwaitiensis*, forming a unique lineage A2. Two strains of *C. globosum* were clustered together with reference strains of *C. globosum*, and *C. bostrychodes*, forming a unique lineage A3.

Among the *Curvularia*, *Exserohilum* and *Bipolaris*, 7 strains formed five clades (**Figure 6**). Results showed that three strains of *E. rostratum* were clustered with reference strains of *E. rostratum*, *E. pedicellatum*, and *E. longirostratum*, forming a unique lineage A1. One strain of *C. brachyspora* was clustered with reference strains

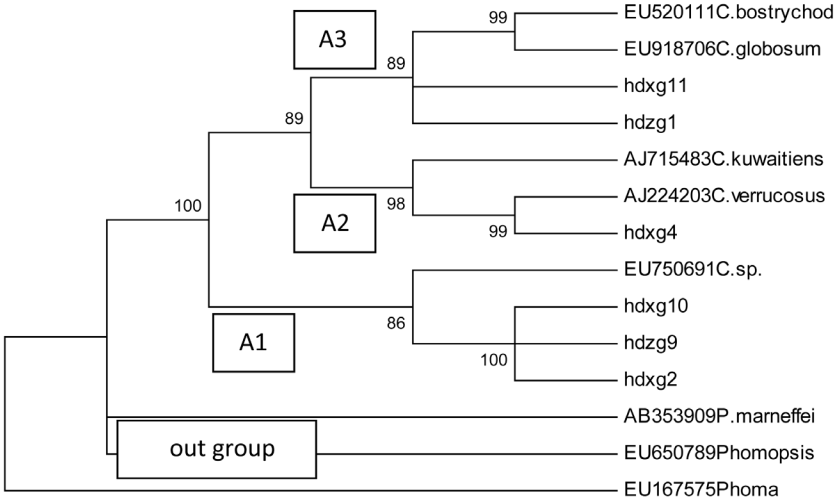

Figure 5. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 6 taxa of *Corynascus* and *Chaetomium*, rooted with *Penicillium marneffei*, *Phomopsis longicolla* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

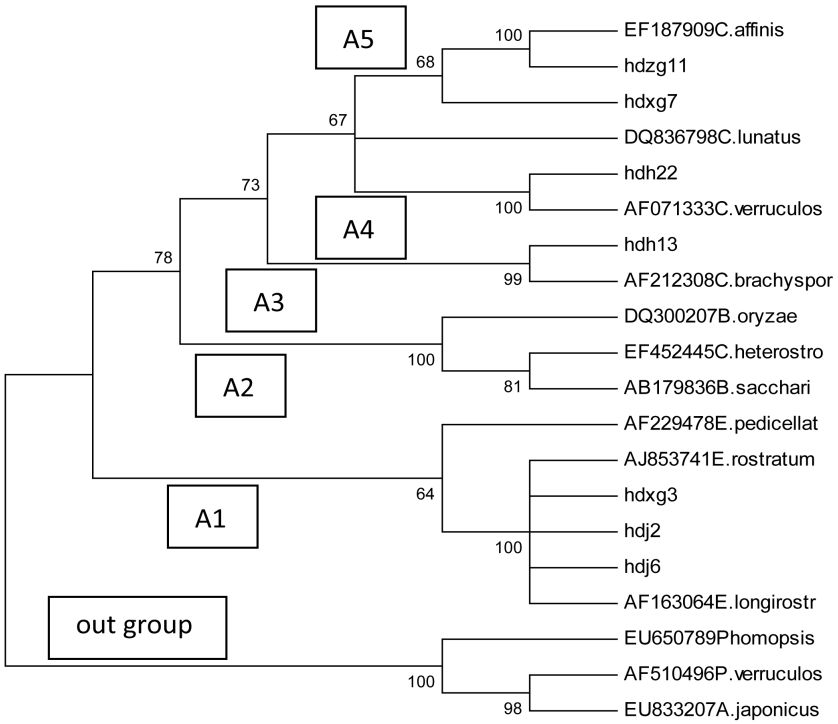

Figure 6. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 17 taxa of *Curvularia*, *Exserohilum* and *Bipolaris*, rooted with *Penicillium verruculosum*, *Phomopsis longicolla* and *Aspergillus japonicus* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

of *C. brachyspora*, forming a unique lineage A3. One strain of *C. verruculosus* was clustered with reference strains of *C. verruculosus*, forming a unique lineage A4. One strain of *C. affinis* was clustered together with reference strains of *C. affinis*, forming a unique lineage A5.

