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A New Disease of Strawberry, Fruit Rot, Caused by *Geotrichum candidum* in China

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Abstract

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A new disease of strawberry (*Fragaria ananassa* Duch.) was discovered in the Lianqiao strawberry planting base in Shaodong County, in Hunan Province, China. In the early disease stage, leaves showed small black spots surrounded by yellow halos, while in the late stage, a white fluffy layer of mold appeared. Fruits were covered with a white layer of mold. The symptoms were observed using *in vitro* inoculation experiments. After the spray-inoculation of stabbed leaves, small black spots surrounded by yellow halos occurred on leaves, with no clear boundary between diseased and healthy areas. In the late stage, disease spots gradually expanded and a white fluffy layer of mold formed under humid conditions. Unstabbed leaves had almost no disease occurrence after spray-inoculation. After the spray-inoculation of stabbed fruits, by the late stage, a dense white layer of mold formed. According to Koch's postulates, the isolated strain was verified as a pathogen. The pathogenic strain, designated SDLQ16, was isolated from diseased fruit by dilution method and tentatively identified as *G. candidum* based on the culture characteristics, morphologies, physio-biochemical analysis, and phylogenetic analysis of the *rDNA-ITS* sequence. The fungus was able to grow on different culture mediums, with a broad range of nutrition. The colonies on PDA medium were raised and pale white, with a neat edge and visible hyphae. The hyphae were friable but the spores were developing. Basal hyphae rapidly grew close to the medium to 3.2–4.2 μm in diameter, with septa and forked branches at acute angles. The solitary or beaded spores with smooth surfaces were 3.5–7.5 μm in length and 3.5–4.5 μm in width. This strain was able to gelatin liquefaction, proteolysis, grease, peptonised milk, urea, and so on. The pathogenicity on strawberry from strong to weak was: fruit > leaf > stem. A BLAST algorithm was used to query SDLQ16's *rDNA-ITS* sequence (cloned and deposited as GenBank number KU373122) against the NCBI database, and it was located in the *Acinetobacter* sp. branch of a phylogenetic tree. SDLQ16 was most closely related to *Geotrichum candidum* ATCC34614 (GQ4580314.1), with a sequence similarity of 99%.

Keywords: *Fragaria ananassa* Duch.; new pathogenic fungi; *Geotrichum candidum*; identification; *rDNA-ITS*

Strawberry (*Fragaria ananassa* Duch.), an herbaceous perennial plant in the genus *Fragaria* and in the family Rosaceae, is highly adaptable, widely cultivated, and extensively distributed. Strawberry is regarded as the "queen of fruit" and is strongly favoured by consumers because of its colourful appearance, sweet, sour taste, aromatic, juicy flesh, and rich nutrient ingredient (Wang *et al.* 2008a; Takahashi *et al.* 2009). Over the past 20 years, the planting area and yield of strawberry have grown

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rapidly. The average annual planting area is 2.5×10^5 hm^2, and the average annual yield is 4.5×10^8 t (FAOSTAT 2013; FAO Statistical database 2012 – http://faostat.fao.org/site/339/default.aspx; USDA Quick Stats 2.0. U.S 2013; Šamec *et al.* 2016). Europe has the greatest strawberry production, followed by the America and, in descending order: Asia, Africa, and Oceania. China is the most important country for strawberry production in Asia. In recent years, there has been a structural adjustment in the planting industry in China. Planting scale of strawberry has been expanded rapidly, because it is a cash crop with a short production cycle, high effectiveness, and high efficiency (Li *et al.* 2004). The planting area and yield of strawberry in China, both ranking first in the world, were 1.1×10^5 hm^2 and 200 t, respectively, in 2010 (Tan *et al.* 2003; Wang *et al.* 2008b; Zhao *et al.* 2012). However, blind large-scale plantings, extensive management modes, climate change, farming system alterations, the disordered introduction of strawberry varieties, the misappropriation, and misuse of chemical pesticides, have resulted in increasingly serious strawberry diseases and the continuous emergence of new diseases (Zhang *et al.* 2010a, b; Walker *et al.* 2011).

