

# Phylogenetic and Morphological Identification of the Novel Pathogen of *Rheum palmatum* Leaf Spot in Gansu, China

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**Abstract** A new leaf spot disease was observed on leaves of *Rheum palmatum* (Chinese rhubarb) in Northwest China (Gansu Province) starting in 2005. A *Septoria*-like fungus was isolated and completion of Koch's postulates confirmed that the fungus was the casual agent of the leaf spot disease. Morphology and molecular methods were combined to identify the pathogen. The fungus produced conidiomata pycnidia and the conidia were 2~5 septate, 61.2~134.1 µm in length and 3.53~5.3 µm in width, which is much larger than the known *Septoria* species that infects Polygonaceae species. Phylogenetic analysis of the internal transcribed spacer region confirmed that this *Septoria*-like fungus is within the *Septoria* genus but distinct from known *Septoria* species. Together, these morphological and phylogenetic data support that the *R. palmatum* infecting *Septoria* strain is a newly-described plant pathogenic species.

**Keywords** Internal transcribed spacer rDNA, Novel pathogen, *Rheum palmatum*, Taxonomy

*Rheum palmatum* L., commonly called Chinese rhubarb, is a medicinal plant. The air-dried root has been used as the traditional Chinese herbal medicine (TCM) for over 2000 years. Although the pharmacological effects of *R. palmatum* are that it is cathartic on the digestive movement of the colon; it was found can also protects the damaged liver, and has antitumor, antibacterial, anti-inflammation, and anti-aging properties, the side effects are nausea, vomiting, and dizziness, etc. [1-3]. *R. palmatum* widely grows either in low altitude area (800 m to 2,000 m) with damp climate or high altitude area (1,200 m to 3,500 m) with bleak climate. In China, it distributed in the southwest, northwest, northeast and north China. Gansu province, located in northwest China, is the main production area of *R. palmatum*.

With the dozens of bioactive substances was found, the use of the Chinese rhubarb become wider and wider. The wild resources can not satisfy the pharmaceutical demands any more leads to the expanding of the cultivation area. Disease problem become the key factor on the yield and quality of the plant. The production of *R. palmatum* was affected by several pathogens, including ring spot (*Ascochyta rhei* Ellis et Everhart), leaf spot (*Septoria polygonorum* Desm.) [4], smut (*Thecaphora schwarzmaniana* Byzova) [5], rust (*Puccinia rhei-palmati* B. Li) [6], gray spot (*Phyllosticta rhei* Ellis et Everhart) [7], powdery mildew (*Erysiphe polygoni* DC.) [8], grey mold (*Botrytis* sp.), root rot (*Fusarium oxysporium* Schl.; *Fusarium* sp.; nematode) [9], and a virus disease (cucumber mosaic cucumovirus) [10]. A new leaf spot, caused by a *Septoria*-like fungus which is different from the reported pathogen, *S. polygonorum*, was first found in Weiyuan, Longxi, and Ming County in 2005 with 30~40% disease incidence and 1~2 disease severity, because of the increasing disease incidence and severity of necrotic symptoms in the main Chinese rhubarb production areas of Gansu, China. The pathogen of the disease need to be confirmed and the control methods as well.

The genus *Septoria* Sacc. (with type *S. cytisi* Desm.) [11] was introduced by Fries in 1819 [4]. The modern prescription of *Septoria* is characterized by pycnidial conidiomata, filiform, hyaline, smooth-walled multi-septate conidia [12-14]. It is among the most common and widespread leaf-spotting fungi worldwide [15, 16]. The identification of this genus is largely based on the host and the biogeography location,

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to date, the genus includes more than 3,000 published names of species and infraspecific taxa [17], most of them are synonyms. Teleomorphs are known for only a relatively small number of *Septoria* species, and in all cases they are species of *Mycosphaerella* Johanson (Dothideales, Ascomycota), a genus with numerous plant pathogenic taxa and over 20 reported anamorph genera [18-20].

The objective of this research is to identify the pathogen, the internal transcribed spacer (rDNA-ITS) region has been amplified for phylogenetic identification purposes. Furthermore, morphological criteria have been taken into consideration to identify the pathogen.