Among the *Phomopsis*, 4 strains formed three clades (**Figure 7**). Results showed that one strain of *P. bougainvilleicola* was clustered with reference strains of *P. bougainvilleicola*, forming a unique lineage A1. One strain of *P. longicolla* was clustered with reference strains of *P. longicolla*, forming a unique lineage A2. Two strains of *P. glabrae* were clustered with reference strains of *P. glabrae*, forming a unique lineage A3.

Among the *Penicillium*, 5 strains formed four clades (**Figure 8**). Results showed that two strains of *P. verruculosum* were clustered with reference strains of *P. verruculosum*, and *P. marneffei*, forming a unique lineage A1.

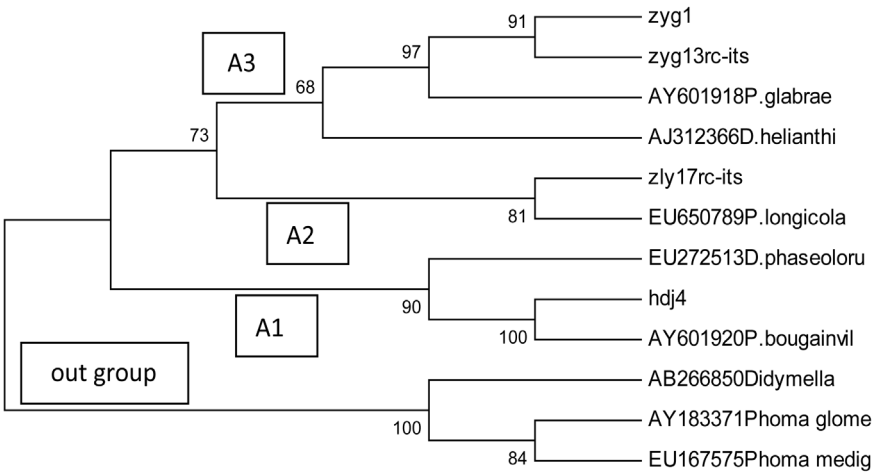

Figure 7. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 9 taxa of *Phomopsis*, rooted with *Didymella bryoniae*, *Phoma glomerata* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

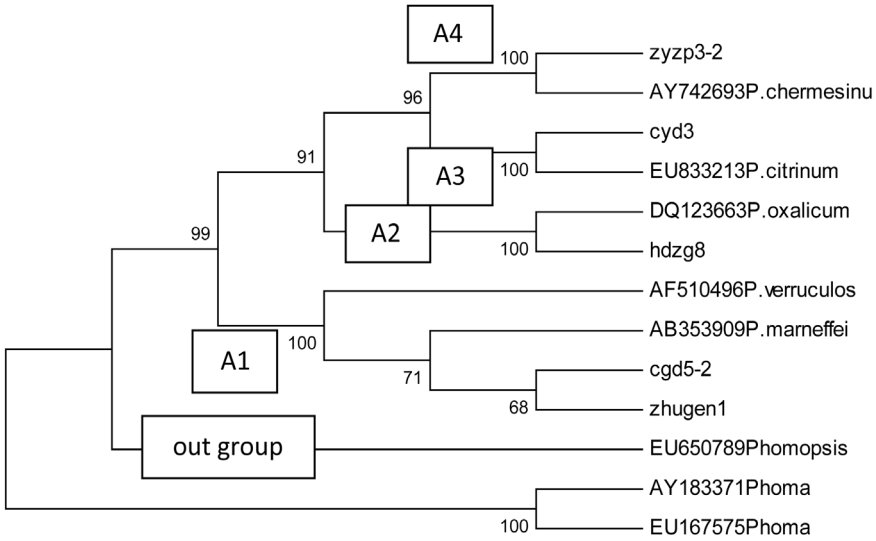

Figure 8. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 10 taxa of *Penicillium*, rooted with *Phomopsis longicolla*, *Phoma glomerata* and *Phoma medicaginis* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

One strain of *P. oxalicum* was clustered with reference strains of *P. oxalicum*, forming a unique lineage A2. One strain of *P. citrinum* was clustered with reference strains of *P. citrinum*, forming a unique lineage A3. One strain of *P. chermesinum* was clustered with reference strains of *P. chermesinum*, forming a unique lineage A4.