The strawberry diseases previously recorded in China mainly included root rot, gray mold, powdery mildew, anthracnose, leaf spot, and brown spot (Behrouz *et al.* 2006; Lv *et al.* 2010; Fang *et al.* 2012; Sylla *et al.* 2013; Asad-Uz-Zaman *et al.* 2015; Campos-Requena *et al.* 2015; Lachhab *et al.* 2015). Recently, we investigated the occurrences of strawberry diseases in 14 regions of Hunan Province, including southern, central, western, northern, and eastern. During this investigation, an unknown new potential disease that infected strawberry fruits was discovered in the Lianqiao strawberry planting base in Shaodong County. Typical diseased fruit samples were taken to the laboratory for identification, and an isolate was grown under culture conditions. The growth was rapid, which excluded powdery mildew pathogens, the growth temperature was low, which excluded Phytophthora fruit rot, and no sclerotia or pycnidia were observed, which excluded southern blight (Li *et al.* 2005; Sylla *et al.* 2013; Eikemo & Stensvand 2015). A thorough review of the literature and historical data found no reports of this disease or any similar diseases. Thus, this disease was considered to be a new disease affecting strawberry.

In present study, we systematically identified the new disease of strawberry through pathogen isolation, culture observations, morphological characterisation, a pathogenicity assay, and an internal transcribed spacer (ITS) sequence analysis. This study provides scientific methods for identifying and diagnosing strawberry disease, and clarifying the pathogen type, which will aid in developing disease prevention and control measures.

MATERIAL AND METHODS

Diseased sample collection. Typical diseased fruit samples were collected from the Lianqiao strawberry planting base in Shaodong County, Hunan Province, China in March–April 2015 on strawberry *Fragaria ananassa* Duch. cv. Hongyan. Fruits were gently placed into sample bags and labelled, including the date. All samples were delivered to the laboratory for identification.

Culture media, We prepared the following culture media according to the method of Fang (1998) using potato dextrose agar medium (PDA; the major composition: potato 200 g, glucose 20 g, agar 18 g, H_2O 1000 ml), beef extract–peptone agar (NA; the major composition: beef extract 3 g, peptone 10 g, NaCl 5 g, agar 18 g, H_2O 1000 ml, pH 7.2–7.4), Gause's No. 1 agar (GSYH; the major composition: soluble starch 20 g, KNO_3 1 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.01g, agar 18 g, H_2O 1000 ml, pH 7.2–7.4), beef extract–nutrient broth (NB; the major composition: beef extract 3 g, peptone 10 g, NaCl 5g, H_2O 1000 ml, pH 7.2–7.4), and Luria–Bertani agar (LB; the major composition: tryptone 10 g, yeast extract 5 g, NaCl 10 g, agar 18 g, H_2O 1000 ml, pH 7.2–7.4).

Strawberry seedlings. Healthy, disease/pest-free seedlings with uniform growth were chosen from the strawberry planting base and transplanted with soil into small experimental pots. The plants were placed in a light incubator (20–23°C, 80–90% humidity) for 3–5 days. After reviving, the plants were used for inoculation and experimentation.

Pathogen isolation. Strain SDLQ16 was isolated by dilution from disease tissue. We utilised the sterile knife to cut morbid and healthy at junctions from typical sick fruits with 4–5 length and 2–3 width. Taken 10 tissues (about 0.5 g) into sterile tube with 4.5 ml of sterile water washed repeatedly, after then we gained dilute spore suspension of 10^{-1}. Put 0.5 ml suspension of 10^{-1} spore suspension into 10 ml tube which has 4.5 ml sterile water and shaking, then we

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gained dilute spore suspension of 10^{-2}. In turn, we got spore suspension of 10^{-6}. Taken 20 μl of 10^{-6} spore suspension into PDA medium which contained streptomycin at a concentration of 40 μg/ml. Afterwards, uniform coated with spreader, after water being aired, the medium were placed upside down and incubated at 20–23°C in the dark for 24–72 hours. Utilised inoculating needle moved single colonies to another PDA medium to pure cultivate. Until gained the pure colonies which meet the requirements of experiments (Fang 1998).

