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Morphological, molecular and pathogenic characterization of *Alternaria longipes*, the fungal pathogen causing leaf spot on *Atractylodes macrocephala*

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Leaf spots were observed on commercial plants of Bai Zhu (*Atractylodes macrocephala* Koidz.), a perennial herb that is used in traditional Chinese medicine in Panan County of Zhejiang Province, China. Approximately, 40% of the plants surveyed in the field showed severe symptoms of leaf spot during the summer of 2011. Fungal isolates obtained from infected leaf tissues were grown on Potato Dextrose Agar (PDA), and morphological characteristics of the colonies and the sporulating apparatus were determined. DNA extracted from the fungal culture was subjected to polymerase chain reaction (PCR) with primers ITS1F/ITS4 and Alt-for/Alt-rev, amplifying the internal transcribed spacer (ITS) regions and *Alternaria* allergen gene (*Alt a 1* gene), respectively. The resulting PCR products were sequenced and compared for homology with other species in GenBank. Comparison of sequences of the *Alt a 1* gene revealed 100% similarity with *Alternaria longipes* (Ellis & Everh.) E. W. Mason. Pathogenicity of the fungal isolate L_3 was confirmed on *A. macrocephala.* Taken together, we concluded that *A. longipes* was the causing agent for the leaf spot on *A. macrocephala*.

Key words: *Alternaria* spp., *Alt a 1*, internal transcribed spacer, morphological characteristics, pathogenicity.

INTRODUCTION

Bai Zhu (*Atractylodes macrocephala* Koidz.) is an herb that has been used in traditional Chinese medicine for centuries with bitter and pungent flavor (Pharmacopeia of People's Republic of China, 2010). The rhizomes of *A. macrocephala* are rich in sesquiterpenes and acetylenic compounds, which can invigorate the spleen, and cure patients with splenic asthenia, anorexia, oedema, excessive perspiration and abnormal fetal movement (Chen, 1987; Huang et al., 1992). *A. macrocephala* is native to regions from Manchuria and Korea to Northern and Eastern China and Japan. It primarily grows in mountain valleys, and a large acreage of this herb is planted in Zhejiang Province of China. As the wild plants are on the verge of extinction, several varieties of this species are cultivated in different areas in China to meet the growing demand for fresh herb. Cultivated species are frequently subjected to diseases including those caused by soil-borne pathogens. Many diseases had been reported on *A. macrocephala*, such as root rot, southern blight and leaf spot (Hu, 2003; Zang et al.,

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Figure 1. Appearance of leaf spot on *A. macrocephala* caused by *A. longipes* (A), and the healthy leaf as contrast (B).

2005; An et al., 2007) causing significant losses in *A. macrocephala* production.

Leaf spot is one of the common diseases in the agricultural production. Many studies showed that leaf spot is caused by fungus pathogens such as *Alternaria*, *Ceratocystis*, *Guignardia*, *Phoma*, etc. And among those pathogens, *Alternaria* account for the most. For instance, leaf spot on *Atractylodes* is caused by *A. tenuissima* (Wang et al., 2007), and *A. brassicae* caused leaf spot on Chinese cabbage (Michereff et al., 2012). Diseases caused by *Alternaria* species are common and these diseases occur worldwide. Both plant pathogenic and saprophytic species of *Alternaria* may cause leaf spot and blight on numerous plant taxa including vegetables, fruit trees and ornamentals (Simmons, 1997; Mirkova and Konstantinova, 2003). Infection by *Alternaria* species typically causes the formation of necrotic lesions, which sometimes have a target-like appearance surrounded by an un-invaded chlorotic halo (Agarwal et al., 1997). For example, *A. alternata* (Fr.) Keissl. is an opportunistic pathogen on numerous host plants causing leaf spots, rots and blights (Droby et al., 1984; Pryor and Michailides, 2002; Maiti et al., 2007). More than 380 hosts including *A. macrocephala* have been recorded to be infected with *Alternaria* species in the USDA Systematic Botany and Mycology Fungus-Host Distribution Database (Mmbaga et al., 2011). *A. tenussima* (Nee.) Wiltshire. has been reported to cause dark leaf spot on *A. lancea* (Thunb.) DC., a plant which is in the same family with *A. macrocephala* (Wang et al., 2007).