Among the *Phoma*, 7 strains formed three clades (**Figure 9**). Results showed that six strains of *Phoma* was clustered with reference strains of *P. glomerata*, and *Didymella bryoniae* (anamorph: *P. cucurbitacearum*), forming a unique lineage A1. One strain of *Phoma* formed a unique lineage A3.

Among the *Aspergillus*, 5 strains formed two clades (**Figure 10**). Results showed that four strains of *A. fumigatus* were clustered with reference strains of *A. fumigatus*, forming a unique lineage A1. One strain of *A. japonicus* was clustered with reference strains of *A. japonicus*, forming a unique lineage A2.

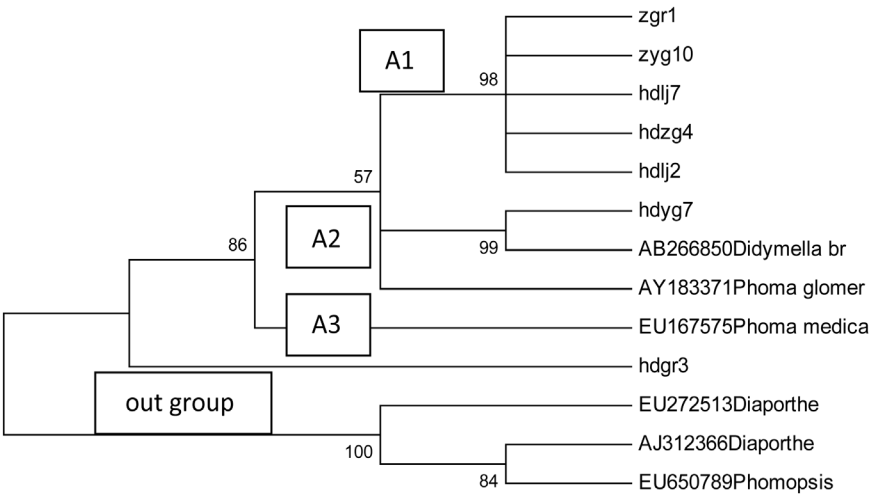

Figure 9. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 10 taxa of *Phoma*, rooted with *Phomopsis longicolla*, *Diaporthe phaseolorum* and *Diaporthe helianthi* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

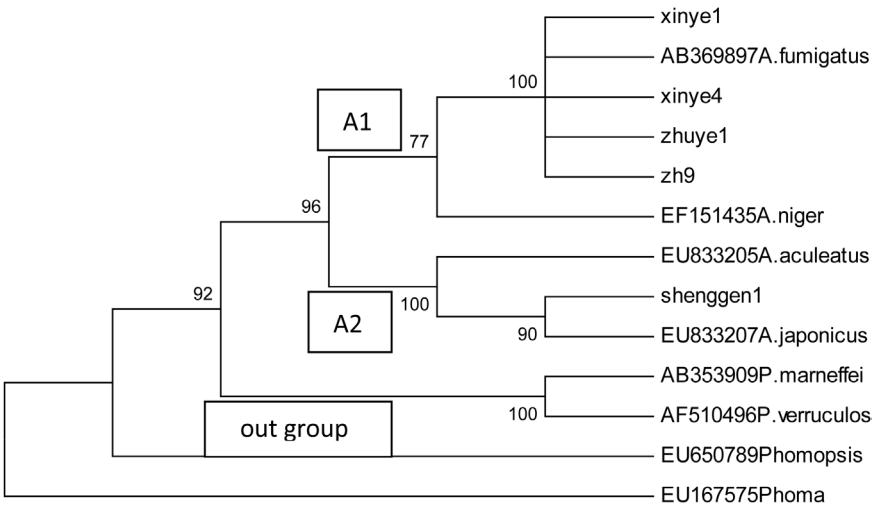

Figure 10. A neighbor-joining tree was generated based on the 5.8S gene and internal transcribed spacer (ITS1 and ITS2) sequences of 9 taxa of *Aspergillus*, rooted with *Phomopsis longicolla*, *Phoma medicaginis*, *Penicillium marneffei* and *Penicillium verruculosum* with Clustal W. The numbers at branches indicated the percentages from 1000 bootstrap replications in which the branch occurred.