## MATERIALS AND METHODS

### Diseased specimen collection and fungal isolate.

Specimens of *Septoria*-like leaf necrotic were collected in 5 different *Rheum* fields in Weiyuan, Gansu, China between 2008 and 2011. These tissues were dried between sheets of newspaper and dispatched by courier for diagnosis and isolation within 1 wk of collection. Leaf tissue with necrotic lesions were surface sterilized in 1% sodium hypochlorite for 3 min and then rinsed three times in sterile distilled water. Small pieces (< 2 mm<sup>2</sup>) of tissue were excised from the lesion margins, placed onto potato dextrose agar (PDA) in darkness. To obtain single-spore colonies, single pycnidia was picked from the surface-sterilized tissue and then placed on a petri plate containing water agar (water 1,000 mL, agar 17 g), distributed across the plate surface evenly using a sterile glass rod, and incubated at 20°C for 12 hr in the dark. Germinating conidia were subsequently removed individually and placed onto PDA in darkness at 20°C. After 20 days, pick the mycelium from the margin of the colony and transferred to PDA.

**Morphology characterization.** A single pycnidia on leaf spot was gently picks up and placed in a small drop of water on a slide. Press gently on the cover slip to allow the dispersion of the conidia. Where possible, conidial measurements were made using conidia from the leaf spots with expelled spores. After the drop of water had evaporated, 1% cotton blue in lactic acid was added and a cover slip applied. Conidia were measured randomly. For curved conidia measurements were made on natural length. The widths of both the conidia were measured at their widest part [21]. Spore dimension and the presence of septa were noted in each of 30 randomly selected conidia from a single sporocarp, observations were made through bright field microscopy at 400 magnification.

**Cultural characteristics.** Mycelium plugs, 7-mm in diam., were removed from margins of colonies which had been growing on PDA in the dark at 15°C for 60 days and inoculated on PDA, malt extract agar (MEA), and oatmeal agar (OA) of 90 mm diam. petri dishes. Three replicates for each media. Dishes were sealed with laboratory film (Parafilm

M, West Berlin, NJ, USA), the isolate were cultured in the dark at 15°C, colony diameters of were measured weekly for 8 wk. The color and the characteristics of the colony were observed after 8 wk of cultivation. Mycelium and fruiting bodies were transferred in a drop of water on a slide, pressed gently under a cover glass and studied with light microscopy. Growth rates were then expressed in mm/day.

**Pathogenicity test.** The pathogenicity of isolate was tested on 10 *R. palmatum* plants. The plants previously grew in the greenhouse in pots for 8 wk. Mycelium and spores were liberated from 30-day-old cultures on PDA by dispensing 10 mL of a 0.1% Tween-80 solution onto each Petri plate and brushing the surface of the culture with a sterile spatula. The concentration of conidial inoculum was adjusted to  $1 \times 10^4$  conidia/mL using a hemocytometer. Plants were inoculated with 10 mL of the inoculum suspension using a hand sprayer. Controls were sprayed with the same volume of 1 µL Tween-80 and distilled water. Plants were incubated by placing the pots in trays that were partially filled with water inside the plastic bags to maintain high humidity. The moist chambers were put at 20°C for 72 hr. The bags were then removed and the plants were placed under diffused room light in the laboratory. Symptoms were observed and recorded at 7 days intervals and isolations were made from lesions after 4 wk.

### DNA extraction and sequencing of the internal transcribed spacer region (ITS) of the ribosomal DNA.

Mycelium was scraped from cultures growing on PDA, put into 100 mL potato dextrose liquid medium (PD; potato 200 g, dextrose 10 g, water 1,000 mL), agitated using an orbital shaker set at 45 rpm and incubated at room temperature under constant illumination by fluorescent lights. Mycelium (100 mg) was removed after 7 days and transferred to a mortar and pestle for grinding with liquid nitrogen. DNA extraction was then carried out using the FastDNAkit (SK1375; Sangon Biotech Co. Ltd., Shanghai, China) following the manufacturer's instructions. The presence of DNA was verified by separation on a 0.8% agarose gel in  $1 \times$  TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) stained with ethidium bromide (0.5 mg/L) and visualized under UV light. The ITS region was amplified using the universal primers, ITS1 and ITS4 [22]. PCR was performed in 50 µL reaction volumes containing 10~100 ng of genomic DNA, 2.5 µL of each primer (10 µM), 0.25 µL dNTP (10 mM), 2.0 unit Supertaq DNA polymerase and 5 µL  $10 \times$  PCR buffer (Sangon Biotech Co. Ltd.). The amplification was performed in a thermocycler (Biometra Tg PCR; Biometra, Göttingen, Germany). The PCR thermal cycle program was as follows: 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 3 min, annealing at 52°C for 45 sec, and elongation at 72°C for 1 min, with a final extension step of 72°C for 8 min. The PCR products, spanning approximately 500 bp of ITS, were checked on 1% agarose electrophoresis gels stained with ethidium bromide. The PCR product was

purified and sequenced using the abovementioned primers by Sangon. The novel sequence was deposited in GenBank of National Center for Biotechnology Information (NCBI).