Cultural characteristics of the pathogen strain. Strain SDLQ16 was inoculated onto PDA, NA, GSYH, and LB and incubated in a temperature-controlled incubator in the dark for 24–120 hours. Colony characteristics were observed, including size, shape (round, rhizoid, prostrate, irregular), edge (neat, diffuse, dense, loose), surface (mouldy, powdery, small black spotty, blossom-like, concentric annular), raised shape (expansion, platform, low convex, convex surface), texture (fatty, filmy, sticky, brittle), colour, ease of picking, extent of binding to medium, production of pigments, and consistency of colour between the front and back sides of the medium.

Morphological characteristics of the pathogen strain. Strain SDLQ16 was inoculated onto thin PDA plates and continuously incubated at 20–23°C for 24–72 hours. First, the basic morphology of the fungus was observed, including the main and secondary hyphal branches, the presence of septa, the position, production and germination of spores, and the size, shape and arrangement of spores. Next, the formation of special structures, such as pycnidia, was examined. Attention was paid to the size, shape, and location of these structures, which helped to identify the fungus (Wei 1979).

ITS sequence analysis and phylogenetic tree construction. Genomic DNA was extracted from the mycelia of strain SDLQ16 using the CTAB (Hexadecyltrimethylammonium bromide) method after 3–5 days culture. The extracted DNA was stored in a refrigerator at –20°C (Liu *et al*. 2005).

The fungal rDNA gene was amplified with the universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC). The PCR reaction contained 0.5 μl of template DNA, 2.5 μl of 10× Buffer (with Mg^{2+}), 1 μl of dNTPs (2.5 mM), 0.2 μl of Taq polymerase, 0.5 μl of IS1, and 0.5 μl of IS4, with ddH_2O added to bring the total volume to 25 μl. The PCR was run as follows: pre-degeneration at 94°C for 4 min, followed by 30 cycles of degeneration at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 10 minutes. The PCR products were held at 4°C until they were removed from the thermal cycler. The target gene fragment was isolated using 1% agarose gel electrophoresis for 20 min at 150 V and 100 mA. The DNA band was recovered and purified using a DNA gel extraction kit and then sequenced by Sangon (Shanghai, China). The sequence was deposited in GenBank to obtain an accession number (KU373122).

The obtained sequence of strain SDLQ16 was compared, using the BLAST algorithm, with other sequences in the NCBI database to identify related sequences and retrieve the sequences of other species for a phylogenetic analysis. Phylogenetic trees were constructed using the maximum likelihood method in MEGA 7.0 (1000 replicates) (Gao *et al*. 2012).

Pathogenicity assay. The pathogenicity of the isolated pure culture was verified by Koch's postulates. In the *in vitro* inoculation experiments, we collected stems, leaves, and semi-mature fruits of a uniform size in the flowering and fruiting periods of strawberry. The samples were surface disinfected and washed with sterile water before use (Wei 1979; Moreno-Garcia & Grey 2012).

The *in vitro* inoculation experiments of stems, leaves, and fruits included two treatments each. In Treatment 1, unstabbed samples were sprayed with the spores suspension, and in Treatment 2, stabbed samples were sprayed with the spores suspension. The inoculated samples were incubated in a light incubator at 20–23°C with 85–90% humidity for 7 days. The incidence of the disease was observed. When obvious symptoms were observed in the inoculated tissue, re-isolation was performed using the diseased tissue. Additionally, an *in vivo* inoculation experiment was performed. Strawberry seedlings were transplanted into small pots and revived before the pathogenicity assay. The other procedures were performed as described in the *in vitro* inoculation experiments.

RESULTS

Isolation and culture characteristics. A pure culture, designated strain SDLQ16, was isolated from strawberry fruit with typical symptoms. At 20–23°C, SDLQ16 was able to grow on different media (e.g., PDA, NA, LB, and GSYH), with a wide

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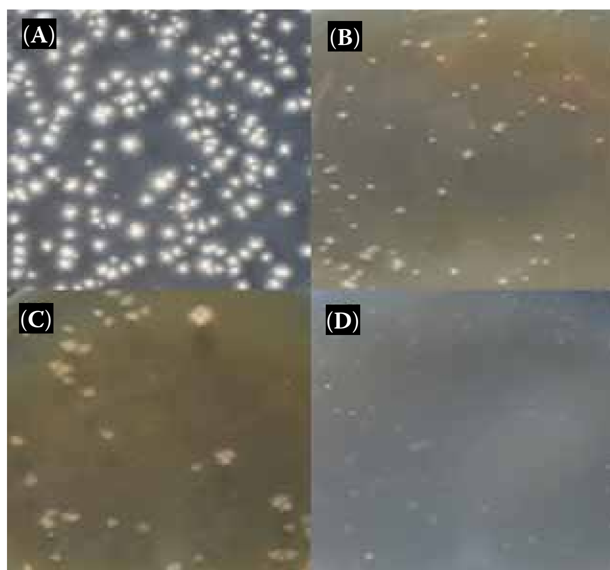