Most species of *Alternaria* exhibit considerable plasticity in morphology depending on cultural conditions such as temperature, light and humidity (Simmons, 1992).

Besides the morphological characteristics, polymerase chain reaction (PCR)-based assays have also been employed for identification of *Alternaria* pathogens (Pryor and Gilbertson, 2000). A PCR assay reported by Konstantinova et al. (2002) can detect *Alternaria* at genus level, but it cannot distinguish any specific species. Zur et al. (2002) developed PCR primers for the internal transcribed spacer (ITS) regions that could detect *A. alternata* or *A. solani* Sorauer., but failed to distinguish between these two species. Hong et al. (2005) found that *Alternaria* allergen (*Alt a 1*) gene can aid resolution among *Alternaria* species, and Shipunov et al. (2008) used primers Alt-rev and Alt-for and had succeeded in distinguishing *A. alternata*, *A. longipes* and *A. tenuissima*.

The main objective of this study was to identify the causal agent of leaf spot on *A. macrocephala*. Morphological examinations, ITS gene and *Alt a 1* gene sequencing, and pathogenicity assays were carried out to characterize the fungi isolated from infected *A. macrocephala* leaves.

MATERIALS AND METHODS

Sampling

Plants of *A. macrocephala* with typical symptoms of leaf spot were collected in 2011 from commercial fields and trial sites across the cropping regions of Panan County, Zhejiang Province, China (Figure 1). Six symptomatic plant materials (from different field) were placed in labeled plastic bags, maintained in a cooler with ice for storage and transportation until the samples were processed in the laboratory.

Fungal isolation, purification and storage

Diseased leaves of *A. macrocephala* were surface sterilized by soaking in ethanol (75%, v/v) for 40 s, followed by 4 min in hypochlorite (1%, w/v) and subsequently soaking in ethanol (75%, v/v) for 30 s again to remove residual hypochlorite, finally rinsed in sterile distilled water three times and dried with sterile filter paper. Leaves were cut into 0.5 cm^2 at the joint section of diseased and healthy tissues and transferred to a plate of malt extract agar (MEA) containing 2% malt extract and 2% agar (w/v) supplemented with streptomycin sulphate at 50 mg/L and quadracycline at 20 mg/L to prevent bacterial growth. Six pieces of leaf tissues were placed on

three plates and incubated at 25°C in the dark. After incubation for 4 days, the fungal hyphae growing from the diseased leaf tissues were cut off from the edge of the colonies and sub-cultured on a plate of potato dextrose agar (PDA) for purification. For storage, the fungal isolates were preserved in 15% (v/v) sterile glycerol containing glucose (10 g/L), yeast extract (1 g/L) and casein hydrolysate (1 g/L) at -80°C.

In this study, a total of eight fungal isolates (referred as L_1, L_2, L_3, L_4, L_5, L_6, L_7 and L_8) were obtained from symptomatic leaves of *A. macrocephala* and all the fungal isolates were preserved at the Research Institute of Subtropical Forestry, Chinese Academy of Forestry, China.

Morphological observations

Isolate L_3 was grown for 7 to 10 days on plates of PDA, water agar (WA) and potato carrot agar (PCA) in an incubator at 25°C. After incubation, cultures were examined for color, margin, texture of colonies and the development of pigments in the agar medium. Conidia produced from 7-day-old fungal cultures on WA plates were mounted on slides and examined for morphological characteristics at magnification of 100× under a compound microscope (Olympus CX41, Tokyo, Japan). The size of conidial spores was based on measurements of 100 conidia. The genus and species of the fungal isolates were identified according to Zhang (2003) and Simmons (1997).