**Sequence alignment and phylogenetic analyses.**

Approximate phylogenetic placements were determined with the Blastn search option of NCBI. The ITS sequences from NCBI were aligned using MAFFT v. 7 (<http://mafft.cbrc.jp/alignment/server/index.html>) [23] and manually improved in BioEditv. 7.0.5.2 [24]. *Dividiella tassiana* (accession No. JF911765) was set as the outgroup. Phylogenetic tree was constructed with MEGA 5 using the neighbor-joining method and the Tajima-Nei distance model.

**RESULTS**

**Observation in the fields.** Occurring on leaves. The symptom initially appears as chlorotic, yellowish spots scatter on the leaf, later the spots expanded to irregular, nearly round lesion, 8~12 mm, gray white in the center and brown to red brown at the edge. The edge thin or wide, a little upheaval, Finally, plenty of black granules (pycnidia) separately scattered on the lesion. When it rains or the humidity of environment is high, the spores expel out of the pycnidia, the lesion were covered with a layer pink to white mold and black empty pycnidia were buried in it (Fig. 1A, B). The lesion is more solid, dry and thick than healthy leaf tissue. Specimens are deposited in the Herbarium of Gansu University of Traditional Chinese Medicine, Lanzhou, China (HTCM).

**Taxonomy.**

*Septoria palmati* Yan Wang, sp. nov. (Plate 2)

Mycobank: MB 817262.

Differs from all other *Septoria* species by its Polygonaceae

host and large spores.

**Type:** China, Gansu Province, Weiyuan county, on leaves of *Rheum palmatum* L. (Polygonaceae), 8 Jul 2008, Yan Wang (holotype, Herbarium specimen in HTCM: 11001215; GenBank: KC874676).

**Etymology:** *Palmati* (meaning “of [*Rheum*] *palmatum*”). Conidiomata pycnidia, semi-immersed, separate, spherical to subglobose, medium brown, (174.9~) 188.1~277.7 (~349.4) (mean, 228.6 μm) in diam. (Fig. 2D). Walls pseudoparenchymatous, 4~6 cells thick, 5~10 μm wide, composed of texture angular cells. Ostiole single, circular, central, 14~21 μm in diam. Conidiophores absent. Conidiogenous cells hyaline, single cell. Conidia hyaline, thick rope, nematode, straight or slightly curved, 2~5 septate, 61.2~134.1 × 3.53~5.3 μm (mean, 89.8 × 4.3 μm) (Fig. 2E and 2F).

Colony grows very slowly (0.33 mm/day), the upper surface of the isolate growing on PDA is powdery and velvet in pink, slightly uplift, conidia form directly from hypha and assembled together to appear yeast-like growth type, mycelium assemble to yellow-brownish to black dormancy organization structure when cultivated for more than 3 mo (Fig. 2A). Colony on OA is white, velvet and slightly uplift (Fig. 2B). The colony cannot grow on MEA, only some mycelium assemble around the previous disk (Fig. 2C). Pycnidia not formed on three tested media.

**Pathogenicity.** *Septoria* isolate TCM-6 was inoculated on *Rheum* plants and symptom can be observed in all repetitions. Initial symptom was small yellow dots that appeared approximately 20 days after inoculation. These dots enlarged to typical necrotic lesions, round or irregular spots, pale gray with red purple margins on the leaves, no symptoms developed on non-inoculated control plants in any of the experiments. The pathogen were isolated again from the lesions, the Koch’s regulation were confirmed.