Figure 1. Colonial morphology of SDLQ16 on the different medium: (**A**) PDA 48 h; (**B**) NA 48 h; (**C**) LB 48 h; (**D**) GSYH 96 hours

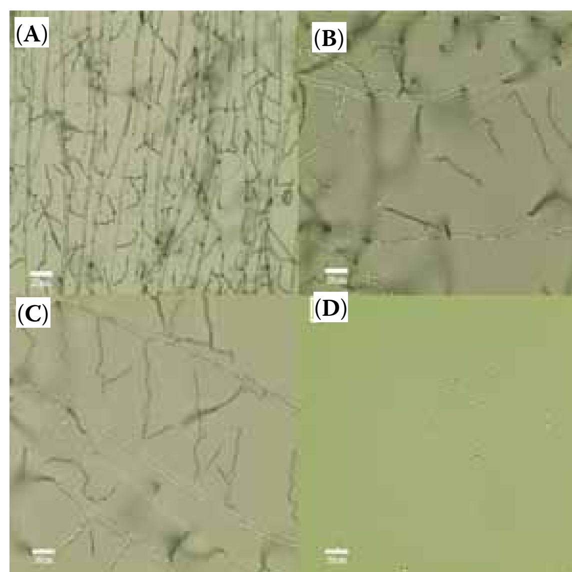

Figure 2. Morphological characteristics of strain SDLQ16 by electron microscope: (**A**) hyphae mycelium, (**B**) branch and branch, (**C**) beaded mycelium, and (**D**) oval spore, round spores or cylindrical spores; 10 × 40

range of nutrition. On PDA, the colonies were raised and pale white, with a neat edge and visible hyphae at the edge; colonies were friable, with no pigments, opaque and not shiny; sporulation was vigorous, and the colony surface was white and powdery. After 6 months of culture, white paste-like secretions appeared on the PDA, but the sexual stage of spores was not observed. On NA, the colonies were raised, with an unapparent colour, neat edge, developed mycelia, weak sporulation, and no pigments, being opaque and not shiny. Additionally, SDLQ16 could grow on LB and showed similar characteristics as observed on the NA medium. On GSYH, the colonies were small, with developed mycelia, weak sporulation, and no pigments, and were opaque and not shiny (Figure 1). The growth rate analysis showed that PDA supported the fastest growth (growth rate 1.49 mm/day), followed by NA (growth rate 1.29 mm/day) and LB (growth rate 1.17 mm/day); GSYH supported the slowest growth (Table 1). These results indicate that strain SDLQ16 has a wide range of nutrition.

Morphological features. Strain SDLQ16 did not form aerial hyphae. Basal hyphae rapidly grew close to the medium, 3.2–4.2 μm in diameter, with septa and forked branches at acute angles (Figure 2A). Single or multiple short hyphae often adhered to the septa at the base of the hyphae (Figure 2B). However, these hyphae were usually short and easily formed septa in a beaded pattern (Figure 2C). When hyphae matured in the late stage, they were fragmented to arthrospores (Figure 2D). Spores, solitary or beaded (Figure 3A), 3.5–7.5 μm in length and 3.5–4.5 μm in width (Figures 3B–C), had smooth surfaces (Figure 3D). Single or multiple short hyphae were rarely observed at the septum on the top of the hyphae. However, when they matured, the top hyphae easily produced septa and formed beaded hyphae. The reproduction mode of strain SDLQ 16 was fession. The colony centre of this strain existed concentric circles. This strain was able to gelatin liquefaction, proteolysis, grease, peptonised milk, urea, and so on. These characteristics were basically consistent with the pattern strain ATCC 34614 (Table 2).