The isolate L_3 was also used to determine the effects of temperature on colony growth. A 5-mm-diameter colony plug from the margin of the L_3 was placed in the center of a 90-mm-diameter PDA petri dish, which incubated separately at 5, 10, 15, 20, 25, 30, 35 and 40°C. Colony diameters were measured after 24, 48, 72 and 96 h.

DNA extraction and PCR amplification

For extraction of fungal genomic DNA, mycelia were scraped from a 10-day-old culture grown on PDA, and placed in a sterile 2 ml eppendorf tube. The Axygen Multisource Genomic DNA Miniprep Kit (Axygen, Hangzhou, China) was used for genomic DNA extraction from the fungal isolates following the manufacturer's instructions. PCR amplification was performed using a Touchgene Thermal Cycler (Barloworld Scientific Ltd, United Kingdom). A total of 50 µl was prepared for each PCR reaction, which consisted of 34.7 µl sterile ddH_2O, 5 µl 10× PCR buffer (Promega), 3 µl $MgCl_2$ (25 mM), 4 µl dNTPs (10 mM in total, 2.5 mM each), 0.5 µl each primer (20 ng/µl), 0.3 µl Taq polymerase (Promega) (5 U/µl), and 2 µl template DNA (20 ng/µl). Thermal cycling conditions involved an initial step of denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 40 s, 55°C for 50 s (for primers ITS1F/ITS4) or 57°C for 30 s (for primers Alt-for/Alt -rev), 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were visualized under UV light after 1.0% agarose gel electrophoresis in 0.5× TBE and being stained with ethidium bromide.

The internal transcribed spacer (ITS) regions of rDNA were amplified using the universal primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993; White et al., 1990). Amplification of *Alt a 1* genes was conducted using primers Alt-for (5'-ACGAGGGTGAYGTAGGCGTC-3') and Alt-rev (5'-ATGCAGTTCACCACCATCGC-3') (Hong et al., 2005), which were designed based on the conserved regions in *Alt a 1* homologs of *A. alternata* and *A. brassicicola* (Schwein.) Wiltshire. The PCR products were purified using a gel band purification kit (Axygen Scientific Inc., Hangzhou, China) and sequenced. The sequences were deposited in GenBank under the accession numbers JQ004404 (ITS) and JQ004405 (ALT).

Sequence alignment and phylogenetic analysis

The resulting ITS and ALT sequences were searched for homology with other genera and species deposited in the GenBank database using NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments were performed using Clustal X program (version 2.0). Phylogenetic analyses were performed with the PHYLIP 3.69 package using Dnadist program. Distance trees were constructed using the neighbor-joining (NJ) method (http://evolution.genetics.washington.edu/phylip.html). Bootstrap values were calculated using the Seqboot (1000 replicates), Neighbor and Consense programs (Yuan et al., 2011).

Pathogenicity test

All isolates were conducted pathogenicity tests, and the tests were performed on detached healthy leaves of *A. macrocephala* grown in greenhouses. Healthy leaves were collected from 2-year-old *A. macrocephala* and maintained in Petri dishes with sterile paper and cotton soaked with sterile water. For each isolate, three fully expanded leaves were inoculated by placing a PDA plug (0.5 cm^2) of the fungal mycelia on upper surfaces of the leaves, and each leaf was slightly wounded on the midrib with a sterile razor blade prior to inoculation. Another three leaves treated with sterile PDA plugs served as the uninoculated control. The Petri dishes were covered and placed under light of a 12 h photoperiod at 25°C and 95% relative humidity for 7 days. The pathogenicity test was repeated three times.