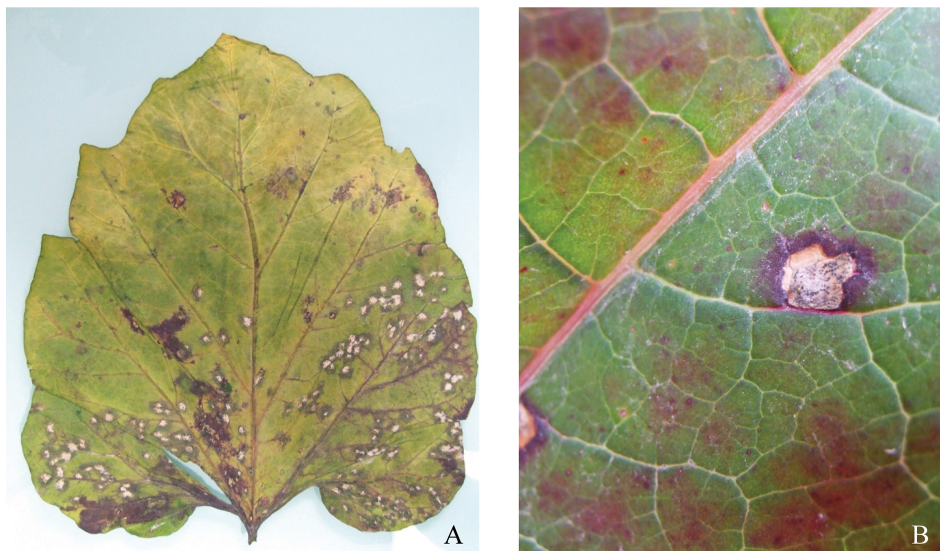
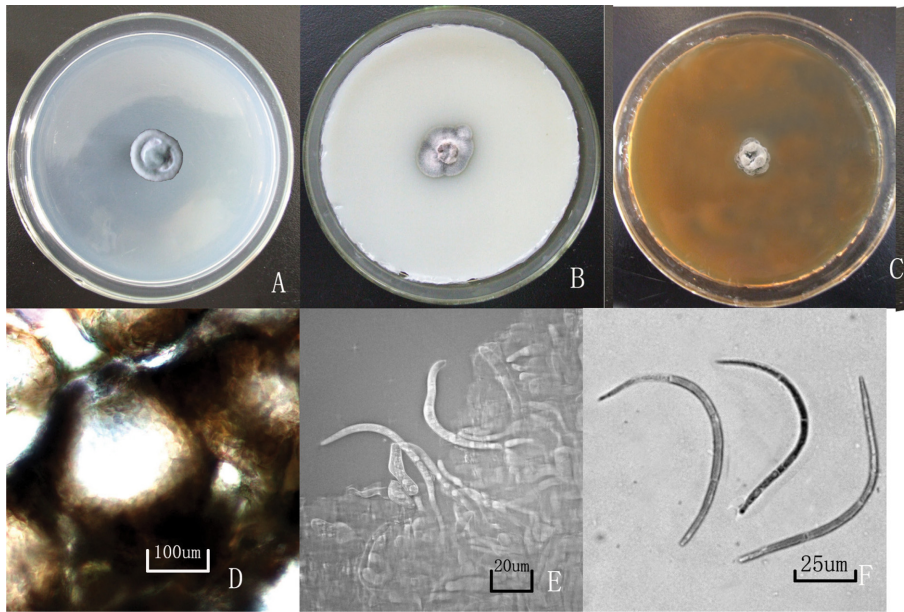
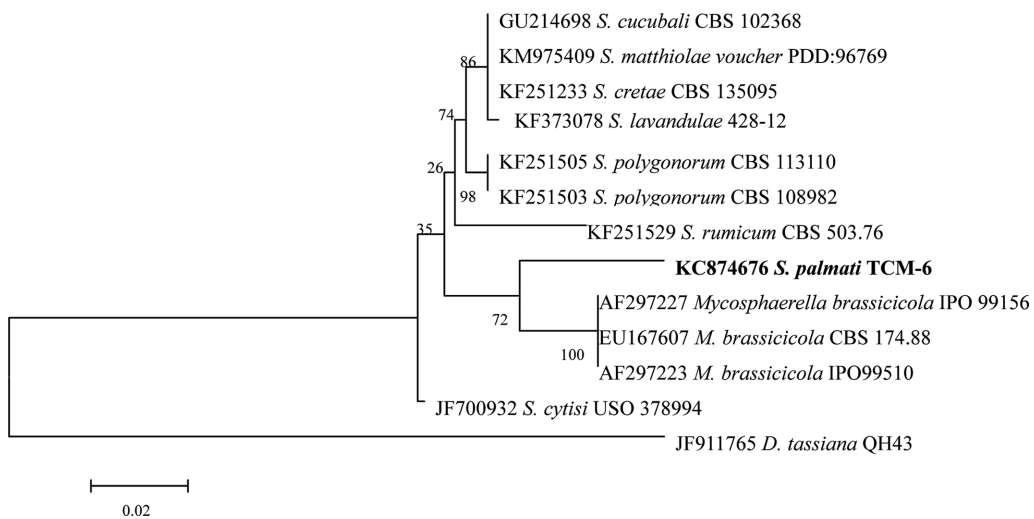


Fig. 1. A, B, Symptoms of leaf spot on *Rheum palmatum* caused by *Septoria*.