Pathogenicity of the pathogen. Stabbed and unstabbed stems, leaves, and semi-mature fruits of

Table 1. Colony growth rate of strain SDLQ16 cultured for 5 days in different culture medium

Culture medium type	Colony diameter (mm)			Average value (mm)	Growth rate (mm/day)
	I	II	III		
PDA	7.30	7.50	7.60	7.47 ± 0.15^{a}	1.49 ± 0.03^{a}
NA	6.50	6.40	6.40	6.43 ± 0.06^{b}	1.29 ± 0.01^{b}
LB	5.80	5.80	6.00	5.87 ± 0.12^{c}	1.17 ± 0.02^{c}
GSYH	2.10	2.30	1.90	2.10 ± 0.20^{d}	0.42 ± 0.04^{d}

Values ± SD followed by different letters indicate significantly different scores in the same phase, according to Duncan's multiple range tests at the P = 0.05 level (SPSS 11.50)

https://doi.org/10.17221/76/2017-PPS

Table 2. Characteristic of strain SDLQ16 morphology and physiological biochemistry in PDA medium

Morphology idex	Strain	
	SDLQ16	ATCC 34614§
Colony colour	milky white	milky white
Spore shape	short rod	rod
Spore size	3.5–7.5 µm	3.0–7.6 µm
Diaphragm	exist	exist
Colony shape	flat	flat
Branch	two fork	two fork
Mycelium width	3.2–4.2 µm	2.6–7.0 µm
Mycelium shape	blanket-like	powdery
Reproduction mode	fissiparity	fissiparity
Colony concentric circles	exist	exist
Gelatin liquefaction	liquefaction	liquefaction
Proteolysis	hydrolysis	hydrolysis
Grease	degradation	degradation
Peptonised milk	peptonisation	peptonisation
Urea	assimilation	assimilation

§standard strain

strawberry were spray-inoculated and cultured for 3–5 days at 85–90% relative humidity. Injured stems underwent a rapid disease occurrence and turned black, with dark spots forming in the late stage (Figure 4A). However, the disease occurrence was the slowest on the stems. Almost no occurrence was observed on uninjured stems (Figure 4B). Injured leaves had a more rapid occurrence, but it was still 1–2 days slower than on fruits. Small black spots surrounded by yellow halos occurred on the leaf surface, with an unapparent boundary between diseased and healthy areas (Figures 4C1–C2). Uninjured leaves underwent slow or no disease occurrence (Figures 4D1–D2). Injured fruits exhibited a rapid occurrence. The fruit colour turned dark red and the fruit surface wilted rapidly; however, fruits did not

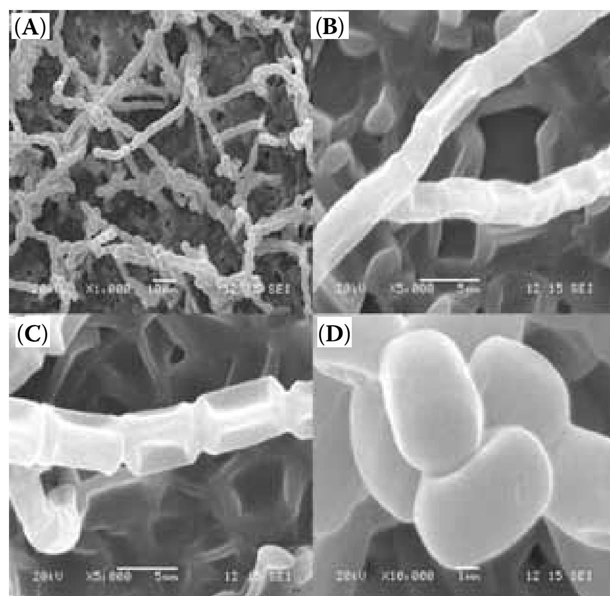

Figure 3. Morphological characteristics of strain SDLQ16 by scanning electron microscope: (**A**) 1000, (**B**) 5000, (**C**) 5000, and (**D**) 10 000×