RESULTS

Cultural and morphological characteristics of the fungal isolates

On PDA, the fungus formed radial colonies of grayish airy mycelia, and the substrate mycelium was dark green with a clear zone observed from the underside of the plate. Colonies on WA plates were radial, sparse with grayish to greenish airy mycelia without clear zones in the substrate. Colonies on PCA were powdery-like and dark grey to olivaceous black (Figure 2A and B). Conidiophores were light brown with one or few regular septa and mostly unbranched. Conidia of the isolates were catenated in long and sometimes branched chains of 5 to 12 spores. The spores were obclavate, dark brown, with 3 to 8 transverse and 0 to 2 longitudinal or oblique septa (Figure 2C and D). The conidia were on average measured 60.2 (20 to 98) × 10.4 (5.8 to 14.2) µm (n = 100). The optimal temperature conditions for the growth of L_3 were assessed, and the diameter growth is shown in Figure 3. The isolates L_3 can grow on a wide range of temperatures from 5 to 35°C and the optimal temperature is 25°C.

Nextly, we do the same test as aforementioned temperature trail from 25 to 30°C, then we found that 26°C is the optimal temperature for L_3 colony growth on PDA.

PCR amplification and DNA sequencing analysis

DNA extracted from the fungal isolate L_3 was amplified by PCR with ITS1F and ITS4 primers resulting in ~560 bp