4. Discussion

Traditional approaches to identifying fungal endophytes rely on microscopic analysis of morphological characteristics. However, significant portions of endophytic isolates consist of sterile mycelia and cannot be identified based on traditional approaches. Phylogenetic analysis of rDNA sequences has been successfully employed for the identification of different morphospecies [21]-[26]. In the present study, there are 49 taxa isolated as endophytic fungi from bitter melon. We conduct molecular phylogenetic analyses of endophytes of bitter melon, and build the phylogenetic tree from ITS sequences between species of the same genus or between the similar genera. Morphological characters, together with DNA sequence information, can be used to identify the endophytic strains.

The endophytic fungi associated with bitter melon comprise a number of cosmopolitan species such as *Alternaria alternata*, *Aspergillus fumigatus*, *Colletotrichum gloeosporioides* and *Curvularia lunata*. All these genera have been previously isolated as endophytes, not only in Taxus species [25]-[28], but also in pharmaceutical plants [5] [6] [29] [30]. *Arthrinium aureum*, *A. marii*, *A. sphaerospermum*, *Corynascus verrucosus*, *Curvularia borreriae*, *C. protuberate*, however, have not been recorded in any host plants in China, suggesting that bitter melon harbours novel and diverse fungi. Most fungi reported as endophytes up to date have been identified as ascomycetes and their anamorphs. Basidiomycetous endophytes have only been reported in a few studies [24] [31]-[34]. Basidiomycetous endophytes, such as *Ceratobasidium* sp., and *C. cornigerum*, are reported in this study for the first time in bitter melon, and the results show that endophytic fungi from bitter melon exhibit a high biodiversity.

Many phylogenetic studies involving fungi rely on the analysis of ribosomal DNA, in particular the internal transcribed spacer (ITS) regions, to assist in separation at the genus and species levels [35] [36]. Based on phylogenetic analysis of endophytes associated with bitter melon, the ITS sequences successfully separate species, such as *F. solani*, *F. kyushuense*, *C. verrucosus*, *C. globosum*, *E. rostratum*, *C. brachyspora*, *C. verruculosus*, *C. affinis*, *P. bougainvilleicola*, *P. longicolla*, *P. glabrae*, *P. verruculosum*, *P. oxalicum*, *P. citrinum*, *P. chermesinum*, *P. glomerata*, *A. fumigatus* and *A. japonicas*; but on the other hand, some isolates belonging to *A. alternata*, *C. gloeosporioides*, *C. cladosporioides*, *C. brasiliense*, *C. convolutum*, *F. proliferatum*, *F. oxysporum*, *F. verticillioides*, *F. equiseti*, *F. camptoceras*, *Xylaria*, require the analysis of others molecular markers to provide better taxonomic resolution. Rivera-Orduña *et al.* (2011) isolated and identified 116 endophytes of *Taxus globosa* (Mexican yew), and found that ITS sequences could not provide enough information to analysis of the isolates belonging to the families Xylariaceae and Pleosporomycetidae [28]. In addition, the fungal endophyte zly-17 is identified as a *Phomopsis* sp., and the phylogenetic analyses based on ITS1, ITS2, and 5.8 ribosomal RNA gene show that this fungal isolate is closely related to *P. longicolla* (GenBank accession number EU650789).

The present study clarifies phylogenetic relationships among endophytes associated with bitter melon. Molecular analyses of rDNA are proven to be useful for identification of endophytes. In the classification of endophytes, it is necessary to consider an integrated approach, such as molecular phylogeny, host, colony growth rate, symptoms, and shape of conidia.

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