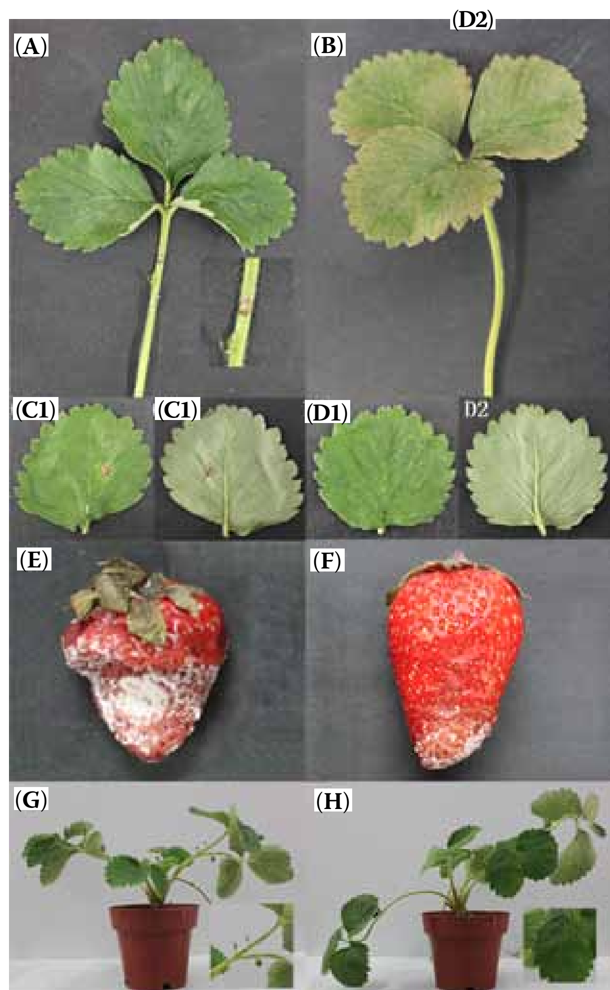

Figure 4. Symptoms of strain SDLQ16 infecting strawberry: (**A**) The inoculated stems with injury *in vitro*; (**B**) The inoculated stems without injury *in vitro*; (**C1**) Positive of the inoculated leaves with injury *in vitro*; (**C2**) Opposite of the inoculated leaves with injury *in vitro*; (**D1**) Positive of the inoculated leaves without injury *in vitro*; (**D2**) Opposite of the inoculated leaves without injury *in vitro*; (**E**) The inoculated stems with injury *in vitro*; (**F**) The inoculated stems without injury *in vitro*; (**G**) The inoculated leaves with injury *in vivo*; (**H**) The inoculated stems with injury *in vivo*

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crack. On the fruit surface, white hyphae appeared in the early stage, and a white layer of mold formed in the late stage. Uninjured fruits underwent a slow occurrence, generally 3–4 days later than injured fruits. The disease signs were not uniform, and the fruit colour turned dark slowly. Fruits were wilted but did not crack. Under humid conditions, the pathogen invaded slowly in the early stage, forming a small amount of hyphae; however, a white layer of mold also formed in the late stage (Figures 4E–F). Spores or hyphae were picked from strawberry stems, leaves, and fruits to re-isolate and purify the pathogen. The same colony characteristics were obtained as for the originally isolated pathogen. The results of the in vivo inoculation experiment were generally consistent with those of the in vitro inoculation experiments (Figures 4G–H).

ITS sequence analysis and phylogenetic tree construction. The genomic DNA of strain SDLQ16 was PCR amplified. The agarose gel electrophoresis of the PCR product revealed a clear 345-bp band. Further sequencing analysis verified that the *rDNA-ITS* sequence of this strain was 350 bp, in agreement with the electrophoresis results. The obtained *rDNA-ITS* sequence was deposited in GenBank under accession number KU373122.

We compared the *rDNA-ITS* sequence of 'SDLQ16', using the BLAST algorithm, with other sequences in the NCBI database and found this strain in the *Geotrichum* sp. branch of a phylogenetic tree. 'SDLQ16' was most closely related to *Geotrichum candidum* ATCC 34614 (GQ4580314.1), with a sequence similarity of 99% (Figure 5). 'SDLQ16' was tentatively identified as *G. candidum* based on the phylogenetic