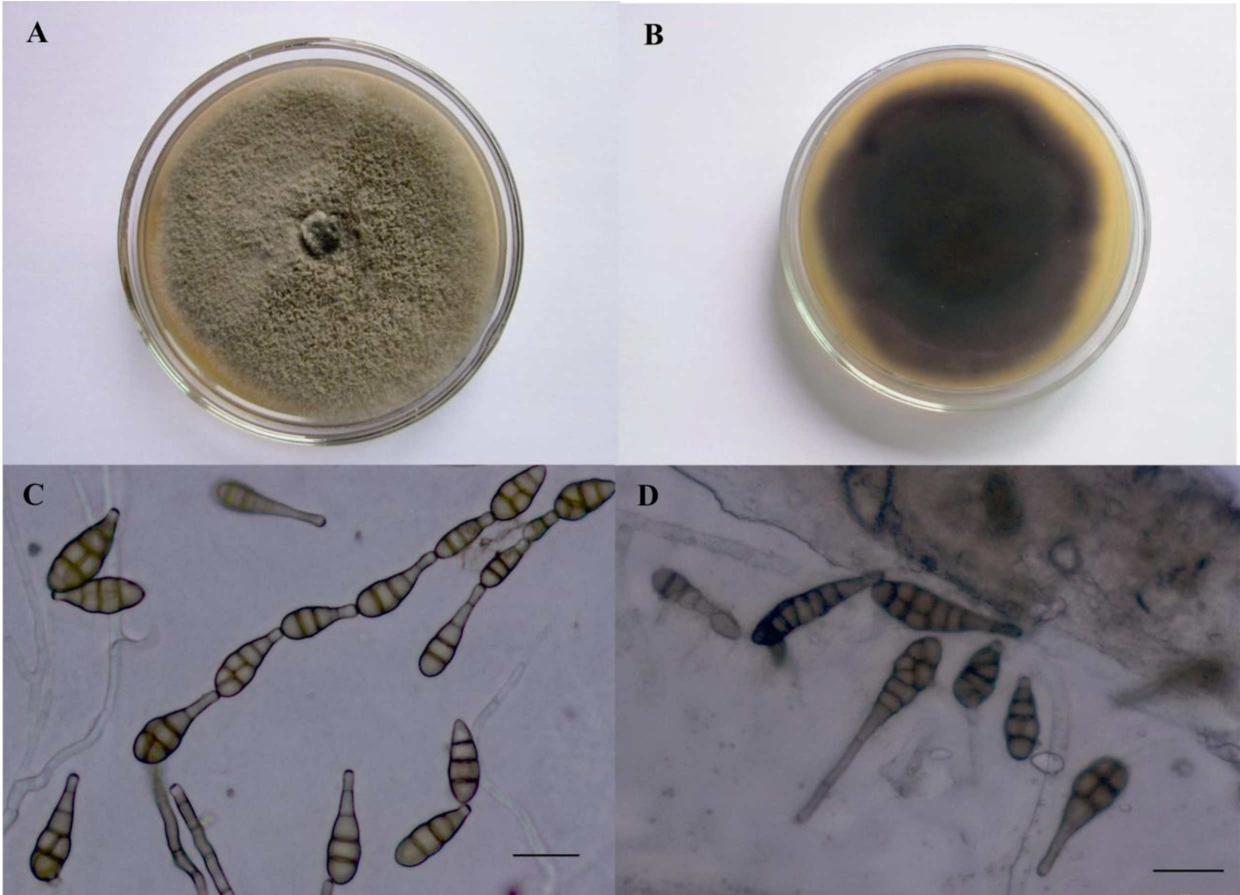

Figure 2. Morphology of the *A. longipes*. (A) Colony of *A. longipes* on PCA after 7 days at 26°C; (B) Colony underside of *A. longipes* on PCA after 7 days at 26°C (C) and (D) Conidiophores and conidia of *A. longipes* on water agar (bar = 20 μm).

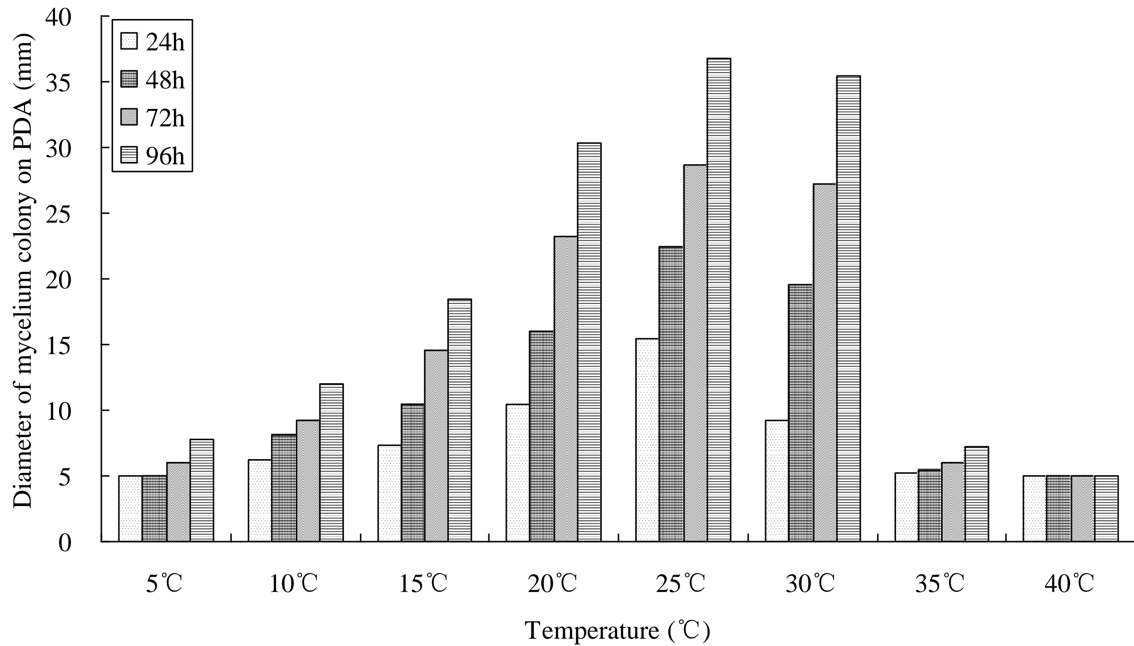

Figure 3. Effect of temperature on growth of *A. longipes*.