**Fig. 2.** *Septoria palmatum*. A~C, Colony on potato dextrose agar, oatmeal agar, and malt extract agar; D, Pycnidia; E, F, Conidia (scale bars: D = 100 μm, E = 20 μm, F = 25 μm).



**Fig. 3.** Bootstrap consensus tree constructed using the neighbor-joining method from 489 bp of ribosomal internal transcribed spacer DNA obtained from the TCM-6 and *Septoria* population. The tree was rooted to *Dividiella tassiana*. The isolate TCM-6 are indicated in bold font.

**Phylogenetic analysis.** Amplification was successfully carried out with the ITS1/ITS4 primers, and fragment of approximately 489 bp was obtained. The ITS alignment contains 13 taxa including the outgroup and the high similarity strains through Blastn search using the ITS sequence in Genbank. The tree was rooted to *D. tassiana* and two sister clades were revealed from the analysis. One clade includes *S. cucubali* (GU214698), *S. matthiolae* voucher (KM975409), *S. cretae* (KF251233), *S. lavandulae*, *S. polygonorum* (KF373078), *S. polygonorum* (KF251505), *S. rumicum* (KF251529). The other clade includes three strains of *Mycosphaerella brassicicola* (AF297227, EU167607,

and AF297223) and TCM-6 as well (Fig. 3). The strain has high similarity with *M. brassicicola*, we assume that the sexual stage of TCM-6 is *Mycosphaerella* sp.

Based on morphological features, pathogenicity test and amplification of ribosomal nuclear sequences, we supposed that the *Septoria* isolate: TCM-6 is a newly-described plant pathogenic species.

## DISCUSSION

The cultural characters of *S. palmati* is hemi-hypha and yeast-like. In *Septoria*, the cultural characters are complex,

asexual and sexual stage are possible be the yeast-like or mycelium type [25]. Kema and Annone's study [26] on 26 strains of *S. tritici* from different part of the world and found nutrition and environment has minor effects on the cultural characters, the strain which is yeast-like style will not form pycnidia on any kind of medias, and in liquid cultural condition, only form spores and short hypha suspension unlike the mycelium balls. To date, only small groups of *Septoria* have been revised with cultural studies [27], the function of this characters is not unclear in the identification of *Septoria* species. So, the relationship between cultural characters and species need to be further studied.

The telemorph stage of *S. palmati* was not observed *in vitro* or *in vivo*. *Mycosphaerella* Johanson is one of the largest genera of ascomycetes [19]. Von Arx [18] accepted 23 genera as known or suspected anamorphs of *Mycosphaerella*, including *Septoria* Sacc. Through Blastn search and sequence alignment *S. palmati* was aligned with several strains of *Mycosphaerella brassicicola*. We suppose that the telemorph of the new species is also *Mycosphaerella* sp.

Beach [28] invested a wide range of species and demonstrated both host and biological specificity in all taxa, he proposed that species of *Septoria* do not have a broad host range, and infectivity rarely beyond two or three related genera. Verkley and Starink-Willemse [15] indicate that the *Septoria* species from Asteraceae are all closely related, and share an evolutionary history within the Mycosphaerellaceae through studies on 14 *Septoria* species pathogenic to Asteraceae by analysing 5.8S ribosomal RNA gene and flanking ITS (ITS1 and ITS2) sequences sequence analyses [15]. So, it is reasonable to identify the taxa according to the host in this genus. Seventeen species have been reported can infect Polygonaceae plants [29]. *S. palmatum* exhibited all of the morphological traits attributed to *Septoria* and conidia 2~5 septate, size 61.2~134.1  $\mu\text{m} \times$  3.53~5.3  $\mu\text{m}$  which is much larger than the other *Septoria* species pathogenic to Polygonaceae, even double size of conidia of *S. polygonorum* (100~150  $\mu\text{m}$  diam., 3~4-septate, hyaline, smooth, 26~46 [38]  $\times$  2.0~2.4  $\mu\text{m}$ ), which was reported as a pathogen of rhubarb leaf spot (*S. polygonorum*) [4], so here we described the isolate *S. palmati* as a new taxa.

The distribution of *S. palmati* thus, may be restricted to the areas where it has been found. However, further surveys are necessary to determine exactly where this new leaf spot pathogen occurs. The occurrence area of the pathogen in Gansu, China is at an narrow area apparently. It might also be present in other locations where the rhubarb were cultivated. Further research work will focus on the pathogenicity rang, the disease circle and control methods, including manipulation of agronomic factors such as plant density and irrigation frequency and timing, to decrease the disease intensity.

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