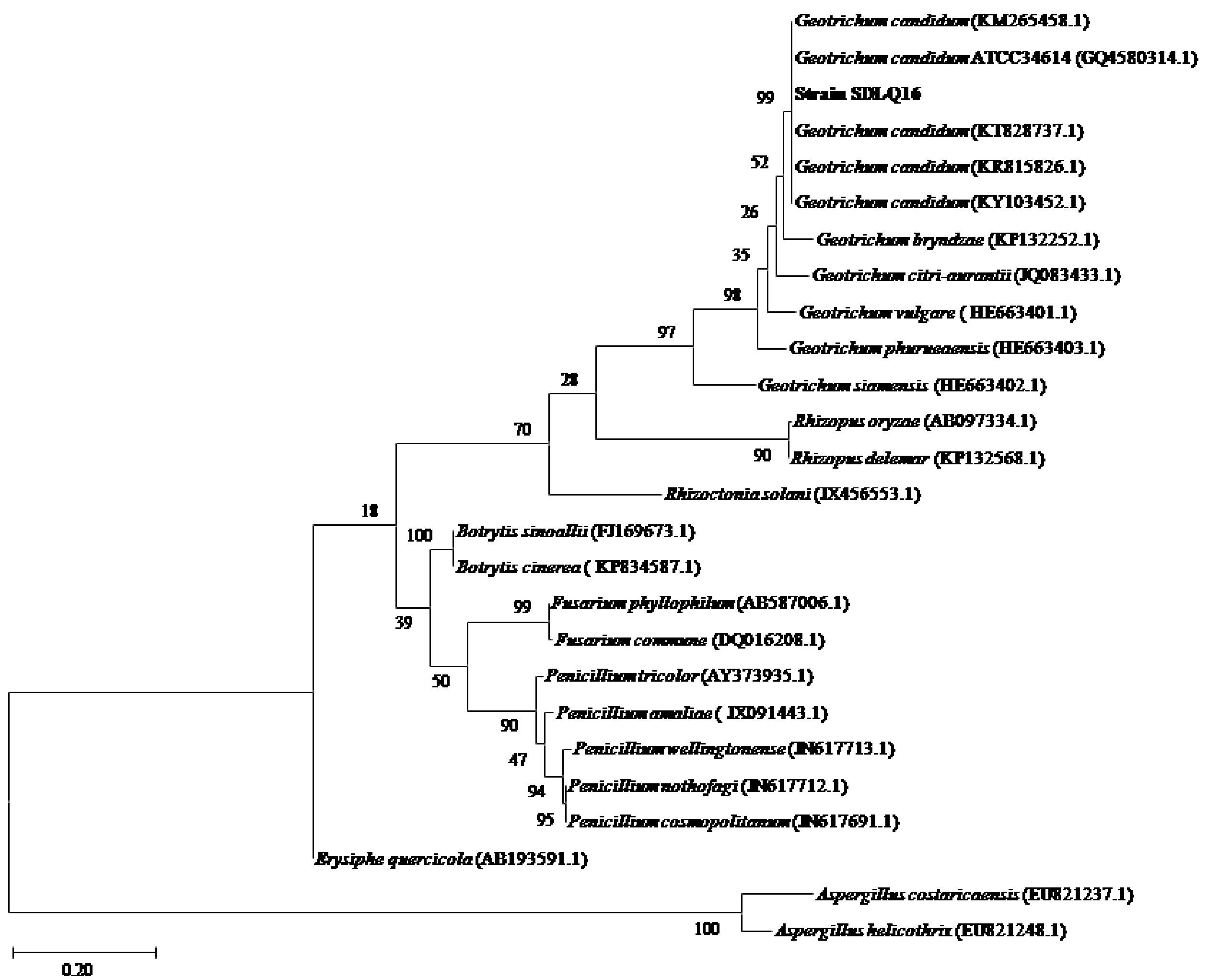

Figure 5. Bootstrap consensus tree constructed using the maximum likelihood method from 345 bp of ribosomal internal transcribed spacer DNA obtained from the SDLQ16 and *Geotrichum* population. The tree was rooted to *Aspergillus helicothrix*. The isolate SDLQ16 are indicated in bold font

https://doi.org/10.17221/76/2017-PPS

analysis of the *rDNA-ITS* sequence, in combination with morphological and culture characteristics and a physico-biochemical analysis.