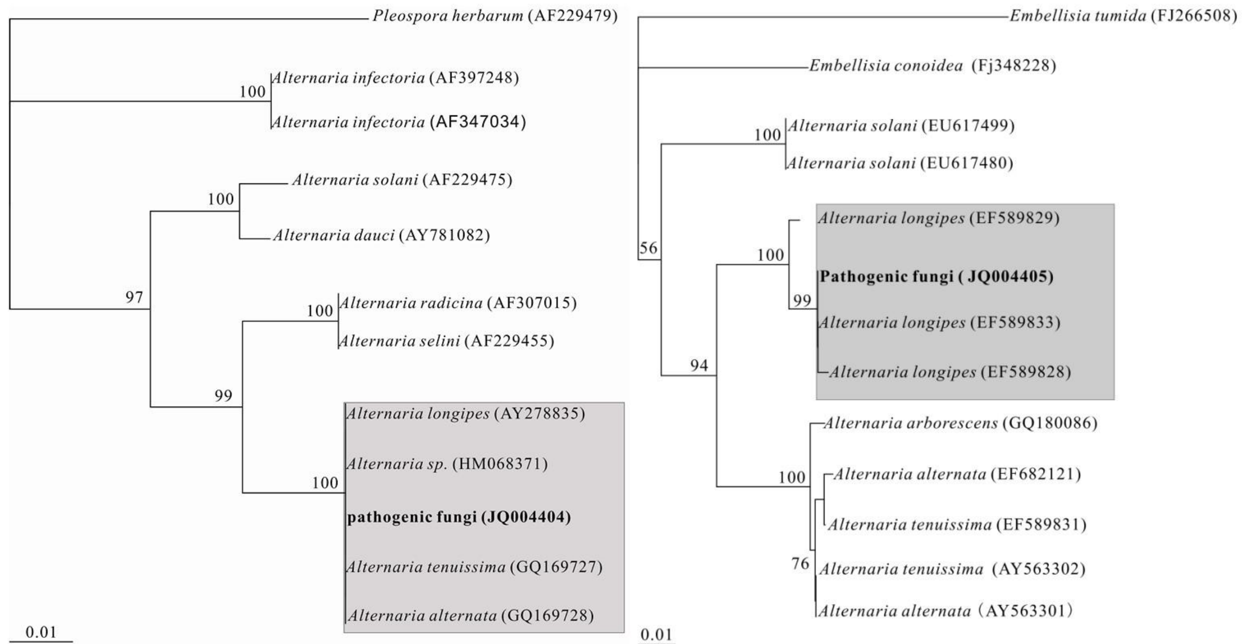

Figure 4. Phylogenetic trees based on ribosomal DNA internal transcribed spacer (ITS) (left) and *Alternaria* allergen (*Alt a 1*) gene sequences (right) of *Alternaria longipes* and other related fungi. *Pleospora herbarum* (AF229479) and *Embellisia tumida* (FJ266508) were included as the outgroups, respectively.

products (GenBank Accession No. JQ004404). As different species of *Alternaria* can share the same sequences in ITS regions, comparison to sequences of ITS regions is not adequate for identifying a specific species of *Alternaria*. For example, an ITS sequence under GenBank Accession No. EF589849 was amplified from the DNA from *A. alternata*, *A. longipes* and *A. tenuissima*, these species of *Alternaria* were not able to be differentiated by their ITS regions (Zur et al., 2002). In our study, phylogenetic analyses with ITS sequences revealed that the *Alternaria* isolate L_3 under GenBank Accession No. JQ004404 was grouped in the same clade with *A. longipes*, *A. tenuissima*, *A. alternata*, and an unknown species of *Alternaria*. Thus, we chose the *Alternaria* allergen gene (*Alt a 1* gene) to further identify the species of *Alternaria* isolated from the diseased leaves of *A. macrocephala* (Hong et al., 2005; Shipunov et al., 2008). Amplification of *Alt a 1* genes was conducted using primers Alt-for/Alt-rev, and the resulting fragments (~ 460 bp) were sequenced and deposited in GenBank (Accession No. JQ004405).