DISCUSSION

Our group has discovered a new disease affecting strawberry. The isolate's identity was determined using cultured characteristics, morphological observations, and molecular biology techniques, and it was verified to be a pathogen, meeting Koch's postulates. The pathogen strain was tentatively identified as *G. candidum.* It belongs to fungus of deuteromycotina, hyphomycetes, hyphomycetales, and moniliaceae (Barnett & Hunter 1998). A review of the literature and historical data showed no research on this pathogen affecting strawberry. Thus, this is the first report of a strawberry disease caused by *G. candidum*.

The traditional classification and identification of plant pathogens are usually based on culture characteristics, colony characteristics, morphological features, and a pathogenicity index. The identification of *Geotrichum* fungi mainly involves colony morphology, the presence of septa in hyphae, hyphal branching, and the angle between main and secondary hyphae, as well as sporulation, and the germination, position, and size of spores. *Geotrichum* are widely distributed, and these fungi show great variation under different geographical and environmental conditions (Liu *et al*. 2011). In recent years, the extensive use of DNA identification techniques has effectively compensated for the shortcomings of traditional classification methods (Sun *et al*. 2015). *rDNA-ITS* is the target most widely used to classify and identify plant pathogenic fungi. The combination of traditional classification methods with DNA-based techniques has substantially improved the accuracy of the identification of plant pathogenic fungi. In particular, for closely related or sibling species of *Geotrichum* that share a close phylogenetic relationship (Blair *et al*. 2008).

G. candidum is a member of the genus *Geotrichum.* The sexual stage (named *Galactomyces geotrichum*) belongs to the family Candida, order Saccharomycetales, and class Hemiascomycetes; the asexual stage (named *G. candidum*) belongs to the genus *Oospora*, family Moniliaceae, and phylum Deuteromycotina. A study indicated that *G. candidum* has more than 10 synonyms (Ma *et al*. 2007). In the present study, we only found the asexual stage of the pathogenic fungus, and no sexual stage was observed during culturing. Further studies are needed to investigate whether the sexual stage of this strain is the same as previously reported. This will include determining how it overwinters and when and under what conditions it germinates, as well as revealing primary infections, the infection conditions, and the mode of transmission in the field. *G. candidum* is strongly virulent towards carrots (Horita & Hatta 2016). Bourret *et al*. (2013) indicated a weak pathogenicity of *G. candidum* to tomatoes that the wounded tomatoes could be infected with *G. candidum* and gotten rot disease at a given tempreature of 20°C. Yaghmour *et al*. (2012) suggested that injury increases the susceptibility of peaches and nectarines to *G. candidum*, and the rot disease appeared after 5 days under a constant 22–26°C. A number of studies have demonstrated that *G. candidum* is the major pathogen of citrus (Eckert 1978; Talibi *et al*. 2012). The present study shows that the pathogenicity of *G. candidum* is not only on different host plants but also has regional differences, which may be related to the pathogenicity genes of *G. candidum* that are present in different areas, resulting in differences in pathogenic performance. However, the causes of the different pathogenicity levels requires further study. In the present research, *G. candidum* could cause strawberry to rot in Shaodong County, Hunan Province, China. This similarity could result from the high water content observed in both peaches and strawberries which belong to different genera of the family Rosaceae, or from regional differences, indicating that *G. candidum* is pathogenic to some plants. Thus, for biological product development, or product degradation or preservation, *G. candidum* should be used with caution, and not arbitrarily discarded, to prevent unnecessary losses.

In addition, under the conditions of low temperature, high humidity, and insufficiency light, the fungus showed a high virulence toward its host, leading to a large disease incidence. Different organs of plants have different sensitivities to the fungi, and the fruit is more sensitive to this fungi than stems and leaves. Thus, the disease rate was high when the pathogen infected wounded tissue, indicating it was a weak pathogen. The lower incidence of disease development on the stem may be the result of the lower stem water content compared with that of the fruit and blade. The cortex thickness, stomatal density, pore size, fruit and leaf ages may also af-

https://doi.org/10.17221/76/2017-PPS

fect the disease rate. Here, *G. candidum* is reported as a new pathogenic fungi of strawberry in China; however, at the present, there is limited research on its treatment or pathogenic mechanisms, such as the biological characteristics, epidemic laws, host range, the mechanisms of infection, fungicides and antagonism-based screenings are seldom used. To increase our understanding of strawberry rot disease, the above aspects require further studies as we work toward disease prevention and control.

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