From the phylogenetic tree generated based on *Alt a 1* homologs, the fungal isolate L_3 was exclusively classified as *A. longipes* with 100% bootstrap support, and *A. tenuissima* and *A. alternata* were clustered in another separate group (Figure 4).

Pathogenicity test

Lesions of leaf spot became visible on leaves of *A. macrocephala* after 72 h of inoculation with the *Alternaria* isolate L_3. The lesions were initially small (<1 mm in diameter), light brown. After 7 days, they became large, semicircular, oval or irregular-shaped with dark brown or black centers surrounded by brown or light brown margins. The symptoms described earlier were the same as those observed on leaves of *A. macrocephala* plants by natural infection in the field, whereas no symptoms developed on the other seven inoculations and control leaves. Then we repeated the isolation, and re-isolation of the fungus from symptomatic leaf tissues (inoculated with L_3) on PDA confirmed that the causal agent was *A. longipes*, fulfilling Koch's postulates.

DISCUSSION

In this report, we described a fungal pathogen that caused leaf spot on *A. macrocephala* including its identification by morphological, molecular and pathogenic characteristics. *A. longipes* is a common pathogen which has been reported to cause brown spot on tobacco (*Nicotiana tabacum* L.), and this disease caused great economic losses in many countries (Welty et al., 1968; Fravel and Spurr, 1977). Symptoms on tobacco first occur on the lower leaves as water-soaked lesions, with the center becoming light lesion surrounded by a yellow halo. Severe spotting leads to necrosis and death of leaves. The lesions are liable to extend during curing of tobacco leaves. Symptoms are much less conspicuous on young leaves. Necrotic lesions also occur on petioles, seed capsules and stems, forming dark brown longitudinally elon-

gated lesions over time. In addition, *A. longipes* was also reported to cause leaf blight on carrots (Vintal et al., 2002) and leaf spot on *Smilax china* (Long et al., 2009). Previous report indicated that *A. alternata* was the pathogen in *A. macrocephala* causing leaf spot (Zang et al., 2005). *A. alternata* and *A. longipes* are very similar in morphology, and they had been obscured in many previous studies.

In order to solve this problem, Simmons (1981) examined the type of specimen of *A. longipes* again, and confirmed they were two different species. de Hoog et al. (2000) described that *A. longipes* had the same ITS profile as *A. alternata*, but differed by darker conidia with longer beaks and mostly smooth-walled conidia. *Alt a 1* is a gene for the Alternaria major allergen, which can reveal greater sequence divergence than ITS and mitochondrial small subunit (mt SSU) rDNA. Results from this study showed that the ITS sequences of *A. longipes*, *A. alternata*, *A. tenuissima* and an unknown species of *Alternaria* were the same with each other with 100% bootstrap. This indicated that the ITS sequences are not able to be solely employed to distinguish among these *Alternaria* species, which was in agreement with Shipunov et al. (2008). In contrast, sequences of *Alt a 1* genes are more useful than ITS regions of rDNA in identification of *Alternaria* species. The phylogenetic tree generated base on *Alt a 1* homologs revealed that the *Alt a 1* gene sequences amplified from the rDNA of the fungal isolates showed 98% similarity with *A. longipes* (Accession No. EF589829) in the GenBank. Therefore, based on the morphological characteristics and molecular characterization, together with pathogenicity confirmation, we concluded that the pathogen responsible for leaf spot on *A. macrocephala* in Zhejiang province of China was *A. longipes*.

As *A. macrocephala* is an important economical crop, there is a large acreage of cultivation in Panan County in Zhejiang Province and other production areas in China. According to our knowledge, this leaf spot commonly occurs in this area, but no effective methods are available for local growers to control this disease problem. Further research is warranted to investigate the epidemiology and to develop strategies for management of this economically important disease.

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